BROAD CLINICAL PHENOTYPES ASSOCIATED WITH TAR-DNA BINDING PROTEIN (*TARDBP*) MUTATIONS IN AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT (Max 150 words)

The finding of TDP-43 as a major component of ubiquitinated protein inclusions in amyotrophic lateral sclerosis (ALS) has led to the identification of 30 mutations in the *TARDBP* gene, encoding TDP-43. All but one are in exon 6, which encodes the glycine-rich domain. The aim of this study was to determine the frequency of *TARDBP* mutations in a large cohort of motor neurone disease (MND) patients from Northern England (42 non-SOD1 FALS, 9 ALS-FTD, 474 SALS, 45 PMA cases). We identified 4 mutations, 2 of which were novel, in 2 familial (FALS) and 2 sporadic (SALS) cases, giving a frequency of *TARDBP* mutations in non-SOD1 FALS of 5% and SALS of 0.4%. Analysis of clinical data identified patients had typical ALS, with limb or bulbar onset, and showed considerable variation in age of onset and rapidity of disease course. However, all cases had an absence of clinically overt cognitive dysfunction.

KEYWORDS: MND, ALS, TDP-43, *TARDBP*, mutation

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is one of the most common adult-onset, neurodegenerative diseases, characterised by progressive cell death of motor neurones (MNs) in the motor cortex, brainstem and spinal cord. Whilst the majority of cases are sporadic (SALS), a familial component is present in 5-10% (FALS), and is usually associated with an autosomal dominant mode of inheritance. To date, the most common cause of FALS is mutation of the *SOD1* gene, although this only accounts for 20% of FALS cases (1). Although other autosomal dominant loci have been identified (2), so far mutations in the two genes senataxin (*SETX*) and VAMP-associated protein B (*VAPB*) have not been found to be a common cause of ALS in large cohorts (3). In contrast, *SOD1* mutations have been found worldwide both in FALS and in apparent SALS cases (4).

Approximately 3 to 10% of patients with ALS also show signs of frontotemporal dementia (FTD), whilst detailed neuropsychological evaluation reveals up to 50% of ALS cases have evidence of cognitive impairment (5-8). Studies of cases with ALS + dementia + Parkinson's disease have revealed mutations in the *MAPT* gene, encoding the microtubule associated protein tau. In addition, two loci on chromosome 9, 9p12-p21 (9, 10) and 9q21-q22 (11), have been identified through genetic linkage studies to harbour genes responsible for ALS+FTD.

Neuropathological examination of both ALS and FTD cases has shown the presence of neuronal cytoplasmic inclusions (NCIs), which stain positive for ubiquitin, but negative for tau and α-synuclein. Neumann and colleagues demonstrated that a major protein component of these inclusions was TAR DNA binding protein (TDP-43) (12). TDP-43, a ubiquitously expressed nuclear protein, was originally identified as a protein that binds the transactive response (TAR) region of DNA of HIV-1 (13). In addition to acting as both a transcriptional repressor and an activator of exon skipping (14), it also reportedly plays a role as a scaffold protein for nuclear bodies, through interaction with survival motor neuron (SMN) protein (15). A further functional role for TDP-43 in motor neurones may be the stabilisation of neurofilament light (NFL) mRNA by direct binding of TDP-43 to the 3' untranslated region (UTR) of NFL (16).

Mackenzie and colleagues, investigating TDP-43 pathology in a cohort of 111 ALS cases, demonstrated that TDP-43 pathology was associated with SALS, FALS (without SOD1 mutation) and ALS + FTD, but not with SOD1-related FALS (17). This was followed by several screening studies where mutations and linkage of ALS was not found associated with the *TARDBP* gene, which encodes the TDP-43 protein (18, 19). However,

Sreedharan and colleagues identified mutations in exon 6 of *TARDBP* in two familial and two sporadic cases (20). This was supported by further studies identifying mutations in exon 6 in both familial and sporadic disease (21-25). The aim of the present study was to determine the frequency of *TARDBP* mutations in our large cohort of MND patients from the North of England and to establish the clinical phenotype associated with these mutations.

