REVIEW

Cu/Zn superoxide dismutase gene mutations in amyotrophic lateral sclerosis: correlation between genotype and clinical features

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Background

Amyotrophic lateral sclerosis (ALS) is a fatal disease in which degeneration of upper and lower motor neurons leads to progressive weakness of bulbar, limb, thoracic, and abdominal muscles with relative sparing of oculomotor muscles and sphincter function. Although the clinical manifestations and pathological changes have been known since the time of Charcot and Joffroy,[1] the cellular and molecular processes leading to the selective loss of motor neurons are not understood. Recent developments in research on ALS, and in particular, the identification of mutations in the gene encoding Cu/Zn superoxide dismutase (CuZnSOD) in patients with the familial form of ALS[2] may provide new treatment strategies. Although familial ALS accounts for only 5%–10% of all patients with ALS,[3 4] it is indistinguishable clinically from sporadic ALS.[5 6] It is likely, therefore, that understanding of the mechanisms leading to motor neuron degeneration in familial ALS may provide fundamental insights into the pathogenesis of the sporadic form as well.

The family of superoxide dismutase enzymes, of which CuZnSOD is one, comprises a series of important physiological antioxidant defence mechanisms in aerobic organisms.[7] Superoxide dismutases inactivate the superoxide radical ($O_2^{\cdot -}$) generated as a byproduct of normal and aberrant metabolic reactions by converting $O_2^{\cdot -}$ into hydrogen peroxide (H_2O_2) and oxygen (O_2):

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

Impaired activity of this reaction will lead to the generation of an excess of superoxide radicals. The potentially damaging role of this excess superoxide lies not in its direct action on biological targets, as superoxide is not capable of setting off free radical chain reactions on its own, but in the formation of much more reactive species—for example, hydroxyl radicals ($\cdot OH$), reactions 2 and 3, and peroxynitrite ($ONOO^-$), reaction 4.

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad (3)$$

$$O_2^{\cdot -} + \cdot NO \rightarrow ONOO^- \quad (4)$$

On the other hand, an excess of these enzymes will increase removal of superoxide with overproduction of H_2O_2 and its derivatives, again resulting in oxidative damage. In addition to its primary role in the inactivation of the superoxide, CuZnSOD is also involved in the generation of the free radicals[8 9] and in the nitration of tyrosine residues.[10] Under normal physiological conditions these reactions are kept to a minimum by relatively low concentrations of H_2O_2 and $\cdot NO$ and by CuZnSOD exercising its essential antioxidant role. However, it seems that ALS associated mutant CuZnSOD enzyme, in an in vitro system at least, becomes a free radical generator to a greater extent than a wild type CuZnSOD.[11]

Mutations in the CuZnSOD gene

The gene that encodes CuZnSOD is located on the long arm of chromosome 21 (21q22·1) and is present as a single copy per haploid genome spanning 11 kb of chromosomal DNA.[12] There are five rather small exons that encode 153 amino acids and four introns that interrupt the coding region. Since 1993, 43 different point mutations involving 31 distinct codons,[2 13–36] a two base pair substitution at codon 6,[37] a deletion at codon 126,[38] and a single base pair substitution in intron 4[25] have been described (fig 1). No mutation has been found in exon 3. The highest rate of substitutions relative to the number of base pairs occurs in exon 4, suggesting that certain areas of the CuZnSOD gene are mutational "hot spots". Gly93, seems to be particularly vulnerable; it is substituted with six new amino acids. Two of these, Asp and Arg, alter the charge equilibrium but it is the introduction of the side chains at position 93 that is critical for the stability of the backbone conformation. Fifteen more mutations result in the charge change but only two (Asp125His, Asn139Lys) are relevant for the electrostatic attraction of the superoxide and which are likely to affect the function of the enzyme.

The mutations can be classified into four categories: (1) mutations that alter the length of the coding sequence; (2) mutations in the active site channel of the enzyme; (3) mutations at Cu binding sites; and (4) mutations that affect the structure of the protein.

