SUPEROXIDE DISMUTASE 1 GENE ANALYSIS IN AMYOTROPHIC LATERAL SCLEROSIS: DETECTION OF A RARE POLYMORPHISM (IVS-III-34) IN TWO TURKISH FAMILIES

by

Aslıhan Özoğuz

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APPROVED BY:

Prof. A. Nazlı Başak.....
(Thesis Supervisor)

Prof. Hande Çağlayan.....

Assoc. Prof. Yeşim Parman.....

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ABSTRACT

SUPEROXIDE DISMUTASE 1 GENE ANALYSIS IN AMYOTROPHIC LATERAL SCLEROSIS: DETECTION OF A RARE POLYMORPHISM (IVS-III-34) IN TWO TURKISH FAMILIES

Amyotrophic Lateral Sclerosis (ALS) is a progressive late-onset neurodegenerative disease. It is clinically characterized by the selective death of motor neurons in the motor cortex, brainstem and spinal cord. ALS is observed with a prevalence of two-six per 100 000 people. Approximately, 10 per cent of ALS cases are familial (FALS), whereas sporadic ALS (SALS) comprises 90 per cent of all ALS patients. However, in terms of clinical features, FALS and SALS are almost indistinguishable. The major locus, comprising 20 per cent of all FALS cases, was localized to chromosome 21q22.1. Superoxide Dismutase 1 (SOD1), a small cytosolic protein, is ubiquitously expressed in most cells. It spans 12 kb of DNA and has five exons and four introns. Since both FALS and SALS cases show similar clinical and pathological features, SOD1, which seems to be the predominant protein responsible for ALS, has been investigated thoroughly.

In the framework of this thesis, the molecular analysis of the SOD1 gene has been established and applied on a group of 41 Turkish ALS patients. PCR-based SSCP studies of the complete SOD1 gene indicated a migration shift in intron 3 in two patients. The DNA sequencing results defined the nucleotide change as A→C transversion in intron 3, position 34. A restriction enzyme assay was applied to 14 family members and 109 healthy controls, and it was found that the IVS-III-34 (A→C) is a previously-defined polymorphism The aetiology of ALS is multifactorial; there is a complex interplay between many pathological and genetic factors. To solve the 'mystery' of pathogenesis of ALS, search for additional causative genes is required. This should not only include the screening of patients or individuals at risk, but also investigation of mechanisms.

ÖZET

AMİYOTROFİK LATERAL SKLEROZ'DA SÜPEROKSİT DİSMUTAZ 1 GEN ANALİZİ: İKİ TÜRK AİLEDE SEYREK BİR POLİMORFİZMİN (IVS-III-34) TANIMLANMASI

Amiyotrofik Lateral Skleroz (ALS) ilerleyici seyir gösteren geç başlangıçlı nörodejenaratif bir hastalıktır. Hastalık, klinikte, motor korteks, beyin sapı ve omurilikteki motor nöronların selektif ölümü ile tanımlanır. ALS, 100 000'de iki-altı kişide gözlenir. ALS olgularının yaklaşık yüzde onu ailesel (FALS) iken, sporadik ALS (SALS) tüm olguların yüzde 90'ını oluşturur. Klinik özellikleri açısından, FALS ve SALS birbirinden ayırt edilemeyecek kadar benzer. Tüm FALS olgularının yüzde 20'sini oluşturan başlıca lokus, kromozom 21'in uzun kolunun 22.1 bölgesine lokalize edilmiştir. Küçük bir sitozolik protein olan Süperoksit Dismutaz 1 (SOD1), birçok hücrede üretilir. SOD1 geni DNA üzerinde 12 kb'lik bir bölgeyi kapsar; beş ekzonu ve dört intronu vardır. FALS ve SALS olguları benzer klinik ve patolojik özellikler gösterdiği için, bugüne kadar, hastalıktan büyük oranda sorumlu olduğu düşünülen SOD1 geni ayrıntılı olarak incelenmiştir.

Bu çalışma çerçevesinde, ALS'nin moleküler analizi laboratuarımızda ilk defa kurulmuş ve toplam 41 ALS hastasına uygulanmıştır. Tüm SOD1 geninin PCR'a dayalı SSCP analizi, iki hastada intron 3'te değişiklik göstermiştir. DNA dizi analizi, bunu intron 3 pozisyon 34'te A→C mutasyonu olarak tanımlamıştır. 14 aile bireyine ve 109 sağlıklı bireye restriksiyon enzim analizi uygulandığında, bu değişikliğin daha önce tanımlanmış bir polimorfizm olduğu anlaşılmıştır.ALS'nin etyolojisi çok faktörlüdür; patolojik ve genetik faktörler arasında karmaşık bir etkileşim vardır. ALS patogenezinin "gizemini" çözmek için, sorumlu olabilecek genlerin araştırılması gerekmektedir. Bu, sadece hastaların ve risk altındaki bireylerin taranması değil, mekanizmaların da araştırılmasını gerektirmektedir.

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LIST OF ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APS	Ammonium Peroxodisulfate
ATP	Adenosine Triphosphate
bp	Basepair
BPB	Bromophenol Blue
CGMP	Cyclic Guanosine Monophospate
DALS	Autosomal Dominant Amyotrophic Lateral Sclerosis
dNTP	Deoxyribonucleotides
e-	Electron
EAAT	Excitatory Amino Acid Neurotransmitter
EDTA	Ethylenediaminetetraacetate
EtBr	Ethidium Bromide
FALS	Familial Amyotrophic Lateral Sclerosis
GA	Golgi apparatus
h	Hour
H_2O_2	Hydrogen Peroxide
IF	Intermediate Filament
IgG	Immunoglobin G
IPC	Insoluble Protein Complexes
kb	Kilobase
kDa	Kilodalton
KSP	Lys-Ser-Pro Phosporylation Sites
LMN	Lower Motor Neuron
min	Minute
MtDNA	Mitochondrial DNA
mV	Milivolt
NF	Neurofilament
NF-H	Heavy Chain Neurofilament
NF-L	Light Chain Neurofilament

NF-M	Medium Chain Neurofilament
NMDA	N-methyl-D-aspartic Acid
NO	Nitric Oxide
NOS	Nitrite Oxide Synthases
OD	Optic Density
Para	Paralysis
PCR	Polymerase Chain Reaction
PD-ALS	Parkinsonism-Amyotrophic Lateral Sclerosis
PMW	Pre-muscle Weakness Stage
RD	Rapid Decline Stage
RE	Restriction Enzyme
RFALS	Autosomal Recessive Juvenile-onset Familial Amyotrophic Lateral Sclerosis
ROS	Reactive Oxygen Species
SALS	Sporadic Amyotrophic Lateral Sclerosis
SD	Slow Decline Stage
SDS	Sodiumdodecylsulphate
SOD1	Superoxide Dismutase1
SOD2	Mitochondrial Superoxide Dismutase
SSCP	Single Strand Conformational Analysis
TBE	Tris-Boric Acid-EDTA
TEMED	N', N', N', N'- tetramethylethylenediamine
U	Unit
UMN	Upper Motor Neuron
UV	Ultraviolet
W	Watt

1. INTRODUCTION

1.1. Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a late onset neurodegenerative disease. It was first described by Charcot in 1869 and named as Lou Gehrig's Disease, after the death of the famous baseball player from ALS in 1930s.

ALS is characterized by the selective death of motor neurons in the motor cortex, brainstem and spinal cord. El Escorial Criteria determines the classification and diagnosis of suspected, possible, probable, and definite ALS cases, based on clinical, electrophysiological and neuropathological examinations (Hand *et al.*, 2002). The clinical picture of ALS relies on the 'center of gravity' of the progression of the disease (Shaw, 2001). While muscle weakness and atrophy are the representatives of lower motor neuron (LMN) degeneration, spasticity is of upper motor neuron (UMN) degeneration. The loss of motor neurons leads to a progressive atrophy of skeletal muscles, hence the term amyotrophic. Although the site of onset is focal, the disease spreads inexorably. Atrophy of the large motor neurons in the spinal cord causes replacement with fibrous astrocytes, which results in the hardening of the anterior and lateral columns, hence the term lateral sclerosis (Hirano, 1991). ALS is a progressive disease, where weakness in extremity muscles results in total paralysis. Generally, sexual functioning, cognitive and sensory functions are preserved. Survival is not affected by age or gender, but rather by the site of symptom onset. Death occurs within two to five years as a result of respiratory failure (Cleveland, 1999).

ALS is observed with a prevalence of two-six per 100 000 people without obvious race-related differences (Yoshida *et al,* 1986). It has a prevalence of five to nine per 100 000. It is proposed that there are 60-70 000 ALS patients worldwide (Pala, 2001). Approximately, 10 per cent of ALS cases are familial (FALS), whereas sporadic ALS (SALS) comprises 90 per cent of all cases (Julien, 2001). However, in terms of clinical features, FALS and SALS are almost indistinguishable. Up to date, only one distinct

phenotype has been reported in the Pacific Islands of Guam, where high incidence of parkinsonism-dementia is observed (Brown *et al.*, 2000).

Although FALS and SALS are clinically identical, minor differences exist. First of all, the age of onset is earlier in FALS, compared SALS. Secondly, the male: female ratio is 1:1 in FALS, whereas it is 1.7:1 in SALS (Veldink *et al.*, 2003). However, the ratio in SALS decreases with increasing age and reaches a 1:1 ratio after 70 years of age. Thirdly, the prognosis of FALS patients is usually longer than SALS patients (Hand *et al,* 2002).

1.2. Molecular Pathology of ALS

In addition to the phenotypic loss of motor function, motor neurons of the affected brain regions and spinal cord exhibit several pathological changes (Morrison *et al*, 2000). Such pathological findings specific for ALS have been grouped according to the four different stages, determined by the time course of muscle strength changes in a mouse model expressing $SOD1^{G93A}$ (Figure 1.1). These are pre-muscle weakness stage (PMW), during which muscle strength is maintained at the normal level, rapid decline stage (RD), during which there is a sharp decline in muscle strength up to 50 per cent, slow decline stage (SD), during which a slow decline in ability is observed and paralysis (Para), during which paralysis of limb muscles have begun and the mouse can no longer hold on to the wire (Kong *et al*, 1998).

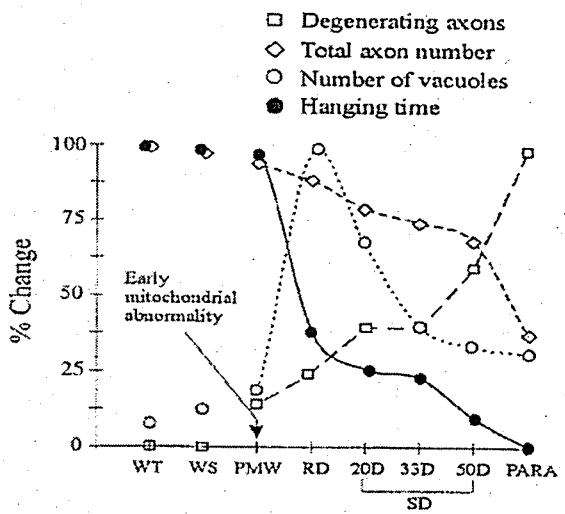

Figure 1.1. Pathological events leading to motor neuron death in $SOD1^{G93A}$ mice (Kong *et al.*,1998)

Changes can be evaluated in different aspects. The sharp decline in muscle activity observed at RD stage is not correlated with a loss of a large number of motor axons, since at the SD stage, a significant number of motor neuron axons are still present (Figure 1.2). It is not until the paralysis stage that a vast majority of motor axons are degenerated (Kong *et al.*, 1998).

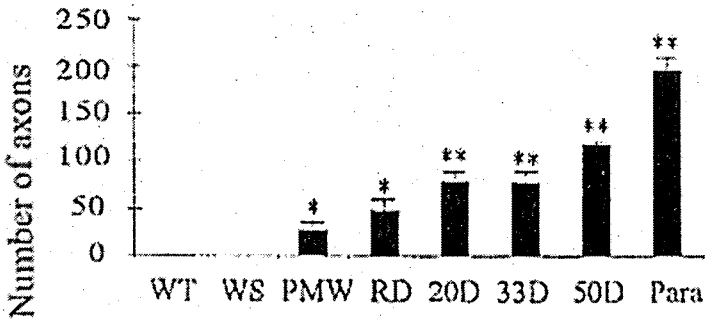

Figure 1.2. Number of degenerating axons in spinal cord motor neurons at different disease stages (Kong *et al,* 1998)

Consistent with the motor axons, there are considerable numbers of motor neurons during RD stage (Figure 1.3). The number declines significantly until the paralysis stage, where most of the large motor neurons are lost (Kong *et al*, 1998).

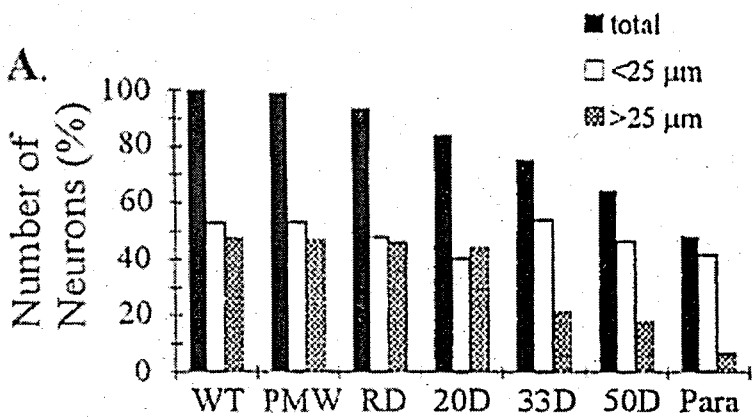

Figure 1.3. Number of motor neurons at different disease stages (Kong *et al*, 1998)

The most predominant feature of the PMW stage is the presence of abnormal mitochondria (Jaarma *et al.,* 2001). Since PMW is the primary stage, abundant mitochondrial changes can be described as the most prominent pathological feature before

the onset of muscle strength decline. Such changes in the morphology can be described as dilated and disorganized cristae, leakage of the outer membrane, broken outer membrane and early vacuoles that still carry remnants of mitochondria (Figure 1.4). Neurofilament accumulations can be observed adjacent to vacuoles in axons, which suggest that failure in slow axonal transport begins before the onset of the RD stage. However, the vacuolation is a transient process: its occurrence is in concordance with the onset of muscle weakness, but the vacuolation decreases in size and number toward the end stage (Kong *et al*, 1998).

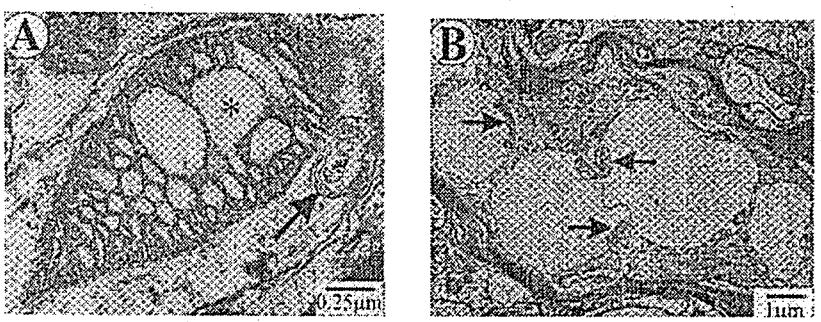

Figure 1.4. Mitochondrial abnormalities at PMW stage. A. a swollen mitochondrion with dilated cristae (*) and leaking outer membrane (arrow); B. early vacuoles in a proximal axon (Kong *et al,* 1998)

Another important histopathological change observed in SALS and FALS cases is the fragmentation of the neuronal Golgi apparatus (GA) (Stieber *et al.*, 2000). Recent experiments on transgenic mice carrying $SOD1^{G93A}$ demonstrated that the fragmentation of GA and appearance of the protein aggregates both occur before the onset of RD stage (Valentine, 2002).

