Supplementary Online Material for

TDP-43 mutations in Familial and Sporadic Amyotrophic Lateral Sclerosis

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Methods

Case ascertainment

Familial cases of ALS were identified through three MND care and research facilities at King's College Hospital and Royal Free Hospital, London, UK, and Concord Hospital, Sydney, Australia. All patients were interviewed and examined by consultant neurologists. A clinical diagnosis of definite or probable ALS was based upon El Escorial criteria (*1*). Consent for research was obtained and blood taken for DNA extraction and for cell line generation. Control samples were matched for ethnicity, age and sex. We identified 154 suitable FALS index cases who had previously been screened and found to be negative for mutations in known ALS genes (*SOD1*, *ANG*, *VAPB* and *CHMP2B*). 372 sporadic cases of ALS identified through King's College and the Australian MND DNA Bank were also included in our mutation screen as well as 872 controls matched for ethnicity, age and sex.

Sequencing

Genomic DNA was extracted from peripheral blood using standard methods. All six exons of *TARDBP* were amplified by the polymerase chain reaction (PCR) and sequenced using oligonucleotide primers (MWG Biotech). Primers were designed to amplify at least 50 base pairs of adjacent intronic sequence (Table **S2**). Amplification conditions are available from the authors on request. Direct sequencing of amplified exons was performed using Big-Dye® Terminator v3.1 sequencing (Applied Biosystems).

Genotyping

SNP genotyping was undertaken using the Affymetrix GeneChip® Mapping 10Kv2.0 XbaI Array containing 10,204 SNP markers following the manufacturer's instructions and apparatus (Affymetrix, Santa Clara, CA, USA). Briefly, 250ng of genomic DNA from each sample was digested with the restriction endonuclease XbaI for 2.5 hours. Digested DNA was ligated to XbaI adapters using T4 ligase for 2.5 hours. Ligated DNA was added to four separate PCRs, amplified, pooled and purified to remove unincorporated dNTPs. The purified PCR product was then fragmented with DNase1, end-labelled with biotin and hybridized to an array for 18 hours in an Affymetrix 640 hybridization oven. Arrays were then washed and stained using a Fluidics Station F450 and then scanned using a GeneArray scanner 3000. The raw microarray feature intensities were processed using the Affymetrix Genotyping Tools software package (GCOS/GTYPE) to derive SNP genotypes, marker order and linear chromosomal location. Microsatellite genotyping was performed using fluorescent dye-labelled primers (MWG Biotech). PCR products were analysed using an ABI 3100 Genetic Analyser and Genotyper software (Applied Biosystems).

Genetic analysis

The SLink v2.65 program (*2*) was used to simulate a genome-wide scan for linkage using the following assumptions: autosomal dominant inheritance with a 65% lifetime risk of disease for all at risk individuals, no phenocopies, a disease gene frequency of 0.0001 and a six allele marker with equal allele frequencies. Parametric multipoint linkage analysis of SNP data was performed using Merlin v1.1 (*3*) with intermarker distances obtained from the Marshfield (*4*) and deCODE (*5*) sex-averaged SNP linkage map. Two-point linkage analysis of microsatellite data was performed using the MLINK program of the LINKAGE package, FASTLINK Version 4.1 (*6, 7*). Parameters for linkage analysis included autosomal dominant inheritance, age-dependent penetrance based upon observed penetrance in family ALS85 (0-30 years, 1%; 31-40 years, 20%; 41-50 years, 40%; 51-60 years, 80%; >60 years, 90%), a disease allele frequency of 0.0001, equal male and female recombination, and equal marker allele frequencies. Multipoint linkage analysis of microsatellite data was performed using the LINKMAP program (FASTLINK Version 4.1) with intermarker distances obtained from the Rutgers sex-averaged linkage map (*8*).

