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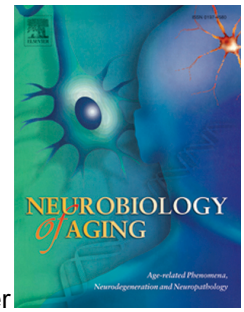

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Genetic analysis of the *SOD1* and *C9ORF72* genes in Hungarian patients with amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the death of motor neurons. To date, more than 20 genes have been implicated in ALS, and of these, the two most frequently mutated are the *superoxide dismutase 1 (SOD1)* gene and the *chromosome 9 open reading frame 72 (C9ORF72)* gene. In this study, we aimed to investigate the contribution of these two Mendelian genes to the development of the disease in Hungarian ALS patients (n=66). Direct sequencing of the *SOD1* gene revealed a novel (p.Lys91ArgfsTer8) and three recurrent heterozygous mutations (p.Val14Met, p.Asp90Ala, p.Leu144Phe) in five patients. The novel p.Lys91ArgfsTer8 mutation led to a frameshift causing the addition of eight new amino acids, including a premature stop codon at position 99. The GGGGCC hexanucleotide repeat expansion of the *C9ORF72* gene was present in one ALS patient. This study represents the first genetic analysis of two major ALS causative genes in a cohort of Hungarian ALS patients and contributes to the further understanding of the genetic and phenotypic diversity of ALS.

Key words: ALS, *SOD1, C9ORF72*, mutation screening, repeat expansion

1. Introduction

Amyotrophic lateral sclerosis (ALS; ORPHA803), also known as ”Lou Gehrig’s disease”, is a fatal, neurodegenerative disorder characterized by the death of motor neurons in the brain, brainstem and spinal cord, resulting in fatal paralysis (Morrison and Harding, 1994). Familial forms account for about 10% of ALS cases, while other cases are sporadic (Strong *et al.,* 1991; Hewitt *et al.,* 2010). Familial forms are mainly transmitted in a Mendelian pattern of autosomal dominant inheritance (Hardiman *et al.,* 2011). Regarding its genetic background, more than 20 genes have been implicated in the development of ALS (ALSoD Database, http://alsod.iop.kcl.ac.uk).

Among the ALS causative genes, *superoxide dismutase 1 (SOD1)* is one of the most commonly mutated genes and accounts for approximately 12–23% of the familial and up to 7% of the sporadic ALS forms (Andersen, 2006). *SOD1* gene encodes the Cu/Zn superoxide dismutase enzyme, which catalyzes the inactivation of superoxide into oxygen and hydrogen peroxide, providing antioxidant defense (Smirnoff 1993). To date, more than 170 mutations have been reported for *SOD1* in the Amyotrophic Lateral Sclerosis Online Genetics Database (ALSoD Database; Abel *et al.,* 2012) since the gene was firstly associated to ALS in 1993 (Rosen *et al.,* 1993). *SOD1* mutations occur in all the five exons of the gene.

Another frequently mutated ALS gene is *chromosome 9 open reading frame 72 (C9ORF72)*, which – in addition to the *SOD1* mutations – is now recognized as the main cause of familial and sporadic ALS (Majounie *et al*., 2012; Gijselinck *et al*., 2012; Ratti *et al*., 2012; Smith *et al*., 2013). A hexanucleotide (GGGGCC) repeat expansion (RE) located in the non-coding region of the gene that can reach up to 4400 units (normal range: 2-23 units) has been identified in patients with ALS and/or frontotemporal dementia. The GGGGCC RE contributes to 23–47% of familial ALS and to 4–5% of sporadic cases (Renton *et al.,* 2011; DeJesus-Hernandez *et al*., 2011; Byrne *et al*., 2012; Ratti *et al*., 2012), with a frequency depending on geographical origin. Although the pathomechanism with which the hexanucleotide RE leads to the development of ALS has not been elucidated completely, both C9orf72 haploinsufficiency gain of function mechanisms (driven by toxicity of sense and antisense RNA transcripts and derived dipeptide repeat proteins) have been reported (Taylor *et al.*, 2016).

