A novel p.Ser108LeufsTer15 SOD1 mutation leading to the formation of a premature stop codon in an apparently sporadic ALS patient: insights into the underlying pathomechanisms

A novel p.Ser108LeufsTer15 *SOD1* mutation leading to the formation of a premature stop codon in an apparently sporadic ALS patient: insights into the underlying pathomechanisms

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Abstract

We report an apparently sporadic Amyotrophic Lateral Sclerosis patient carrying a heterozygous novel frameshift *SOD1* mutation (p.Ser108LeufsTer15), predicted to cause a premature protein truncation. RT-PCR analysis of *SOD1* mRNA and WI analysis of PBMC demonstrated that mRNA from the mutant allele is expressed at levels similar to those of the wild type allele, but the truncated protein is undetectable in the insoluble fraction and after proteasome inhibition. Accordingly, the dismutation activity in erythrocytes is halved. The mutation can theoretically result in a neopeptide that is heavily positive-charged compared to wild type SOD1 (wtSOD1). The mutant protein – if synthesized – should accordingly have less repulsive forces, resulting in an increased propensity to aggregate. The mutation will result in the loss of residues essential to bind the catalytic copper and the stabilizing zinc, to stabilize the monomer, and to form a stable dimer interface. We suggest that the predicted mutant protein could be a highly unstable, aggregation-prone protein with no superoxide dismutation capability, unable to form a heterodimer with wtSOD1 and with prion-like behavior.

Keywords: Amyotrophic Lateral Sclerosis, *SOD1*, truncated protein, prion-like behaviour

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease of the adult life affecting both upper and lower motor neurons, frequently leading to death within 3-5 years, due to failure of the respiratory muscles. *SOD1* mutations account for 12-23% of familial ALS (fALS) cases and ~1% of apparently sporadic (sALS) cases (Renton et al., 2014). While most of the reported *SOD1* mutations are missense mutations, some 20 insertion, deletion and splice-site mutations predicted to result in a change of the length of the putative mutant polypeptides have also been reported (Andersen et al., 2003, 1997; Corrado et al., 2006; Hosler et al., 1996; Hu et al., 2012; Jackson et al., 1997; Nakashima et al., 1995; Orrell et al., 1997; Pramatarova et al., 1994; Watanabe et al., 2001, 2000, 1997; Zu et al., 1997). Noticeably, in some of these studies the mutant protein could not be found in autopsy brain tissue (Watanabe et al., 1997), in erythrocytes or lymphoblastoid cells (Zu et al., 1997). We here report an apparently sALS patient carrying a heterozygous novel frameshift *SOD1* mutation (c.320_321insT; p.Ser108LeufsTer15 or p.Leu106fs*15 according to the traditional nomenclature, omitting the initial methionine), predicted to cause a premature truncation of the protein.

2. Methods

2.1 Genetic analysis

Following approval by the institutional ethical review board and with written informed consent from the patient, whole venous blood was collected in EDTA-containing vacuum tubes and the DNA extracted using standard procedures. The five coding exons and 50bp of the flanking intron-exon boundaries of *SOD1* were PCR amplified, sequenced using the Big-Dye Terminator v3.1 sequencing kit (Applied Biosystems Inc.), and run on an ABIPrism 3130 genetic analyzer.

2.2 SOD1 enzymatic activity

The SOD1 dismutation activity was analysed in erythrocytes by the direct spectrophotometric method using potassium superoxide as previously described (Andersen et al., 1998; Marklund, 1976). The activity was related to the content of hemoglobin in the erythrocyte lysates. Further details are reported in Supplemental Methods.

2.3 PBMC isolation

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-anticoagulated venous blood by Lymphoprep™ (Axis-Shield, Oslo, Norway) by density-gradient centrifugation as previously described (De Marco et al., 2011). In brief, blood sample diluted (1:1) in phosphate-buffered saline (PBS) was slowly layered over Lymphoprep™ and centrifuged at 600*g* without brake at room temperature for 20 min. PBMC layer was collected, pelleted and washed twice with PBS.

2.4 Extraction of detergent-insoluble proteins

An aliquot of PBMC was resuspended in an ice-cold buffer containing 0.05 M HEPES pH 7.3, 0.15 M NaCl, 0.1 M EDTA, 0.1 mM DTT, 1% protease inhibitor cocktail, 1% Triton X-100 and centrifuged at 15000g for 15 min at 4°C. The obtained supernatant was designated the soluble fraction; the pellet was washed twice with PBS and designated the insoluble fraction. Both fractions were treated with a urea buffer (8 M deionized urea, 0.05 M Tris-HCl pH 6.8, 5% w/v sodium dodecyl sulfate, 2% v/v 2-mercaptoethanol).

