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SOD1 C6W mutation and exon deletion in an amyotrophic lateral sclerosis patient

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Abstract

Background: Despite the genetic heterogeneity in familial ALS (FALS), SOD1 gene mutations are the most frequent cause of FALS, accounting for around 20% of familial cases and sporadic cases. Mutant forms of SOD1 exhibit toxicity that promotes the death of motor neurons. In case of FALS protein aggregates are produced in the motor neurons in patients, which is probably associated to mitochondria.

Methods: In this study, we cloned the SOD1 gene, using reverse transcription Polymerase Chain Reaction (RT-PCR) method, from a 79 years-old man diagnosis as sporadic form of ALS who had shown unusual rapid progression of disease and a matched control individual. The RNA was extracted from the available lymphocytes. pET28a expression system and BL21 chemically competent *Escherichia coli* strain as host were used for protein expression.

Results: DNA sequencing data showed that both heterozygosis C to G transition at nucleotide position 21 leading to a C6W changing at protein level and a deletion at nucleotides position 73 to 169 leading to complete deletion of exon two.

Conclusions: Based on this case study, it appears that SOD-1 mutation and its protein aggregation is associated with the progression of ALS.

Keywords: Sporadic amyotrophic lateral sclerosis (SALS), Familial amyotrophic lateral sclerosis (FALS), Cu/Zn Superoxide Dismutase 1 (SOD1), Exon deletion and Point mutation

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disorder characterized by the death of motor neurons in the cerebral cortex, brainstem, and spinal cord. ALS occurs in both familial and sporadic forms (Rowland et al., 2001). The majority of cases are sporadic (SALS), whereas Familial ALS (FALS) represents 5-10% of ALS cases, that is usually expressed as an autosomal-dominant trait that is clinically indistinguishable from sporadic cases. In 15-20% of FALS cases and in 2-7% of SALS, the disease is linked to mutations in Cu/Zn superoxide dismutase gene (SOD1) (Rosen et al., 1993, Mulder et al., 1986, Cudkowicz et al., 1997).

The genetics of FALS is complex. At least six major genes, seven minor genes, eight different loci, and an increasing number of susceptibility or modifying genes have been

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identified to be involved in FALS (Kunst, 2004; Simpson et al., 2006, Lomen-Hoerth, 2008; Kasperaviciute et al., 2007; Majoor-Krakauer et al., 2003; Kabashi et al., 2008; Van et al., 2007; Gros-Louis et al., 2006; Schymick et al., 2007; Mitchell et al., 2007; Orrell., 2007; Gamez et al., 2006; Sreedharan et al., 2008; Gwinn et al., 2007; Sleegers et al., 2008; Siddique et al., 2008; Shaw, 2001; Momeni et al., 2006; Valdmanis and Rouleau., 2008; Valdmanis et al., 2007; Pasinelli and Brown, 2006; Przedborski et al., 2003; Dunckley et al.,2007). The FALS linked to the SOD1 gene is designated as ALS1 (Mendelian Inheritance in Man (MIM): 105400). There is a clear allelic heterogeneity in ALS1and at least156 different mutations in SOD1 have been described to date (http://alsod.iop.kcl.ac.uk/misc/dataDownload.aspx#C). Some mutations are associated with a long survival time, while others are linked to a very rapid disease progression.

D90A has been considered as the most common mutation worldwide. However, the most frequent mutation in North America is A4V, which accounts for 50% of North American ALS1 families. Other SOD1 mutations have been described as being restricted to mutations for specific populations (private mutations). Most mutations described in the SOD1 are missense mutations. Seventeen nonsense and non-amino acid altering mutations have also been reported (Syriani et al.,2009, Luigetti et al., 2011, Andersen , 2006, Boukaftane et al., 1998, Andersen et al., 2003,Watanabe et al., 2000).

In addition, protein aggregates and inclusions are a common pathological feature of many neurological disorders such as Huntington, Alzheimer and Parkinson (Taylor et al., 2005). In these neurodegenerative diseases, misfolding, aggregation, and precipitation of proteins appears to be directly related to neurotoxicity. Mutant forms of SOD1 exhibit toxicity that promotes the death of motor neurons. It is now well assessed that, in neural tissues of SOD1-linked FALS patients, visible protein aggregates are present that contain aggregated SOD1 and that these aggregates are essentially associated to mitochondria (Pasinelli et al., 2004, Ohi et al., 2004).

In this study, we report heterozygosis sporadic SOD1 C6W mutation and a heterozygosis deletion at nucleotides position 73 to 169 leading to complete deletion of exon two, in an Iranian patient affected by sporadic ALS that disclosed an unusual rapid progression with death occurring after 11 months from the onset of symptoms. Furthermore, we report that healthy control's human SOD1, when lacking both its metal ions, forms stable, and soluble protein in solutions but its mutant, patient's SOD1 protein, was seen as both soluble and an

aggregated structure in precipitate.