In this study, we have investigated the contributions of the two most commonly mutated ALS genes, *SOD1* and *C9ORF72*, to the pathogenesis of the disease in Hungarian patients (n=66). This study represents the first genetic screening of ALS in Hungary, which adds novel data to the genetic and phenotypic diversity of this disease.

2. Patients and methods

2.1 Investigated individuals

The unrelated patients (n=66) included in this study were recruited from the Department of Neurology, University of Szeged, Szeged, Hungary, between 2010 and 2016. All patients fulfilled the El Escorial criteria for ALS (Brooks *et al*., 2000). Of the 66 cases, only one patient reported other affected family members; the other 65 cases were considered sporadic. All patients were of Hungarian ancestry. The study was approved by the Internal Ethical Review Board of the University of Szeged. Written informed consent was obtained from all patients, and the study was conducted according to the Principles of the Declaration of Helsinki.

2.2 Genetic analyses

Blood samples were collected from all the enrolled individuals (n=66), and genomic DNA was isolated using a BioRobot EZ1 DSP Workstation (QIAGEN; Godollo, Hungary). The entire coding region of the *SOD1* gene and the flanking introns were amplified (primer sequences used were taken from the UCSC Genome Browser www.genome.ucsc.edu). Direct sequencing of the PCR products was performed on an ABI 3100 sequencer and compared with the wild-type gene sequences at the Ensemble Genome Browser (http://ensemble.org). To identify known variations, we used ALS Online Genetics Database (http://alsod.iop.kcl.ac.uk/) (Abel *et al*., 2012), 1000 Genomes Database (www.1000genomes.org/), dbSNP (http://www.ncbi.nlm.nih.gov/project/SNP) and Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org). To predict the functional effects of novel mutations, the sequence variations were assessed by *in silico* prediction programs, such as SIFT (http://sift.bii.a-star.edu.sg/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2) and Mutation Taster (http://mutationtaster.org). To examine possible effects of the mutations on the three-dimensional (3-D) structure of the SOD1 protein, we used the Swiss-Model protein structure homology-modeling server (http://swissmodel.expasy.org/; SOD1 Protein Data Bank accession number 4b3e.1.A).

A two-step protocol was followed for the detection of the GGGGCC hexanucleotide RE in the *C9ORF72* gene. Fragment length analysis was performed using GeneMapper ID v3.2.1., and the samples producing a single peak product were further analyzed in the second step by repeat-primed PCR using an 310 ABI Prism Genetic Analyzer (Applied Biosystems). The peaks were visualized using GeneMapper ID v3.2.1.

software (Akimoto *et al.*, 2014). The presence of the GGGGCC RE was observed as a saw-tooth pattern with 6-base pair periodicity.

To determine whether the single individual carrying the GGGGCC RE identified in this study also carried the "risk" haplotype, we selected the rs3849942 variant to be used as a marker for the "risk" haplotype for the patient and control genotypes. Rs3849942 genotyping was based on allelic discrimination assays using TaqMan chemistry following the manufacturer's instructions (Life Technologies; Budapest, Hungary).

3. Results

The direct DNA sequencing approach identified four different mutations of the *SOD1* gene in 5 ALS patients: three known heterozygous missense mutations (c.43G>A p.Val14Met; c.272A>C p.Asp90Ala; c.435G>C p.Leu144Phe) and one novel mutation (c.275_276delAA, p.Lys91ArgfsTer8) (Table 1).

The detected novel heterozygous mutation (c.275_276delAA, p.Lys91ArgfsTer8) is located in the fourth exon of the *SOD1* gene (Figure 1a) and led to a frameshift with the insertion of 8 novel amino acids and the formation of premature stop codon at the new amino acid position 99. Analysis using Mutation Taster software predicted that the p.Lys91ArgfsTer8 mutation causes severe truncation of the encoded enzyme, and, thus, we hypothesize that this mutation is likely to be pathogenic (Figure 1b). The pathogenic role of the p.Lys91ArgfsTer8 mutation is further supported by the fact that it interferes with the integrity of the Cys57-Cys146 disulfide bond, and results in the weakening of the dimer interface.