1.3. Molecular Genetics of ALS

The major locus, comprising 20% of all FALS cases, has been localized to chromosome 21q22.1; this locus encodes the Superoxide Dismutase1 (SOD1) enzyme (Siddique *et al*, 1993).

In most cases, pattern of inheritance is autosomal dominant (DALS). These cases can be further subcategorized as ALS1 (localized on chromosome 21), ALS4 (localized on chromosome 9q34) (Chance *et al,* 1998) and ALS3 (locus yet to be determined). Also, a

locus for a dominant form of ALS has been identified on chromosome Xp11-q12 (Hong *et al,* 1998).

Autosomal recessive juvenile-onset familial amyotrophic lateral sclerosis (RFALS) is rare but has been reported in places with high consanguinity, such as Tunisia (Hentati *et al,* 1989). The subcategories are ALS2 (chromosome 2q33) and ALS5 (chromosome 15q15.1-q21.2) (Siddique *et al,* 1996).

In a small number of SALS patients, mutations in SOD1 have been reported. However, other gene/genes are believed to be responsible for SALS.

In search of understanding ALS, since both FALS and SALS show similar clinical and pathological characteristics, SOD1, which seems to be the predominant protein responsible for the disease, has been investigated thoroughly.

1.4. Superoxide Dismutase 1(SOD1)

Superoxide Dismutase1 (SOD1), one of three mammalian superoxide dismutases, is a small cytosolic protein, ubiquitously expressed in most cells, including red blood cells (Siddique Estéves *et al.*, 1996). It is particularly abundant in neurons, comprising approximately 1% of total cytosolic protein (Cleveland, 1999). In humans, it is located on chromosome 21q22.1 and spans 12 kb of DNA. It has five exons and four introns.

The genomic organization is surprisingly similar among species. The promoter region comprises TATA and CCAAT boxes, as well as several highly conserved GC-rich regions. Such a high incidence of homology in the 5′ flanking region implies that intense evolutionary factors have preserved key regulatory regions for this gene. The 3′ end has poly A sequences that determine the establishment of two mature mRNA transcripts of 0.7 and 0.9 kb (Zelko *et al,* 2002).

The five exons encode a 21 kDa protein of 153 highly conserved amino acids, which functions as a homomer. The polypeptide chain is folded into a flattened cylinder of eight strands of antiparallel β-structure, which are arranged in two interlocking Greek key motifs to form the β-barrel (Siddique *et al.*, 1996). Inside this β barrel extends two large nonhelical loops (Figure 1.5). One of these loops contains the essential residues for the

establishment of the electrostatic guidance of O_2^-, while the second one contributes to the dimer interface. Stable dimers are formed by strong hydrophobic interactions between individual monomers at the interface. At the end of the β-barrel, each monomer contains a cave-like active site, embedding one atom of copper and zinc. Copper (Cu^{+2}) binding is coordinated by four histidine residues (His 46, His48, His63 and His 120), while zinc (Zn^{+2}) is coordinated by three histidines (His63, His71, His80) and one aspartic acid (Asp83). Thus, His63 is in interaction with both atoms, forming a histidine bridge. Cu^{+2} is essential for the dismutase activity, whereas Zn^{+2} is involved in maintaining pH stability of the dismutation reaction and in the rapid dissociation of the H_2O_2 produced (Valentine *et al*, 1999).

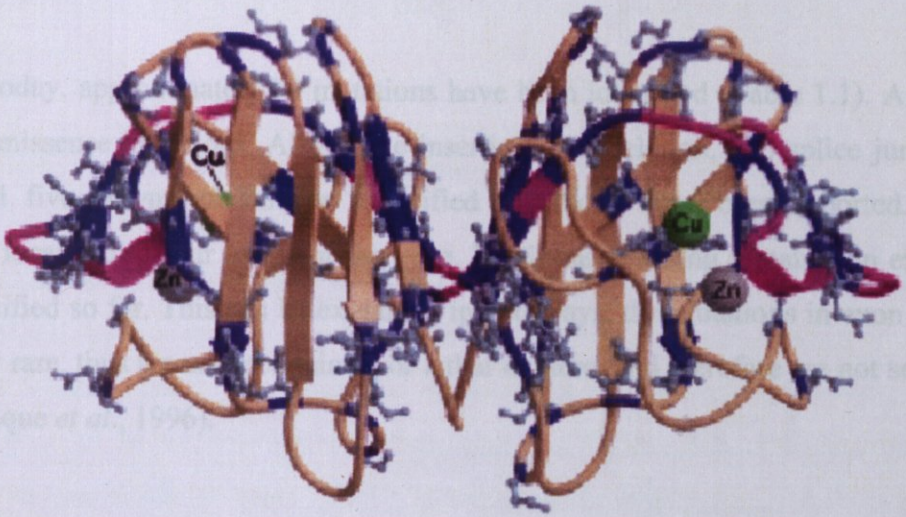

Figure 1.5. Three-dimensional structure of Superoxide Dismutase 1 (SOD1) (Beckman *et al*, 2001)

The major function of the enzyme is to detoxify O_2^- by forming O_2 and H_2O_2. H_2O_2 is then further detoxified to H_2O by either glutathione peroxidase or catalase (Figure 1.6). In this respect, O_2^- is guided to the Cu^{+2}-containing active site through a positively-charged electrostatic guidance channel. This channel is established by twenty-one highly conserved amino acids, and the positive charge is produced by the amino acids Lys122, Lys134 and Arg 143. Arg143 stabilizes the position of O_2^- in relation to the Cu^{+2} atom. The electrostatic guidance channel narrows down in a stepwise fashion from a 24A° to a 10A° width and ends up in an opening of less than 4A° just above the Cu^{+2} atom. Such a

structure enables selective access of small negatively charged O_2^- to the active site. The dismutase activity occurs at a rate of 2×10^9 M^{-1} sec^{-1} (Siddique *et al.*, 1996).

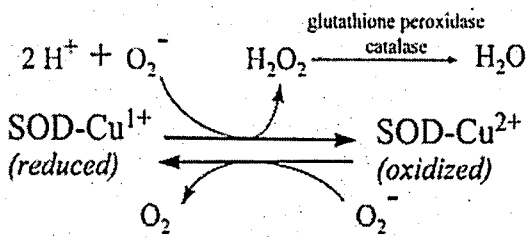

Figure 1.6. SOD1-mediated dismutation of superoxide (Cleveland, 1999)

1.5. Mutations in SOD1 Gene

Until today, approximately 90 mutations have been identified (Table 1.1). Among these, 83 are missense mutations. Also, three insertion, two deletion, two splice junction mutations and five polymorphisms or unverified mutations have been reported. The mutations are localized to four exons of the gene. No disease-causing mutation in exon 3 has been identified so far. This can be explained in two ways: the mutations in exon 3 are benign or very rare, thus are not ascertained, or lethal in utero, and therefore are not seen in patients (Siddique *et al.*, 1996).

Physical properties of SOD1 (half-life, stability, protein solubility, etc.) differ greatly between various SOD1 mutations, but they don't appear to correlate with severity of the disease (Elliott, 2001). Different mutations do not exhibit different clinical phenotypes. Similarly, FALS patients with SOD1 mutation are clinically almost indistinguishable from FALS without SOD1 mutation or SALS patients (Cole *et al*, 1999). Different mutations don't seem to affect the onset of the disease. However, they do have an affect on the progression. In FALS families, the most common mutation is A4V, which exhibits an aggressive progression; death occurs usually within 1.2 years from the age of onset. Duration for most SOD1 mutations ranges from three to five years, while H46R and G37R have the longest duration of about 18-20 years. Still, even patients of the same family with the same SOD1 mutation can exhibit significant phenotypic differences (Siddique *et al*, 1996).

All SOD1 mutations behave as autosomal dominant traits, except D90A. Haplotype analysis, including Belgium, France, North America, England and Australia, have revealed that it is derived from a common ancestor 43 generations ago and has been distributed throughout the world by migration (Al-Chalabi *et al*, 1998). In non-Scandinavian populations, it exhibits an autosomal dominant effect. However, in Sweden and Finland, 2.5 per cent of the populations are heterozygous carriers, and homozygous carriers of D90A have a significantly increased risk of developing ALS, compared to D90A heterozygotes and wild-type homozygous individuals (Själander *et al.*, 1998). In this situation, D90A causes the FALS phenotype in an autosomal recessive manner. The duration of the disease for these heterozygotes is longer than non-Scandinavian heterozygotes (Siddique *et al*, 1996). It is proposed that in these populations, a factor is co-inherited, which protects the heterozygotes from the deleterious effects of the mutation (Robberecht, 2000).

1.6. Possible Mechanisms in ALS

The cytoplasmic dismutase activity of mice carrying a mutation in the SOD1 gene is usually 30-70% of the normal activity (Siddique *et al*, 1996). Such a decrease may result from the random dimerization of mutant and wild-type heterodimers, which are highly unstable. Formation of an unstable structure also decreases the half-life of the protein: the half-life of the normal SOD1 protein is about 30 hours, whereas half-life of A4V is about seven hours (Siddique *et al.*, 1996).

However, studies with transgenic and knockout mice revealed, that the basic mechanism leading to FALS or SALS phenotype is not the reduction in dismutase activity (Brown, 1998). Although several mutations, such as A4V or G85R, have reduced dismutase activity, others, such as G37R, G93A or D90A establish normal levels. There is no correlation between the phenotype and the extent of residual SOD1 dismutase activity. While the deletion of both alleles, thus the absence of normal SOD1 protein, causes no change in phenotype, transgenic mice expressing a mutant protein show progressive motor neuron loss. Similarly, the overexpression of the wild-type protein exhibits no phenotypic difference, whereas the overexpression of mutant SOD1 produces a lethal, paralytic state (Elliott, 2001). These results imply that mutant SOD1-induced disease is not a consequence of a reduction in dismutase activity, but rather a 'gain-of-function'.

1.6.1. Oxidative Damage

Oxidative damage is the degeneration of cells due to excess amount of reactive oxygen species (ROS) or nitric oxide (NO). In ALS, two different hypotheses, regarding oxidative damage, are proposed.

1.6.1.1. Peroxidase Hypothesis: In addition to the dismutase activity, SOD1 has a free-radical generating function, where it utilizes its own product, H_2O_2, as a substrate to generate hydroxyl radicals in a Fenton type reaction. Hydroxyl radicals are highly reactive species that can damage macromolecules in cells. Under certain conditions, SOD1, by using its peroxidase activity, can act as a prooxidant (Bär, 2000). However, this reaction is self-limiting. After a few catalytic steps, both the dismutase and the peroxidase activities of SOD1 are inactivated by the newly generated hydroxyl ions (Roe *et al*, 2002).

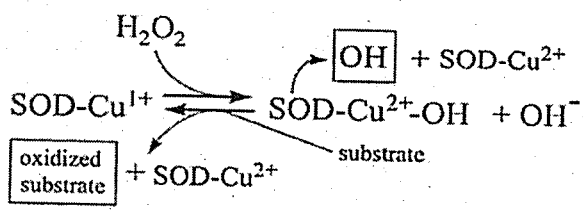

Figure 1.7. Schematic representation of the peroxidase hypothesis (Cleveland, 1999)

On the other hand, most FALS mutations exhibit enhanced peroxidase activity. These mutations alter the highly conserved interactions within the homodimer structure. Change in Zn^{+2}-binding capacity leads to destabilization of the protein backbone. For example, the G93A mutation is located in a loop at the end of the β barrel. Destabilization of the β barrel backbone causes the opening of the active channel (Yim *et al*, 1996). As a result, Cu^{+2} located at the active site becomes more accessible to H_2O_2. Such an interaction causes an increase in the generation of hydroxyl radicals (Figure 1.7).

To determine the enhanced free-radical generating function and its oxidative damage, leading to motor neuron degeneration, a series of experiments were constructed (Figure 1.8). In the first step, it was ensured that all the mutant and wild-type SOD1 enzymes had the equal enzymatic activity. Then, measurement of the free-radical

generating activities of SOD1 by spectrophotometer revealed the following descending order: A4V, G93A, D90A, wt, heat-inactivated wt (Kang *et al*, 2000).

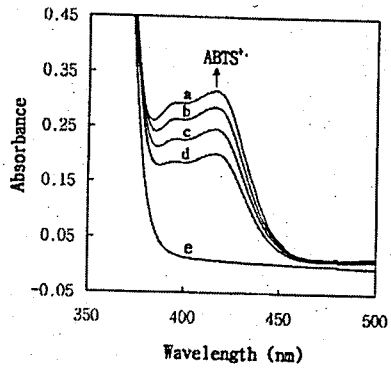

Figure 1.8. Electronic absorption of ABTS, formed in a solution containing H_2O_2 and a. $SOD1^{G93A}$, b. $SOD1^{D90A}$, c. wild-type SOD1, d. heat-inactivated wild-type SOD1. $ABTS^+$ is a chromogen (Kang *et al*, 2000)

In the second step, Cu^{+2} release was measured (Figure 1.9). The order was again the same.

These two results can be interpreted in the concept of oxidative damage. Higher concentrations of hydroxyl radicals, generated by enhanced peroxidase activity of the mutant SOD1 enzymes, cause damage to cellular macromolecules, including SOD1 itself. As a result, SOD1 is inactivated and Cu^{+2} is released (Kang *et al*, 2000).

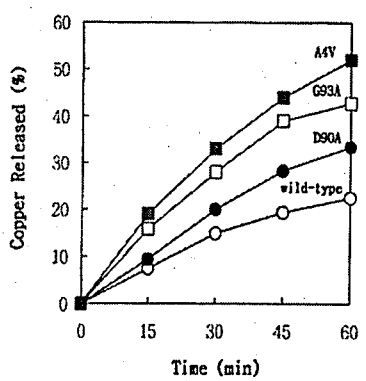

Figure 1.9. Release of copper ions from wild-type and $SOD1^{D90A}$, $SOD1^{G93A}$ and $SOD1^{A4V}$ mutant mice in the presence of H_2O_2 (Kang *et al*, 2000)

However, similar evidence for hydroxyl radicals is not found in many other transgenic mouse models at any stage of the disease. Also, vitamin E doesn't appear to alter the outcome of the disease (Ahmed *et al.*, 2000). These results indicate that either different mutations produce disease via different mechanisms or the enhanced peroxidase activity is not necessary for neuronal death.

1.6.1.2. Peroxynitrite hypothesis: Another hypothesis that has been related to oxidative damage is the peroxynitrite hypothesis. Some mutations in FALS cause the clumsy binding of Cu^{+2} to the active site, leading to unwanted reactions in cells. An example is the production of peroxynitrite (ONOO-) by the interaction of O_2^- and NO.

NO is a free radical synthesized by nitrite oxide synthases (NOS) in a Ca^{+2}-dependent manner (Estéves *et al.*, 2002). Thus, its activity can be upregulated in conditions, where intracellular Ca^{+2} is raised, such as during glutamate excitotoxicity, which is significantly observed in ALS. NO can act as a protective factor by enhancing cGMP synthesis. On the contrary, it can cause neuronal injury through the formation of secondary oxidants or by the inhibition of mitochondrial respiration (Estéves *et al.*, 2000).

