MOLECULAR INVESTIGATION OF AMYTROPHIC LATERAL SCLEROSIS

Aslıhan Özoğuz
March, 2009
OUTLINE

- Introduction:
  - Clinical Features of ALS
  - Genetics of ALS
- Aim
- Materials & Methods
- Results:
  - SOD1 Gene Analysis
  - D90A Haplotype Analysis
- Conclusions & Future Perspectives
Motor Neurons

- Motor neurons send their signals from their cell bodies though the axons to the muscle which results in the movement of that muscle.
CLINICAL FEATURES

- late onset neurodegenerative disease which causes selective death of motor neurons in the motor cortex, brainstem and spinal cord
- Loss of motor neurons → progressive atrophy of skeletal muscles → AMYOTROPHIC
- Progressive → total paralysis
- Survival not affected by age or gender, but rather by site of symptom onset
- Death within 2-5 years as a result of respiratory failure
2-6 per 100 000 people without obvious race-related differences

10% → Familial ALS (FALS)
90% → Sporadic ALS (SALS)

In terms of clinical features, FALS and SALS are almost indistinguishable.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene (Protein/Function)</th>
<th>Heredity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Typical ALS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALS1</td>
<td>SOD1</td>
<td>Dominant</td>
</tr>
<tr>
<td>ALS3</td>
<td>Unknown</td>
<td>Dominant</td>
</tr>
<tr>
<td>ALS6</td>
<td>Unknown</td>
<td>Dominant</td>
</tr>
<tr>
<td>ALS7</td>
<td>Unknown</td>
<td>Dominant</td>
</tr>
<tr>
<td><strong>ALS with dementia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALS-FTD</td>
<td>Unknown</td>
<td>Dominant</td>
</tr>
<tr>
<td>ALS-FTDP</td>
<td>MAPT (Tau)</td>
<td>Dominant</td>
</tr>
<tr>
<td><strong>Typical/Atypical ALS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALS8</td>
<td>VAPB</td>
<td>Dominant</td>
</tr>
<tr>
<td>Progressive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lower m.n. disease</td>
<td>DCTN1</td>
<td>Dominant</td>
</tr>
<tr>
<td><strong>Other Motor Neuron Diseases Sometimes Referred to as ALS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALS2</td>
<td>Alsin</td>
<td>Recessive</td>
</tr>
<tr>
<td>ALS4</td>
<td>SETX</td>
<td>Dominant</td>
</tr>
<tr>
<td>ALS5</td>
<td>Unknown</td>
<td>Recessive</td>
</tr>
</tbody>
</table>
GENETICS OF ALS
SOD1 : the first and major gene

1993

chromosome 21

- Major gene in ALS:
  - It is responsible for 20% of all familial forms (accounts for 2% of all ALS cases).
  - ALS patients with SOD1-mutations present Classical ALS phenotype.
**Superoxide Dismutase 1 (SOD1)**

- Small cytosolic protein, ubiquitously expressed in most cells
- Abundant in neurons (1% of total cytosolic protein)
- 12 kb of DNA, 5 exons and 4 introns; chrom. 21q22.1
- 21 kDa protein, 153 highly conserved aa
Function of SOD1

$O_2^- \xrightarrow{\text{SOD1}} H_2O_2 + O_2$

$\text{Peroxidase/catalase}$

$H_2O + \frac{1}{2} O_2$
SOD1 Gene Mutations

- >125 mutations scattered all over the gene
  - 114: causative; 6: silent mutations; 5: polymorphisms
SOD1 Mutations

- Physical properties of SOD1 (half-life, stability, protein solubility) differ between various SOD1 mutations.

- The dismutase activity of mice carrying a SOD1 mutation → 30-70% of the normal activity

- Random dimerization → unstable → half-life (↓)
  - Normal SOD1 → 30 hours
  - A4V → 7 hours

- Patients of the same family with the same SOD1 mutation can exhibit significant phenotypic differences.
‘Basic mechanism leading to FALS and SALS is not the reduction in dismutase activity’

- Phenotype $\neq$ the extent of residual SOD1 dismutase activity

- Knock-out mice $\rightarrow$ no change in phenotype
  Transgenic mice expressing mutant protein $\rightarrow$ progressive motor neuron loss

- Overexpression of wt SOD1 $\rightarrow$ no phenotypic differences
  Overexpression of mutant SOD1 $\rightarrow$ lethal paralytic state
Mutant SOD1-induced disease is NOT a consequence of a reduction in the dismutase activity, but rather ‘a gain of function’
ALS Pathogenesis

- A. Oxidative Damage
- B. Glutamate-induced Excitotoxicity
- C. Neurofilament Disorganization
- D. Protein Aggregation
- E. Mitochondrial Involvement
Several mechanisms are suggested in the pathogenesis of ALS.
SOD1 Gene Mutations & Transmission

- When there is a mutation in one of the alleles of the SOD1 gene, ALS phenotype is observed.

