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Original Contribution

Thioredoxin reductase 1 haplotypes modify familial amyotrophic lateral sclerosis onset

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ABSTRACT

Thioredoxin reductase 1 is a key enzyme in cellular redox processes, which are known to play a role in the pathogenesis of familial amyotrophic lateral sclerosis (FALS). The gene *TXNRD1* was therefore screened for association with FALS. Resequencing of the exons and flanking regions identified 19 single-nucleotide polymorphisms (SNPs) of which 2, the intronic SNPs rs6539137 and rs4630362, were significantly associated with FALS. However, no association of rs6539137 with sporadic ALS was detected. The *TXNRD1* haplotypes were reconstructed using the EH and PHASE 2.1 programs and also showed an association with FALS. Bayesian analysis of these SNP combinations, carried out using the BIMBAM program, indicated that rs10861192 strongly augmented this association. Indeed the haplotypes with minor alleles at both rs10861192 and rs6539137, although present in FALS, were totally absent from controls. Patients with the minor allele of rs6539137 were also associated with an early age at onset, which was decreased by 8 years. Furthermore the shift of onset was more pronounced in males and not significant in females. These results show that *TXNRD1* may act as an important modifier gene of FALS and indicate that the additional thiol-redox system genes, thioredoxin and the peroxiredoxins, should also be investigated in FALS and other neurological disorders.

Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative condition that selectively affects neurons of the motor system in the spinal cord, brain stem, and cerebral cortex, causing progressive muscle weakness, atrophy, paralysis, and bulbar dysfunction, leading to death within 3–5 years in most cases. The etiology of the disease is unknown and treatment is largely palliative. The discovery of disease-associated mutations in the superoxide dismutase gene (*SOD1*) in a subset of cases of familial ALS (FALS) [1,2] prompted extensive research into the role of oxidative stress in the pathogenesis of FALS cases and in animal models expressing these mutations [3–5]. Oxidative modification of proteins by carbonylation, nitration, and bound crotonaldehyde has been detected in the spinal cord of the mutant *SOD1* mouse model [5–8]. In ALS, glutathione peroxidase and peroxiredoxin 2 (Prdx 2) proteins are up-regulated in motor neurons [9], and increased levels of Prdx 6 protein are found in astrocytes [10]. Conversely, Prdx 3 and 4 transcripts appear down-regulated in motor neurons [11]. Gene expression profiling in ALS spinal cord showed other changes in redox enzymes: thioredoxin was elevated sixfold, lysyl oxidase was also increased, and levels of flavin monooxygenase mRNA were decreased [12]. Thioredoxin expression was also elevated in muscle from presymptomatic mutant SOD1 mice [13] and has previously been shown to be markedly up-regulated after injury of the hypoglossal nerve [14].

The thioredoxin system consists of three components that are found in most organisms: the 12-kDa dithiol/disulfide oxidoreductase enzyme thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH. Thioredoxin is reduced by TrxR1 (EC 1.6.4.5) containing the highly reductive selenocysteine active site using NADPH as a source of reducing equivalents. These two enzymes function at the core of antioxidant defense and thiol redox control of the cell [14,15]. TrxR1 is a 495-amino-acid protein, encoded by the *TXNRD1* gene, which maps to 12q23–q24.1 and has 40% identity to glutathione reductase [16]. TrxR is widely expressed and is abundant in the central nervous system. The role of TrxR as an antioxidant in the cell is shown by its ability to reduce a wide range of substrates in addition to Trx; for instance, it reduces ribonucleotide reductase, involved in deoxyribonucleotide production, and the p53 protein [17,18]. TrxR1 activity enhances DNA binding of the transcription factor NF-kappaB, which stimulates T cell mitogenesis [19]. In addition, TrxR reduces a number of nonprotein molecules such as lipoamide, lipoic acid, ascorbate, hydrogen peroxide, lipid hydroperoxides, alloxane, and the lipid-soluble antioxidant ubiquinone Q10 [15,20]. Selenium-compromised TrxR leads to rapid cell death, showing its importance in cell survival [21], mediated in part by inhibiting the apoptosis signal-regulating kinase, ASK1 [22], and by regenerating peroxiredoxins [23]. Indeed selenium levels are decreased in patients with advanced ALS [24,25],

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which would decrease TrxR activity [26]. Based on emerging evidence for the important contribution of the thioredoxin system to motor neuron function, we investigated the role of TrxR as a potential modifier gene in ALS.

Substantial phenotype differences, for example, in the age at onset and duration of disease, are a common feature of *SOD1* mutations occurring within and between families, indicating that other genetic and environmental factors play a key role in the disease phenotype. Modifier genes have been implicated in other human diseases, in which they have been found to reduce the penetrance or influence the age at onset of a given disease [27,28]. Modifier genes that delay the age at onset of disease in G86R *SOD1* mice have been identified in a chromosomal region containing the *Smn* (survival motor neuron) gene [29]. FALS cases with V148G *SOD1* and null for ciliary neurotrophic factor gene ($CNTF^{-/-}$) showed an earlier onset of disease than V148G *SOD1* family members with wild-type *CNTF,* and this was replicated in the G93A mouse model [30]. The CNTF null mutation is, however, not common and this association with ALS was not found in other studies [31,32]. Evidence for a *cis*-activating disease modifier in close proximity to *SOD1* has been reported for the D90A *SOD1* recessive haplotype [33]. The influence of modifier genes affecting the redox state in the *SOD1* mutant mouse model has been examined in the NADPH oxidases [34], and knockouts of *Nox1* and *2,* especially of *Nox2,* improved survival rates and delayed disease onset and progression [35].