MUTATIONS THAT ALTER THE LENGTH OF THE CODING SEQUENCE

The two bp deletion in codon 126 (TTG to

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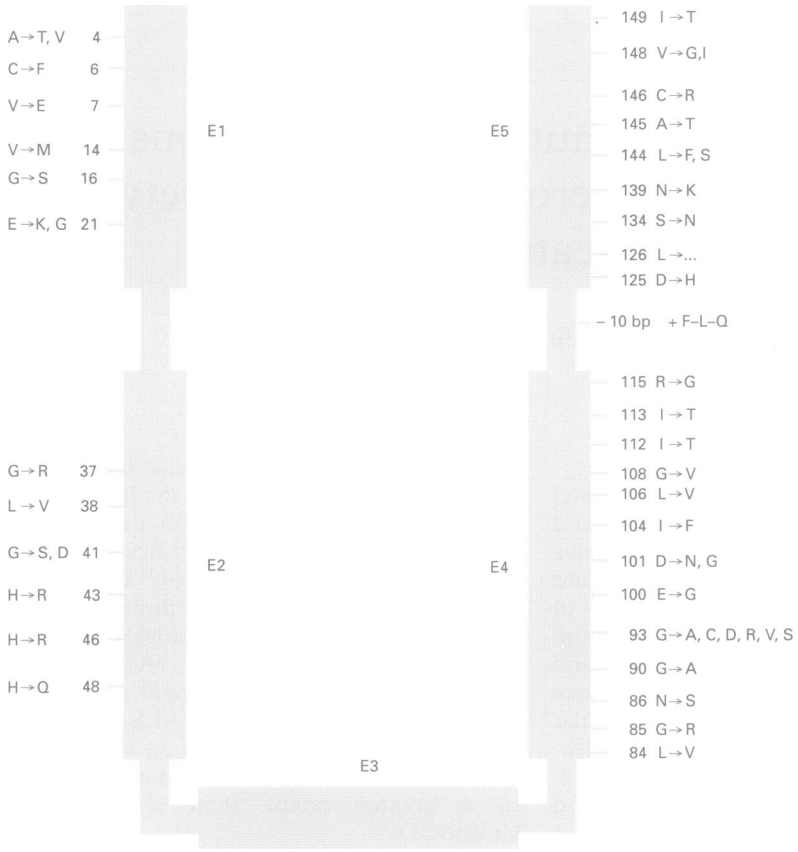

Figure 1 CuZnSOD mutations in familial ALS. The structure of the gene is shown diagrammatically. E1-E5 = exons 1 to 5; amino acid substitutions are indicated at appropriate codons according to the accepted international nomenclature.

**G) and single bp substitution in intron 4 (T to G), 10 bp upstream of the first bp of exon 5, alter the length of the coding sequence and cause changes in the size of translated protein. The first predicts the formation of a stop codon at position 131 and a loss of the electrostatic loop and a dimer contact region of the CuZnSOD protein whereas the second results in an alternatively spliced mRNA causing the introduction of three amino acids between exon 4 and 5 while conserving the open reading frame of exon 5.

MUTATIONS IN THE ACTIVE SITE CHANNEL OF THE ENZYME

The active site channel is formed between the electrostatic loop (residues 121–144) and a loop made up of the disulphide and the Zn ligand subloop regions (residues 49–84). In addition to the mentioned Asp125His and Asn139Lys mutations, four other mutations are found in the active site channel and these are Ser134Asn, Leu144Ser, and Leu144Phe as well as Leu84Val. The side chain of Ser134 is critical as it helps control conformation of the electrostatic loop; Leu144, although not so critical, is involved together with Val14, Leu38, Ile35, and His43 in the core packing at one end of the β-barrel. Interestingly, mutations are known also to occur in Val14, Leu38, and His43. A mutation in Leu84 is also expected to result in the defective packing as Leu84 tightly interacts with some residues of which two (Asp101, Ile104) are also mutated. In addition, Leu84 can indirectly destabilise the Cu ligand His46.

MUTATIONS AT Cu BINDING SITES

The essential part of the CuZnSOD dismutation reaction lies in the ability of the redox-active Cu centre to oxidise superoxide in one state and to reduce it in another. This Cu centre is stabilised by four His residues (His46, His48, His63, and His120). His63 also links Cu with Zn and is the one which is involved in the dismutation reaction. His48 and His120 also seem to play an important part in the loop conformation and interaction between structural elements. Mutations have been found in His46 and His48 but not in His63 and His120.