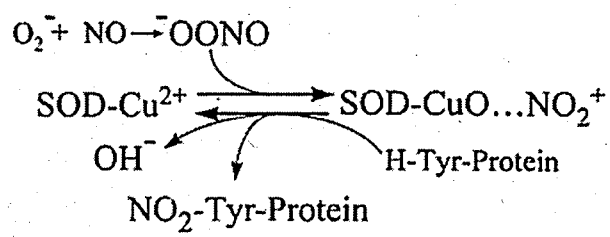

Figure 1.10. Schematic representation of the peroxynitrite hypothesis (Cleveland, 1999)

In ALS cases, following the synthesis of peroxynitrite is its catalysis by mutant SOD1. The end product is 3'nitrotyrosine (Figure 1.10). $SOD1^{G37R}$ transgenic mice exhibit significantly higher levels of 3'nitrotyrosine/nitrotyrosine levels with respect to normal controls or hSOD76 mice. Similarly, immunochemical analysis of the spinal cord sections of $SOD1^{G93A}$ mice showed nitrotyrosine inclusions (Figure 1.11) (Jung *et al.*, 2001).

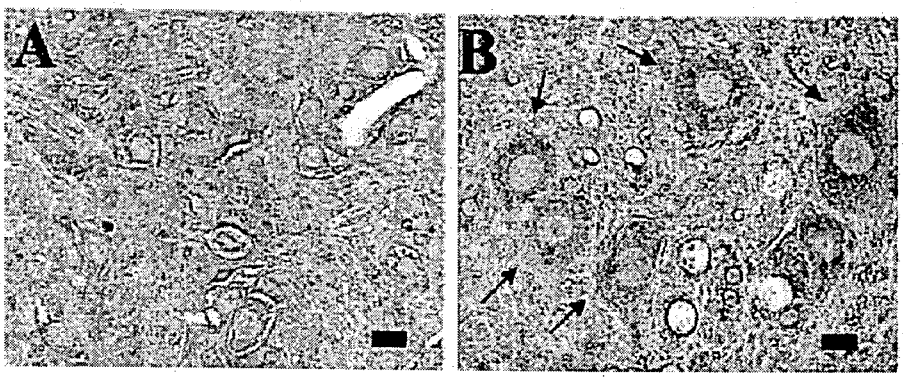

Figure 1.11. Immunochemical staining of nitrotyrosine in spinal cord sections of a. wild-type and b. $SOD1^{G93A}$ mice (Jung *et al.*, 2001)

Also, in the case of Zn^{+2}-deficiency, mutant subunits of SOD1 fail to bind or retain Zn^{+2} efficiently (Liohev *et al.*, 2003). For example, A4V mutant exhibits 30 fold decrease in Zn^{+2}-binding ability (Elliott, 2001). In this case, rather than acting as a scavenger of O_2^-, Zn^{+2}-deficient SOD1 steals e- from cellular antioxidants and transfer these e- to O_2 to produce O_2^- (Williamson *et al.*, 2000). Then, this superoxide combines with NO to form peroxynitrite.

This end product can cause series of actions that will lead to apoptosis of cells. For example, structural proteins that form NFs are particularly prone to tyrosine nitration by peroxynitrite. Dissembled NFs are exceptionally vulnerable to nitration. A small fraction of nitrated subunits can disrupt the assembly of non-nitrated NFs and this would contribute to the aberrant gathering of NFs in soma and axons. Such an interaction between NFs and Zn^{+2}-deficient SOD1 enters a vicious cycle: As more Zn^{+2} is bound to NFs, more Zn^{+2}-deficient SOD1 accumulates and more peroxynitrite is generated; peroxynitrite nitrates NFs, causing more NFs to accumulate until enough peroxynitrite is produced to trigger apoptosis of the cell (Morrison *et al.*, 2000).

On the other hand, both mechanisms regarding oxidative damage by the peroxynitrite reaction are faced with contradictory experimental results. In a cell culture model, limiting NO synthesis by inhibition of NOS by 14-folds was predicted to ameliorate disease. However, neither the onset nor the progression of the disease was altered (Cleveland *et al.*, 2001). Also, so far no mutation has been identified in any of the four

residues that directly coordinate Zn^{+2} (Elliott, 2001). Thus, the predominant role of oxidative damage in ALS pathogenesis should still be considered with caution.

1.6.2. Glutamate-induced Excitotoxicity

Glutamate-induced excitotoxicity is another potential contributor to ALS pathogenesis. Glutamate is the major excitatory amino acid (EAA) neurotransmitter in the central nervous system (Brown *et al.,* 2000). There are two types of receptors that glutamate acts through: the G protein-coupled receptors, which when activated, enhance the release of intracellular calcium stores, and the glutamate-gated ion channels, which are recognized by their sensitivity or insensitivity to N-methyl-D-aspartic acid (NMDA) (Rownad *et al.*, 2001). The latter can be subcategorized as NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainite receptors.

All NMDA receptors have very high Ca^{+2} conductances. They are not only ligand-gated but also voltage-gated channels. At resting membrane potential, the ion channel is blocked by extracellular Mg^{+2}, which prevents ion transduction even if glutamate is bounded. Since this blockage is voltage-dependent, postsynaptic depolarization facilitates NMDA receptor activation. As a result, NMDA receptor is activated only under conditions of coincident agonist binding and postsynaptic depolarization (Brown *et al.,* 2000).

AMPA and kainite receptors are the primary regulators of fast excitatory neurotransmission. They are heteromeric complexes assembled by various combinations of four different subunits (GluR1-GluR4). Most of them are only permeable to Na^+ and K^+, whereas heteromers, lacking GluR2 subunits, are permeable to Ca^{+2} ions (Trounce *et al.*, 2003). Although most synapses possess few Ca^{+2}-permeable AMPA or kainite receptors, under stress, selective degeneration of the GluR2 AMPA receptor subunit is triggered. Increased numbers of Ca^{+2}-permeable AMPA channels permit rapid Zn^{+2} and Ca^{+2} entries (Weiss *et al.*, 2000).

The binding of glutamate to the receptors on the post-synaptic membrane opens up the associated ion channels, resulting in the entry of Na^+ and H_2O (Figure 1.12). This depolarization, leading to the opening of NMDA receptor-linked Na^+-Ca^{+2} channels,

facilitates Ca^{+2} influx. Excess activation by glutamate can cause cell death via increase in Ca^{+2} level (Julien, 2001). Thus, it should be removed. The activity of glutamate at the synaptic cleft is regulated by receptor inactivation and high-affinity glutamate up-take by transporter proteins, named excitatory amino acid transporters, EAAT.

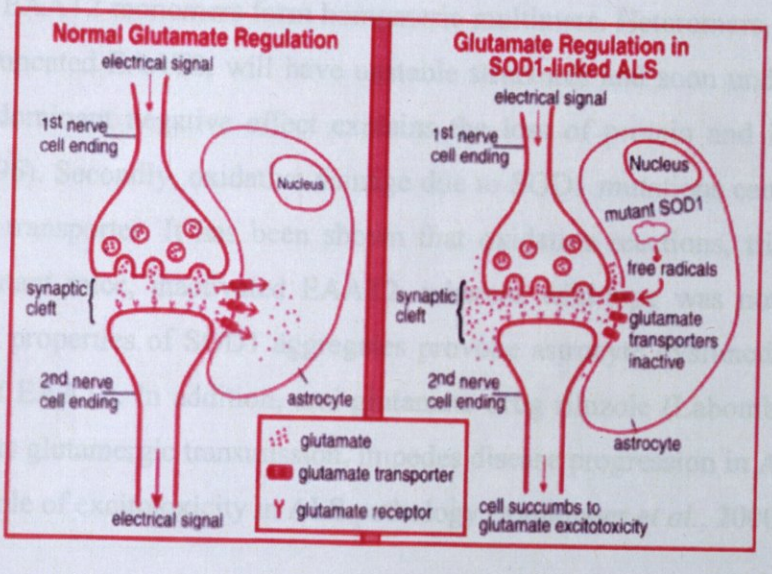

Figure 1.12. Glutamate release mechanism in normal and ALS cases

So far, five EAAT (EAAT1-5) have been identified. Among these, EAAT2 is expressed specifically in astrocytes (Maragakis *et al.*, 2001). In a large quantity of ALS patients, elevated levels of glutamate have been detected in the cerebrospinal fluid. Also, 60-70% of SALS patients were shown to have a loss of 35-95% of EAAT2 protein in the motor cortex and spinal cord, the regions specifically affected in ALS (Cluskey *et al.*, 2001). Furthermore, EAAT2 null mice develop excitotoxic neuronal degeneration, while antisense knockdown, a situation analogous to human ALS, show progressive paralysis in motor neuron degeneration. Thus, the loss of EAAT2 receptors aroused attention in search of pathogenesis of ALS.

Interestingly, it has been shown that while the protein level is decreased, the mRNA level is unchanged (Lin *et al.*, 1998). cDNA isolated from motor neurons of ALS patients revealed aberrant transcripts. In Northern blot analysis, these could not be distinguished, so led to the wrong consumption that the 'normal' EAAT level was unchanged. Thus, it can be concluded that the aberrant translation or post-translational processing are the reasons of decline in protein level (Deitch *et al.*, 2002).

There are two predominant aberrant transcripts: one resulting from depletion of exon 9, named 'exon-skipping' RNA, and the other resulting from skipping of intron 7, 'intron retention' RNA (Meyer *et al.*, 1999).

These abnormal transcripts can be effective in two different pathways. First, under normal conditions, EAAT2 monomers form homomeric multimers. Heteromers, consisting of wild-type and truncated EAAT2, will have unstable structures and soon undergo rapid degradation. This dominant negative effect explains the loss of protein and its activity (Haugeto *et al.*, 1996). Secondly, oxidative damage due to SOD1 mutations can cause the inactivation of the transporter. It has been shown that oxidative reactions, triggered by H_2O_2 in SOD1 mutant mice, inactivated EAAT2, whereas wild-type was not affected. Alternatively, toxic properties of SOD1 aggregates provoke astrocyte dysfunction, which affects the levels of EAAT2. In addition, anti-glutamate drug riluzole (Labomblez *et al.*, 1996), which inhibits glutamergic transmission, impedes disease progression in ALS cases, implying a crucial role of excitotoxicity in ALS pathology (Meininger *et al.*, 2000).

However, absence of aberrant EAAT2 mRNA or loss of EAAT2 had not been reported in a subset of ALS patients, who had pathological confirmation of motor neuron loss. Similarly, these two transcripts were not found in G37R transgenic mice (Lin *et al.*. 1998). Thus, it can be concluded that aberrant EAAT2 transcripts are not the primary reason, but rather secondary to motor neuron degeneration or astrocyte response or both.

1.6.3. Neurofilaments

An important pathological hallmark of both SALS and FALS cases is the intermediate filament (IF) accumulations in perikaryon and axons of motor neurons. Neurofilaments (NF), the major type of IF in motor neurons, are cytoskeletal proteins that are subcategorized as light (NF-L, 61 kDa), medium (NF-M, 90 kDa) and heavy (NF-H, 115 kDa) (Julien *et al.*, 2000). All IF proteins have an alpha-helical rod domain, flanked by non-alpha-helical N-terminal 'head' and C-terminal 'tail' domains. The rod domains consist of 310 amino acid residues. While NF-L forms the core of the NF neurofibril, the tail domains of NF-M and NF-H spread radically from the neurofibril to establish the spacing between adjacent neurofibrils and interactions with other proteins. NF-M and NF-H have lysine-serine-proline (KSP) repeats at the C-terminus, which are

phosphorylated by serine-threonine kinases. Phosphorylated NFs settle in axons to modulate the packing density of neurofibrils. 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 (Lee *et al.*, 1996).

In 1994, codon deletions in the tail of NF-H domain were described for the first time in five sporadic cases (Figure 1.13). Recently, four novel codon deletions and one insertion of 84 bp in the same tail domain have been reported (Al-Chalabi *et al.*, 1999). However, such variations in the NF-H tail domain can be a primary cause for a small percentage (1%) of ALS patients, so it is rather a risk factor than a primary cause.

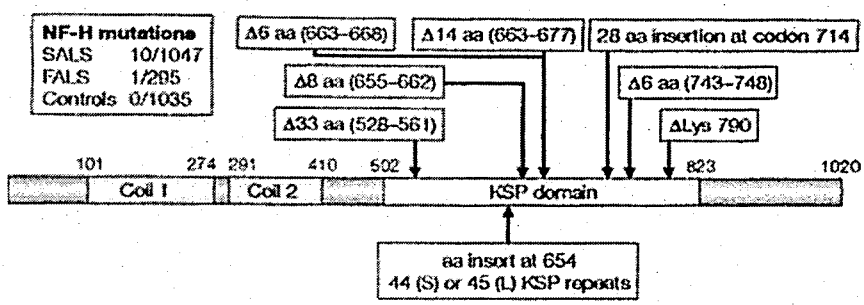

Figure 1.13. ALS-related mutations in NF-H (Cleveland *et al.*,2001)

Neurofilaments are detected in the form of spheroids in motor neurons of ALS patients. Accumulations can occur as a result of deregulation of IF protein synthesis, proteolysis, defective axonal transport, abnormal phosphorylation and other modifications. It has been shown that deregulation of the expression of NF-L has resulted in the reduction of NF-L mRNA level. Since NF-L is in charge of establishing the proper neurofilament structure, its absence provokes the sequestration of NF-H and NF-M in the cell bodies of motor neurons (Figure 1.14c) (Jacomy *et al.*, 1999).

It has been demonstrated that NF-L, NF-M and NF-H knockout mice appear normal in terms of clinical picture and development. This indicates that NFs aren't obligatory for nervous system development. However, NF-L knockout and NF-M/NF-H knockout mice lose 50% of the motor neurons (Julien, 1999). In NF-L knockout mice, this causes 50% reduction in conduction velocity, while sequestration of unassembled NF-L in

perikarya and alteration in axonal transport of slow components are observed in the latter (Williamson *et al.*, 1999). In the absence of NF-L, NF-M and NF-H cannot assemble into 10 nm filaments, which results in severe axonal hypotrophy. Thus, deficiencies in NF proteins aren't completely innocuous.

Overexpression of any of the neurofilaments in mouse models causes perikaryal NF accumulation, reminiscence of those found in human ALS, atrophy of axons and altered axonal conductance without motor neuron death even in two-year-old mice (Figure 1.14b) (Kong *et al.*, 2000). Remarkably, perikaryal swellings and axonal transport defects in mice overexpressing human NF-H were rescued by the overexpression of NF-L in a dosage-dependent fashion. These results emphasize the importance of subunit stoichiometry for proper NF assembly and transport (Rowland, 2000).

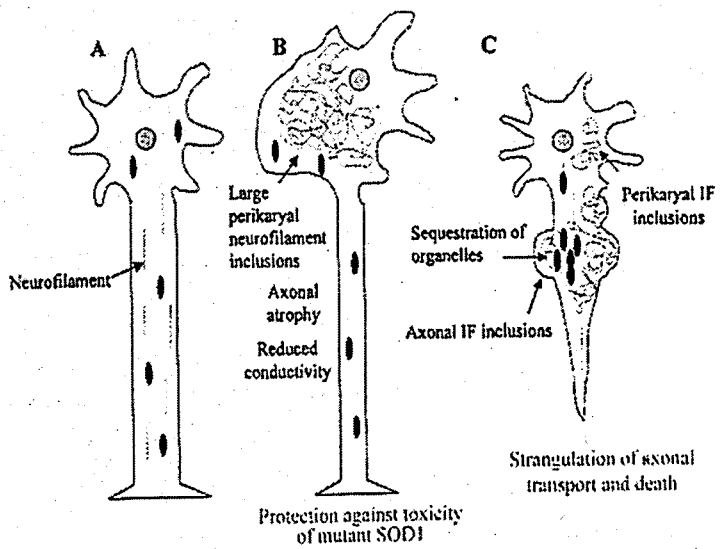

Figure 1.14. Formation and effects of IF inclusions. A. normal motor protein, b. the overexpression of wild-type NF-H, c. the expression of mutant NF-L (Julien, 2001)

Interestingly, $SOD1^{G37R}$ mice overexpressing NF-H show a 65% increase in lifespan, while NF-L null transgenic mice with $SOD1^{G93R}$ mice shows a 15% increase (Beauliea *et al.*, 2000). In both cases, perikaryal NF accumulations are present. However, they are well-tolerated and indeed neuroprotective. This may be explained by two hypotheses. The first one claims that NFs act as Ca^{+2} chelators. They have multiple Ca^{+2}-binding sites. Thus, by scavengering the toxic amount of Ca^{+2} that accumulated due

to glutamate excitotoxicity, the motor neurons are rescued. Accordingly, overexpression of Ca^{+2}-binding-protein calbindin D_{28K} was recently reported to confer protection against death of cultured motor neurons expressing mutant SOD1 (Clumskey *et al.*, 2001). The second hypothesis, regarding the protective effect of overexpression of NF-H, emphasizes the role of acting as a sink for ROS. This way, the destructive effects of these toxic molecules are reduced (Couillard-Despres *et al.*, 1998). However, no definite change in pattern or amount of protein-bound neurotoxicity has been detected so far.