To identify modifier effects in FALS, the association of a set of *TXNRD1* single-nucleotide polymorphisms (SNPs) with clinical phenotypes was investigated in this study. Single-site and haplotype statistical tests showed that rs6539137 in intron 4 (denoted SNP4 in this study) and rs4630362 in intron 12 (SNP6) were the most significantly associated with disease. Moreover, the minor allele of SNP4 was significantly associated with an earlier age at onset in male FALS cases but not in females or sporadic ALS (SALS) patients.

Materials and methods

Subjects

A diagnosis of ALS was confirmed clinically in affected individuals with evidence of both upper and lower motor neuron involvement according to El Escorial criteria as defined by the World Federation of Neurology. Each FALS sample was an index case obtained from a different kindred. Controls with a similar ratio of males to females were obtained from spouses. This study has been approved by the Riverside Research Ethics Committee (Hammersmith Hospitals NHS Trust) with appropriate informed consent from subjects.

Extraction of DNA from blood

DNA was extracted from whole blood or the buffy coat layer using a blood and tissue DNA extraction kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions except for the elution of the DNA from the spin columns, which was carried out in 200 µl distilled H_2O instead of Qiagen's elution buffer.

Amplification of genomic DNA

The 15 exons of *TXNRD1* were amplified from genomic DNA using primers (Sigma-Genosys, Pampisford, Cambridgeshire, UK) annealing to the regions flanking the exons. Polymerase chain reactions (PCRs) were performed using Platinum Taq DNA polymerase (Invitrogen, Paisley, UK) according to the manufacturer's instructions in a total volume of 100 µl. Primers were designed from the nucleotide sequence data obtained from the National Center for Biotechnology Information.

Purification and sequencing of PCR products

The PCR products (100 µl) were purified using the Nucleospin extraction kit (Clontech, Cowley, Oxford, UK) according to the manufacturer's instructions except that the PCR products were eluted off the columns in 50 µl distilled H_2O instead of the supplied elution buffer. The samples were then ethanol precipitated and resuspended in 30 µl distilled H_2O. Purified PCR products of *TXNRD1* were sequenced in both the forward and the reverse directions. Reactions were carried out using the ABI Prism BigDye terminator kit (Applied Biosystems, Warrington, Cheshire, UK) according to the manufacturer's instructions. Once the cycling reaction was complete the samples were purified by ethanol precipitation. The dry pellets were then mixed with 12 µl HiDi formamide (Applied Biosystems) before being loaded onto the AB3100 (Applied Biosystems) sequencer.

Genotyping of SNPs 1–6

SNPs 1 to 5 are within known restriction sites that were digested with *Taq*I, *Fat*I, *Afl*III, *Dra*I, and *Mse*I, respectively. All restriction enzymes were obtained from New England Biolabs (Hitchin, Hertfordshire, UK) and were used according to the manufacturer's instructions. The digested products were then separated on 1–2% agarose gels (molecular biology grade; VWR, Poole, Dorset) for 1 h at 200 V and visualized using ethidium bromide (0.5 µg/ml) with a gel documentation system (Gel Doc 2000; Bio-Rad UK, Hemel Hempstead Hertfordshire, UK). Sequencing was used to type SNP6, in which no restriction site was found.

Statistics

Fisher's exact test was used for allelic and genotypic associations of single SNPs with 2×2 and 2×3 contingency tables (quantitativeskills.com/sisa/statistics/fisher.htm), as well as to test for departures from Hardy–Weinberg equilibrium. The Cochran–Armitage test was used to test for trends in the genotypes [36]. The estimates of D' and r^2 for measuring the linkage disequilibrium between two SNPs were calculated as previously described [37,38]. Two SNP haplotype frequencies and their log likelihoods were estimated using EH [39] and these were used in a model-free test of disease association, in which $2(\ln L_{case}+\ln L_{control}-\ln L_{combined})$ gives a χ^2-based test statistic with (n haplotypes − 1) degrees of freedom [39]. A Bayesian statistical method for haplotype reconstruction based on all six SNPs of *TXNRD1* employed PHASE version 2.1 software [40], which also estimated a case–control p value by using 1000 permutations to test for similarity between the FALS and the control haplotypes. In addition Bayes factors for disease associations with individual SNPs and their combined effects were calculated using the BIMBAM program, which also estimates single SNP p-values [41]. The Bayes factor (BF) corresponds to a "weight of evidence" in support of a hypothesis using Jeffrey's scale of six categories, where BF<1 is negative support; BF=1–3 is no support; BF=3–10 is substantial support; BF=10–30 is strong evidence; BF=30–100 is very strong evidence, and BF>100 is decisive. This was set up in the case–control mode and multi-SNP modes with 100,000 iterations, for which cases were coded as 1 and controls as 0. For the SNP imputation run, the *TXNRD1* genotype data of 44 SNPs from HapMap (hapmap.org) was obtained as a "dumped" file and used as an additional input file of controls. BIMBAM was run in the case–control mode with 100,000 imputations and those SNPs with a $p<0.05$ and a $BF>BF_{SNP6}$ were considered significant. The program was run three times, and SNPs that were significant in at least two of the three runs are listed under Results. The effects of SNP4 alleles and gender on age at onset of FALS were tested using the log-rank test and displayed by Kaplan–Meier plots using XLSTAT software (xlstat.com). Hazards ratios and