Cloning and site-specific mutagenesis

A human cDNA clone encoding full-length wild type TDP-43 was used as a template for cloning. An HA tag was added to the C-terminus and a c-myc tag to the N-terminus of the cDNA by PCR. The tagged cDNA was then used to generate mutants M337V and Q331K using the primer sets given in table S3. Direct sequencing confirmed successful mutagenesis. Both wild-type and mutant cDNAs were then cloned into pCI-neo for expression studies in cells and chick embryo.

In vitro Cell culture, DNA transfections, Western blotting and Immunocytochemistry

Chinese hamster ovary (CHO) cells were maintained as monolayers in Glutamax (GIBCO) supplemented with 100units/ml penicillin-streptomycin and 10% foetal calf serum (FCS). Cultures were maintained at 37ºC and 5% CO_2. One day before transfection the cells were seeded at 50-70% confluence in 6-well plates. Fugene HD was used for transfection following the manufacturer's protocols.

Cells were cultured for a further 48 h before protein extraction in 1× TEN (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 100mM NaCl) with 1% Nonidet P40 and proteinase inhibitor cocktail (Roche). Extracts were briefly sonicated and then centrifuged at 100,000*g* for 60 min to isolate the cytoplasmic fraction. SDS-PAGE of protein samples was conducted using 4-12% gradient gels (Invitrogen). Electrophoresed proteins were transferred to nitrocellulose membranes and then probed using the following antibodies: rabbit polyclonal anti-TDP-43 (Proteintech, 1:1000); mouse monoclonal anti-myc (NEB, 1:1000); and rabbit monoclonal anti-HA (Sigma 1:5000). After washing, membranes were incubated in the dark in 0.1% Tween-20 in blocking solution with 1:10,000 fluorescent secondary antibodies: IRDye 680 and 800 anti-rabbit and anti-mouse (LI-COR, Biosciences, NE, USA). Images were acquired with the Odyssey infrared imaging system (LI-COR, Biosciences, NE, USA) and analyzed as outlined by the manufacturer.

Cells were also grown on glass coverslips. 48 hours after transfection cells were fixed in 4% paraformaldehyde, permeabilised with 0.5% Triton X-100 and blocked in 10% FCS before overnight incubation at 4ºC with primary antibodies as used above: anti-myc (1:1000), anti-HA (1:5000). After washing in phosphate buffered saline (PBS) cells were incubated with fluorescent secondary antibodies

(anti-rabbit Alexa Fluor 488, 1:2000 and anti-mouse Alexa Fluor 568, 1:2000, Invitrogen). Images were captured using an Axiovert 4.0 (Carl Zeiss, UK) microscope using Image-Pro Express 6.0 (MediaCybernetics). Adobe Photoshop 7.0 (Adobe Systems, Inc., USA) was used to process the images.

Chick embryo studies

In ovo electroporation: Pathogen-free white Leghorn chicken eggs were obtained from Henry Stewart and Co. Ltd. Eggs were stored at room temperature for no longer than 5 days before use. 52 hours before electroporation the eggs were transferred to an incubator (39ºC, 70% humidity) for development. Embryos were staged according to the Hamburger and Hamilton criteria (*9*) and electroporated at HH stage 14. On the day of electroporation a small window was cut in the shell to expose the embryo. PBS containing 0.1% Penicillin-Streptromycin (Invitrogen) was added to keep the embryos moist. Plasmid DNA was dissolved in ddH_2O to give a final concentration of 2.5 µg/µl. Visualisation of DNA during injection was aided by adding 0.5 µl of 10 mg/ml fast green to 2 µl of DNA solution. A modified method of (*10*) was applied. 1nl of DNA was injected into the lumen of the neural tube using a picospritzer (Intracell). Two platinum electrodes of 0.3 mm diameter and 0.5 mm exposed tip length were placed parallel to the spinal cord. A 3-4 mm gap was maintained between electrodes. A CUY21-EDIT electroporator was used to generate square pulses (3 pulses at 15 volts, pulse length 50 milliseconds with 150 milliseconds interval). Eggs were then sealed and incubated for 24-48 hours. Embryos were then dissected out and fixed using 4% paraformaldehyde for 1 hour at room temperature.