  - Autosomal Dominant inheritance

- One of the exceptions: D90A
Aspartic acid at position 90 (D90) is a non-sheet element which is a part of a convoluted chain that connects one β-sheet to another.
D90A Mutation

- It is unique among human genetic disorders that the same mutation exhibits both dominant and recessive patterns of inheritance.

- Non-Scandinavian populations: AD inheritance

- Scandinavian: AR inheritance (Andersen, 1996)
  - 2-3% of the Northern Scandinavia: heterozygous D90A mutation carrier - phenotypically normal

- Recessive D90A ALS patients share a highly distinctive, reproducible clinical phenotype, where
  - The initial symptoms is in the lower limbs
  - Progression is very slow with a median age of survival of 11.7 years.

- By contrast, SOD1 heterozygotes from dominant pedigrees (Non-Scandinavian) present a clinical phenotype of typical ALS, associated with other dominant SOD1 mutations and SALS.
Current data suggest that the risk of a D90A carrier in Northern Scandinavia developing ALS is no higher than the risk of normal individuals in the Scandinavian population.

However, the studies regarding the frequency of the D90A in non-Scandinavian countries show that heterozygosity is associated with an increased risk of ALS.

In addition, transgenic mice overexpressing D90A develop motor neuron degeneration, indicating that the D90A allele is not simply a benign variant that is a marker for Scandinavian ancestry.

Another possible explanation for the absence of a phenotype in D90A carriers in Northern Scandinavia is that, for some reason, expression levels of SOD1 are reduced in this group.

This seems unlikely since D90A heterozygotes from recessive and dominant D90A pedigrees show no differences in cerebrospinal fluid or erythrocyte SOD1 protein levels.
It is proposed that in Scandinavian populations, a factor is co-inherited which protects the heterozygotes from the deleterious effects of the mutation.

(Robberecht, 2000)
The initial study (Parton et al., 2002):

- confirmed significant over-representation of the common haplotype in D90A-ALS cases, relative to controls.
- All carriers of the recessive haplotype share a relatively small region across SOD1 that has been spared from recombination.
- Proposal: second mutation reduces the transcription of D90A, such that two copies are required to cause disease in those carrying the recessive haplotype.
- This could explain how D90A homozygotes demonstrate prolonged survival, in contrast to the dose-dependent progression of mutant SOD1 transgenic mice.
A further study (Broom et al., 2006):

- The overlap region between markers Shylock and Goneril contains two known genes (SOD1 and KIAA1172) and two hypothetical genes (LOC150051 and FBXW1BP1).

- The promoters and coding sequences from four tightly linked genes were sequenced.

- 15 sequence variations were identified; however, none of these was positively associated with D90A homozygosity.
AIM OF THE STUDY

- **SOD1:**
  - Since FALS and SALS are clinically almost indistinguishable,
  - ALS patients with SOD1 mutation clinically present a typical ALS,
  - Define the genotype of Turkish ALS patients for the first time in Turkey by SOD1 gene analysis

- **D90A:**
  - Searching/narrowing down the indicated region for a putative protective factor
MATERIALS & METHODS

- 149 SALS cases
- 33 FALS cases
- These samples have been sent from university hospitals all over Turkey.
- 26 Scandinavian ALS cases, known to carry D90A mutation.
- These samples have been sent by Peter Andersen, University of Umea, Sweden.
### A. SOD1 GENE ANALYSIS

Each exon of the SOD1 gene was amplified by PCR.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Annealing (°C)</th>
<th>[MgCl₂]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62.0</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>2</td>
<td>52.2</td>
<td>2 mM</td>
</tr>
<tr>
<td>3</td>
<td>54.5</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>4</td>
<td>59.4</td>
<td>2 mM</td>
</tr>
<tr>
<td>5</td>
<td>57.3</td>
<td>2 mM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TTCCGTTGCACTCTCCGGAACCGGCTCGCAAAACAGGCCT</td>
</tr>
<tr>
<td></td>
<td>TTCAGAAACTCTCTCCAACCTTACGTTAGGGGCTACTCTAGT</td>
</tr>
<tr>
<td></td>
<td>TGGGAACCTTTAATTCATAAATTAGTATAACCATATGAAAACTCCA</td>
</tr>
<tr>
<td></td>
<td>CATCAGCCCTAATCCATCTGACGACTAACAATCAAAGTGA</td>
</tr>
<tr>
<td></td>
<td>AGTGTATTACTTGACAGGCCCATCTTAGA</td>
</tr>
</tbody>
</table>
Each exon of the SOD1 gene was amplified by PCR.

