

Research article

Human CCS gene: genomic organization and exclusion as a candidate for amyotrophic lateral sclerosis (ALS)

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Abstract

Background: Amyotrophic lateral sclerosis (ALS) is a progressive lethal disorder of large motor neurons of the spinal cord and brain. In approximately 20% of the familial and 2% of sporadic cases the disease is due to a defect in the gene encoding the cytosolic antioxidant enzyme Cu, Zn-superoxide dismutase (SOD1). The underlying molecular defect is known only in a very small portion of the remaining cases and therefore involvement of other genes is likely. As SOD1 receives copper, essential for its normal function, by the copper chaperone, CCS (Copper Chaperone for SOD), we considered CCS as a potential candidate gene for ALS.

Results: We have characterized the genomic organization of CCS and determined exon-intron boundaries. The 823 bp coding region of the CCS is organized in 8 exons. We have evaluated involvement of the CCS in ALS by sequencing the entire coding region for mutations in 20 sporadic ALS patients.

Conclusions: No causative mutations for the ALS have been detected in the CCS gene in 20 sporadic ALS patients analyzed, but an intragenic single nucleotide polymorphism has been identified.

Background

Copper serves as a cofactor for a number of oxygen-processing enzymes involved in diverse metabolic processes, but the metal is highly toxic as a free ion. Upon entering the cell the copper is bound to a number of copper chelating transporters (copper chaperones), which guide the copper ion to different cellular locations while protecting the cellular environment from the toxic effect of

copper. Copper chaperones are highly specific for their targets and they participate directly in the loading of the metal into the recipient molecule. One of the copper chaperones is CCS (Copper Chaperone for SOD), which targets copper to Cu, Zn-superoxide dismutase (SOD1) [1].

Superoxide dismutases are the major antioxidant enzymes involved in free radical scavenging. These enzymes exist in three distinct forms, where two of them contain copper (SOD1 and SOD3), and the third one contains manganese (SOD2). SOD1 (Cu, Zn-superoxide dismutase) is located in the cytoplasm and peroxisomes, and the metal is inserted into the enzyme in the cytosol by means of the chaperone CCS after the enzyme has been synthesized [1].

The CCS gene has been localized to 11q13 and three functional domains have been described in the 274 amino acid long protein product [2,3]. Domain I (1–85) is the copper binding site with the consensus MTCXXC motif that is conserved in several other copper binding proteins including HAH1, ATP7A/ATP7B and SOD1. This domain is important in scavenging Cu from the cells and accepting the metal from the transporters. Domain II (86–234) has sequence homology to the SOD1 domain, which is associated with Cu and Zn binding. Domain III (235–274) contains a CXC motif, which is a copper site essential for CCS-SOD1 transmetalation reaction. CCS interacts with SOD1 by two putative metal binding sites at the opposite ends via domains I and III [4]. It has been shown that copper chaperone CCS can interact both with the wild type and the SOD1 mutant in amyotrophic lateral sclerosis (ALS) [5].

ALS is a progressive fatal disorder, which results from the degeneration of motor neurons in the brain and spinal cord. ALS is one of the most common adult-onset neurodegenerative disorders, leading to death within 2–5 years of clinical onset. Approximately 10% of ALS patients are familial cases and approximately 15–20% of these familial cases is associated with mutations in the SOD1 gene [6–8]. Recently, homozygous mutations have been described in the *alsin* gene, located at 2q33, and a new locus

has been proposed in a 8-Mb region on chromosome 18q21 in different families with various forms of FALS [9–12]. In sporadic cases of ALS de novo mutations in the SOD1 gene has only occasionally been observed [13–15]. Apparent heterogeneous distribution of ALS patients with SOD1 gene mutations among different populations suggests that other genetic loci should be involved in the disease.

In brain CCS and SOD1 co-localize within the same cell types [15] and SOD1 can not acquire its Cu in the absence of the CCS [16]. Due to its biological function we have considered CCS as a potential candidate gene for ALS. Herein we report the exon/intron structure of the human CCS and the mutational analysis of the entire coding region of the gene in 20 individuals affected with ALS.

Results and discussion

Genomic organization of CCS

The genomic sequence of the CCS was obtained from the sequence information of unordered fragments of two BAC clones RP11-527H7 (AC019220.3) and CMB9-75A11 (AP000481) which were identified through a BLAST [www.ncbi.nlm.nih.gov:80/blast/] search of the NCBI High-Throughput Genomic Sequences (HTGS) Database using the cDNA sequence of the CCS gene (accession no: AF002210). Correct orientation of the sequence and the final order of the gene elements, coding and non-coding regions were assembled by alignment of the CCS cDNA sequence with genomic sequences. Exon-intron boundaries were established by the identification of the conserved splice site consensus sequences (table 1) and the results were verified by sequencing exon-intron boundaries using genomic DNA and intron specific primers placed within at least 25 bp away from the exons (table 2).