Cryosections: Fixed embryos were washed with PBST (PBS + 0.1% Triton X-100) 3 times for 30 minutes each at room temperature and processed through ascending concentrations of sucrose (10%, 20%, 30%) for 30 minutes each at room temperature. They were finally washed in 1:1 30% Sucrose:OCT and then in OCT for 30 minutes each at room temperature. Embryos were then embedded in OCT, flash-frozen in iso-Pentane (Sigma) using liquid nitrogen and sectioned using a cryostat maintaining a thickness of 20 µm to 25 µm.

Immunofluorescence: Frozen sections were washed in PBS, permeabilised in PBST, blocked using 10% FCS/PBS for 1 hour and incubated with primary antibodies overnight at 4°C (Sigma rabbit anti-HA, 1:1000; NEB mouse anti-myc, 1:1000). Sections were washed again before incubation with fluorescent secondary antibodies for 1 hour at room temperature (anti-rabbit Alexa Fluor 488, 1:500 and anti-mouse Alexa Fluor 568, 1:500, Invitrogen). After further washes sections were mounted using Vectashield. Images were captured and processed as described for CHO cells.

TUNEL: Apoptotic cell death was visualized on frozen sections by nuclear DNA fragmentation analysis using a DeadEnd Colorimetric TUNEL System (Promega Corporation, USA) with horseradish peroxidase-labeled streptavidin according to the manufacturer's protocol.

Supplementary Figures

Figure S1. Chromosome 15 linkage results for kindred ALS85

Genome-wide linkage analysis with SNPs suggested linkage to a locus on chromosome 15 in kindred ALS85. Fine-mapping with microsatellite markers across this region did not support linkage to this locus (see table S1).

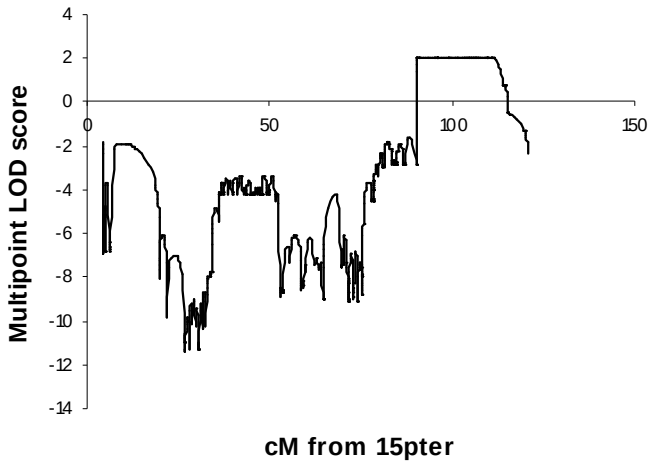


Table S1. LOD scores for kindred ALS85.

Fine-mapping of loci implicated in the genome-wide scan was conducted with microsatellite markers. Two-point linkage analysis of kindred ALS85 demonstrated contiguous markers with a LOD score greater than 1.0 (in bold) on chromosome 1p36 (encompassing *TARDBP)* but not 15q23. Furthermore, no haplotype could be made from the chromosome 15 markers.