MATERIALS AND METHODS

The Sheffield MND Blood DNA Bank contains DNA extracted from 37 FALS, 8 ALS-FTD, 407 SALS and 34 progressive muscular atrophy (PMA) cases. Additional DNA samples were isolated from 5 non-SOD1 FALS, 1 ALS-FTD, 67 SALS and 11 PMA cases identified in the Sheffield Brain Tissue Bank, providing a cohort of 42 non-SOD1 FALS, 9 ALS-FTD, 474 SALS and 45 PMA cases. ALS patients had definite or probable ALS as defined by the El Escorial criteria. Cases with SOD1 mutations were excluded from the analysis. DNA was extracted from blood using the Nucleon BACC Genomic Extraction kit (Tepnel, UK) according to the manufacturer's protocol, whilst DNA was extracted from fresh frozen cerebellar samples using the Soft Tissue DNA Extraction Kit (Tepnel, UK). Control DNA (n=183) was extracted from blood donated by partners or unrelated carers of MND patients. Additional controls were obtained from the Birmingham MND DNA bank (n=316). All samples were from UK Caucasians. The South Sheffield Research Ethics Committee approved the study and informed consent has been obtained for all cases.

The *TARDBP* gene comprises of 6 exons of which the first exon is non-coding(24). Primers were designed to amplify the 6 exons (Ensembl transcript ID: ENST00000240185) as well as the intron/exon boundaries (Table 1). PCR products were treated with ExoSAP-IT (GE Healthcare) before bi-directional sequencing using BigDye Terminator v3.1 (ABI)

according to the manufacturer's protocol. The resulting reactions were electrophoresed on a DNA Analyser 3730 capillary sequencer (ABI). The chromatographs were analysed by Sequencher (Gene Codes Corporation, USA) and any potential mutations validated by sequencing a second PCR reaction.

To screen for the presence of the exon 6 nucleotide changes in the general population, 499 neurologically normal age-matched Caucasian controls were bi-directionally sequenced as described above. Control DNA samples (n=183) were also bi-directionally sequenced to screen for the exon 3 nucleotide change.

To establish the effect of the identified TARDBP mutations, quantitative PCR (QPCR) of CDK6 was performed on fibroblasts derived from patients carrying TARDBP mutations. RNA was extracted from 3 mutant TARDBP fibroblasts (p.G287S, p.A321V, p.M337V) and 6 control fibroblast cultures using the RNeasy Mini Kit, according to manufacturer's protocol (Qiagen). Following cDNA synthesis using Superscript II (Invitrogen), QPCR was used to determine the expression levels of CDK6 (Assay ID Hs01026372_m1, Applied Biosystems) in each of the samples. ACTB (Applied Biosystems) was used as the endogenous control. Reactions were run on an MX3000 (Stratagene) and data analysed using MX Pro. An unpaired T-test was used to establish if CDK6 expression levels were significantly different to controls.

RESULTS

Mutation screening of the 6 exons of the *TARDBP* gene, including intron/exon boundaries, in 42 FALS, 9 ALS-FTD, 474 SALS and 45 PMA cases identified 4 mutations in exon 6, of which 2 were novel. In addition, we identified 2 novel SNPs in the 5'UTR, 3 novel synonymous SNPs, 1 previously published synonymous SNP in the coding region and 3

intronic substitutions. A further non-synonymous change was found in a patient and a control (Table 2).

The 4 *TARDBP* mutations were found in 2 FALS cases and 2 SALS cases. In the first family (FALS1), the index case showed a c.1009A>G substitution leading to p.Met377Val (Fig 1a). Screening of family members showed that the sister of the index case, who is also affected with ALS, carries the mutation, whilst an unaffected brother was homozygous for the wild type allele (Fig 1b). In the second family (FALS2), a c.1043G>T substitution was found in the index case, leading to a p.Gly348Val amino acid change (Fig 2a). Screening of a brother, who was also affected with the disease, showed he also carried the mutation (Fig 2b). The two mutations found in SALS cases were a c.859G>A substitution which results in a p.Gly287Ser amino acid alteration (SALS1) and a c.962C>T, which leads to a p.Ala321Val substitution (SALS2) (Fig 3). None of these four mutations were found in 499 neurologically normal controls.