2.5 Treatment of PBMC with MG132

Another aliquot of PBMC from the patient was resuspended in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Milan, Italy) and 1X penicillin/streptomycin cocktail (Sigma-Aldrich, Milan, Italy) and incubated in a 24-wells plate (5x105 cells/well) at 37°C and 5% CO_2. Cells were exposed to 0.01 M of the proteasome inhibitor MG132 (Sigma-Aldrich, Milan, Italy) for 3 and 12 hours. At the end of incubation, cells were washed twice with PBS and treated for immunoblot experiments as described below.

2.6 WI analysis

PBMC were lysed in the above mentioned urea buffer. 50 µg of protein extract was loaded on each lane of polyacrylamide 4-15% precast gel (BioRad, Milan, Italy). Membranes were blocked with 2% bovine serum albumin (BSA) in Tris-buffered saline added with 0.02% (v/v) Tween 20 (TBST buffer). Membranes were exposed overnight at 4°C to the rabbit polyclonal SOD1 antibody FL-154 (Santa Cruz Biotechnology, Heidelberg, Germany) raised against the full-length human SOD1 protein, to the goat polyclonal SOD1 antibody N-19 (Santa Cruz Biotechnology, Heidelberg, Germany) targeting the N-terminal region of human SOD1, and to the rabbit polyclonal SOD1 antibody A303-811 (Bethyl Laboratories, Montgomery, TX, USA) raised specifically against an epitope between amino acids 54 and 104. Probed blots were then incubated at 4°C with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany), washed in TBST buffer and incubated with enhanced chemiluminescent reagent (Advansta, Menlo Park, CA, USA). The immunostained bands were visualized using C-Digit® Blot Scanner (Licor Biosciences, Bad Homburg, Germany). Membranes were reprobed with an antibody against β-actin (Sigma-Aldrich, Milan, Italy) for equal protein loading. An antibody against ubiquitin (Santa Cruz Biotechnology, Heidelberg, Germany) was used to ascertain the efficacy of the treatment with MG132. The relative intensity of each protein band was quantified using Image Studio Digits v.5.0 (Licor Biosciences, Bad Homburg, Germany).

2.7 RT-PCR analysis of SOD1 mRNA and cDNA analysis

Total RNA was extracted using TriZol (Invitrogen) from lymphocytes of the patient and from two control individuals (a healthy person and an ALS patient heterozygous for a p.Asp109Tyr *SOD1* mutation). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was amplified by end point PCR, using two primers mapping on exons flanking the one carrying the mutation (forward primer 5'- GGC CTG CAT GGA TTC CAT GTT CAT G-3', starting at codon 42 in exon 2; reverse primer 5'- CCC AAT TAC ACC ACA AGC CAA ACG AC-3', starting at codon 151 in exon 5).

To analyze whether the mRNAs corresponding to the two *SOD1* alleles were expressed at similar levels, we sequenced the amplified products.

3. Case report

3.1 Clinical depiction

The patient is a woman who developed dysarthria with tongue atrophy at the age of 64, followed by dysphagia. The course was slowly progressive and a progressive bulbar palsy diagnosis was made two years after symptom onset. One year after diagnosis she had developed tetraparesis with brisk deep-tendon reflexes on the left side, left-sided Babinski sign and bilateral ankle clonus. A neuropsychological assessment was normal. Four years after onset the patient displayed a walker-assisted gait and used nocturnal non-invasive ventilation. At this time-point a brain MRI-Diffusion Tensor Imaging showed reduced Fractional Anisotropy along the corticospinal tracts, suggestive of axonal damage; the spectroscopy of the primary motor cortex evidenced a decrease of the NAA/Cr

ratio, in agreement with neuronal loss. A brain ^{18}F-FDG-PET showed asymmetric tracer uptake in the temporal poles (left<right). Family history was negative for neurodegenerative conditions. Her father died at 77 years, her mother at 73. Her sister is alive and 70 years old. A paternal uncle died at 80. Two paternal aunts died at 62 and 95, respectively. A paternal aunt is alive and 92 years old. A maternal uncle died at 84. Her daughter (44) and her son (47) are healthy. Parents and grandparents were from the province of Pavia, Lombardia, Italy. DNA of the relatives was not available. The genetic analysis identified a heterozygous insertion of a thymine in exon 4 (c.320_321insT), predicted to cause a frameshift and the introduction of a neopeptide sequence of 15 aminoacids followed by a premature stop codon (p.Ser108LeufsTer15). The patient tested negative for a panel of other ALS-related genes (details available upon request). The patient is alive, approximately 7 years from symptom onset.