Patient History

A 79 year old Iranian man was admitted with gradually progressive weakness involving left upper extremity, right upper extremity and legs sequentially and in a few months period. On examination, arms and hands were very weak and wasted; legs were mildly affected with normal tone and bulk. Cranial nerves and tongue were normal. Deep tendon reflexes were generally absent. Plantar reflex was downward. Fasciculation were seen in limb and axial muscles. Mental status was normal. Sensory examination was also normal. Sensory nerve studies were normal. Motor conduction velocities were slightly reduced with very low amplitude compound muscle action potentials. Electromyography revealed widespread denervation, fasciculation and re-innervation involving cervical, thoracic and lumbar innervated muscles. Facial and tongue muscles were normal and the diagnosis of motor neuron disease was made (Brooks et al., 2000). There was no significant past medical history and the family history was negative for neurological diseases. Laboratory studies were normal and cervical MRI showed mild C3-5 disc protrusion. The disease course was rapidly progressive and he died 4 months after diagnosis (11 months after onset) with respiratory failure and superimposed infection.

Before collecting blood sample, informed consent was obtained from the patient and the healthy individual.

Materials and Methods

Isolation of Lymphocytes from Peripheral Blood

Five ml of blood obtained from each individual was diluted with Hank′s solution at a ratio of 1:2 within one hour of extraction and slowly layered onto a 15-ml screw-cap tube containing 5 ml Ficolymph (Baharafshan co. Tehran, Iran). The tubes were centrifuged for 20 minutes at 1000 × g, after which the lymphocyte-containing layer was collected into a new centrifuge tube using a sterile pipette. The lymphocyte mix was then diluted in 10 ml Hank′s solution and centrifuged for 10 minutes at 440 × g. The supernatant was discarded, 5 ml Hank′s solution was added, the pellet was mixed gently in this buffer, and the mixture was allowed to sit for about 45 second. The mixture was gently pipette and then centrifuged at 230 × g for 15 minutes. The supernatant was discarded, and the pellet was suspended in RPMI 1640 medium (Baharafshan co. Tehran, Iran) supplemented with L-glutamine (Heidari 2009).

Strains and Culture Conditions

Escherichia coli DH5α (Pasteur Inc. Tehran, Iran) was used for general nucleic acid

operation. *E. coli* BL21 (DE3)-RP (Pasteur Inc. Tehran, Iran) was used as the host strain for high-level protein expression in combination with vector pET28a (Pasteur Ins. Tehran, Iran). Bacterial culture, plasmid DNA purification, cloning and transformation were all according to Sambrook and Russell (Sambrook 2001).

RNA Extraction and RT-PCR

Total RNA was extracted from lymphocytes of both patient and healthy control using RNX RNA extraction kit (Cinnagen Co. Tehran, Iran) and was dissolved in 20 µl DEPC- treated water (the DEPC water was heat inactivated after 12 hours of incubation). The extracted RNA was immediately frozen at -80 ˚C, but 50 µg of each RNA samples were reverse transcribed in a 20 µl reverse transcriptase (RT) master mix using Revert^{TM}AID reverse transcriptase (Fermentase). RNA copied to cDNA using oligo$_{18}$(dT) primer (Fermentase) (Liedtke 1994).

Cloning of SOD1 gene from healthy control and SALS patient

Forward and reverse designed primers were: forward primer, 5′-GATCGGATCCATGGC GACGAAGGCGGTGTG-3′; reverse primer, 5′-AGCTAAGCTTTTATTGGGCGATTCCT ATTACACCACAAG-3′; which had DNA cleavage nucleotide sits for restriction endonucleases *Bam*HI and *Hin*dIII (Fermentase), γ-actin gene primers were used as internal control. Standard PCR amplification was performed with EX Taq (Takara Biotechnology) at 94˚C 5 min (1 cycle); 94˚C 30 s, 57˚C 45s, and 72˚C 1min (30 cycle) and a final extension of 72˚C 10 min, using cDNA of SOD1as template to generate a band clarified on an ethidium bromide (0.001%) 1% agarose gel, which was then removed from the gel and extracted using the Qiagen extraction kit (Qiagen Inc.). Both amplified DNA and vector were cut with restriction endonucleases *Bam*HI and *Hin*dIII and then ligation reaction was performed on 30 µg cut DNA and 90 µg cut vector using T_4DNA Ligase kit (Fermentase). The resultant plasmid then transformed to DH5α bacterial cells and plated on kanamycin (50 µg/ml) treated LB agar plates. Colonies were selected and then grown overnight in kanamycin enriched LB media, purified and sequenced using standard T7 promoter and terminator primers.