While SOD1-mediated disease is alleviated by NF-L knockout and NF-H transgenic mice, the expression of NF-H-β-galactosidase, a fusion protein that causes the trapping of NFs in neuronal perikarya in $SOD1^{G37R}$ mice (Williamson *et al.*, 1998), exhibited no beneficial effect in terms of lifespan. Such an apparent discrepancy may be due to the very high levels of mutant SOD1 in mice lines used for these studies. These lines overexpress $SOD1^{G37R}$ by 12 folds, whereas the mice for the NF-H studies overexpress the mutant SOD1 by 5 folds (Julien *et al.*, 2000). This assumption has been supported by a recent experiment, conducted with $NF\text{-}H^{43}$ and $NF\text{-}H^{44}$ (Couillard-Despres *et al.*, 1998). $NF\text{-}H^{43}$, which codes for 43 Lys-Ser-Pro (KSP) phosporylation sites causes more perikaryal accumulations and depletion of axonal conductivity, while $NF\text{-}H^{44}$, which codes for 44 KSP repeats, have less perikaryal swelling and more axonal NFs. The increased synthesis of $NF\text{-}H^{43}$ has more beneficial effects than $NF\text{-}H^{44}$. This difference can be explained by the specific localization of the accumulations.

Thus, it can be concluded that maximum slowing of disease, or increasing the lifespan can be achieved with marked reduction in axonal NF and increased perikaryal levels of both NF and all three subtypes, especially NF-H. However, it should be noted that protection is possible only when mutant SOD1 proteins are expressed at moderate levels.

1.6.4. Protein Aggregates

Aggregates, common findings for many neurodegenerative diseases, are believed to be important contributors to ALS pathology: all examples of SOD1 mutant mice develop prominent, cytoplasmic, intracellular inclusions in motor neurons (Brujin et al., 1998). Such formations are present in both FALS, which results from mutations in SOD1, and SALS.

Recently, in a mouse model, using $SOD1^{G93A}$, it has been shown that the aggregation of SOD1 into high molecular weight, insoluble protein complexes (IPC) is an early event in pathology (Johnston *et al.*, 2000). These IPCs are sequestered into inclusion bodies, resembling aggresomes. Aggresomes can be assembled by the retrograde transport of a variety of mutant cytosolic proteins on microtubules. These aggregates are substrates for dyain-mediated transport. So, the increasing burden of IPCs can interfere with microtubule-dependent axonal transport of other substances, which are necessary for viability (Figure 1.15). Such a hypothesis is in concordance with the evidence of defects in slow transport in mutant SOD1 mice (Julien, 2001).

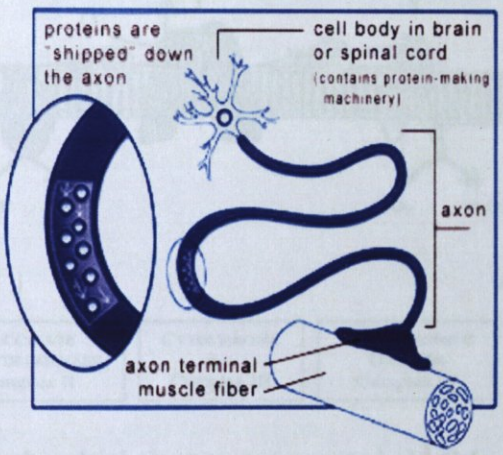

Figure 1.15. Formation of aggregates in ALS

Also, other mechanisms can contribute to toxicity of SOD1 aggregates. Toxicity can arise either as a result of the aberrant chemistry mediated by misfolded aggregated mutants or of the loss of essential components, such as protein degradation machinery or protein folding chaperons, by coprecipitation (Selverstone *et al.*, 2002).

1.6.5. Mitochondrial involvement

Mitochondria are powerhouses of cells due to their ATP-producing ability. They are also involved in buffering of intracellular Ca^{+2} and triggering apoptosis (Menzies *et al.*, 2002). Motor neurons are in need of constant energy in order to produce action potentials. Thus, they are significantly affected by any perturbations in mitochondrial energy production.

ATP production is achieved via the creation of an electrochemical gradient across the mitochondrial inner membrane, which is essentially impermeable (Figure 1.16). The gradient is established by pumping of the H^+ ion through the protein complexes, located at the inner membrane. NADH or FADH donate electrons to complex I or II, respectively. Next, they are passed through a series of redox reactions that provide the energy for the transfer of H ions across the membrane. The final electron acceptor is O_2, which is then reduced to form H_2O. The H^+ gradient created in this process drives the synthesis of ATP via complex V (Menzies *et al.*, 2002).

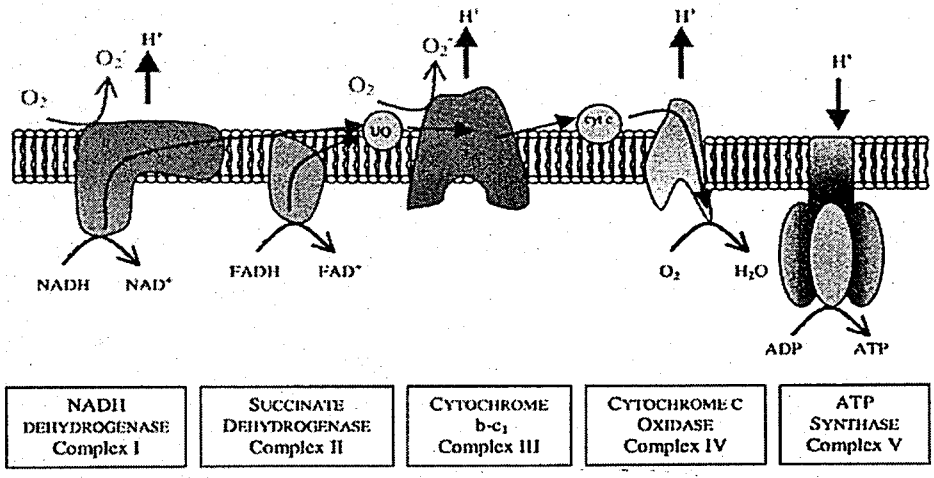

Figure 1.16. Mitochondrial electron transport chain (Menzies *et al.*, 2002)

Mitochondria have their own superoxide dismutase enzyme, SOD2, located at the mitochondrial matrix (Zelko *et al.*, 2002). So far, no mutation in SOD2 has been reported in ALS. Also, SOD1 is located at the intermembrane of the organelle, in order to remove the superoxide released into this space, by the ubisemiquinone anion (Okado-Matsumoto *et al.*, 2002). Since SOD2 cannot reach this space, the presence of SOD1 is important for mitochondria.

Mitochondria are vulnerable to oxidative stress. In ALS cases, the measurement of the activities of the mitochondrial electron transfer chain complex II and complex IV show a significant decrease compared to normal, whereas the activity levels for complex I and complex III are not significantly altered. Such an inhibition is accompanied by changes in morphology and cellular energy metabolism (Menzies *et al.*, 2002).

Mitochondrial DNA (mtDNA) is very sensitive to free radical damage due to its close localization to a major site of free radical production, its lack of protective histones and less effective repair mechanisms compared to nuclear DNA (Menzies *et al.*, 2002). In this respect, a so-called 'common' deletion of 4977 bp in mtDNA has been shown to be present in the motor cortex in 11-fold higher concentration with respect to controls (Dhaliwal *et al.*., 2000). Also, mutant SOD1 locates abundantly to the external side of the outer mitochondrial membrane, where it may interfere with the trafficking of substrates and ions between mitochondria and other cellular components and import of proteins from the cytosol.

The involvement of mitochondria in ALS pathogenesis can be hypothesized depending on the findings in changes in morphology and function of the organelle. These include conglomerate of dark abnormal mitochondria in anterior horn motor neurons of SALS patients, the presence of mitochondrially-derived vacuoles in axons and dentrites, increased O_2^- radical damage in motor neurons and a selective deficiency of NADH:CoQ oxidoreductase in skeletal muscles of SALS patients (Vielhaber *et al.*, 2000). Recently, creatine, which improves cellular energy stores and maintains ATP level, was shown to have a neuroprotective effect in $SOD1^{G93A}$ mice (Klivenyi *et al.*, 1999). This hypothesis proposes that decline in energy production may result in increase sensitivity of neurons to glutamate until a point is reached, where normal glutamate causes an excitotoxic effect. Reduction in ATP level will aggravate this situation until a certain point where decrease in cellular ATP level can no longer be tolerated by the neuron. The neuron cannot continue to maintain its membrane potential, since the ion transporters responsible for stabilizing the membrane potential are ATP-dependent. This depolarization activates NMDA receptors, causing an influx of Ca^{+2} and Na^+ into the cell (Figure 1.17). Such an influx will have multiple consequences. First of all, it will depolarize the cell to a greater extent. Secondly, excess Ca^{+2} would be more toxic for the cell, thus should be removed. However, the influx of Na^+ would impair the function of Na^+-Ca^{+2} transporters. Therefore, Ca^{+2} can only be removed via ATP-dependent transporters, which results in the draining of more ATP supplies from the cell. Also, increased levels of Ca^{+2} activate cellular proteases, lipases and endonucleases. This triggers further production of free radicals and leads to oxidative damage in the cell. Oxidative stress-mediated mitochondrial dysfunction also provokes the release of cytochrome c to the cytosol, which activates caspase cascade, leading to apoptosis (Menzies *et al.*, 2002).

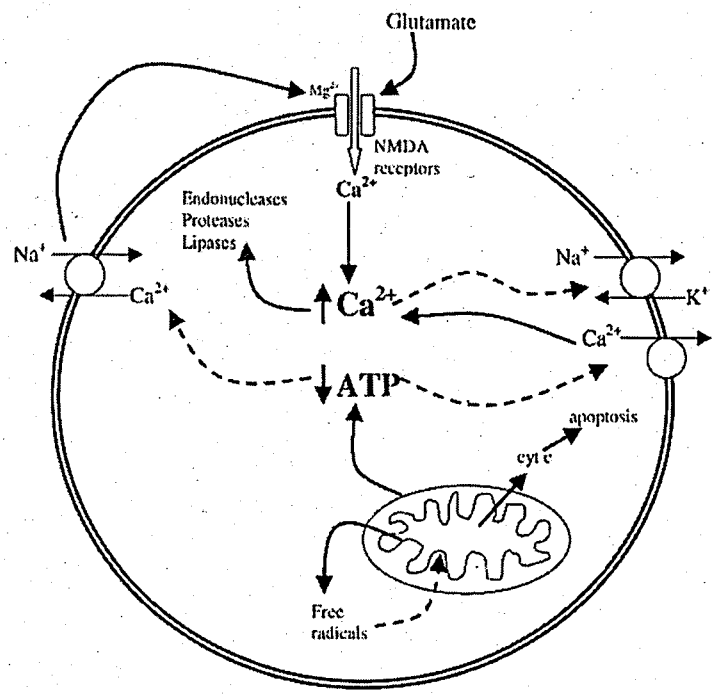

Figure 1.17. Mitochondrial involvement in energy-linked excitotoxicity. Solid arrows represent causative or activating damage. Dashed arrows represent inhibitory effects (Menzies *et al.*, 2002)

1.6.6. Other Possible Contributors to ALS Pathology

The presence of other factors has been proposed in search of a possible mechanism that can explain remaining FALS and SALS cases.

1.6.6.1. Environmental Causes

Exposure to heavy metals, such as aluminum and mercury, has been considered in the context of ALS. The starting point was the detection of high incidence of ALS in Guam population, where dietary habits were shown to include food toxins like those found in the cycad plants. However, the recent decline in incidence of PD-ALS attributed to dietary habits suggests a strong environmental basis with a limited genetic influence (Figlewicz *et al.*, 1994).

1.6.6.2. Autoimmunity: In spinal cords of ALS patients, activated microglia and T cells were detected. These patients had IgG antibodies against motor neurons. Similarly, in SALS patients, antibodies against voltage-gated Ca^{+2} channels were observed, which can alter the function and activate Ca^{+2} influx (Appel *et al.*, 1993). However, immunotherapy has not been effective in any ALS patients. Also, recent experiments have revealed controversial results (Hand *et al.*, 2002).

1.6.6.3. Viral Infections: Chronic viral infection is another hypothesis proposed to explain the etiology of ALS, especially for SALS cases (Rowland *et al.*, 2001). Berger et al detected enterovirus RNA in the spinal cords of some ALS patients (Berger *et al.*, 2000), but that observation has not been confirmed (Walker *et al.*, 2001). ALS was reported in a small group of HIV patients, which was not statistically significant. Recently, a very sensitive technique helped to detect enterovirus RNA sequences in over 88% of ALS spinal cords compared to 3% of normals (Hand *et al.*, 2002). Furthermore, reverse transcriptase activity not attributable to known retroviruses was discovered in a significant proportion of ALS patients, so further work is required to determine the source of the activity (Rowland *et al.*, 2001).

1.7. ALS in Turkey

There is no known cure for ALS, and there are few effective pharmacological interventions for the disease; unfortunately, clinically beneficial treatment remains limited. Considering these disease characteristics, physchosocial and genetic interventions might, at present, be the most promising means by which the ALS patients can be assisted.

In 2002, the ALS-Motor Neuron Disease Association of Turkey (ALS-MNH) was established by a group of neurologists and ALS patients. The main purpose of the association is to gather both the sufferers (patients) and the caregivers (family members and neurologists) under the same roof for a fast and efficient exchange of recent clinical findings and caregiving strategies. It also aims to arise the attention of the community to ALS. In this respect, an informative web site (www.als.org.tr) has been constructed. This site includes general information on ALS, caregiving strategies, recent publications and a platform for patients and their family members to share their feelings and problems. Also, 3-month periodicals are published and posted to members of the association regularly.

The expected number of ALS patients in Turkey is between 3500-5000. There are several specialized centers, e.g. Neurology Departments of Medical Schools of Hacettepe, Istanbul, Cerrahpaşa and Çukurova Universities, in which clinical diagnosis, primary care and treatment, as well as psychological support is given to ALS patients. However, to the best of our knowledge, thus far, no research has been conducted, regarding the molecular analysis and genetics of ALS in Turkey.

II. PURPOSE

In the framework of this thesis, 41 Turkish patients with ALS were referred to our unit for SOD1 gene analysis. Two of these patients were consanguineous; the remaining 39 were individual cases. Only a few SALS patients were described thus far, who carry a SOD1 gene mutation; however, the mainstream experimental strategy in the investigation of SALS patients does not differ from FALS patients and starts with the analysis of the SOD1 gene mutations.

The aim of this study was:

- to establish the methods used in the molecular analysis of the SOD1 gene in our laboratory, and
- to apply these methods to a group of Turkish ALS patients.