Marker	**LOD score at recombination fraction**							**cM**
	0.00	**0.01**	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**	
Chr 1p36								
D1S468	-0.67	-0.61	-0.43	-0.28	-0.11	-0.04	-0.01	8.76
D1S2893	0.65	0.68	0.74	0.73	0.56	0.30	0.08	11.63
D1S2795	**1.31**	1.29	1.23	1.13	0.86	0.52	0.17	13.82
D1S2642	**2.25**	2.20	2.00	1.74	1.20	0.66	0.21	16.77
D1S2663	**2.47**	2.42	2.24	1.99	1.46	0.87	0.28	17.45
D1S2694	**1.73**	1.69	1.53	1.33	0.90	0.46	0.12	17.80
D1S450	**2.73**	2.68	2.48	2.21	1.64	0.99	0.33	22.14
D1S2667	**2.26**	2.21	2.02	1.77	1.25	0.72	0.23	24.75
D1S489	-1.43	-1.33	-1.02	-0.74	-0.37	-0.16	-0.04	27.27
D1S228	-1.37	-1.27	-0.97	-0.70	-0.34	-0.14	-0.03	30.31
D1S2834	-5.36	-0.05	0.49	0.59	0.47	0.25	0.06	31.53
D1S3669	-5.27	-0.28	0.38	0.58	0.58	0.38	0.13	40.29
D1S2644	-0.27	-0.20	0.02	0.18	0.29	0.24	0.10	44.25
D1S2725	-5.37	0.51	1.03	1.10	0.92	0.56	0.17	49.12
Chr 15q23-q26								
D15S1050	-6.77	-2.40	-1.10	-0.58	-0.17	-0.03	0.00	76.61
D15S114	-4.16	-1.40	-0.70	-0.41	-0.13	-0.01	0.01	78.41
D15S989	-4.15	-1.35	-0.67	-0.38	-0.12	-0.01	0.01	82.70
D15S969	-3.80	-1.06	-0.27	0.03	0.19	0.14	0.04	82.70
D15S205	-6.17	-1.74	-0.50	-0.07	0.18	0.16	0.06	87.45
D15S201	-3.18	-1.15	-0.39	-0.08	0.13	0.13	0.05	88.41
D15S1045	**1.05**	1.03	0.93	0.81	0.56	0.30	0.09	93.98
D15S202	-2.68	-0.47	0.15	0.34	0.38	0.24	0.07	94.62
D15S183	-2.90	-0.62	-0.00	0.19	0.24	0.15	0.04	95.31
D15S158	0.41	0.41	0.40	0.38	0.29	0.16	0.05	97.03
D15S652	-0.22	-0.22	-0.21	-0.19	-0.12	-0.06	-0.01	98.56
D15S1004	0.08	0.09	0.10	0.11	0.09	0.05	0.01	106.17
D15S1038	0.81	0.80	0.75	0.67	0.48	0.27	0.08	109.39
D15S657	-0.19	-0.18	-0.15	-0.10	0.00	0.03	0.01	114.40
D15S212	**1.54**	1.52	1.43	1.30	0.96	0.55	0.15	119.23
D15S985	0.85	0.84	0.78	0.70	0.49	0.26	0.06	121.77
D15S966	**1.54**	1.52	1.43	1.30	0.96	0.55	0.15	122.56
D15S87	-0.24	-0.24	-0.23	-0.21	-0.16	-0.09	-0.02	126.53
D15S642	-0.24	-0.24	-0.23	-0.21	-0.16	-0.09	-0.02	131.77

Table S2.

Primer pairs used to amplify exons and splice sites of *TARDBP*.

Exon	Forward	Reverse
1	TCAATCTTCAGCTTTTCAGGC	TGCCAGGACCTAACGACGCT
2	GAACTCTGACATGGTTTGGGT	TTTCAGGAGACATTCTGCCA
3	TGCCAAGTTTTCAGTGTCTTA	AGGGAACATAGTGATACCCCA
4	TTAAGCCACTGCATCCAGTTG	CCCTGCCGCTATCTTTTCTAA
5	GGCGAATGATTTTGTTATATC	CGGGACATATCGTTAAGGAGA
6	TGCTTATTTTTCCTCTGGCT	CTCCACACTGAACAAACCAA

Table S3.

Primers used for cloning and mutagenesis.