Several different colonies were sequenced and after nucleotide sequence confirmation by DNA sequencing, pET28a vectors was transformed to *E. coli BL21* (DE3)-RP for high level protein expression. The recombinant protein has His tag at the end of N terminus which could be purified with Ni-NTA Sepharose (Qiagene, hilden, Germany) column. The cells were grown at 37°C until

the absorbance at 600 nm reached 0.6. Lactose 4 mM and Isopropyl β-Dthiogalactopyranoside (IPTG) were added to the culture at final concentrations of 0.5, 1, and 1.5 mM. The cells were further grown at 22°C and 30°C for 6 h and 16 h on shaking incubator at 250 rpm. Bacteria were harvested by centrifugation (10,000×g, 5 min, 4°C) and stored at −20°C for further use.

Bacterial cell wall was disruption of using sonication for 140s seven bursts of 20s each at 70W on ice. SOD1 protein has been purified with Ni-NTA Sepharose column. Where indicated SDS-PAGE and Urea-PAGE was performed for purification of protein (Jeong et al, 2007).

Results

Sequencing and nucleotide alignment of patient and healthy control SOD1 genes in several different colonies detected as a heterozygosis novel mutation and also a heterozygosis deletion.

SOD1 gene sequencing of the sample from healthy control showed a100% identity with the presented subject SOD1 nucleotide sequence in NCBI (National Center for Biotechnology Information) Gen Bank. However the sequencing data of the patient showed a heterozygosis C to G nucleotide transition at position 21 leading to a C6W sequence change at protein level which caused by converting of TGC codon to TGG. The heterozygosis deletion at nucleotides position 73 to 169 leading to complete deletion of exon two was recorded (Figure 1).

Healthy control SOD1 protein has 154 amino acid residues but in case of the patient sample, following frame shift due to the deletion resulted in a truncated protein with 55 amino acid residues and also a complete form of 154 amino acid residues (Figure 2).

Following protein expression, the supernatant and precipitate from both healthy control and patient were subjected to SDS-PAGE. SOD1 was a homodimer protein with a single disulfide bond linking two monomer together (~32 kDa). However, in case of control sample, due to reduction of disulfide bond by β-Mercaptoethanol (present in protein loading dye) SOD1 was seen as a sharp single band (~13 kDa) as a monodimer in both supernatant and precipitate.

In case of the patient samples two protein bands were seen, one in each colony, a normal band of about 13 kD both in supernatant and precipitate in some colonies and a truncated band of about 6 kDa in the urea-PAGE which was carried out on other colonies (Figures 3,4).

Fig. 1. The nucleotide sequence of superoxide Dismutase-1 gene in the patient. The patient SOD1 gene nucleotide sequence has a C to G nucleotide transition at position 21, and a deletion at nucleotides position 73 to 169 leading to complete deletion of exon two and one nucleotide from exon three.

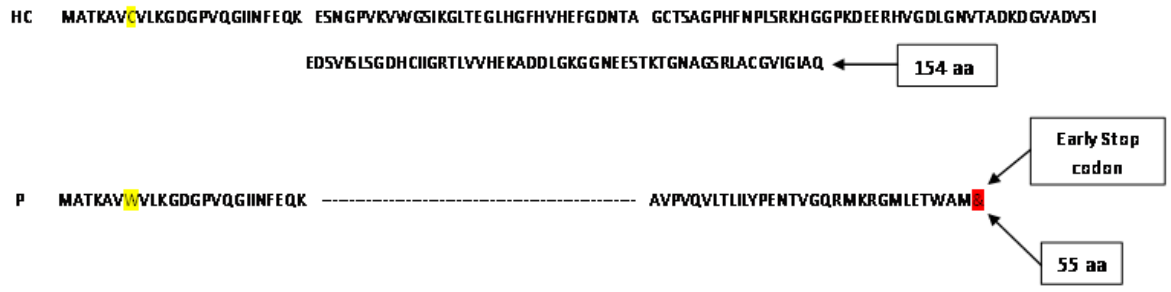

Fig. 2. The nucleotide sequence of superoxide Dismutase-1 gene in healthy control. Healthy control SOD1 protein has 154 amino acid residues but in the patient a truncated protein with 55 amino acid residues (HC: Healthy control, P: Patient).