3.2 WI analysis

Detection of SOD1 protein was assessed in PBMC using an antibody against full-length SOD1, an antibody against the N-terminal region of the protein as well as an antibody recognizing a SOD1 epitope between amino acids 54 and 104. All three antibodies detected the full-length SOD1 at about 15-20 kDa. The intensity of SOD1 band in the proband was lower than that of the bands detected in control individuals (Figure 1A). In quantitative terms, the intensity of SOD1 in the proband was ~50% of the mean intensity detected in three neurologically unaffected subjects and in three individuals with sALS (Figure 1B). The SOD1 N-terminal antibody revealed an additional faint band in the lower molecular mass range (~10 kDa) in all cases analyzed. None of the antibodies tested revealed any band specific for the protein encoded by the allele carrying the p.Ser108LeufsTer15 mutation (Figure 1A).

The wild type SOD1 (wtSOD1) was detected only in the soluble fraction of PBMC. The lower molecular mass band found in the insoluble fraction was not specific for the p.Ser108LeufsTer15, since a similar band was detected also in an ALS patient without *SOD1* mutations (Figure 2A). Immunoblot of PBMC from the proband and an ALS patient without *SOD1* mutations incubated with the proteasome inhibitor MG132 did not reveal any band ascribable to the product of the mutated allele either in the lower or in the higher molecular mass range. The efficacy of MG132 treatment was confirmed by the increase of intensity of the smear revealed by the ubiquitin-targeted antibody in treated cells as compared to untreated cells (Figure 2B).

3.3 RT-PCR analysis of SOD1 mRNA

To test whether the absence of the truncated protein in WI was due to degradation of the mutated transcript through nonsense-mediated mRNA decay, we assessed whether this transcript was normally represented as compared to the wild type, by amplifying *SOD1* cDNA and direct sequencing. The product of end point PCR obtained from patient RNA was not qualitatively different from products obtained from the two controls (a healthy person and an ALS patient with a p.Asp109Tyr *SOD1* mutation), suggesting that mutation has no effect on pre-mRNA processing within the explored region (Figure 3A). Considering the small sequence difference between wild type and mutant alleles and the distance of mutations from primers, end-point PCR would be expected to produce the same ratio between normal and mutant sequences as obtained after reverse transcription.

The patient's electropherogram revealed a systematic doubling of peaks, starting exactly in correspondence of the single nucleotide deletion detected in genomic DNA. In comparison, the wild type control did not reveal double peaks, while the p.Asp109Tyr patient revealed only one double peak exactly corresponding to the identified heterozygous mutation (Figure 3B).

From these results we conclude that the allele carrying the p.Ser108LeufsTer15 mutation is expressed at levels similar to those of the wild type allele.

3.4 SOD1 enzymatic activity

The dismutation activity in erythrocytes was halved at 26.46 U/mg Hb (reference 55,36±6,22 U/mg Hb, from n = 170 female controls who were spouses of ALS patients and healthy control individuals) and comes from the patient's intact wtSOD1.

4. Discussion

We report a sALS patient carrying a novel p.Ser108LeufsTer15 *SOD1* mutation, predicted to lead to the formation of a truncated protein of 121 amino acids. A summary of the characteristics of the reported non-sense *SOD1* mutations causing the introduction of a premature stop codon is listed in Table 1. Both familial and sporadic cases have been found with a wide variability of age of onset and disease duration. The present case is the first one with bulbar onset.

RT-PCR analysis of *SOD1* mRNA and WI analysis of the patient's PBMC demonstrated that mRNA transcribed from the allele carrying the frameshift mutation is expressed at levels similar to those of the wild type allele, but noticeably the truncated protein is undetectable in the cell lysate, also when searched in the insoluble fraction and after proteasome inhibition. Accordingly, the dismutation activity in erythrocytes is halved.