Description	**Sequence**
n-terminal myc-tagged forward primer with Sal I restriction site	GCGGCGGTCGACATGGAGCAAAAGCTCATTTCTGAAGAGGACT TGTCTGAATATATTCGGGTAACCGAAGATGAG
c-terminal HA-tagged reverse primer with Sal I restriction site	TTGGTTGTCGACCTAAGCATAATCAGGGACATCATAAGGATACA TTCCCCAGCCAGAAGAC
$TDP43_{Q331K}$ forward mutagenesis primer	CCAGGCAGCACTAAAGAGCAGTTGGGGTATGATG
$TDP43_{Q331K}$ reverse mutagenesis primer	CCATCATACCCCAACTGCTCTTTAGTGCTGCCTG
$TDP43_{M337V}$ forward mutagenesis primer	GCAGTTGGGGTATGGTGGGCATGTTAGCCAG
$TDP43_{M337V}$ reverse mutagenesis primer	GGCTGGCTAACATGCCCACCATACCCCAACTG

Figure S2. SNP in a control individual.

This exon 3 SNP is predicted to substitute alanine for valine (A90V).

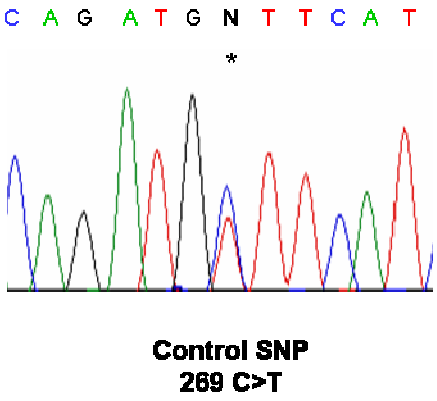


Figure S3. Whole western blot probed for myc (CHO cell cytoplasmic lysate)

NT = non-transfected, CAT = chloramphenicol acetyl transferase transfected,

W = WT; Q = Q331K; M = M337V.