MUTATIONS THAT AFFECT ENZYME STRUCTURE

Thirty seven of the known mutations seem to affect the structure of the enzyme. The mentioned Leu84, His48, and Ser134 residues also play an important part in the preservation of the structure; in addition to its packing interactions Leu84 anchors the Zn loop whereas His48 and Ser134 have a role in controlling loop conformation. Removal of critical hydrogen bridges at His43, Asn86, and Arg115 can also be expected to affect loop conformation. His43 and Arg115 stabilise Greek key topology of loops III and VI respectively, whereas Asn86 bonds to the electrostatic loop VII. Left handed backbone conformation favourable only to Gly is destabilised by mutations at residues 37, 41, 85, and 93. Mutations at Val7, Ile112, Ile113, Arg115, Val148, and Ile149 are likely to disrupt dimer interaction whereas disruption of a subunit fold might be expected to occur by mutating residues localised in the β-barrel; 19 of them are mutated. Defective packing of Val14, Leu38, Asp101, and Ile104 is likely to contribute additionally to the destabilisation of the β-barrel.

SEQUENCE CONSERVATION

Amino acid sequences of 19 known CuZnSODs show a high degree of homology; the sequence conservation is almost as great as that of eukaryotic cytochrome *c*.[39] There are 21 invariant residues in key positions related to maintenance of the β-barrel fold, the active site structure including the electrostatic channel loop and dimer contacts. Five of those (Gly16, His46, His48, Leu106, and Cys146) are substituted with other amino acids in patients with familial ALS. His46 and His48 are Cu ligands; Gly16 and Leu106 are responsible for maintaining the β-barrel fold whereas the S-S bridge between Cys146 and Cys57 maintains dimer contact by binding the disulphide region to the β-barrel. Eleven of 19 nearly invariant residues, which are defined as found in 18/19 sequences,[39] are mutated and these account for a third of all so far reported. These are mutations in the active site channel (Leu84, Asp125, Ser134, and Asn139), mutations in Gly residues 37, 85 and 93, and Asn86Ser, Arg115Gly, Leu126, and Ala145Thr.