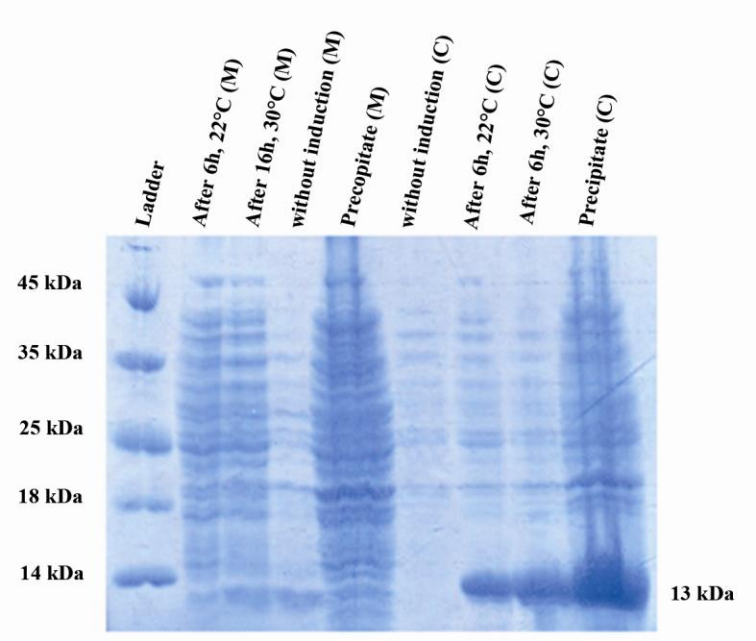

Fig. 3. Purified superoxide Dismutase-1 protein from ALS patient and control sample. Healthy control SOD1 is observed as a sharp single band (~ 13 kDa) as a mondimer in both supernatant and precipitate. No protein band was seen in the patient sample neither in supernatant nor the precipitate (M: Mutant, C: Control).

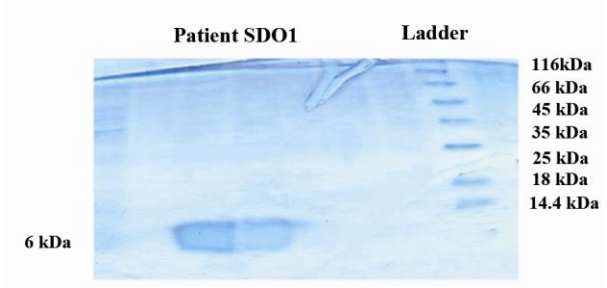

Fig. 4. Purification of Superoxide Dismutase-1 from the patient's sample. Protein was precipitated on Urea-PAGE. The Patient SOD1 is observed as a single band of about 6 kDa.

Discussion

In this report, we describe a 79-year-old man suffering from sporadic ALS with SOD1 heterozygosis C6W mutation and a heterozygosis deletion from nucleotide position 72 to 169 disclosing a rapid progression of the disease. Furthermore, three other similar nucleotide substitutions, which lead to C6G, C6F, and C6S mutations at SOD1 protein level, similar to those reported previously [http://alsod.iop.kcl.ac.uk/misc/dataDownload.aspx#C1]. The C6W mutation and complete exon 2 deletion in the patient with SALS whose disease had unusual rapid progression is reported for the first time in this paper.

Presence of both point mutation and exon

deletion on the same plasmid that was confirmed by repeating the assay, may suggest that both the changes occur on the same allele of SOD1 gene. Furthermore, it seems the occurrence of these two mutations on the same allele together may be responsible for unusual rapid progression of the disease and early death of the patient compared with other ALS patients.

It has been reported that genetic background has a large influence on lifespan in SOD1 mutant mouse model of ALS (Heiman-patterson et al., 2011). Hence the distribution of SOD1 mutations in different ethnic groups, and clarifying the genotype-phenotype correlation in patients with SOD1 associated with a given mutation could be an important issue.

Eukaryotic SOD1 is a 32 kDa homodimeric metalloenzyme, found predominantly in the cytosol, but also in the mitochondrial intermembrane space, nucleus, and peroxisomes which dismutase superoxide radical to dioxygen and hydrogen peroxide. Comparison of SOD-1 from the healthy control and the patient's sample showed that SOD1 protein is in the form of aggregated structure in precipitate. Nevertheless, the protein in normal sample was lacking both its metal ions, forms stable, and soluble protein in solutions but bearing mutations.

A few frameshift and nonsense mutations have also been reported in FALS cases as well as sporadic ALS (Hosler et al., 1996; Pramatarova et al., 1994; Zu et al., 1997; Nakashima et al., 1995; Jackson et al., 1997; Sapp et al., 1995; Andersen et al., 1997; Orrell et al., 1997). These mutation were predominantly present in exon 4, intron 4, and exon 5 (Watanabe et al., 2001). The patient's SOD1 protein was a relatively smaller truncated protein compared to that reported by others having 55 amino acid residues.

The overall data indicate that that aggregation of SOD1 protein is linked to the progression and pathogenesis of SOD1 linked ALS.

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