Our results are in agreement with earlier reports. Watanabe and collaborators (Watanabe et al., 1997) described a Japanese pedigree with a two base pair deletion in the 126th codon in exon 5 of *SOD1*, leading to a frameshift and a premature stop in codon 130. The presence of mRNA from the

allele carrying the mutation was found in fibroblasts from one of the patients, although the mutant protein was not found either in erythrocytes or in brain tissue of the individuals carrying the mutation. Furthermore, this mutation caused reduced dismutation activity in erythrocytes of the mutation carriers. Zu and collaborators (Zu et al., 1997) reported two novel *SOD1* mutations associated with fALS: a p.Leu126X mutation, causing the termination of the protein at residue 125, and an intronic mutation upstream of the intron 4 – exon 5 junction, leading to the truncation of 35 amino acids from the C-terminus. For both mutations the truncated protein was not found in patient's erythrocytes and lymphoblastoid cells. The presence of *SOD1* mRNA was demonstrated in patient's lymphoblastoid cells only for the intronic mutation. Halved superoxide dismutation activity in erythrocytes has been reported also in four members of a Danish family heterozygous for the p.Gly127insTGGG mutation. This mutation introduces a neopeptide GGQRWK-sequence followed by a premature stop codon in position 133 (Andersen et al., 1997). Using an antibody raised against the novel epitope, minute (<0.5% of SOD1 content in controls) amount of the mutant protein could be detected in spinal cord of an autopsied patient (Jonsson et al., 2004).

The question arises whether the p.Ser108LeufsTer15 mutation is causative. The patient had no family history of ALS or FTD, but reduced disease penetrance has been reported in *SOD1*-related ALS, also in association with a non-sense mutation (Andersen et al., 1997). Another possibility is that the p.Ser108LeufsTer15 is a *de novo* mutation which has been reported previously in ALS (Alexander et al., 2002). How these mutations scattered all over the SOD1 molecule can result in a characteristic pattern of neurodegeneration has until now remained enigmatic.

The absence of the product of the allele carrying the p.Ser108LeufsTer15 may be due to the high instability of the encoded protein. Human SOD1 is an extraordinarily stable protein, but many mutant forms exhibit decreased stability (Valentine Annu Rev Biochem 2005). Figure 4 shows the comparison between the wtSOD1 protein and the mutant protein predicted to be encoded by the allele carrying the mutation p.Ser108LeufsTer15. In the mutant protein, the loss of Cys146 prevents the formation of the intrasubunit disulfide bond that is critical for stabilizing the overall structure of

wtSOD1. Furthermore, the p.Ser108LeufsTer15 mutation results in the loss of His120 essential for binding the catalytic copper, the absence of Asp124 precluding the formation of the hydrogen bond important for binding the stabilizing zinc, and finally the loss of the hydrophobic residues at the very carboxy-terminal end preventing the formation of a stable dimer interface (Valentine Annu Rev Biochem 2005)+Lindberg et al. 2005.

SOD1 protein catalyzes the conversion of the highly toxic O_2^- anion to the less toxic H_2O_2 and to O_2. Being only the product of the wild type allele present in the proband, aloss of function may cause insufficient degradation of Reactive Oxygen Species (ROS), leading to cell damage. Particularly, ROS are known to hamper axonal transport (Kaur, S. J., McKeown, S. R. & Rashid, S. Mutant SOD1 mediated pathogenesis of Amyotrophic Lateral Sclerosis. *Gene* **577,** 109–118 (2016)). In a study of mouse models of ALS, it was found an accumulation of free radicals in the mitochondrial intermembrane space, in association with the lowest SOD1 levels (De Vos, K. J. *et al.* Familial amyotrophic lateral sclerosis-linked SOD1 mutants perturb fast axonal transport to reduce axonal mitochondria content. *Hum. Mol. Genet.* **16,** 2720–2728 (2007)). It has been hypothesized that such mitochondrial damage harms distal axons of motor neurons (Kaur, S. J., McKeown, S. R. & Rashid, S. Mutant SOD1 mediated pathogenesis of Amyotrophic Lateral Sclerosis. *Gene* **577,** 109–118 (2016)). Oxidative stress may also lead to misfolding and consequent aggregation of cellular proteins (Bozzo, F., Mirra, A. & Carrì, M. T. Oxidative stress and mitochondrial damage in the pathogenesis of ALS: New perspectives. *Neurosci. Lett.* **636,** 3–8 (2017)). Besides, an increasing body of literature supports the relationship between chronic oxidative stress and RNA dysmetabolism through the formation of stress granules sequestrating RNA-binding proteins (Bozzo, F., Mirra, A. & Carrì, M. T. Oxidative stress and mitochondrial damage in the pathogenesis of ALS: New perspectives. *Neurosci. Lett.* **636,** 3–8 (2017)).