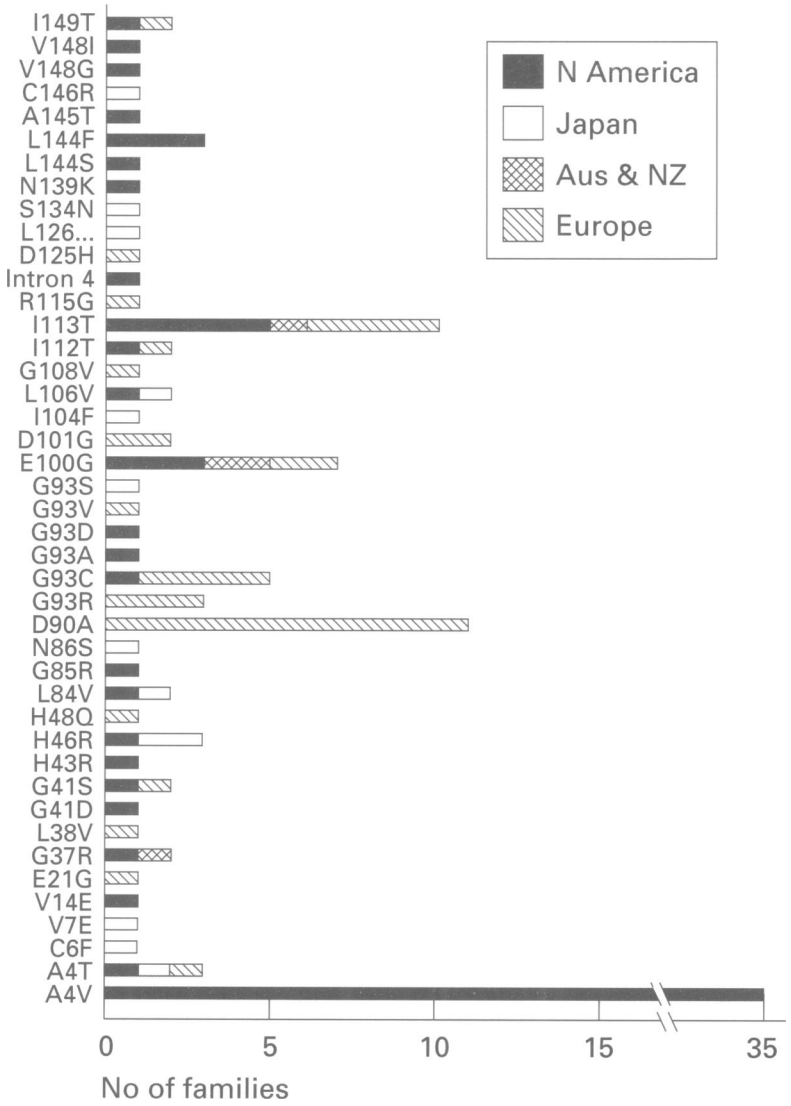

Figure 2 Geographical location of patients with familial ALS with CuZnSOD mutations.

Glu100 and Asp90 are conserved only in four and seven, respectively, of 19 species. Humans, pigs, rats, and mice CuZn SODs have Glu at position 100 whereas Asp90 is homologous in humans, bovine species, sheep, horses, and swordfish, and *Neurospora crassa* and *Saccharomyces cerevisae*. Other mutation sites show greater (for example, Ala4 89·5%) or lower (for example, Cys6 31·6%) degrees of homology.

Epidemiology of the CuZnSOD gene mutations

World wide, 121 families have been identified with mutations in the CuZnSOD gene (fig 2).[2 13-32 37 40-56] (Also Robberecht W and Enayat *et al*, unpublished data.) Some members from 35 families carry Ala → Val substitution at codon 4 but the exact number has not been reported. Bowling and associates[41] reported 58 affected patients from 14 families with Ala4Val mutation but they did not specify the number of asymptomatic carriers. In some families the asymptomatic members can account for a large proportion of subjects who carry the particular mutation.[42 46 49 51] Although carrying the mutation increases the risk of developing ALS, some members who carry the CuZnSOD gene mutation may not develop the disease, reflecting the variable penetrance of the mutation. As screening for the mutations in the CuZnSOD gene is currently done mainly for research purposes, it is rather difficult to know how many subjects have mutations in the CuZnSOD gene. Although the Ala4Val mutation is the most frequent overall, it is only found in patients from North America. By contrast, the same mutations (fig 2) occur in several families of different ethnic origin. For example, the Ala4Thr mutation has been identified in Japanese, North American, and Cypriot families[16 27 51] suggesting that these are independent mutation events. Two mutations (Glu100Gly and Ile113Thr) are of particular interest as they are found in British,[26 28 30 45 52] (and Enayat *et al*, unpublished data) North American,[13 41 54] Australian,[44] and New Zealand[49] families and hence may have a common ancestor, and it will be interesting to explore this in more detail.

The Asp90Ala mutation is the only homozygous mutation associated with familial ALS. It has been found in nine families in Finland and northern Sweden, and 30 of 37 persons homozygous for this mutation had evidence of ALS.[23 56] However, patients from the Belgian families who are heterozygous for the same mutation have developed ALS.[55]

Mutations have been reported in "apparently" sporadic cases with ALS (table 1). Although the CuZnSOD gene might have a high new mutation frequency, it is more likely that these mutations are due to their low penetrance especially when family history is incomplete. Indeed, the six Scottish patients with Ile113Thr mutation share a common haplotype with a shared ancestor some 10 generations ago (RJ Swingler, personal communication).

Mutations in the CuZnSOD gene have not been found in normal population[2 19 22 25] or in series of several hundred sporadic cases with ALS,[2 19 25 40] or among the patients with ALS and parkinsonsim-dementia of Guam.[57] Familial and sporadic patients with Parkinson's disease do not have mutations in the CuZnSOD gene either.[58]

Table 1 Mutations in the CuZnSOD gene found in patients with "apparently" sporadic ALS

Codon	Amino acid change	No of patients	Country
16	Gly→Ser	1	Japan[36]
21	Glu→Lys	1	Scotland[33]
90	Asp→Ala	10	Scandinavia[23, 56] Belgium[55] England (Enayat *et al* unpublished)
101	Asp→Asn	1	Scotland[34]
113	Ile→Thr	4	North America[27] Scotland[33, 35]