The novel p.Lys91ArgfsTer8 *SOD1* mutation was not present in 110 healthy controls of Hungarian ancestry that we investigated, neither it is represented in mutation databases, including Single Nucleotide Polymorphism Database (http://www.ncbi.nlm.nih.gov/projects/SNP/), 1000 Genomes (www.1000genomes.org/), Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org) and the ALS Online Genetics Database (http://alsod.iop.kcl.ac.uk/) (Abel et al., 2012).

This novel mutation was carried by a female Hungarian patient suffering from sporadic ALS with the latest onset but the fastest progression of the disease (Table 1). She had been cured from a breast cancer eight years before the onset of ALS and proved to be tumor free on following checkups. As first symptoms, the lower extremities became clumsy and weak because of spasticity and muscle atrophy. The calculated ALS Functional Rating Scale R (ALSFRS-R score; Cedarbaum *et al.*, 1999) was 39/48 at the first examination, which was carried out four months after the appearance of the first symptoms. Then,

the signs of upper and lower motor neuron damage developed fast together with bulbar and pseudobulbar symptoms. She was given 2x50 mg riluzole/day, nevertheless, her status deteriorated quickly, and she became tetraplegic with dysarthria and dysphagia. Respiratory failure developed due to the weakened respiratory muscles and she died at home.

The SOD1 p.Leu144Phe mutation is located in the fifth exon of the gene and was identified in two female ALS patients. One of the patients reported that her maternal grandfather had suffered from non-progressive paralysis and weakness for 20 years. The other patient reported that her paternal grandmother had suffered from a disease similar to her own. In this study, only the latter patient was therefore considered as having a familial form of the disease. She was the youngest one at the disease onset.

The p.Val14Met mutation is located in the first exon of the *SOD1* gene and was present in an affected female patient who reported no family history of ALS. Her first symptoms appeared at the age of 62. This patient showed lower and upper motor neuron signs. The disease course was progressive and led to the patient's death within one year after disease onset.

The p.Asp90Ala mutation, which is the most prevalent *SOD1* mutation in Europe (Andersen *et al.*, 1995; Al-Chalabi *et al*., 1998; Andersen 2001), is located in the fourth exon of the *SOD1* gene and was present in a female patient. The patient also carried the rs111273304 splice-donor variant (c.239+2T>A), which is of unknown significance, in heterozygous form. This patient had clinical features typically associated with this genotype, including a relatively long survival after onset (Andersen 2006).

Analysis of the *C9orf72* gene identified GGGGCC RE in one out of 66 ALS patients. The average repeat number based on fragment-length analysis was 5 (range 2–17 repeats) in the remaining 65 patients, none of whom carried repeat expansion. The patient with the repeat expansion also carried the rs3849942 risk allele, which was previously described as a part of the Finnish "risk" haplotype (Laaksovirta *et al.,* 2010).

This latter patient (Table 1) reported the first symptoms of ALS six months before she presented to the neurological unit at age 65 with gradually increasing foot drop on the left side due to peroneal weakness. The ALSFRS-R was 44/48. Three years before admission, an adenoma of one of the parathyroid glands causing hyperparathyroidism was surgically removed but on admission the level of the parathyroid hormone in her serum was within normal ranges. A debate whether hyperparathyroidism can mimic ALS has been ongoing for the last 50 years (Jackson *et al*., 1998).

4. Discussion

In this study, we analyzed *SOD1* and *C9ORF72* genes in a cohort of 66 Hungarian ALS patients, including one single case with a reported familial history for the disease. We identified a novel and likely disease-causing heterozygous frameshift mutation (p.Lys91ArgfsTer8) in the *SOD1* gene. Three other heterozygous recurrent *SOD1* missense mutations (p.Val14Met, p.Asp90Ala and p.Leu144Phe) were also detected. *SOD1* mutations were detected in 7.5% (5/66) of our cohort, in line with literature data reporting that *SOD1* mutations account for approximately 0–7% patients with sporadic disease (Andersen, 2006).