As above said, in a subject carrying the mutation p.Gly127insTGGG, which is rather similar to the p.Ser108LeufsTer15, small amounts of the mutant protein were found in the nervous tissue using an antibody specific for the neopeptide sequence introduced by the mutation (Jonsson et al., 2004). It

cannot be excluded that the protein derived from the p.Ser108LeufsTer15 mutation has a conformation very different from the wtSOD1 used to immunize the rabbits/goats (the animals were immunized with normally folded wtSOD1 and not misfolded SOD1). Therefore, small amounts of the mutant protein might be actually present, at least in the nervous tissue, in the proband. The p.Ser108LeufsTer15 mutant can theoretically result in a neopeptide sequence 106Leu-Leu-Arg-Arg-Pro-Leu-His-His-Trp-Pro-His-Thr-Gly-Gly-Pro-stop, that is heavily positive-charged compared to wtSOD1. This sequence will reduce the overall net negative charge of SOD1 to almost zero (0) whereas the wtSOD1 monomer has a net repulsive charge of about -6 under physiological conditions. The mutant protein should accordingly have less repulsive forces resulting in a greatly increased propensity to aggregate compared to wtSOD1. Furthermore, the reduction of repulsive charge of the p.Ser108LeufsTer15 mutant is in line with the finding that a reduction of repulsive charge overall is higher in SOD1-associated ALS than in other inherited protein disorders (Sandelin et al., 2007). Finally, a number of studies now suggest that minute amounts of mutant SOD1 misfold thereby forming cytoplasmic prion-like species with the ability for templated cell to cell propagation throughout the motor system (Ayers et al., 2016; Bidhendi et al., 2016). The discovery of the p.Ser108LeufsTer15 mutant might help in elucidating which sequence of the SOD1 molecule is essential for prion-like propagation.

The data collected in this study suggest that the p.Ser108LeufsTer15 is causative. Two main pathophysiological hypotheses arise about the pathogenic role of the p.Ser108LeufsTer15 mutation. On one hand, the low levels of SOD1, probably due to the instability of the product of the mutant allele, could make motor neurons more vulnerable to oxidative stress and other related pathomechanisms On the other hand, the mutant protein, even if present in minimal amounts, may trigger toxic mechanisms in the nervous tissue as a consequence of its propensity to aggregate.

Conflicts of interests

Drs. Canosa, De Marco, Lomartire, Rinaudo, Di Cunto, Turco, Barberis, Brunetti, Casale, Moglia, Calvo, and Marklund report no conflicts of interest.

Dr. Peter M. Andersen receives honoraria from Biogen Idec and Neuroimmune for advisory board tasks and counselling on clinical trials. He receives numerous research grants from the Swedish Science Council and private foundations and patient organizations in Sweden and Norway.

Dr. Gabriele Mora reports no conflicts of interest.

Dr. Chiò serves on the editorial advisory board of Amyotrophic Lateral Sclerosis and has received research support from the Italian Ministry of Health (Ricerca Finalizzata), Regione Piemonte (Ricerca Finalizzata), University of Turin, Fondazione Vialli e Mauro onlus, and the European Commission (Health Seventh Framework Programme); he serves on scientific advisory boards for Biogen Idec and Italfarmaco.