3. MATERIALS

3.1. Blood Samples

3.1.1. Blood Samples of ALS Patients

A total of 41 ALS patients were analyzed in the framework of this thesis. Two of these patients were siblings, the remaining 39 were individual cases. Blood samples of 26 ALS patients were provided from the Department of Neurology at Çukurova University, seven from İzmir SSK Education Hospital, five from the Department of Neurology at İstanbul University, Çapa Medical School, two from Şişli Etfal Hospital and one from Bakırköy State Hospital for Psychiatric and Neurological Diseases.

3.1.2. Blood Samples of Normal Individuals

DNA samples of 109 apparently healthy Turkish individuals were used as negative controls in the analysis of intron 3 polymorphism. Additionally, blood samples of family members of two patients were collected for the same purpose.

3.2. Oligonucleotide Primers

3.2.1. Primers for PCR Amplification and DNA Sequencing

The primer sequences for the amplification of all five exons of the SOD1 gene were as described in Deng *et al.*, 1993. They were purchased from Rafigen, İstanbul. The sequences of the primers used in the framework of this thesis are listed in Table 3.1. The primers used also for DNA sequencing are shown in bold.

3.3. Enzymes

Taq DNA polymerase : 5 U/µl, (Promega, USA)

Hind6I : 10 U/µl, (Promega, USA)

Buffer Y^+/Tango : 10X Enzyme Buffer for Hind6I
Promega, USA

Table 3.1. Oligonucleotide primer pairs used in PCR and DNA sequencing

Primer Name	**Primer Sequence**
Exon 1 Forward	F-5′ TTC CGT TGC AGT CCT CGG AAC
Exon 1 Reverse	R-5′ CGG CCT CGC AAA CAA GCC T
Exon 2 Forward	F-5′ TTC AGA AAC TCT CTC CAA CTT
Exon 2 Reverse	R-5′ ACG TTA GGG GCT ACT CTA GT
Exon 3 Forward	**F-5′ TGG GAA CTT TAA TTC ATA ATT**
Exon 3 Reverse	**R-5′ AGT ATA CCA TAT GAA CTC CA**
Exon 4 Forward	F-5′ CAT CAG CCC TAA TCC ATC TGA
Exon 4 Reverse	R-5′ CCG ACT AAC AAT CAA AGT GA
Exon 5 Forward	F-5′ AGT GAT TTAC TTG ACA GCC CA
Exon 5 Reverse	R-5′ TTC TAC AGC TAG CAG GAT AAC A

3.4. Buffers and Solutions

3.4.1. DNA Extraction

Lysis Buffer : 155 mM NH_4Cl
10 mM $KHCO_3$
1 mM Na_2EDTA (pH 7.4)

Nuclease Buffer : 10 mM Tris-HCl (pH 8.0)
400 mM NaCl
2 mM Na_2EDTA (pH 7.4)

Sodiumdodecylsulphate (SDS) : 10 per cent SDS (w/v) (pH 7.2)

Proteinase K	:	20 mg/ml in H_2O
Sodium Chloride (NaCl)	:	5 M saturated stock solution
Ethanol	:	Absolute Ethanol Riedel de-Häen, Germany
TE Buffer	:	20 mM Tris-HCl (pH 8.0) 1 mM Na_2EDTA (pH 8.0)

3.4.2. Polymerase Chain Reaction (PCR)

10 X $MgCl_2$ Free Buffer	:	100 mM Tris-HCl 500 mM KCl Promega, USA
$MgCl_2$	:	25 mM in dH_2O Promega, USA
Deoxyribonucleotides (dNTP)	:	100 mM of each dNTP Promega, USA

3.4.3. Agarose Gel Eletrophoresis

10 X TBE Buffer	:	0.89 M Tris-Base 0.89 M Boric acid 20 mM Na_2EDTA (pH 8.3)
Ethidium Bromide (EtBr)	:	10 mg/ml
1 or 2 per cent Agarose Gel	:	1 or 2 per cent Agarose in 0.5X TBE Buffer, containing 0.5 µg/ml Ethidium Bromide

10 X Loading Buffer	:	2.5 mg/ml Bromophenol Blue (BPB) 1 per cent SDS in glycerol Promega, USA
DNA Ladder	:	100 bp Promega, USA 50 bp MBI Fermentas, Lithunia

3.4.4. Polyacrylamide Gel Electrophoresis

25 per cent Acrylamide Stock	:	24.5 per cent Acrylamide 0.5 per cent N, N'-methylenebisacrylamide
Ammonium Peroxodisulfate	:	10 per cent APS (w/v) in dH_2O
TEMED	:	Sigma, USA
SSCP Loading Dye	:	10 ml Formamide 200 µl 0.5 M EDTA (pH 8) 15 mg Xylene Cyanole 3 mg Bromophenol Blue

3.4.5. Silver-staining

Ethanol	:	10% Ethanol (Riedel de-Häen, Germany)
Nitric Acid (HNO_3)	:	1 per cent HNO_3 Atabay Chemicals, Türkiye
Silver Nitrate ($AgNO_3$)	: :	0.2 per cent $AgNO_3$ in dH_2O Merck, Germany

Formaldehyde (40 per cent)	:	40 per cent (w/v) Carlo Erba Reagent, Spain
Sodium Carbonate (Na_2CO_3)	:	3 per cent in dH_2O Merck, Germany
Acetic acid (CH_3COOH)	:	10 per cent CH_3COOH Merck, Germany

3.5. Equipment

Autoclave	:	Model MAC-601 Eyela, Japan
Balances	:	Electronic Balance Model VA124 Gec Avery, UK Electronic Balance Model CC081 Gec Avery, UK
Centrifuges	:	Centrifuge 5415C Eppendorf, Germany Universal 16R Hettich, Germany
Deep Freezers (-20°C)	:	Bosch, Germany
Documentation System	:	BioDoc Video Documentation System Biometra, Germany
Electrophoretic Equipments	:	Horizon 58, Model 200 BRL, USA Multigel-Long Biometra, Germany

Gel Drier	:	Biometra, USA Easy Breeze Drying Frame, Hoefer Scientific Instruments, Germany
Heat Blocks	:	Thermostat Heater 5320 Eppendorf, Germany
Magnetic Stirrer	:	Chiltern Hotplate Magnetic Stirrer, HS31 UK
Ovens	:	Microwave Oven Vestel, Türkiye EN400 Nuve, Türkiye 56°C LEEC, UK
Power Supplies	:	ECPS 3000/150 Constant Power Supply Pharmacia, Sweden Model 200 BRL, USA
Refrigerator	:	4°C Medicool Sanyo, Japan
Spectrophotometer	:	CE 5502 Scanning Double Beam 5000 Series CECIL Elegant Technology, UK
Thermocyclers	:	Techne Progene, UK Techne Gradient Progene, UK

Water Purification	:	Millipore, USA

4. METHODS

4.1. Analysis of Genomic DNA

4.1.1. DNA Extraction from White Blood Cells

In order to extract genomic DNA from white blood cells (leukocytes), 10 ml of peripheral blood samples were collected in anticoagulant (K_2EDTA) containing tubes. First, 30 ml cold lysis buffer was added on the blood samples to lyse the membrane of leukocytes. After incubation of the samples at 4°C for 15 minutes, they were centrifuged at 5K for 10 minutes at 4°C. This results in the precipitation of the cellular nuclei. After discarding the supernatant, the pellet was resuspended in 10 ml cold lysis buffer and centrifuged again at 5K for 10 minutes at 4°C. The nuclear pellet was resuspended in lysis buffer and centrifuged at the same conditions until a clean pellet was obtained. In order to lyse the nuclear envelope, the nuclear pellet was resuspended in nuclease buffer. The nuclear proteins were degraded by treating the suspension with proteinase K (150 µg/ml) and SDS (0.14 per cent). Then, the suspension was incubated at either 37°C for overnight, or 56°C for three hours. Following the incubation, the degraded proteins were precipitated by treating the suspension with 5 ml of cold distilled water and 5 ml of 5M NaCl solution. The tubes were shaken vigorously and centrifuged at 5K for 20 minutes at room temperature, which resulted in the precipitation of proteins in the pellet. The supernatant, containing the genomic DNA, was transferred into a new Falcon tube. In order to precipitate the DNA, two volumes of cold absolute ethanol were added to the supernatant. The tubes were gently inverted until DNA threads were visible. By fishing out with a pipette, the DNA was quickly taken into an eppendorf tube and left to dry. When all ethanol was evaporated, the DNA was dissolved in TE buffer and stored at 4°C.

4.1.2. Analysis by Agarose Gel Electrophoresis

Genomic DNA was analyzed on 1 per cent agarose gel, which was prepared by dissolving 1 g of agarose in 100 ml 0.5X TBE buffer. Agarose was dissolved in TBE buffer by boiling it in a microwave. EtBr, which intercalates into DNA and thus enables its

visualization under UV light, was added into the agarose solution with a final concentration of 0.5 μg/ml. Then, the homogenous mixture was poured onto an electrophoresis plate and left to polymerize at room temperature. For loading, DNA samples were mixed with 10X loading dye to a final concentration of 1X. The gel was placed into an electrophoresis tank, containing 0.5X TBE and run at 150 volt for 15-20 minutes, depending on the size of the expected fragment. When migration was completed, the gels were visualized under UV light and documented.

4.1.3. Analysis by Spectrophotometer

The DNA concentration was also measured by a spectrophotometer. 50 μg of double stranded DNA has an absorbance of 1.0 at 260 nm (OD_{260}). Each sample was diluted to a factor and the absorbance was read at 260 nm in a spectrophotometer. The concentration was calculated by the following equation:

50 μg/ml x OD_{260} x dilution factor = concentration of genomic DNA (μg/ml)

4.2. Investigation of Mutations

4.2.1. PCR Amplifications of Exons

Five exons of the SOD1 gene were amplified by specific primers (Table 3.1). Each PCR reaction was prepared in a 25μl volume, containing 1X Mg^{+2} free reaction buffer, 2 or 2.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer, 1 unit of Taq polymerase and 50 ng of genomic DNA at exon-specific conditions shown in table 4.1.

The amplifications were done by the following PCR programme:

94°C for 4 minutes
94°C for 30 seconds (denaturation)
52.2- 62°C for 30 seconds (annealing) } 32 cycles
72°C for 30 seconds (extension)
72°C for 8 minutes

The PCR products were run on two per cent agarose gels. A 5 µl aliquot of each product was mixed with 5 µl 1X loading dye and run at 150 volt with a 100 bp DNA ladder for 15-20 minutes. The gels were visualized under UV light and documented.

Table 4.1. PCR conditions for each exon of the SOD1 gene

Exon Number	Annealing Temperature (°C)	[$Mgcl_2$]
1	62.0	2.5 mM
2	52.2	2 mM
3	54.5	2.5 mM
4	59.4	2 mM
5	57.3	2 mM

4.2.2. Single Strand Conformational Analysis (SSCP)

4.2.2.1. Preparation of Polyacrylamide Gels: The glass plates were cleaned with 70 per cent ethanol. They were assembled by clamps, placed on both sides, and 0.75 mm spacers in-between. Each gel was prepared by mixing 20 ml 12.5 or 15 per cent SSCP solution with 200 µl APS and 15 ml TEMED and poured between the glass plates. A 20-well comb was inserted and the gel was left to polymerase at room temperature for 40-45 minutes.

4.2.2.2. SSCP Gel Electrophoresis: For all exons, the samples were run with positive controls, which had been obtained from Northwestern University Medical School Neuromuscular Disease Division Laboratories, Chicago, USA. The 'identities' of the positive controls are shown in Table 4.2.

The samples were prepared by mixing 5 µl PCR products with 5 µl SSCP loading dye. The products were denaturated at 95°C for 10 minutes and kept in ice until loading. From each denaturated sample, 10 µl aliquots were run at 4 °C at specific conditions listed in Table 4.3.

Table 4.2. The 'identities' of the positive controls used in SSCP gel electrophoresis

Exon	Change in nucleotide	Change in amino acid
Pos1A	GCC→ CTC	Codon 4 Ala→ Val
Pos1B	GCC→ ACC	Codon 4 Ala→ Thr
Pos1C	GTG→ ATG	Codon 14 Val→ Met
Pos1D	GGC→ GCC	Codon 16 Gly→ Ala
Pos1E	CAG→ CTG	Codon 22 Gln→ Leu
Pos2A	GGC→ GAC	Codon 41 Gly→ Asp
Pos2B	CAT→ CGT	Codon 46 His→ Arg
Pos2C	CAT→ CGT	Codon 48 His→ Arg
Pos3A	Hetero (IVS-34)	Intron 3 A→ C
Pos3B	Homo	Intronic
Pos4A	TTG→ GTG	Codon 84 Leu→ Val
Pos4B	GGC→ CGC	Codon 85 Gly→ Arg
Pos4C	GGT→ GCT	Codon 93 Gly→ Ala
Pos4D	GAA→ GGA	Codon 100 Glu→ Gly
Pos4F	ATT→ ACT	Codon 113 Ile→ Thr
$Pos4G_1$	GAC→ GCC	Codon 90 Asp→ Ala
$Pos4G_2$	GAC→ GCC	
Pos4H	GAA→ AAA	Codon 100 Glu→ Lys
Pos4I	GGT→ GAT	Codon 93 Gly→ Asp
Pos4J	TTG→ TTC	Codon 84 Leu→ Phe
Pos4K	GTG→ ATG	Codon 97 Val→ Met
Pos4L	GTG→ CTG	Codon 118 Val→ Leu
Pos4M	GTG→ ATG	Codon 87 Val→ Met
Pos4O	GGT→ TGC	Codon 93 Gly→ Cys
Pos5A	GTA→ GGA	Codon 148 Val → Gly
Pos5B	TTG→ TTC	Codon 144 Leu → Phe
Pos5C	TTG ..G	Codon 126 Leu → Stop

Table 4.3. Electrophoresis conditions for the SOD1 gene

Exon	Concentration	Running Condition
Pos1A, Pos1B, Pos1C, Pos1D, Pos1E	12.5 per cent	5W for 2h
Pos2A, Pos2B	12.5 per cent	6W for 2h
Pos2C	12.5 per cent	5W for 2h
Pos3A, Pos3B	12.5 per cent	2W for 3h15min
Pos4A, Pos4B, Pos4C, Pos4D, Pos4F, Pos4G$_1$, Pos4G$_2$, Pos4H, Pos4I, Pos4J, Pos4K, Pos4L, Pos4M	12.5 per cent	6W for 3h15min
Pos4O	12.5 per cent	3W for 3h
Pos5A, Pos5B	15 per cent	9 W for 2h30min

4.2.2.3. Silver-staining: After electrophoresis, the gel was taken out from the glass plates carefully. The staining protocol follows as: fixation for 10 minutes with 10 per cent ethanol, oxidation for 3 minutes with one per cent nitric acid solution, washing three times with distilled water for 1 minute, staining for 20 minutes with silver solution, washing two times with distilled water for 1 minute, developing with sodium carbonate solution until bands appear and stopping the staining with 10 per cent acetic acid. The gels were covered with wet cellophane sheets and dried at room temperature.

4.2.3. DNA Sequencing

Two samples, which showed aberrant migration pattern in SSCP analysis for exon 3, were subjected to DNA sequencing (Iontek, Istanbul). The results were obtained online as pdf files in chromatograms.