Table 1: Exon intron structure of the CCS gene

Exon	Exon Size (bp)	3' Splice acceptor	5' Splice donor	Intron size (kb)	Nucleotide position	AA position	
1	82		GCACGgtgagggtcgag	0.341	1 – 82	1 – 13	domain I
2	73	ccgcccttcagTTGGA	GGCAGgtaagaacaggg	5.402	83 – 135	14 – 37	
3	138	ctttcttgccagGTGTC	GTTGCgtgagtgaccac	0.205	136 – 273	38 – 83	
4	178	aataccttcagAGAAT	AACAGgtgagttgtctg	0.852	274 – 451	84 – 143	domain II
5	61	tttctttcttagCTGTG	ACCGGgtaagtgtctgt	4.786	452 – 512	144 – 163	
6	77	atctcattccagCACCG	TGAAGgtaaggtggaaa	0.072	513 – 589	164 – 189	
7	102	ctcccctcaagGTGTG	GAGAGgtgagtggtctc	0.109	590 – 691	190 – 224	
8	318	attcttctgcagGTTGG			692 – 1009	225 – 274	domain III

Table 2: Primers and PCR conditions used for analysis of CCS gene

Exon	Forward	Reverse	Product Size (bp)	Annealing Temp (°C)*
1	CGCCGCGCTGGTTGGTG	CGGCGAGGCTCCACACGAAG	138	57
2	CGATCATCGGTTGGT	TAATAGATTCGCCTGGTC	173	51
3	CAGCCTCTGTTGCCCTCT	ACCTTCCTCAAAGCCACTCC	285	57
4	CAAGCCAGCCCAAGTGTCTA	TGAGGGCCCAAGTGTGAAA	271	57
5	CCTGTGAGCTGCTTTCCT	TCTGAGACTTTGGGTCCTCT	159	57
6	ATAGCAGCTTGGCACCAC	GATGATGGACCCTGTTAGGA	184	57
7	TGGGCACCCTTCCTAACAG	ACCATCCGCAGAGCACAG	201	57
8	CACCCCTGACCACACATT	ATAAGGGGCATGGAGACAA	379	57

*PCR conditions: initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C (30 s), annealing (30 s), extension at 72°C (40 s); final extension at 72°C for 7 min.

The 823 bp translated region of the CCS gene was organized in 8 exons and from the translation start site to the translation termination site it spans 12798 bp of genomic DNA (table 1). ATG translation start codon was in the 43 bp exon 1. The smallest exon was the 61 bp exon 5, while the largest exon was the last exon (exon 8) which contained the 150 bp translated sequence, TGA translation termination site, 163 bp 3'-untranslated sequence and a polyadenylation site. The splice donor and acceptor sites in all exons conformed to the AG/GT rule [18,19]. The sizes of the introns were determined from the published sequences of the two BAC clones (Table 1). The smallest intron was the 72-bp intron 6 and the largest was intron 2, which was 5402 bp long. All introns were type 0 (uninterrupted codon).

Database analysis [http://www.friutfly.org/cgi-bin/seq_tools/promoter.pl] predicted two transcription start sites at -300 to -250 bp and -1606 to -1565 bp within 1713 bp of genomic sequence upstream to the translation initiation codon. In order to roughly determine the extent of the 5' untranslated region of the CCS gene, a series of RT-PCRs have been performed using 7 different forward primers residing within a sequence of 1670 bp upstream to the ATG start codon (starting at nucleotide positions -1672, -1442, -871, -603, -302, -214 and -38, respectively) and the same reverse primer (5'-CGTGCAGAGGGTCCC-3', starting from nucleotide position +22 of the CCS gene) downstream to the start codon. In these reactions cDNA synthesized from human heart was used and genomic DNA served as control. These analyses suggested a transcription start site between nucleotides 588 and 302 upstream to the start codon. In the meanwhile, the results also suggested presence of another gene in the opposite direction.

600 bp region upstream to the translation start site of CCS gene was analyzed for presence of transcription factor

binding sites using Genomatics Genomatics MatInspector Professional program [http://www.genomatics.de]. Sites for activator protein, zinc finger interaction domain, E-box binding factor, X-box binding factor, myeloid zinc finger 1 factor, myeloblast determining factor and winged helix protein binding site have been detected within 430 bp in the 5' region of the gene with 100% core similarity and 95% matrix similarity.