94°C  5 min
94°C  30 sec
Δ°C  30 sec  32 cycles
72°C  30 sec
72°C  8 min.

PCR products were analyzed by DNA Sequencing.
RESULTS: SOD1 Analysis in SALS Patients-1

- AO: 19
- Initial symptom: weakness in left leg
  - followed by weakness in right leg within a month
  - unable to walk at the end of two months
  - fast progressive atrophy in legs and arms
  - ex in less than 1 year
DNA Sequencing revealed CAC→TAC transversion in exon 3 position 71 (His71Tyr).
RESULTS: SOD1 Analysis in SALS Patients-1 H71Y

- Interestingly, the father and the younger brother also carry the same mutation, while the mother and the younger sister have normal alleles.

- The father, (currently being at the age of 36), doesn’t exhibit a disease phenotype yet.

- The disease was initiated at a very early age and progressed very fastly.

- The father is still phenotypically normal, and when these facts are considered, it is proposed that there has been an increase in penetrance.
RESULTS: SOD1 Analysis in SALS Patients

- AO: 28
- Initial symptom: weakness in right shoulder
  - followed by weakness in right arm, right leg and left shoulder within a month
  - very fast progressive atrophy in legs and arms
  - ex within 4 months after diagnosis
RESULTS: SOD1 Analysis in SALS Patients-2 N86S

- DNA Sequencing revealed AAT→AGT transversion in exon 4 position 86 (Asn86Ser) in a homozygous state.
RESULTS: SOD1 Analysis in SALS Patients-2

N86S

- Since the index case carries the mutation in a homozygous state, both of the parents should be carries of the mutant allele.
- This mutation has been associated only with dominant inheritance and heterozygotes develop typical ALS in middle age.
- The mother died of cirrhosis at the age of 28.
- The father is now 54 years old → expected to develop the disease??
- The uncle died of a disease with a clinical phenotype similar to ALS.
Assuming a toxic gain of function, a double dose of mutant SOD1 would be expected to result in a more severe phenotype.

It is a striking observation that all patients with a mutation at codons 84–86, a small domain on the periphery of the molecule, have rapid severe disease progression.

Previous reports have shown that N86S mutations do not affect the enzymatic activity of SOD1.
RESULTS: SOD1 Analysis in SALS Patients

IVS-III-34 (A→C) Intrinsic Transversion

- IVS-III-34 A→C sequence variation was detected in 6 SALS patients.
RESULTS: SOD1 Analysis in SALS Patients
IVS-III-34 (A→C) Intronc Transversion

- One SALS patient carried two changes in the SOD1 gene:
  - IVS-III-34 (A→C) exon1 position 4: GCC→TCC A4S

![Graphs showing the changes in exon and intron regions]
RESULTS: SOD1 Analysis in SALS&FALS Patients
IVS-III-34 (A→C) Intrinsic Transversion

- IVS-III-34 A→C sequence variation was detected in a total of 6 SALS and 1 FALS patients.

- Patients: 3.8 % → (7/182 (149 SALS+33 FALS))
  Controls: 3.4 % → (4/118)

- The frequency of this intrinsic variation is not significantly different in patients and controls:
  POLYMORPHISM (consistent with literature: 4%) (Siddique, 1993).
RESULTS: SOD1 Analysis in FALS Patients-

- AO: 52
- Origin: Balkan region
- First symptoms: weakness in legs, difficulty in walking
- Recently (having the disease for 6 years): walking with cane, can eat on her own, slow progression
RESULTS: SOD1 Analysis in FALS Patients-1

L144F

- DNA Sequencing revealed TTG→TTC transition in exon 5 position 144 (Leu144Phe).
RESULTS: SOD1 Analysis in FALS Patients

- Origin: Milas, Muğla; AO: 49, current age: 54
- Initial symptoms: weakness in right leg, imbalance, some difficulty in swallowing, depression
- Recently (having the disease for 5 years): walking with a cane, more difficulty in swallowing but can still eat soft food
  - slow progression
RESULTS: SOD1 Analysis in FALS Patients-2

- Parents are first cousins (consanguinity)
- Older sister died of ALS
  - AO: 48
  - Duration: short due to bad care-giving
RESULTS: SOD1 Analysis in FALS Patients-2