Functional impact of the CuZnSOD mutations and clinical correlation

To date there has been no comprenhesive attempt to correlate the genotype of patients with familial ALS with CuZnSOD mutations and their phenotypic characteristics. The question arises as to whether it is possible to

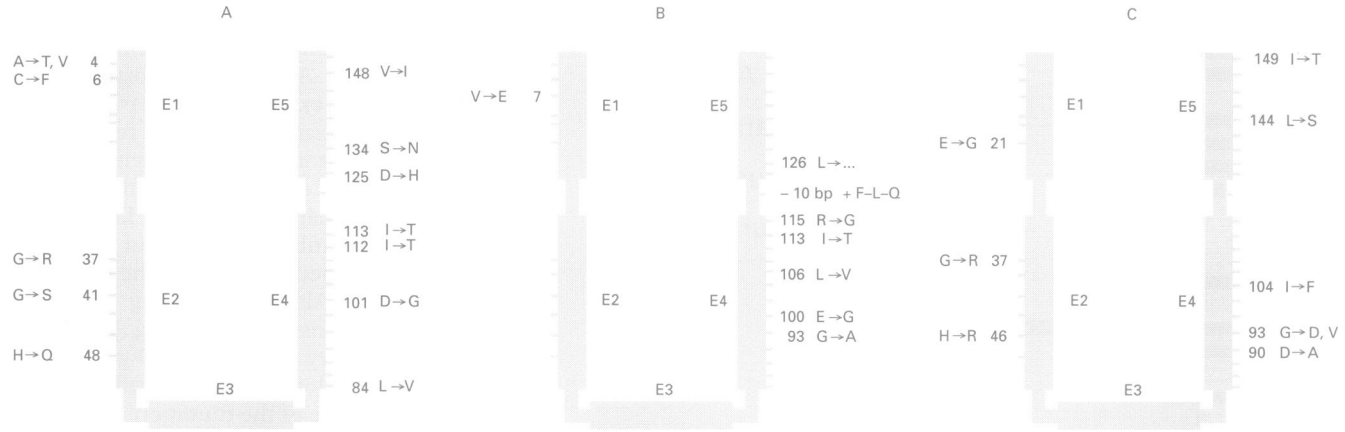

Figure 3 CuZnSOD gene mutations associated with (A) rapidly progressive (< two years), (B) classic (two to five years), and (C) relatively benign (> five years) forms of ALS.

predict the age at onset, distribution, and likely prognosis on the basis of the particular genetic defect. Some attempts, however, have been made to correlate the activity of CuZnSOD in extracts of red blood cells or lymphoblastoid cell lines with age of disease onset and its duration.[41 48 53] These have not produced convincing evidence that severity of disease arises from the loss of enzymatic activity. This is not surprising as the activity of enzymes in critical tissues in other neurological disorders may not be reflected by their activity in erythrocytes.[59 60]

The presenting symptoms in patients with familial ALS are similar to those with sporadic ALS; most of the cases present with the first symptoms in the limbs and only around 20% present with bulbar symptoms.[5] These symptoms may develop earlier in life in patients with familial ALS than in the those with sporadic ALS.[61 62] Early onset (below 50 years) is associated with longer survival[63] and some younger patients with familial ALS tend to run a slightly longer course.[61] Nevertheless, survival of patients with familial ALS tends to be shorter than in patients with sporadic disease.[5] Familial ALS occurs in men and women at roughly the same frequency[61 62] whereas in sporadic ALS more men are affected than women.[5] Interestingly, in some families with the CuZnSOD gene mutations women seem to be more often affected than men,[42 47 52] or only the women are affected.[54] This may, of course, reflect a bias of small numbers. In a Japanese family with a Leu84Val mutation disease appears at an earlier age in subsequent generations only in men whereas onset in women varies between 60 and 76 years.[21] Neither sex nor generation has been reported to influence the age of onset in other families with the CuZnSOD gene mutations. However, variability in the age of onset within families is characteristic of most of the CuZnSOD families. Identification of genetic or environment modifying factors may explain this phenomenon.