4.2.4. Restriction Enzyme Analysis

In order to confirm the polymorphism in intron 3, two patient samples, which had shown the same aberrant migration pattern in SSCP analysis, their family members and 109 Turkish control individuals were subjected to restriction enzyme analysis. Same set of primers were used. The region was amplified in 25 µl volume, containing 1X Mg^{+2} free

reaction buffer, 2.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer, 1 unit of Taq polymerase and 50 ng of genomic DNA. The PCR reaction was carried out in the following conditions:

94°C for 4 minutes
94°C for 30 seconds (denaturation)
54.5°C for 30 seconds (annealing) } 32 cycles
72°C for 30 seconds (extension)
72°C for 8 minutes

The amplified products were applied to a two per cent agarose gels with a 100 bp DNA ladder. The gel was allowed to run at 150 volt for 15 minutes and visualized under UV light.

R.E. digestion was carried out in 10 µl volume containing 1 µl 10X buffer, 1 unit of HhaI or Hind6I restriction enzyme (Table 4.4) and 8 µl of each PCR product. The tubes were placed in 37°C incubator for overnight. The samples were loaded to two per cent agarose gels with 50 bp ladder. Agarose gels were run at 150 volt for 15 minutes and visualized under UV light.

Table 4.4. Digestion site of Hind6I on intron 3 of SOD1 gene

Restriction Enzyme	**Wild-type Sequence**	**Mutant Sequence and Digestion Site**
HhaI	ATC AAT GGC GAT ACG TTT	ATC AAT GGC G↓**C**T ACG TTT
Hind6I	ATC AAT GGC GAT ACG TTT	ATC AAT GGC G↓**C**T ACG TTT

5. RESULTS

5.1. PCR Amplification of All Exons

All exons of the SOD1 gene of forty-one patients were amplified as described in 4.2.1. The size of each PCR product is shown in Table 5.1. The amplifications of the five exons are shown in Figure 5.1. through Figure 5.5.

Table 5.1. The size of PCR products

Exon Number	PCR Size (bp)
1	156
2	207
3	182
4	236
5	216

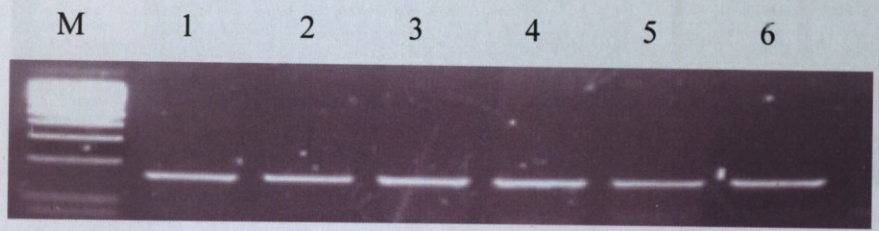

Figure 5.1. PCR amplification of exon 1. M: 100 bp ladder; lanes 1-6: ALS samples

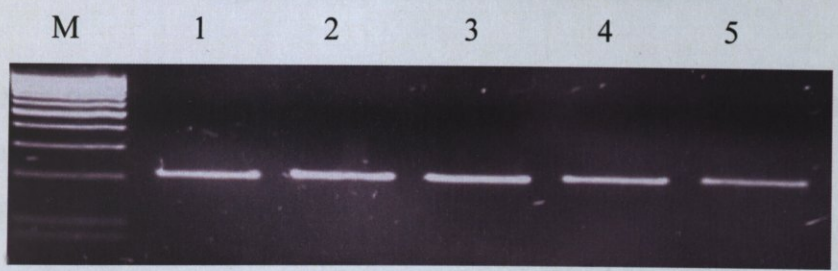

Figure 5.2. PCR amplification of exon 2. M: 100 bp ladder; lanes 1-5: ALS samples

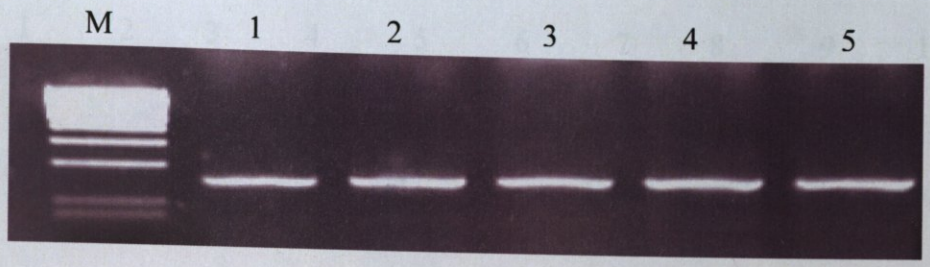

Figure 5.3. PCR amplification of exon 3. M: 100 bp ladder; lanes 1-5: ALS samples

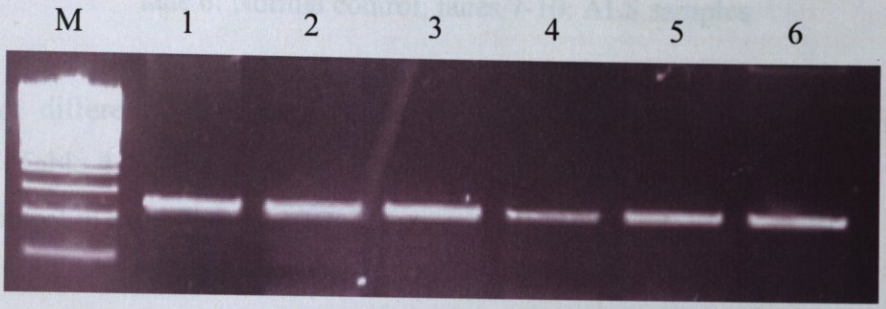

Figure 5.4. PCR amplification of exon 4. M: 100 bp ladder; lanes 1-6: ALS samples

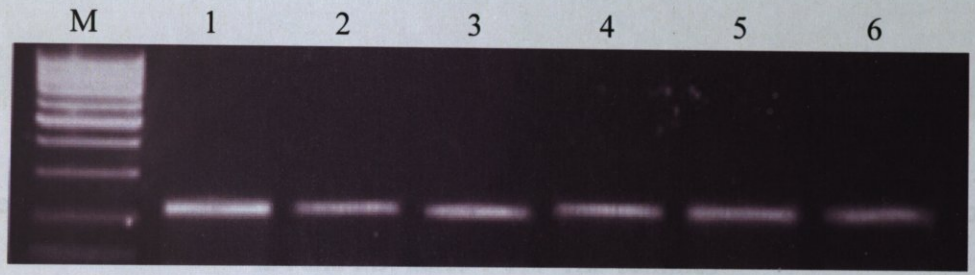

Figure 5.5. PCR amplification of exon 4. M: 100 bp ladder; lanes 1-6: ALS samples

5.2. Single Strand Conformational Analysis

For each patient, the PCR products of all exons were run on SSCP gels with one normal control; all positive controls, belonging to that exon, were also run on this gel. SSCP analysis of exon 1 is shown in Figure 5.6.

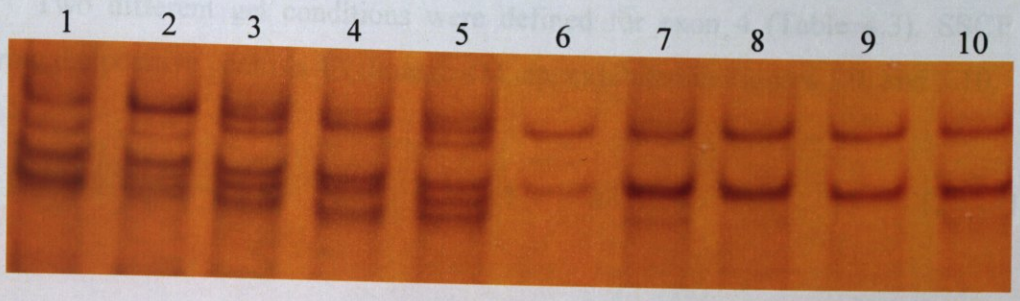

Figure 5.6. SSCP analysis of exon 1. Lanes 1-5: Positive controls 1A, 1B, 1C, 1D and 1E; lane 6: Normal control; lanes 7-10: ALS samples

Two different gel running conditions were defined for three different exon 2 mutations (Table 4.3). The positive controls 2A and 2B are shown in Figure 5.7. Another positive control in exon 2, named 2C, is shown in Figure 5.8.

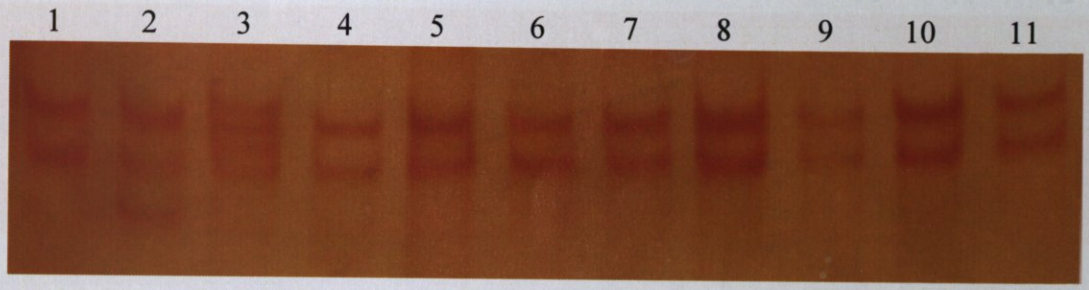

Figure 5.7. SSCP analysis of exon 2. Lane 1: Normal control; lanes 2-3: Positive controls 2A and 2B; lanes 4-11: ALS samples

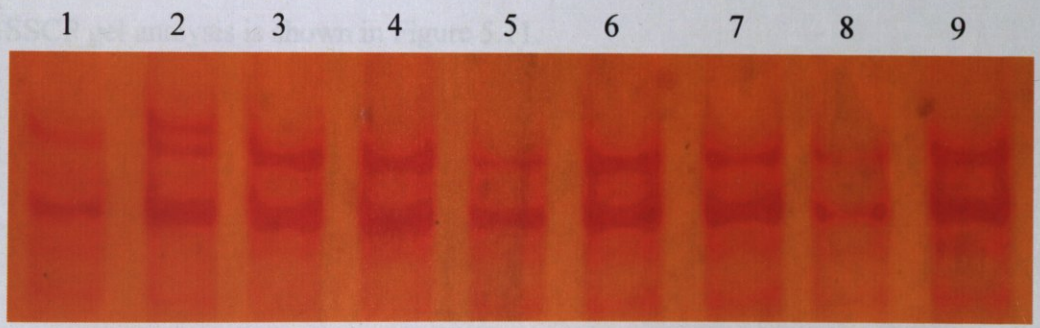

Figure 5.8. SSCP analysis of 2C. Lane 1: Normal control; lane 2: Positive control 2C; lanes 3-9: ALS samples

Two different gel conditions were defined for exon 4 (Table 4.3). SSCP gels, showing the positive controls 4A through 4M, are exhibited in Figures 5.9. and 5.10.

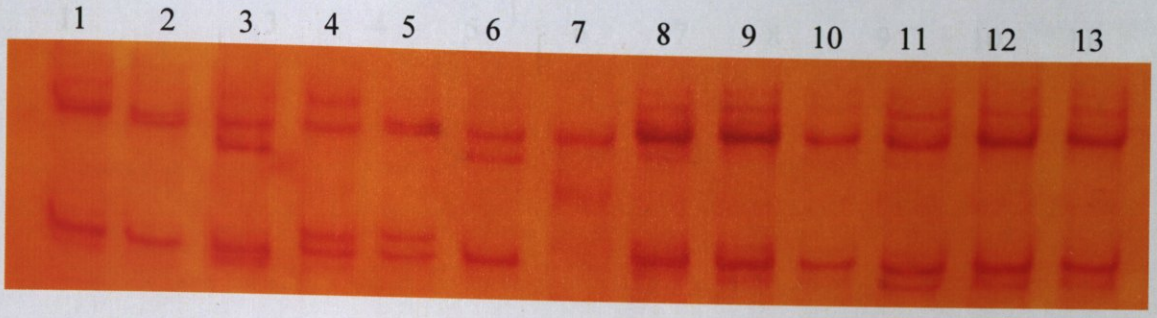

Figure 5.9. SSCP analysis of exon 4. Lane1: Normal control; lanes 2-7: Positive controls 4A, 4B, 4C, 4D, 4F and $4G_1$; lanes 8-13: ALS samples

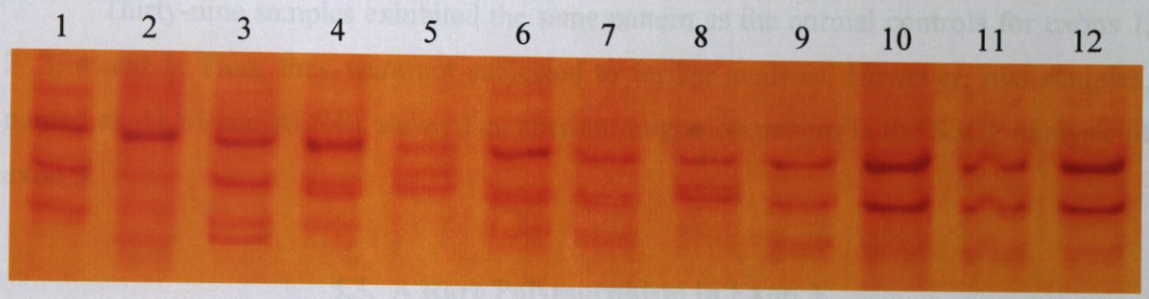

Figure 5.10. SSCP analysis of exon 4. Lane 1: Normal control; lanes 2-8: Positive controls $4G_2$, 4H, 4I, 4J, 4K, 4L and 4M; lanes 9-12: ALS samples

The last positive control in exon 4, 4O, was run at a different condition (Table 4.3). The SSCP gel analysis is shown in Figure 5.11.

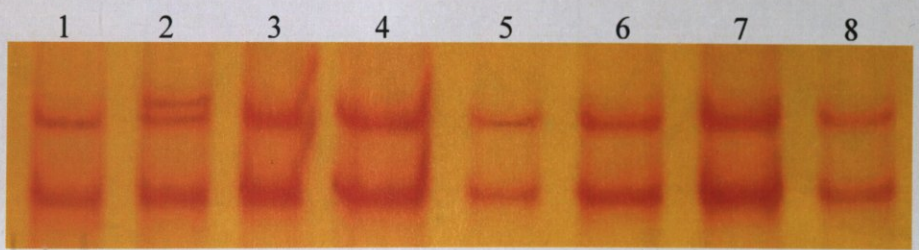

Figure 5.11. SSCP analysis of 4O. Lane 1: Normal control; lane 2: Positive control 4O; lanes 3-8: ALS samples

The samples were run on a 15 per cent SSCP gel for the detection of exon 5 mutations. In Figure 5.12., the SSCP analysis of exon 5 is exhibited.

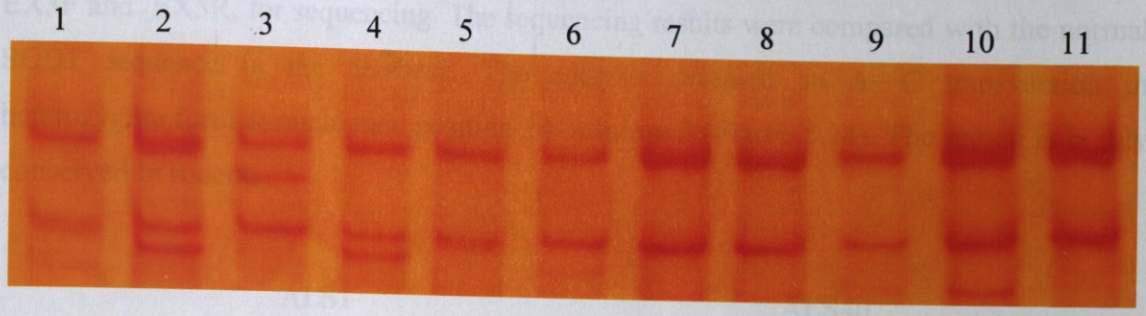

Figure 5.12. 15 per cent SSCP gel for exon 5. Lane1: Normal control; lanes 2-4: Positive controls 5A, 5B and 5C; lanes 5-11: ALS samples

Thirty-nine samples exhibited the same pattern as the normal controls for exons 1, 2, 3, 4 and 5. Thus, they were not subjected to further analysis. However, two samples, named as ALS1 and ALS40, showed an aberrant migration pattern in the SSCP analysis of exon 3.