The identified novel p.Lys91ArgfsTer8 *SOD1* mutation is associated with a typical ALS phenotype characterized by lower and upper motor neuron signs. The disease had a late onset at the age of 67 years and progressive course leading to the death of the patient within one year after the onset of the signs and symptoms of the disease. The two-base-pair deletion of the p.Lys91ArgfsTer8 *SOD1* mutation leads to a frameshift with the formation of a premature stop codon after the insertion of eight novel amino acids, causing a severe truncation of the protein (Figure 1b). This truncation abolishes the integrity of the intrachain C57–C146 disulfide bridge. Although most mutations in the *SOD1* gene are missense ones, a few deletions and insertions have been described previously causing truncations of different sizes and in most cases affecting also the C57-C146 disulfide bridge (http://alsod.iop.kcl.ac.uk/; Abel *et al*., 2012). The most comprehensively studied truncation mutant is the Gly127insTGGG (G127X), carriers of which developed signs of motor neuron degeneration with a rapid disease course (Jonsson *et al*., 2004). These observations correlate well with the clinical manifestations and course of ALS in the investigated Hungarian patient with the truncating p.Lys91ArgfsTer8 *SOD1* mutation. SOD1 protein with truncating mutations exhibits structural instability causing misfolding in the mutated enzyme (Jonsson *et al*., 2004) which can consequently aggregate in motor neurons and lead to the development of ALS (Forsberg *et al*., 2011).

With the exception of the p.Asp90Ala *SOD1* mutation, the detected recurrent mutations are all associated with typical ALS phenotypes, characterized by lower and upper motor neuron signs, late onset and progressive disease course. In the case of the patient with the p.Asp90Ala heterozygous mutation, lower limb involvement and relatively long duration of the disease course was detected similarly to cases reported previously (Robberecht *et al.,* 1996; Andersen *et al.,* 2006). The p.Asp90Ala mutation is the most common *SOD1* mutation in Europe (Andersen 2001), and it can be inherited in either a dominant or recessive manner (Robberecht *et al.,* 1996).

The p.Leu144Phe missense mutation, which is the most prevalent mutation in the Balkan region (ALSoD), was detected in a familial and in a sporadic case. The clinical symptoms and the course of the disease were similar in these two patients. To note, the patient with a positive family history developed ALS symptoms at a relatively early age, whereas onset for the other patient was late. Although a significant number of patients with this mutation have lower limb onset (Corcia *et al.,* 2011), both of our patients developed upper and lower motor neuron signs. The rare p.Val14Met mutation was detected in an apparently sporadic case in a female patient with upper and lower motor neuron signs. None of the *SOD1* mutations detected in these ALS patients was identified in 110 healthy Hungarian controls.

C9orf72 repeat expansion was detected only in one patient of 66. The patient carrying the RE variant also carried the previously described rs3849942 risk allele (Laaksovirta *et al.,* 2010; DeJesus-Hernandez *et al.,* 2011) in heterozygous form. According to earlier findings, the "A" allele of SNP rs3849942 was significantly associated to the expanded *C9ORF72* allele (DeJesus-Hernandez *et al.,* 2011). We screened 110 controls of Hungarian origin to establish the allele frequencies in this genomic position. The allele frequency of the minor allele in the Hungarian population (MAF (A) = 0.18) correlates well with the data from 1000 Genomes Database (www.1000genomes.org/). We observed that the average repeat number was 5 (range: 2–17 repeats) in the remaining 65 patients who did not carry repeat expansions.

In conclusion, we performed the first genetic analysis of *SOD1* and *C9ORF72* genes in a cohort of Hungarian ALS patients. Our study further widens the geographic range for the origin of disease-causing heterozygous missense and frameshift mutations of the *SOD1* gene, which have already been implicated in ALS patients from different countries of origin. In this study, we identified the hexanucleotide RE of the *C9ORF72* gene only in one sporadic patient. These results suggest that the frequency of RE observed in the Hungarian ALS patients (1,5%) is significantly lower than in Western European populations (Majounie *et al.,* 2012; Ratti *et al.,* 2012; Fogh *et al.,* 2014), further demonstrating that the frequency of genetic factors for ALS varies among different geographic regions.

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Conflict of interest

None to declare.

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Figure legends

Figure 1. A novel p.Lys91ArgfsTer8 mutation in the Cu-Zn superoxide dismutase 1 (*SOD1*) gene identified in a Hungarian ALS patient

(a) The p.Lys91ArgfsTer8 novel frameshift mutation in the *SOD1* gene was identified by direct sequencing. **(b)** The crystallographic model shows that the novel frameshift mutation causes a severe truncation of the protein.