The mean age of onset of disease varies from as early as 28·0 years[31] to 61·2 years,[41] with the youngest patient developing symptoms at the age of 6.[31] When the same mutation (for example, Gly37Arg, His46Arg, Ile112Thr, Ile113Thr) is present in more than one family the mean age of onset in each of these families seems to be similar. However, there might also be a difference of up to 15 years in the mean onset of the disease in families with the same mutation.[51]

CuZnSOD gene mutations are associated with a rapidly progressive form (fig 3A), a classic form (fig 3B), and a relatively benign form of ALS (fig 3C). 13 CuZnSOD gene mutations have been identified in families with rapidly progressive ALS (fig 3A).[16 21 28 30–32 37 41 42 51 53] (And Enayat *et al*, unpublished data.) Additionally, two North American families with Val14Met and Leu84Val mutations[27] and an Australian Ile113Thr family[44] seem to have an aggressive course. In different families the same mutation can be associated with a different clinical course—for example, a one year duration in the Australian Gly37Arg family by contrast with an American family with the same mutation manifesting a mild form of ALS with a mean duration of 12 years.[53] Ile113Thr is another mutation which may be associated with rapidly progressive ALS[41] or with more classic ALS.[54] This mutation is also one which shows variation in duration of disease within the family; survival in family members ranged from three to 20 years.[52] In a Leu38Val family survival of five patients was 2·0 (SD) 0·7 years but there was also a patient who survived for 10 years.[64] The disease in some of the Belgian patients with the Asp90Ala mutation showed a rapid course of one year but prolonged survival as well; patients are still alive after four and nine years.[55] Three more CuZnSOD gene mutations (Gly93Arg,[48] Asn86Ser,[15] Glu100Gly[30]) also show wide variation within families in duration of the disease.

A relatively mild form of ALS is associated with a number of CuZnSOD mutations (fig 3C) with all but a few patients living longer than eight years after the onset of the disease.[25 28–31 41 47 53 56] Most of these patients presented with ALS at around 45 or even earlier. However, the patients in these families with onset in their 60s or later had a relatively good prognosis as well. Patients with (apparently)

sporadic ALS with homozygous Asp90Ala mutation have the same benign form of ALS as those with familial ALS who are homozygous for the Asp90Ala mutation.[56] However, some of the patients with sporadic ALS who are heterozygous for the Asp90Ala mutation present with classic,[55] (and Enayat *et al*, unpublished data) or even rapidly progressive ALS.[23]

There is thus no clear correlation between the site of mutations in the CuZnSOD gene and their predicted effects on enzyme function, or the age of onset or duration of ALS. However, it is striking that all CuZnSOD gene mutations for which clinical data are available are associated with onset in the limb rather than in the bulbar musculature. It is conceivable that the apparent lack of correlation between the site of mutations and clinical features of the disease reflects the incompletness of data. In 56 of the known 121 families there is no information available on the number of affected people, age of onset, duration of disease, and site of onset. Twelve of these mutations are particularly interesting as they appear in more than one family and clinical data relating to some of these families are presented in this review. It will be important to know if all families with the Ala4Val mutation behave similarly to the 14 families reported by Bowling and associates[41] or if Scottish and American Ile113Thr families show the variability of the English Ile113Thr family.[52] Of course, it will be of the utmost importance to learn if all these patients with familial ALS presented with limb onset.

There is no clear relation between mutations in highly conserved regions of the gene (indicating functionally important residues) and the age of onset or severity. Mutations in these critical residues might be expected to cause rapidly progressive ALS, and although some are associated with a rapid course—for example, Gly37Arg, His48Gln, Leu84Val, and Ser134Asn—others are linked to mild forms of ALS—for example, Gly37Arg, His46Arg, and Gly93Asp. In addition, mutations in less highly conserved sites (hence less important for normal functioning of the enzyme) are associated with rapidly progressive ALS—for example, the Cys6Phe mutation.