Mutation analysis of CCS in ALS patients

The entire protein-coding region of CCS was screened for mutations in 20 ALS patients. In exon 8 a silent mutation (847G>A) was detected in five Danish patients, where one patient was homozygous and four were heterozygous. Further screening of 25 Danish individuals revealed this SNP in 9 chromosomes. The 847G>A transition was also predicted as a candidate SNP by statistical analysis of human expressed sequence tags [20] and reported in the NCBI, Single Nucleotide Polymorphism database (ID 58140, [http://www.ncbi.nlm.nih.gov/SNP/]). The frequency of this polymorphism in the Danish population analyzed in the present study was calculated as 0.19 (15/80 independent chromosomes). Statistical analysis by Fishers test showed no significant difference between this value and the previously predicted frequency (LeeLab SNP database, [http://www.bioinformatics.ucla.edu/snp/]). In the NCBI SNP Database there were two other predicted SNPs within exon 8 of CCS (ID 55000 and 57614), those SNPs were not detected among the 90 chromosomes analyzed in the present study.

Conclusions

Sporadic patients comprise the majority of ALS cases (90%) and the underlying genetic component is known in only a small number of sporadic cases. Codon deletions or insertion in the KSP repeat motif of the neurofilament *NF-H* gene has been identified in ~1% of the sporadic ALS cases [21]. Segregation of DNA markers flanking the CCS

gene was previously investigated in two FALS families but no evidence of linkage was shown in these two families [22]. Very recently the role of CCS in FALS has been studied by ablating the CCS gene in FALS-linked SOD1-mutant mice. Although the absence of CCS led to a significant reduction in the amount of copper loaded mutant-SOD1, it did not modify the onset and progression of the disease in FALS-linked SOD1-mutant mice [23]. In the present study, we could not detect any disease causing mutations in CCS among 20 sporadic ALS patients we have analyzed. The genomic organization of the CCS gene and the intra-genic SNP described in this study can be used in investigating further ALS patients/families.

Materials and methods

20 sporadic cases of ALS (5 Greek and 15 Danish patients) were investigated. The patient cohort was composed of 8 females and 12 males aged from 46 to 84. All the cases met the criteria for ALS with both upper and lower motor neuron involvement. The patients did not have any mutations in the SOD1 gene.

Exons of the CCS gene were PCR amplified using intron specific primers and 20 ng genomic DNA (table 2). PCR was performed in a 15 µl reaction mixture containing 2.5 mM each dNTP, 12 pmol each primer, 0.1 U *Taq* DNA polymerase (Amersham Life Science Inc.), 1 × reaction buffer in a thermal cycler (MJ Research). For PCR conditions see table 2. Amplification products were gel purified and sequencing was performed on both strands with the same amplification primers using Thermo Sequenase Radiolabelled Terminator Cycle Sequencing kit (Amersham Life Science Inc.) in a thermal cycler (MJ research).

The single nucleotide polymorphism (SNP) in exon 8 (847G>A), which destroyed a Fnu4HI recognition site was screened in 25 healthy unrelated Danes using a PCR induced restriction site assay. Following PCR amplification of exon 8 (table 2), the 384 bp-PCR product was digested with Fnu4H (New England Biolabs) producing 302 bp and 82 bp fragments in the 847G allele, and 165 bp, 137 bp and 82 bp fragments in the 847A allele. The fragments separated on a 2% agarose gel were visualized by ethidium bromide staining.