5.3. A Rare Polymorphism in Exon 3

The SSCP analysis of ALS1 nad ALS40 patients revealed the same pattern as the positive control 3A. The corresponding gel is shown in Figure 5.13.; the migration shift is indicated by the arrow.

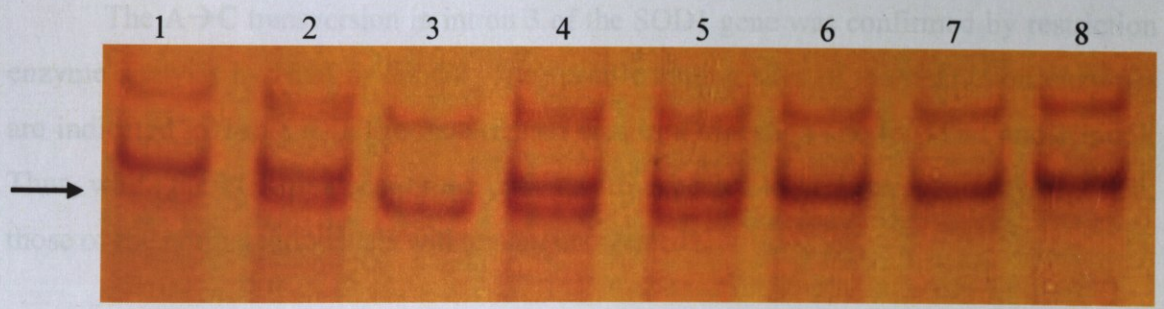

Figure 5.13. SSCP analysis of exon 3. Lane1: Normal control; lanes 2-3: Positive controls 3A and 3B; lane 4: ALS1; lane5: ALS40; lanes 6-8: ALS samples

5.4. Sequencing of Exon 3

Exon 3 of patients ALS1 and ALS40 was amplified with the same PCR primers, EX3F and EX3R, for sequencing. The sequencing results were compared with the normal SOD1 sequence in the database. The analysis revealed an A→C transversion in heterozygote form at nucleotide position 34 in intron 3 (Figure 5.14). The region is highly conserved in rodents.

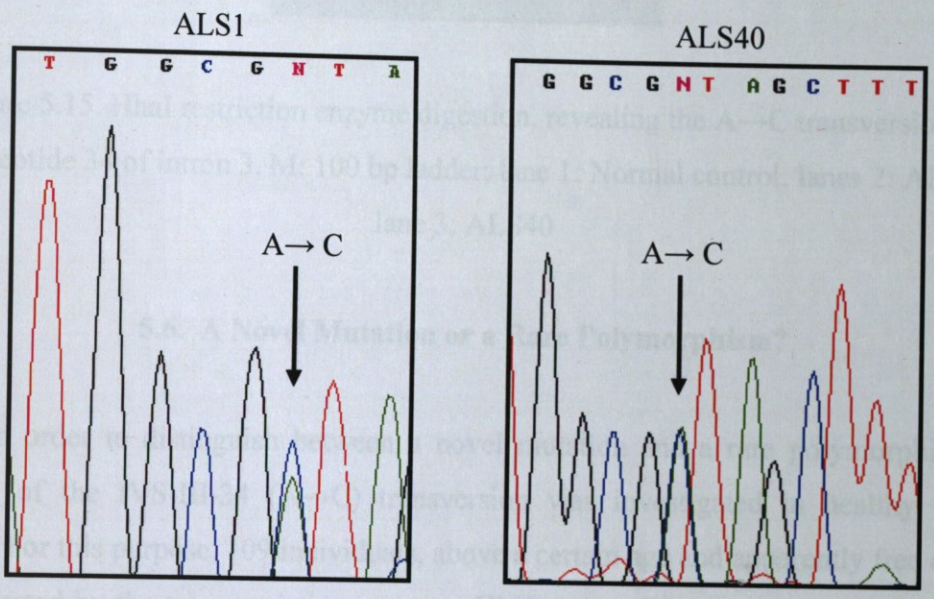

Figure 5.14. Chromatograms, showing the sequencing profiles of ALS1 and ALS40

5.5. Restriction Analysis of the Mutant Site

The A→C transversion in intron 3 of the SOD1 gene was confirmed by restriction enzyme analysis by HhaI or Hind6I. The specific cutting sites of the restriction enzymes are indicated in Table 4.4. The transversion creates a digestion site for HhaI and Hind6I. Thus, while the PCR products of the patients are expected to be heterogenously digested, those of the normal individuals will remain undigested.

Restriction of the PCR products of both patients with HhaI produced one undigested 182 bp fragment and two fragments of 154 bp and 28 bp, meaning that they are heterozygotes for this change (Figure 5.15).

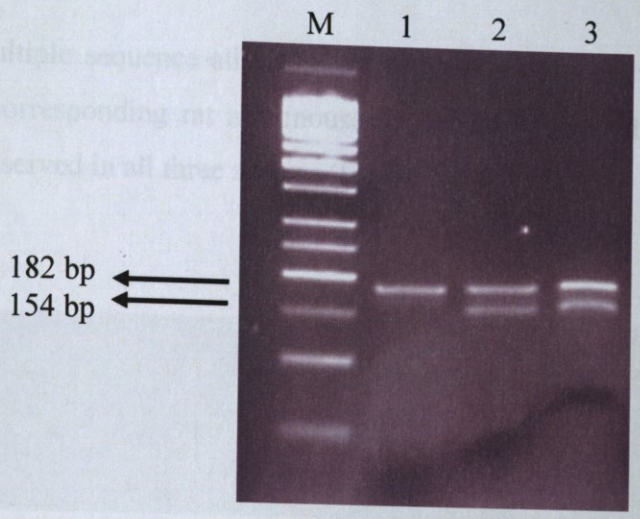

Figure 5.15. HhaI restriction enzyme digestion, revealing the A→C transversion in nucleotide 34 of intron 3. M: 100 bp ladder; lane 1: Normal control; lanes 2: ALS1; lane 3: ALS40

5.6. A Novel Mutation or a Rare Polymorphism?

In order to distinguish between a novel mutation and a rare polymorphism, the presence of the IVS-III-34 (A→C) transversion was investigated in healthy Turkish samples. For this purpose, 109 individuals, above a certain age and apparently free of ALS, were digested by the two restriction enzymes HhaI and/or Hind6I; none of them revealed the IVS-III-34 (A→C) transversion (Figure 5.16).

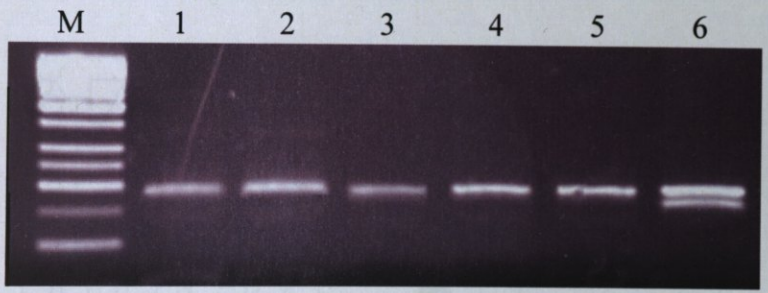

Figure 5.16. HhaI restriction enzyme analysis of healthy individuals. M: 50 bp ladder; lanes 1-5: Healthy samples; lane 6: ALS1

Multiple sequence alignment of this part of the human SOD1 intron 3 sequence with the corresponding rat and mouse sequences showed the position 34 (A→C) to be highly conserved in all three species (Figure 5.17).

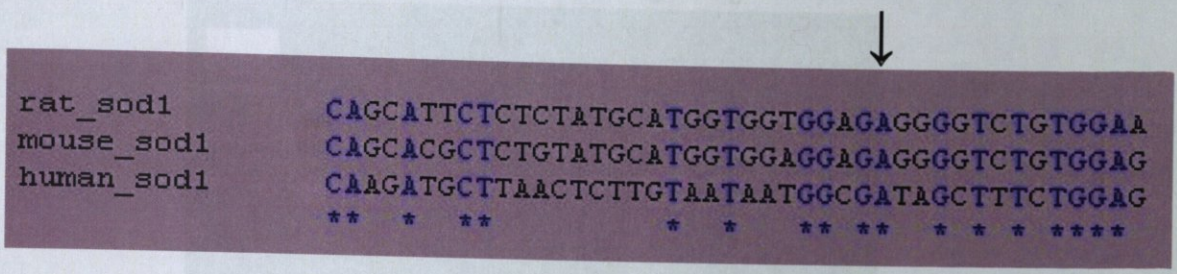

Figure 5.17. Multiple sequence alignment of a part of intron 3 nucleotide sequence. The nucleotide in interest is indicated by the arrow and the (*) shows the conserved regions.

These results, being indicative of a novel mutation, encouraged a further investigation on the family members of Patient ALS40 and Patient ALS1.

5.7. Investigation of ALS40 and ALS1 Families

ALS40: The family tree is shown in Figure 5.18. Blood samples were collected from six members, including two siblings and four children, designated in the family tree by the numbers 1-6. Informed consent was obtained.

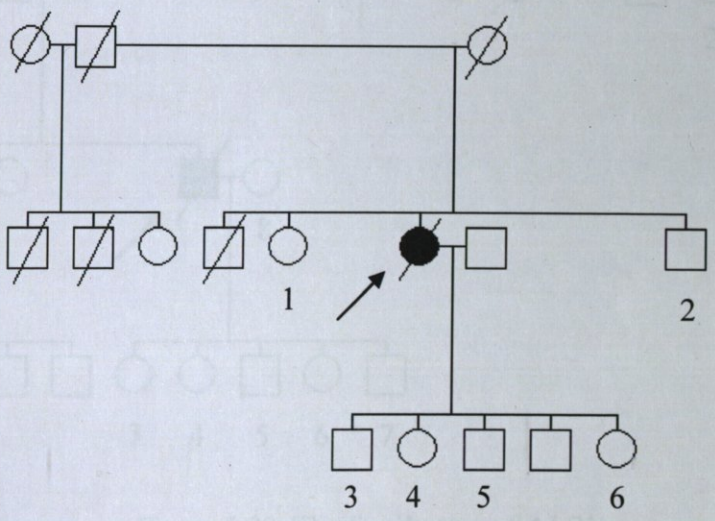

Figure 5.18. The family tree of ALS40

The restriction enzyme digestion results are shown in Figure 5.19. None of the family members revealed the specific nucleotide change.

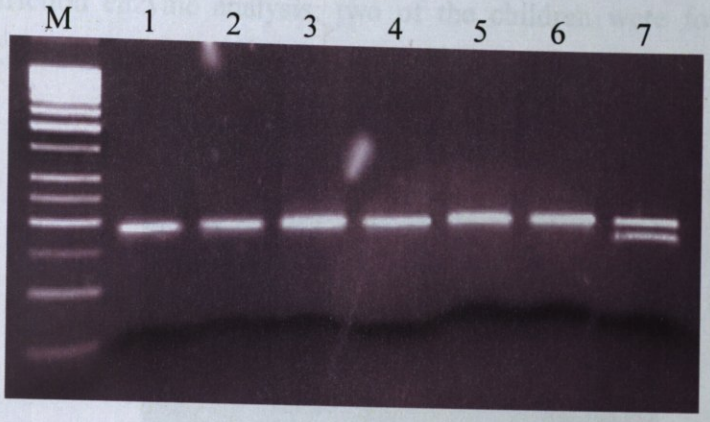

Figure 5.19. HhaI restriction enzyme digestion of the family members of ALS40. M: 50 bp ladder; lanes 1-6: The family members, as in order shown in Figure 5.18; lane 7: ALS40

ALS1: Blood samples were collected from eight individuals, designated by numbers 1-8 in the family tree (Figure 5.20). Informed consent was obtained.

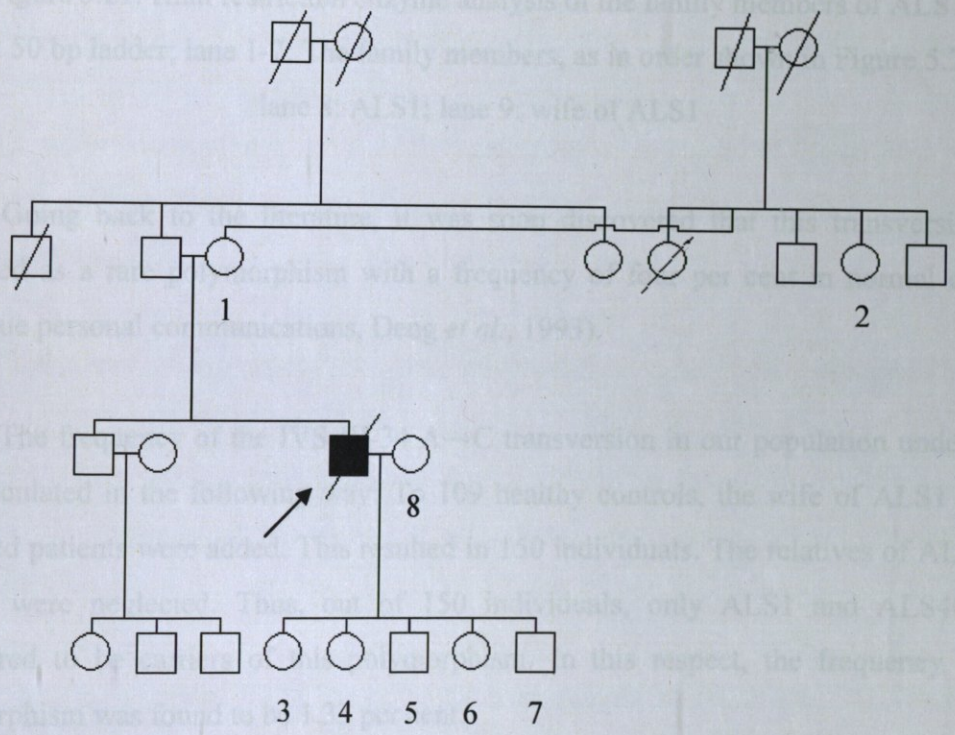

Figure 5.20. The family tree of ALS1

The analysis of the upper generation of patient ALS1 (mother and aunt) showed the presence of the IVS-III-34 A→C transversion in these two individuals, indicating a possible polymorphism (Figure 5.21). In the next step, the wife and five children were subjected to restriction enzyme analysis; two of the children were found to carry the IVS-III-34 A→C transversion.

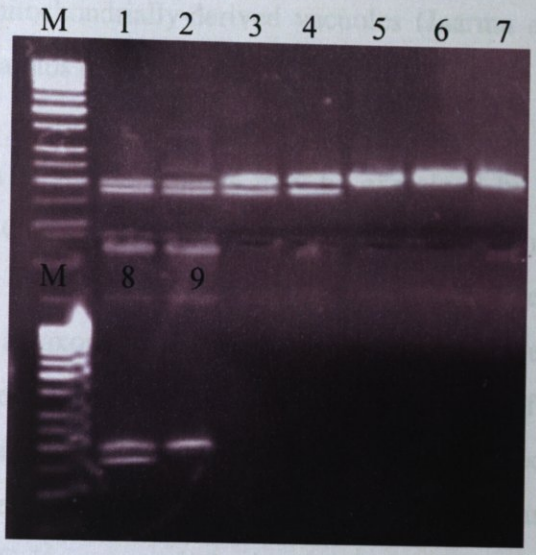

Figure 5.21. HhaI restriction enzyme analysis of the family members of ALS1. M: 50 bp ladder; lane 1-7: The family members, as in order shown in Figure 5.20.; lane 8: ALS1; lane 9: wife of ALS1

Going back to the literature, it was soon discovered that this transversion was described as a rare polymorphism with a frequency of four per cent in normal controls (Siddique personal communications, Deng *et al.*, 1993).