The evidence outlined suggests that the phenotype of subjects with CuZnSOD mutations is determined by factors other than the site of the mutation. These factors could be environmental, or genetic, or both. A search for genetic modifying factors may be the most productive approach, given the failure of epidemiological studies to identify significant environmental risk factors.[65] It could be argued that there is selection bias with respect to families with CuZnSOD gene mutation as they were sought specifically for linkage analysis, and probably reflect highly penetrant mutations. Caution is therefore needed in making genotype/phenotype correlations and population based studies are necessary to understand the complex interactions between gene-gene and gene-environment.[45]

Pathology

There have been few reports to date on the molecular pathology of ALS associated with CuZnSOD gene mutations.[54 66–68] Many of the patients have shown neurofilamentous accumulations in lower motor neurons, and in one patient there were also neurofilamentous accumulations in Betz cells of the primary motor cortex.[67] Ubiquitinated inclusions in anterior horn cells have also been noted in some of these patients and in one family, these inclusions were also labelled by antibody against CuZnSOD.[69] Proximal axonal swellings containing bundles of neurofilaments have long been recognised as a feature of both sporadic and familial ALS, and alterations in the phosphorylation state of neurofilaments in anterior horn cells have been described.[70] The nature of the relation between CuZnSOD mutations and neurofilamentous inclusions is unclear, but it is possible that the mutations lead to altered neurofilament function, or to changes in axonal transport. It is interesting that mutations of neurofilament genes have been identified in a few patients with (apparently) sporadic ALS,[71] and in transgenic mice certain neurofilament gene mutations also lead to progressive loss of motor neurons.[72] At present there is no evidence that neurofilamentous inclusions and axonal spheroids represent a primary pathogenic change in familial or sporadic ALS, but neurofilament dysfunction possibly triggered by CuZnSOD mutations may none the less contribute to neuronal damage. Finally, it is worth nothing that intraneuronal vacuoles, which are prominent in transgenic models, are absent in familial ALS with CuZnSOD mutations.

In summary, the molecular pathology of familial ALS with CuZnSOD mutations differs in some striking respects from the findings in transgenic mice (table 2).

Possible mechanisms of motor neuron damage due to CuZnSOD mutations

CuZnSOD is a homodimer and heterozygous mutations are expected to cause a 50% reduction in enzymatic activity. Activity of CuZnSOD in patients with familial ALS with some CuZnSOD mutations is reduced to 25%–80% of normal in red blood cells,[13 16 17 19 24 31 32 37 40 41 46–50] lymphoblasts,[22 40 73 74] fibroblasts,[21] and brain[40 75] (fig 4). In some patients, however, the mutant dimer may have a dominant negative effect on the activity and longevity of the wildtype subunit[48] although this is an exception rather than a rule, and the activity of CuZnSOD in red blood cells of patients with ALS homozygous for the Asp90Ala mutation is essentially normal.[23] The specific activity of the enzyme is also fully retained in transfected COS-1 cells with the human CuZnSOD gene carrying the Gly37Arg mutation.[73] When the same mutation is introduced into transgenic mice the animals develop features of lower motor neuron disease with CuZnSOD in the spinal cord increased sevenfold to 14-fold, whereas mice expressing the wild type human CuZnSOD

Table 2 Neuropathology in patients with familial ALS with CuZnSOD gene mutations and transgenic mice expressing the mutant CuZnSOD gene

CuZnSOD mutation	Bunina bodies	LBI inclusions	UbIR inclusions	Vacuolation	Neurofilament accumulation		Multisystem degeneration
					Intraneuronal	Axonal	
Human A4T, H48Q, E100G, I113T	−	+	+ or −	−	+	+	Posterior columns and spinocerebellar tracts; Clarke's column
Transgenic hG93A	−	−	−	+	+	−	Posterior columns, red nucleus, reticular formation, interpeduncular nucleus
Transgenic hG37R	−	−	−	+	+/−	+/−	Dorsal root, thalamus, hypothalamus, pyriform cortex, olfactory bulb
Transgenic mG85R	−	−	?	−	?	?	Thalamus, hypothalamus, superior colliculus, cerebellar nuclei, basal ganglia