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Reference number	Mutation	Predicted protein length (amino acids)	fALS/sALS	Origin	Age at onset (ys)	Onset (S/B)	Sex (F/M)	Cognitive impairment	Other features	Survival
(Nakashima et al., 1995; Pramatarova et al., 1994; Watanabe et al., 2001, 2000, 1997)	p.Leu126delTT	130	fALS (7 subjects)	Japan	22-54 (mean 42)	S (5 patients) Unknown (2 patients)	3 F 4 M	NA	Predominant LMN signs; extraocular eye movement disturbance and urinary disturbance in advanced stages	1.5-11 ys (mean 2)
(Hosler et al., 1996)	p.Glu133del	152	sALS (1 subject)	NA	42	S	NA	NA	NA	>2 ys
(Jackson et al., 1997)	p.Val118 insAAAAC	121	sALS (1 subject)	NA	36.6	NA	NA	NA	NA	16 ms
(Andersen et al., 1997)	p.Gly127 insTGGG	132	fALS (3 subjects)	Denmark	41-59 (mean 50)	S	2F 1M	NA	Decreased sense of vibration in the left hand and foot in one case; myalgia in two cases; four healthy carriers of the mutation in the family (aged 45-67 ys)	30-41 ms (mean 34)
(Zu et al., 1997)	p.Leu126X	125	fALS (1 subject)	NA	58	S	M	NA	NA	4 ys
(Zu et al., 1997)	A-G-intron4-11bp	123	fALS (1 subject)	NA	72	S	M	NA	NA	NA
(Orrell et al., 1997)	p.Glu132insTT	132	fALS (2 subjects)	United Kingdom	40-50	NA	NA	NA	NA	< 2 ys
(Andersen et al., 2003)	p.Gly141X	140	fALS (1 subject)	NA	34	S	M	NA	Lower back pain at the onset	5 ys
(Corrado et al., 2006)	p.Lys136X	135	sALS (1 subject)	Italy	45	S	M	NA	NA	NA
(Hu et al., 2012)	InsAexon2	35	sALS	China	19-43	NA	11 F	NA	Predominant LMN signs; relatively extended life expectancy	12.2 ± 9.8 ys

			(15 subjects)		(mean 30.3)		4 M			

Table 1. Review of published papers reporting *SOD1* mutations causing the onset of a premature stop codon with clinical characteristics of carriers. The mutations have been indicated according to the traditional nomenclature in the table and in the text, as they were reported in the published papers. Abbreviations. Onset S (spinal), B (bulbar); Sex F (female), M (male); LMN (lower motor neuron); ys (years); ms (months); NA (not available).

Figure legends

Figure 1. Western immunoblot analysis of SOD1 protein in peripheral blood mononuclear cells (PBMC). (A) SOD1 protein levels were evaluated in PBMC from three neurologically unaffected subjects (ctrl #1, ctrl #2, ctrl #3), the proband and three patients with sporadic ALS (ALS #1, ALS #2, ALS #3), one of which carried a *SOD1* missense mutation (SOD1 D109Y) using a polyclonal antibody against full-length SOD1, an antibody against the N-terminal region of the protein as well as an antibody raised against an epitope between amino acids 54 and 104 of human SOD1. β-actin was used as a measure of equal protein loading. (B) The bar graph illustrates the mean values of SOD1expression , in the three groups considered (ctrl, proband, ALS) revealed with the three antibodies used. SOD1 levels are expressed as a percentage respect to the mean value of the ctrl group, which was arbitrarily set at a value of 100. Error bars: standard deviation; **: $p<0.01$.

Figure 2. Detection of the p.Ser108LeufsTer15 SOD1 in the insoluble fraction of proband's PBMC as well as in proband's PBMC subjected to proteasome inhibition. (A) Western immunoblot analysis of SOD1 protein in the detergent-soluble and insoluble fractions from PBMC of the proband and of a sALS patient without SOD1 mutations. (B) Western immunoblot analysis of SOD1 protein in PBMC of the proband and of a sALS patient without SOD1 mutations untreated and treated with the proteasome inhibitor MG132.
SOD1 protein levels were evaluated using a polyclonal antibody against full-length SOD1 as well as an antibody against the N-terminal region of the protein. β-actin was used as a measure of equal protein loading. Ubiquitin antibody was used to ascertain the efficacy of treatment with MG132.
*: aspecific band.

Figure 3. RT-PCR analysis of *SOD1* mRNA in the index case. (A) Results of end-point PCR performed in duplicate from patient RNA (lanes p.Ser108LeufsTer15, samples 1 and 2) and from

RNA of the indicated controls. (B) Results of Sanger-based sequence analysis performed on the proband's and controls' cDNA. The point of mutation in the proband's electropherogram is indicated by an arrow. An asterisk indicates the point of mutation in the electropherogram of the p.Asp109Tyr patient.

Figure 4. Comparison between wild type SOD1 and the protein encoded by the allele carrying the frameshift mutation. The figure reports the amino acid sequence of wild SOD1 and p.Ser108LeufsTer15 SOD1 as well as α-helix and β-sheet structures, the sites involved in binding copper and zinc ions and the sites responsible for intramolecular disulfide bond (SH). In comparison to wild type SOD1, the mutant protein lacks the amino acid sequence 122-154. The amino acid sequence 108-121 of the mutated protein differs respect to that of wild type SOD1.