- DNA Sequencing revealed GAC → GCC transversion in exon 4 position 90 (Asp90Ala) in a homozygous state.
- The mother is heterozygous at the same position; however, at the age of 80 and still phenotypically healthy!!!
Collection of blood samples from other family members

- The father of the index case should also be heterozygous for D90A mutation.
Homozygosity in the Turkish patient resembles the pattern observed in Scandinavia.

derived from a common ancestor 43 generations ago and has been distributed throughout Europe by Viking migration.
B. FURTHER INVESTIGATION OF THE D90A HOMOZYGOUS FALS PATIENT BY HAPLOTYPING ANALYSIS

- In this study, we aim to genotype recessive D90A cases for six closely-linked polymorphic markers.
STS (sequence tagged sites):
- are important for physical mapping because the presence of that sequence can be easily assayed by PCR.
- Most are nonpolymorphic and subchromosominal locations are unique.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>D21S213</td>
<td>TAGAGGCTTTGAATGGGGCTGG</td>
<td>GTGTTTTTAGACACACACACCC</td>
</tr>
<tr>
<td>D21S219</td>
<td>GCCTCCTTGACCTTTTGGC</td>
<td>GCTGCAAGCCTT CCTACATT</td>
</tr>
<tr>
<td>D21S224</td>
<td>GATCTATGGCTAACCACCTAA</td>
<td>ATGATAATGTTCCTCACTGTTT</td>
</tr>
<tr>
<td>D21S263</td>
<td>GTTAAGGGTGAAATGGGCTT</td>
<td>TGGAGATAGCATCACACTAACA</td>
</tr>
<tr>
<td>D21S1270</td>
<td>CCCACTGTATTATTCAGGGC</td>
<td>ACACACACACACACACATGC</td>
</tr>
<tr>
<td>D21S272</td>
<td>GAAAGAGCAATATAGAGCAGACA</td>
<td>TATATTCAGTGATTCTTTGGC</td>
</tr>
</tbody>
</table>

- D21S213: 152 bp
- D21S219: 173 bp
- D21S224: 119 bp
- D21S263: 330 bp
- D21S1270: 174 bp
- D21S272: 205 bp
25 µl PCR mix:
10X reaction buffer (including 2.5 mM MgCl\(_2\)), 2.5 mM dNTP, 5M Betaine, 1.3% DMSO, 20 pmol of each primer, 1 U Taq polymerase and 100 ng genomic DNA

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>80°C</td>
<td>hold</td>
</tr>
<tr>
<td>94°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Δ°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>72°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

40 cycles

<table>
<thead>
<tr>
<th>Markers</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D21S213</td>
<td>56.6</td>
</tr>
<tr>
<td>D21S219</td>
<td>53.4</td>
</tr>
<tr>
<td>D21S224</td>
<td>52.2</td>
</tr>
<tr>
<td>D21S263</td>
<td>53.4</td>
</tr>
<tr>
<td>D21S272</td>
<td>56.7</td>
</tr>
</tbody>
</table>
PCR amplification of D21S213

PCR amplification of D21S224

PCR amplification of D21S219

PCR amplification of D21S272

PCR amplification of D21S263

PCR amplification of D21S219

PCR amplification of D21S272
D21S213: TC repeat

D21S219: GT repeat

D21S224: CA repeat

D21S263: CA repeat

D21S272: CA repeat
PAGE Analysis

- 8% instagel; 55W; 2h30min

D21S272: CA repeat
D21S224: CA repeat
When Swedish samples are concerned, we haven’t been informed about the genotypes of the markers → blind-study

Since we do not have controls, it was important to sequence the samples for the comparison in the further steps.

In order to be able to include our results to a larger study, involving various European populations (Sweden, Finland, France, Russia, etc.), it is important to determine the exact number of repeats for each marker.

Thus, GeneScan strategy, using FAM-labelled primers, is being performed.
Preliminary Results of the GeneScan Analysis

D21S213

D90A heterozygote: 135/145 bp

12/17 TC repeats
Preliminary Results of the GeneScan Analysis

D21S224

both alleles normal: 127/129 bp

D90A heterozygote: 127/129

18/19 CA repeat
CONCLUSIONS & FUTURE PERSPECTIVES

PART 1: SOD1 Gene Analysis:
- Thus far, 5 mutations/182 cases have been identified → 2.7%
- This ratio is in concordance with the literature, stating that SOD1 is responsible for 2% of all ALS cases.