gene had similar CuZnSOD values but no disease.[76] Transgenic mice expressing other CuZnSOD mutants, both human and murine, also develop motor neuron degeneration and either have normal or increased amounts of CuZnSOD in the brain.[77-78] Overexpression of the wild type human CuZnSOD gene causes oxidative damage[79] and could contribute to degenerative changes seen in Down's syndrome,[80] but overexpression of the wild type CuZnSOD gene in transgenic mice is not associated with neuronal degeneration.[77] The mechanism by which overexpression of mutant CuZnSOD leads to loss of motor neurons in transgenic mice is unclear but may not involve inadequate handling of superoxide. It is possible that mutant enzyme may precipitate to form toxic cytoplasmic aggregates due to its conformational changes and shorter half life, facilitate nitration of protein tyrosine residues by peroxynitrite, or fail to appropriately shield Cu from its powerful toxic effects.[81] Recent in vitro studies also suggest that mutant CuZnSOD enzyme possesses greater peroxidative activity than wild enzyme, thus leading to increased production of hydroxyl radicals from H_2O_2.[11] This peroxidation reaction seems to be prevented by Cu chelators[11] and by supplementing a diet of CuZnSOD transgenic mice with vitamin E and selenium.[82]

Conclusion

Mutations in the CuZnSOD gene have been convincingly linked to familial ALS, and can be presumed to be causative. The mechanisms by which the mutations lead to selective neuronal damage remain unclear. It is by no means certain that free radical damage or changes in Cu^{2+} homeostasis are to blame. New insights are urgently needed if rational new therapies are to be designed.

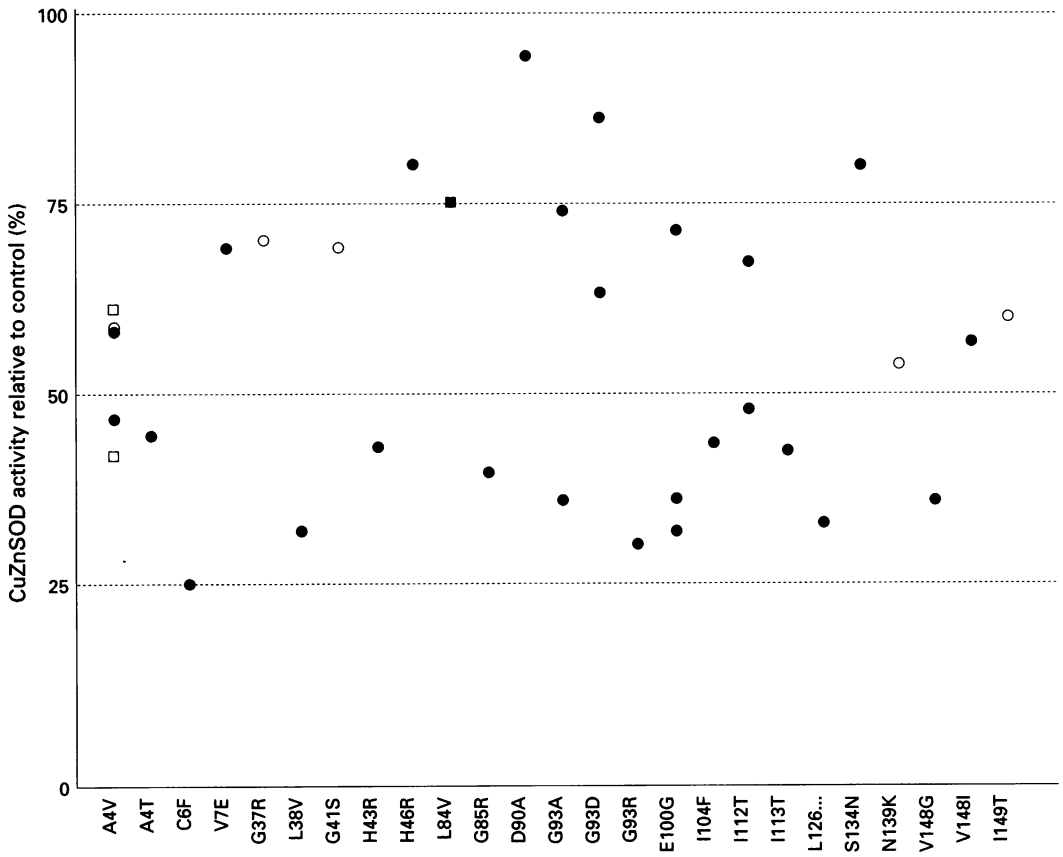

Figure 4 CuZnSOD activity in red blood cells (●), lymphoblasts (○), brain (□), and fibroblasts (■) of patients with familial ALS with CuZnSOD gene mutations compared with control values.

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