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References

- O'Halloran TV, Culotta VC: **Metallochaperones, an intracellular shuttle service for metal ions.** *J Biol Chem* 2000, **275**:25057-60
- Moore SDP, Chen MM, Cox DW: **Cloning and mapping of murine superoxide dismutase copper chaperone (Ccsd) and mapping of the human ortholog.** *Cytogenet Cell Genet* 2000, **88**:35-37
- Bartnikas TB, Waggoner DJ, Casareno RLB, Gaedigk R, White RA, Gitlin JD: **Chromosomal localization of CCS, the copper chaperone for Cu/Zn superoxide dismutase.** *Mammalian Genome* 2000, **11**:409-411
- Eisses JF, Stasser JP, Ralle M, Kaplan JH, Blackburn NJ: **Domains I and III of the human copper chaperone for superoxide dismutase interact via a cysteine-bridged dicopper (I) cluster.** *Biochemistry* 2000, **39**:7337-7342
- Casareno RLB, Waggoner D, Gitlin JD: **The copper chaperone CCS directly interacts with copper/zinc superoxide dismutase.** *J Biol Chem* 1998, **273**:23625-23628
- Watanabe Y, Kuno N, Kono Y, Nanba E, Ohama E, Nakashima K, Takahashi K: **Absence of the mutant SOD1 in familial amyotrophic lateral sclerosis (FALS) with two base pair deletion in the SOD1 gene.** *Acta Neurol Scand* 1997, **95**:167-72
- Boukafane Y, Khoris J, Moulard B, Salachas F, Meininger V, Malafosse A, Camu W, Rouleau GA: **Identification of six novel SOD1 gene mutations in familial amyotrophic lateral sclerosis.** *Can J Neurol Sci* 1998, **25**(3):192-6
- Gestri D, Cecchi C, Tedde A, Latorraca S, Orlacchio A, Grassi E, Massaro AM, Liguri G, George-Hyslop PH, Sorbi S: **Lack of SOD1 gene mutations and activity alterations in two Italian families with amyotrophic lateral sclerosis.** *Neurosci Lett* 2000, **289**(3):157-60
- Shaw PJ: **Genetic inroads in familial ALS.** *Nat Genet* 2001, **29**:103-104
- Hadano S, Hand CK, Osuga H, Yanagisawa Y, Otomo A, Devon RS, Miyamoto N, Showguchi-Miyata J, Okada Y, Singaraja R, et al: **A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2.** *Nat Genet* 2001, **29**(2):166-173
- Yang Y, Hentati A, Deng HX, Dabbagh O, Sasaki T, Hirano M, Hung WY, Ouahchi K, Yan J, Azim AC, et al: **The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis.** *Nat Genet* 2001, **29**(2):160-5
- Hand CK, Khoris J, Salachas F, Gros-Louis F, Lopes AA, Mayeux-Portas V, Brown Jr RH Jr, Meininger V, Camu W, Rouleau GA: **A novel locus for familial amyotrophic lateral sclerosis, on chromosome 18q.** *Am J Hum Genet* 2002, **70**(1):251-6
- Jones CT, Swingle RJ, Brock DJ: **Identification of a novel SOD1 mutation in an apparently sporadic amyotrophic lateral sclerosis patient and the detection of Ile113Thr in three others.** *Hum Mol Genet.* 1994, **3**(4):649-50
- Jones CT, Swingle RJ, Simpson SA, Brock DJ: **Superoxide dismutase mutations in an unselected cohort of Scottish amyotrophic lateral sclerosis patients.** *J Med Genet* 1995, **32**:290-292
- Gellera C, Castellotti B, Riggio MC, Silani V, Morandi L, Testa D, Casali C, Taroni F, Di Donato S, Zeviani M, et al: **Superoxide dismutase gene mutations in Italian patients with familial and sporadic amyotrophic lateral sclerosis: identification of three novel missense mutations.** *Neuromuscul Disord* 2001, **11**(4):404-10
- Rothstein JD, Dykes-Hoberg M, Corson LB, Becker M, Cleveland DW, Price DL, Culotta VC, Wong PC: **The copper chaperone CCS is abundant in neurons and astrocytes in human and rodent brain.** *J Neurochem* 1999, **72**(1):422-9
- Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV: **Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase.** *Science* 1999, **284**:805-808
- Shapiro MB, Senapathy P: **RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression.** *Nucleic Acids Res* 1987, **15**:7155-7174
- Mount SM: **A catalogue of splice junction sequences.** *Nucleic Acids Res* 1982, **10**:459-472
- Irizarry K, Kustanovich V, Li C, Brown N, Nelson S, Wong W, Lee CJ: **Genome-wide analysis of single-nucleotide polymorphisms in human expressed sequences.** *Nat Genet* 2000, **26**(2):233-236
- Al-Chalabi A., Andersen PM, Nilsson P, Chioza B, Andersson JL, Russ C, Shaw CE, Powell JF, Leigh PN: **Deletions of the heavy neurofilament subunit tail in amyotrophic lateral sclerosis.** *Hum Mol Genet* 1999, **8**(2):157-164
- Orlacchio A, Kawarai T, Massaro AM, St George-Hyslop PH, Sorbi S: **Absence of linkage between familial amyotrophic lateral sclerosis and copper chaperone for the superoxide dis-**

- mutase gene locus in two Italian pedigrees.** *Neurosci Lett* 2000, **285(2):83-86**
23. Subramaniam JR, Lyons WE, Liu J, Bartnikas TB, Rothstein J, Price DL, Cleveland DW, Gitlin JD, Wong PC: **Mutant SOD1 causes motor neuron disease independent of copper chaperone-mediated copper loading.** *Nat Neurosci* 2002, **5(4):301-7**

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