Table 1. Clinical data of Hungarian ALS patients carrying pathogenic variants in *SOD1* and *C9orf72* genes

Gene	Age of onset/gender	Mutation	Disease signs	Disease duration, years	Other diseases	ALS family history	Reference
SOD1	62/Female	p.Val14Met	LMN, UMN	1	Lumbar disc protrusions, atherosclerosis	No	Deng *et al*. 1995
SOD1	63/Female	p.Asp90Ala	LMN, UMN, B	12	Lumbar disc protrusions	No	Andersen *et al*. 1995
SOD1	67/Female	p.Lys91ArgfsTer8	LMN, UMN, B, PB	1	Breast cancer, hypertension, hypercholesterolemia, spondylosis and lumbar disc protrusions	No	*This study*
SOD1	29/Female	p.Leu144Phe	LMN, UMN	4	None reported	Yes	Deng *et al*. 1993
SOD1	46/Female	p.Leu144Phe	LMN, UMN, B	3	Lumbar disc protrusions	No	Deng *et al*. 1993
C9orf72	65/Female	Repeat Expansion	LMN, UMN	0,5	Hyperparathyroidism, multiple lipomas	No	Renton *et al*., 2011; DeJesus *et al*.,2011

Figure 1 a

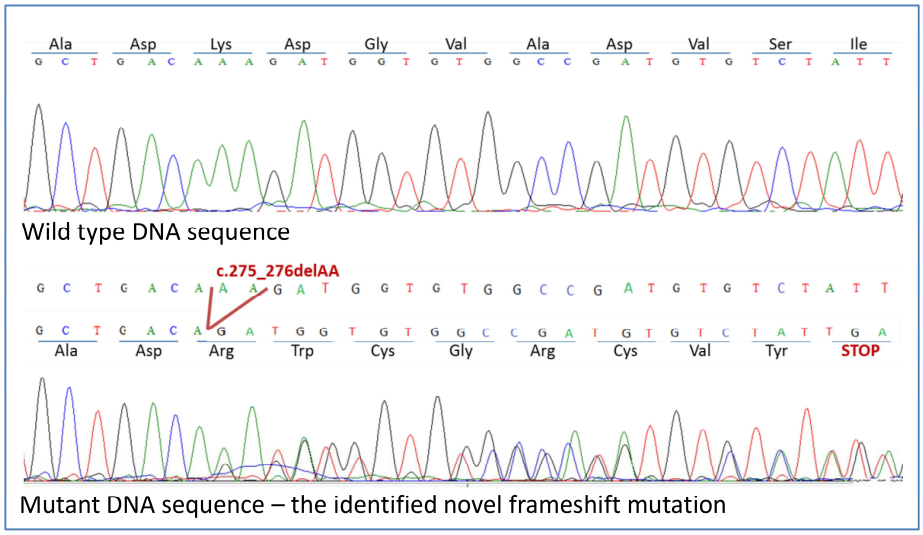
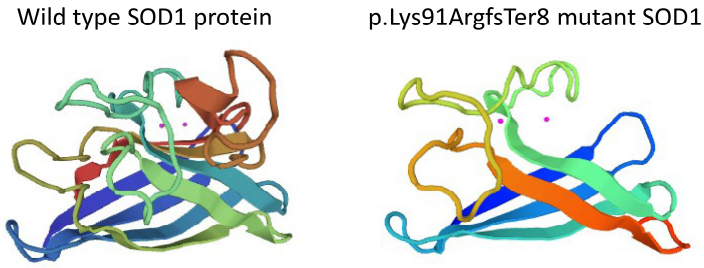


Figure 1 b



Highlights

- A novel (K91RfsX8) and three recurrent mutations (V14M, D90A, L144F) were detected
- The novel K91RfsX8 mutation causes severe truncation of the encoded enzyme
- The repeat expansion of the *C9ORF72* gene was present in one Hungarian patient
- First genetic analysis of two major ALS causative genes in Hungarian patients.