Major bands labelled with arrows

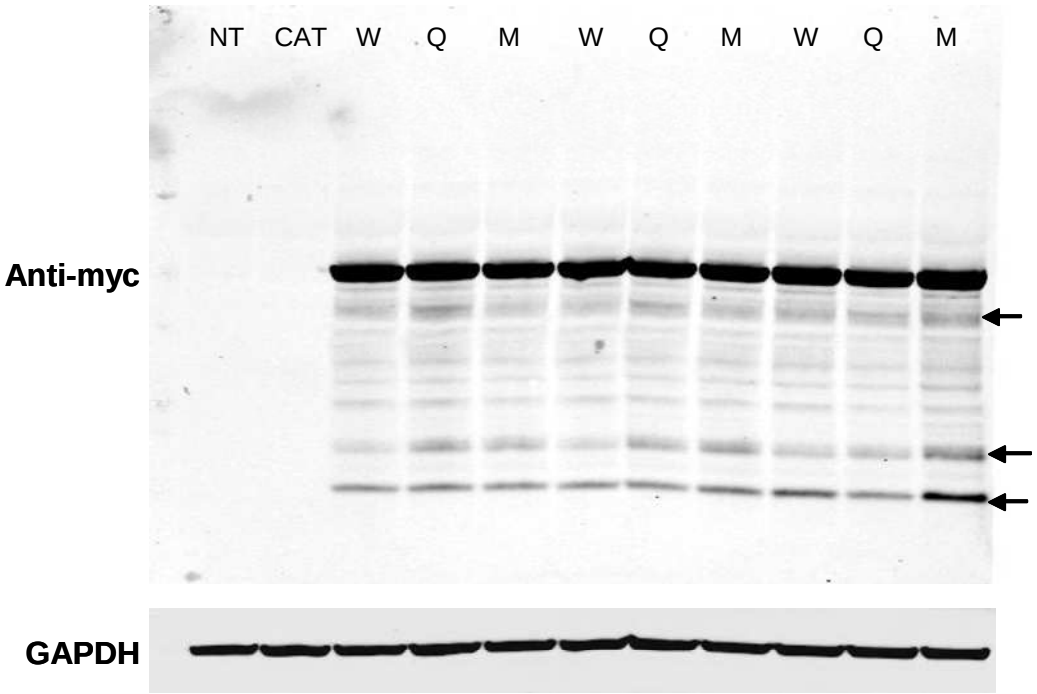
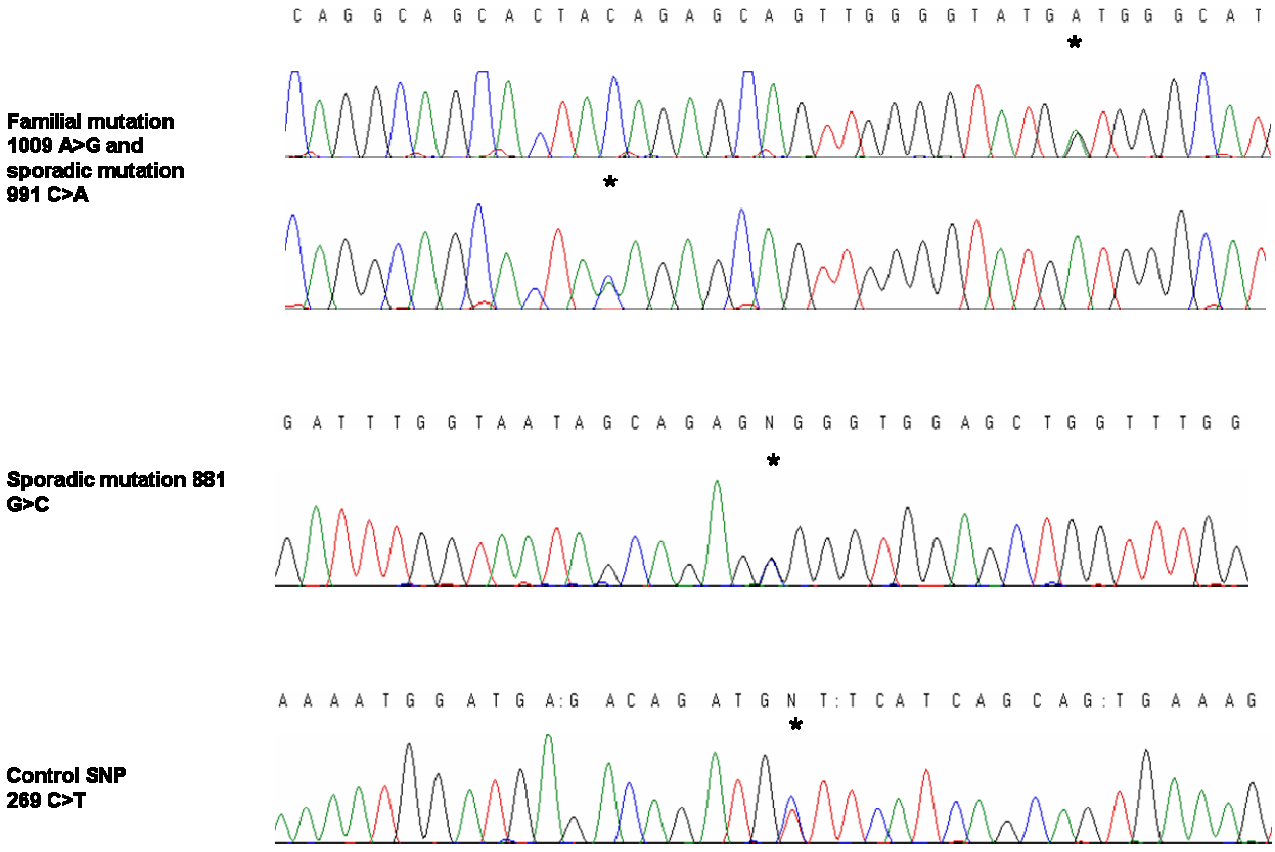


Figure S4. Chromatograms of identified mutations and control SNP



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