The frequency of the IVS-III-34 A→C transversion in our population under study was calculated in the following way: To 109 healthy controls, the wife of ALS1 and 40 unrelated patients were added. This resulted in 150 individuals. The relatives of ALS1 and ALS40 were neglected. Thus, out of 150 individuals, only ALS1 and ALS40 were considered to be carriers of this polymorphism. In this respect, the frequency of this polymorphism was found to be 1.33 per cent.

6. DISCUSSION

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease, predominantly initiated in mid-adult life and characterized by the selective death of motor neurons (Cleveland, 1999). The most predominant neuropathological changes are the appearance of abnormal mitochondrially-derived vacuoles (Jaarma *et al.*, 2001) and the fragmentation of Golgi apparatus (Stieber *et al.*, 2000).

The selective death of motor neurons may be explained by three reasons. First, Superoxide Dismutase 1 (SOD1) is abundantly produced in motor neurons and the amount increases with age (Brown *et al.*, 2001). Secondly, motor neurons require high levels of energy for activities, such as axonal transport. These cells may have axons of even one meter, which contain 99 per cent of the cell's cytoplasm. In case of any perturbation in mitochondria's energy production system, axonal transport or metabolic impairment, the cell will be seriously affected (Brown *et al.*, 2001). Thirdly, motor neurons are exclusively sensitive to high levels of Ca^{+2} upon excitotoxic stimulation. The cytosol has poor Ca^{+2}-buffering capacity, so the accumulation of Ca^{+2} cannot be dealt easily. In contrast, neurons, which are unaffected in ALS, such as the oculomotor nucleus or Onuf's nucleus, have Ca^{+2}-binding proteins, calbindin-D28K and parvalbumin (Cluskey *et al.*, 2001). In addition, motor neurons have more AMPA receptors with respect to dorsal horn neurons. In case of ALS, the composition of these receptors is altered and they become more permeable (Bär, 2000).

There was no consistent proposed mechanism, regarding the pathogenesis of the disease, until the major breakthrough in 1993, when a consortium of researchers discovered that approximately 20 per cent of autosomal dominant FALS cases were localized on chromosome 21, encoding Superoxide Dismutase 1 (SOD1), a very well-characterized enzyme that protects cells from damage by free radicals.

The major reasons for SOD1 being the predominant candidate gene for FALS are:

- its proximity to a FALS locus mapped to chromosome 21q22.1 in a subset of FALS families (Siddique *et al.*, 1993),
- decreased SOD1 activity in cerebrospinal fluid of some ALS patients (Siddique *et al.*, 1996),
- the important function of SOD1 in free radical homeostasis, and
- the apparent role of free radicals in neurodegeneration.

The underlying cause of ALS remains unknown. Although it comprises only 10% of cases, the familial form of ALS, and thus SOD1, is commonly used in genetic studies, basically due to the clinical and pathological similarities of FALS and SALS cases. Identification of FALS genes may lead to a greater understanding of the mechanisms of cell death in all forms of ALS.

The mechanism by which mutations in SOD1 brings about motor neuron injury is not yet totally understood, but is considered to be a toxic gain of function, rather than a loss of normal function. Two supporting findings are (i) the identification of both decreased and normal levels of SOD1 dismutase activity in the case of SOD1 mutations and (ii) the observation of ALS-like clinical phenotype in transgenic mice overexpressing FALS-linked mutations, while an unaffected clinical picture is present in mice overexpressing normal SOD1 (Gurnet *et al.*, 1997).

The combination of genetic, pathological and biochemical studies have brought out possible mechanisms that may provoke the disease or contribute to the disease:

- oxidative damage, an idea emerged after the discovery that the mutations in SOD1 are a primary cause in ALS,
- excitotoxic death due to glutamate,
- axonal strangulation from neurofilament disorganization, an idea supported by the abnormal accumulation of neurofilaments as a pathological feature in many SALS and SOD1-mediated FALS cases,
- toxicity due to intracellular protein aggregation and failure in protein folding and ubiquitination mechanisms, a common feature of SOD1-linked ALS cases, and

- the involvement of mitochondria, an idea dependent on the requirements of high energy levels of motor neurons and the neuropathological changes in mitochondria.

Being the predominant antioxidant in neurons, the disturbance in free radical homeostasis has been considered the major cause of mutations in the SOD1 gene. The insufficiency/deficiency of SOD1 in scavenging superoxide ions causes accumulating oxidative damage. So far, two oxidative pathways have been proposed: (i) the enhancement of hydroxyl radical production in a Fenton-like reaction, and (ii) the nitration of tyrosines using peroxynitrite. In both cases, mutations in SOD1 gene cause the destabilization of the protein. The opening of the active site and the entrance of H_2O_2 are observed, which result in an increase in the generation of hydroxyl radicals. Similarly, in the cases of the clumsy binding of Cu^{+2} (Kang *et al.*, 2000) and Zn^{+2} deficiencies (Elliott, 2001), SOD1 enhances the production of O_2^-, which then combines with NO to form peroxynitrite.

While confirming results have been obtained in oxidative damage hypothesis, contradictory experimental results are also present. First of all, similar evidence for hydroxyl radicals is not found in many transgenic mouse models at any stage of the disease. Also, vitamin E, an important antioxidant, does not appear to alter the outcome of the disease (Ahmed *et al.*, 2000). These results indicate that either different mutations produce disease via different mechanisms, or the enhanced peroxidase activity is not necessary for neuronal death. Similarly, the mechanism, regarding oxidative damage by the peroxynitrite reaction, is faced with contradictory experimental results. In a cell culture model, limiting NO synthesis by inhibition of NOS by 14-folds was predicted to ameliorate disease. However, neither the onset, nor the progression of the disease were altered (Cleveland *et al.*, 2001). Also, so far no mutations have been identified in any of the four residues that directly coordinate Zn^{+2}. Thus, the predominant role of oxidative damage in ALS pathogenesis should still be considered with caution (Elliott, 2001).

Glutamate-induced excitotoxicity has also been a favorably hypothesis after the detection of elevated levels of glutamate in cerebrospinal fluid of ALS patients. This has been explained by the partial loss of one of the excitatory amino acid transporters, named as EAAT2 (Cluskey *et al.*, 2001). In the absence of this transmitter, continuous activation

of the post-synaptic membrane, by high levels of glutamate, causes cell death via increased intracellular Ca^{+2} levels.

Considering this mechanism, riluzole has been proposed as an effective treatment. It inhibits synaptic glutamate release. It was shown to extend survival for a very modest time in transgenic mice models (Labomblez *et al.*, 1996). Unfortunately, in ALS patients, no apparent slowing in loss of strength or improvement in muscle function has been detected. Also, the absence of aberrant EAAT2 mRNA or loss of EAAT2 had not been detected in a subset of ALS patients, who had pathological confirmation of motor neuron loss. Similarly, these two transcripts were not found in $SOD1^{G37R}$ transgenic mice (Lin *et al.*, 1998). Thus, it can be concluded that aberrant EAAT2 transcripts are not the primary reason, but rather secondary to motor neuron degeneration or astrocyte response or both.

A key pathological finding in both SALS and FALS is the accumulation of neurofilaments in the soma and proximal axons of neurons. Neurofilaments are essential for the establishment of the proper structure. The discovery of a set of mutations in SALS patients (one per cent), but not in any controls, led to the assumption that damage to neurofilaments may play a role in ALS pathogenesis (Al-Chalabi *et al.*, 1999). Later, many mouse models, regarding all three subunits, have been constructed. The overexpression of any of the subunits results in the disassembly of the subunit stoichiometry, axonal atrophy and slowing of axonal transport (Kong *et al.*, 2000). However, increase in life span has been observed in mice carrying the overexpression of NF-H (Beauliea *et al.*, 2000). Thus, there is a thin line between the protective and detrimental effects of neurofilaments. At this point, it is not understood whether neurofilament accumulations are the cause or consequence of neuronal dysfunction. However, even if they are not capable of initiating the disease, they are at least important risk factors in some SALS cases.

The presence of cytoplasmic inclusions in all SOD1 mice arose the attention to intracellular protein aggregates (Brujin *et al.*, 1998). They appear before the onset of clinical symptoms. In some cases, they even are the first pathological findings and accumulate in abundance during progression (Cleveland *et al.*, 2001).

The possible involvement of mitochondria in ALS pathogenesis mainly depends on the morphological (Jaarma *et al.*, 2001) and functional changes in the organelle (Menzies *et al.*, 2002). The reduction in ATP-producing ability of the mitochondria is inversely correlated with glutamate-mediated excitation. The continuous depolarization of the cellular membrane and accumulation of Ca^{+2} result in neuronal death (Menzies *et al.*, 2002). In this respect, 'mitochondria-glutamate-induced excitotocity collaboration' seems to have a role in pathogenesis, rather than mitochondria being the predominant factor alone. Considering the loss in energy production, Klivenyi *et al.* tested creatine on transgenic mice to enhance the level of phosphocreatine and therefore provide a buffer for the energy level (Klivenyi *et al.*, 1999). An improvement in motor performance as well as survival time was reported. However, this study did not involve controls. Also, the proper dose in humans was not detected, and there had been reports of renal damage in case of use of large quantities (Rowland, 2000).

Today, in the concept of understanding the pathogenesis of ALS, it seems more probable that the death of motor neurons are the result of the convergence of the factors, here within stated as 'probable contributors'. However, it should be noted that the current knowledge of pathogenic mechanism of ALS is mostly based on studies with mutant SOD1, which is responsible for only two per cent of all ALS cases. For the vast majority of ALS patients, the primary cause of disease is unknown. Thus, there is a need to identify new genes associated with familial forms of ALS.

Linkage analysis in FALS is problematic due to the late onset and age-dependent penetrance of the disease and the relatively short survival of affected individuals. Thus, large pedigrees with several affected individuals available for study are not obtained frequently. Despite these challenges, five other ALS loci have been identified to date; these have been localized to chromosomes 2q33 (Hentati *et al.*, 1989), 9q34 (Chance *et al.*, 1998), 15q15.1-21.2 (Siddique *et al.*, 1993), 17q21-22 and Xp11-q12 (Hong *et al.*, 1998).

In a small number of SALS patients, a set of mutations in the NF-H gene was found. Single cases have been reported with a mutation in EAAT2, cytochrome c and APEX nuclease genes (Hand *et al.*, 2002). The Apo E has been studied as a candidate risk factor gene. While two groups had reported an increased risk of developing bulbar onset

ALS in the presence of Apo E4 allele, other groups were unable to confirm this. Deletions in the SMN2 gene were shown to be more frequent in ALS patients with respect to controls. Furthermore, a mutation in leukemia inhibitory factor (LIF) has been reported in a set of ALS patients but not in controls (Robberecht, 2000). The identification of such genetics may help to clarify the pathogenesis of the disease.

In the framework of this study, 41 ALS samples were examined for possible mutations in the SOD1 gene. Among these 41 patients, there were two first degree relatives and the remaining 39 were individual cases. The SSCP patterns for all patients were normal, except for two SALS patients (ALS1 and ALS40), who exhibited an abnormal pattern in the SSCP analysis. DNA sequencing results defined this nucleotide change to be an A→C transversion in intron 3, position 34 which was reported in the literature as a rare polymorphism with a frequency of 4 per cent. However, in the Turkish population under study, the frequency of this polymorphism was found to be much lower (1.33 per cent). Thus the nature of the IVS-III-34 (A→C) transversion is still questioned: it should be further investigated by increasing the sample sizes analyzed.

The absence of any SOD1 mutations in this group of patients was not too surprising; thus far, only a few SALS patients were reported to exhibit a SOD1 mutation in the literature. However, the mainstream experimental strategy in the investigation of SALS patients starts with the analysis of the SOD1 gene.

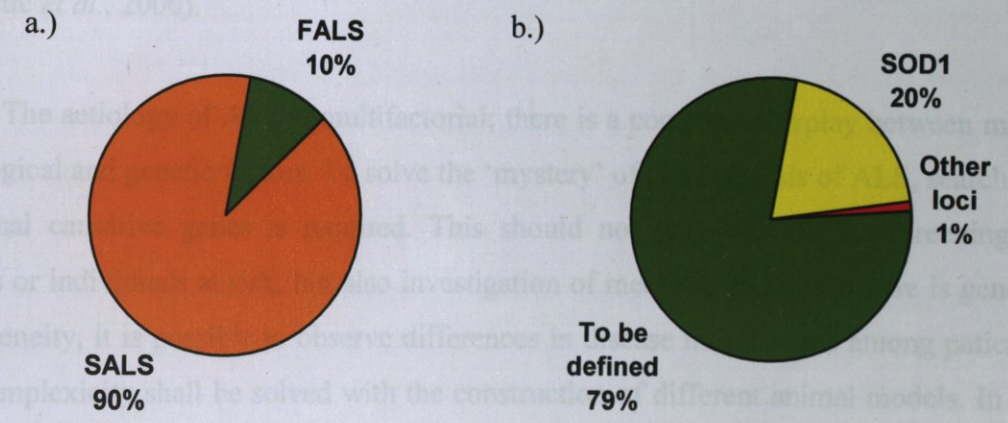

Figure 6.1. a. Relative distribution of familial (FALS) and sporadic (SALS) ALS cases; b. Relative distribution of SOD1 cases in FALS

FALS cases comprise about 10 per cent of the total ALS cases (Figure 6.1a), and only 20 per cent of this 10 per cent group carries a mutation in the SOD1 gene (Figure 6.1b). However, these values have been defined among American, British and Japanese families. In the continental European ALS populations, less SOD1 gene mutations have been identified, which indicates an apparent heterogeneity in the distribution of genetic abnormalities in different ethnic groups. In the study of Gestri *et al.*, two unrelated Italian families with FALS were analyzed for the SOD1 mutations, and no mutation was detected in the living patients of both families (Gestri *et al.*, 2000). Also, the activity of the SOD1 enzyme was measured to be not different in patients and subjects at risk with respect to healthy controls. Similar studies were also performed by another Italian group in 1999 (Malaspina *et al.*, 1999). Although larger numbers of patient groups are needed to confirm these results, it can be hypothesized that there is a heterogeneity in the distribution of ALS abnormalities in different ethnic groups. In this respect, when both the low percentage and heterogeneity hypothesis are considered, the lack of SOD1 gene mutations in the FALS cases of our study can be reasoned.

Since no effective treatment exists in ALS, SOD1 genetic testing provides patients only with a molecular diagnosis. Testing may also offer some prognostic guidelines and has the potential to alter genetic counseling, e.g. identifying a mutation in an apparent SALS patient. Some other patients, positive for SOD1 mutations, may find it necessary to know that ALS research is focused on understanding the SOD1 disease mechanism (Gaudette *et al.*, 2000).

The aetiology of ALS is multifactorial; there is a complex interplay between many pathological and genetic factors. To solve the 'mystery' of pathogenesis of ALS, search for additional causative genes is required. This should not only include the screening of patients or individuals at risk, but also investigation of mechanisms. Since there is genetic heterogeneity, it is possible to observe differences in disease mechanisms among patients. This complexicity shall be solved with the construction of different animal models. In the future, it is hoped that the identification of the disease-causing mechanisms will help developing more effective diagnosis, and hopefully curative therapies.

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