PART 2: D90A Haplotype Analysis:
- GeneScan analysis of the 6 markers for our D90A-homozygote patient and the his family members will be completed.
- The same approach will be performed for Scandinavian D90A cases.
- The results will be analyzed/compared.
- Meanwhile; collection of blood samples from the same region:
  - In case of a new D90A case, it will be included to the study.
  - In the further step, the study can be expanded with the use of new
This study is being established under the project supported by TUBITAK.

“Nörodejeneratif Hastalıklara Örnek Olarak Amiyotrofik Lateral Skleroz’un (ALS) Genetik, Moleküler ve Hücresel Yapısının İleriye Dönük Erken Tanı ve Tedavi Olanaklarının Geliştirilmesi Açısından İncelenmesi”
Thank you for listening... 😊
A. Oxidative Damage

1. **Peroxidase Hypothesis:**

- SOD1 utilizes its own product, $\text{H}_2\text{O}_2$, as a substrate to produce hydroxyl radicals.

- Self-limiting; after a few catalytic steps, the peroxidase activity of SOD1 is inactivated by the newly formed hydroxyl ions.

- FALS mutations exhibit enhanced peroxidase activity.
Change in Zn$^{+2}$-binding capacity

Destabilization of the protein backbone

The opening of the active channel

Cu$^{+2}$ located at the active site becomes more accessible to H$_2$O$_2$

Such an interaction causes an increase in the generation of hydroxyl radicals
A. Oxidative Damage

2. Peroxynitrite Hypothesis:
   - Some mutations cause the clumsy binding of Cu$^{+2}$ to the active site peroxynitrite by the interaction of O$_2^-$ and NO.

- **NO:**
  - reactive and unstable free radical gas
  - synthesized by nitrite oxide synthases (NOS) in a Ca$^{+2}$-dependent manner.
  - its activity can be upregulated in conditions, where intracellular Ca$^{+2}$ is raised, such as during glutamate excitotoxicity, which is significantly observed in ALS.
This end product can cause series of actions that will lead to apoptosis of cells.

NFs are particularly prone to tyrosine nitration by peroxynitrite.

Nitrated subunits can disrupt the assembly of non-nitrated NFs and this would contribute to the aberrant gathering of NFs in soma and axons.

Figure. Immunochemical analysis of the spinal cord sections of
a. wild type, b. SOD1<sup>G93A</sup> mice.
B. Glutamate-induced Excitotoxicity

- Glutamate is the major excitatory neurotransmitter in CNS.
Excess activation by glutamate can cause cell death via increase in Ca\(^{+2}\) level → it should be removed.

The activity of glutamate at the synaptic cleft is regulated by
- receptor inactivation
- glutamate up-take by transporter proteins, named excitatory amino acid transporters, EAAT.

In a large quantity of ALS patients,
- elevated levels of glutamate have been detected in the cerebrospinal fluid which causes an increase in the cytoplasmic Ca\(^{+2}\) level through the activation of Ca\(^{+2}\)-permeable NMDA receptors, Ca\(^{+2}\)-permeable AMPA receptors and metabotropic receptors.
- 60-70% of SALS patients have 35-95% decrease in EAAT2 protein level in the motor cortex and spinal cord, the regions specifically affected in ALS.
C. Neurofilaments

- the major type of IF in motor neurons
- Cytoskeletal proteins which confers intracellular scaffold and mechanical stability: NF-H, NF-M, NF-L
- NF-L is responsible for forming heterodimers with NF-M and NF-H to establish the proper 10 nm filaments, while NF-H and NF-M control axonal caliber.
- In several studies, mutations in NF genes have been identified in SALS cases.
NF-L, NF-M and NF-H knock-out mice appear normal in terms of clinical picture and development.

Overexpression of any subunit causes perikaryal NF inclusions, axonal atrophy and reduced conductivity.
D. Protein Aggregates

- All SOD1 mutant mice develop prominent cytoplasmic inclusions in motor neurons.

- These develop by the onset of clinical phenotype and increase in abundance during disease progression.

- The aggregates can interfere with microtubule-dependent axonal transport of other substances, which are necessary for viability.

- Toxicity can be the result of:
  - slow axonal transport
  - coprecipitation of important components which are necessary for cellular functions
E. Mitochondrial Involvement

- Powerhouses of the cell due to their ATP-producing ability
- Involved in buffering of intracellular Ca$^{+2}$ and triggering apoptosis.
- Motor neurons are in need of constant energy in order to produce action potentials.
Possible involvement of mitochondria in ALS pathogenesis depends on the morphological and functional changes in the organelle → reduction in energy production