In addition, in a third SALS case (SALS3) an exon 3 c.269C>T substitution was identified, which encodes the amino acid alteration p.Ala90Val. Screening of controls also identified this mutation in a male aged 78 years at time of donation. Since it has also been published previously in controls, this substitution is likely to represent a non-pathogenic polymorphism (18, 20, 21). Novel synonymous SNPs were identified in 3 SALS cases in exon 2 (p.Leu27), exon 3 (p.Ser104) and exon 4 (p.Lys137), along with two SALS cases carrying the previously reported p.Ala66 SNP in exon 2 (18, 21, 23, 26-28).

Functional implications of the mutations:

The five amino acid substitutions were run through several databases to predict the effects of the alteration on the structure and function of the protein (Table 3). The two FALS

mutations p.M337V and p.G348V and the novel SALS mutation p.A321V are all predicted to be pathological according to PMut, and the two FALS mutation are also likely to be damaging, according to PolyPhen. In contrast, the G287S substitution found in SALS1 is listed as benign by PolyPhen and neutral by PMut, as is the A90V variant, found in both a case and control.

In order to establish the functional implications of the TARDBP mutations in-vivo, we obtained fibroblast cultures from 3 ALS patients, carrying the p.G287S, p.A321V and p.M337V. TDP43 has been shown to repress the expression of cyclin-dependent kinase 6 (CDK6), via GT repeats in the CDK6 sequence (29). QPCR showed that CDK6 transcript levels were increased in the mutant TARDBP fibroblast samples, compared to levels in controls (Fig 4), suggesting that the three mutant TDP43 proteins were unable to repress CDK6.

Clinical phenotypes:

These are summarised in Table 4.

FALS1: (Fig 1)

The index case (II.1) developed mild dysarthria at the age of 57 years. Subsequently he went on to develop a mild distal weakness with loss of dexterity in the left hand and painless left foot drop. The signs were of a classical ALS phenotype with mixed upper and lower motor signs in the bulbar and limb regions. Disease progression has been slow and 48 months since the onset of symptoms the individual remains ambulant with assistance, manages an oral diet with consistency changes, and is free from respiratory symptoms. The ALS functional rating scale (ALSFRS) score at his last visit was 32/40. Forced vital capacity (FVC) measurements are unreliable due to his bulbar disease but daytime pCO2 measurements are normal.

A sister (II.2) at 42 years old, developed progressive weakness of the right hand. Within three years the individual had similar problems in the left arm and had developed a spastic dysarthria and mild dysphagia. Seven years into the illness the lower limbs were affected with slow walking only possible over short distances. Nine years after onset of symptoms the individual was effectively wheel chair bound and had begun to develop symptoms of respiratory insufficiency. Over the next decade the disease slowly progressed and the individual died of pneumonia, 17 years after disease onset.

A brother (II.4) developed ALS at the age of 31 and experienced rapid disease progression, dying only 9 months after the onset of symptoms. The daughter of II.4, III.1, has a progressive neurodegenerative disorder affecting the pyramidal and cerebellar systems. III.1 was born by Ventouse extraction at term after a normal pregnancy and initially achieved normal milestones, walking by 11 months. However by the age of 2.5 years language and intellectual impairment was apparent. Her walking deteriorated over a decade until she was largely confined to a wheelchair. In addition, at the age of 12 years she developed generalised seizures. The major features on examination were a spastic tetraparesis with relatively preserved power, a concomitant convergent strabismus, and intellectual impairment. Investigations revealed an atrophic cerebellum on MRI scan, with other investigations normal, including nerve conduction studies, electro-myography (EMG), lysosomal enzymes, genetic screening of SCA 1-3, SCA 6, SCA7, Frataxin and DRPLA.

The mother (I.2) of the index case, developed progressive loss of voice and limb weakness due to MND at the age of 55 and died of the illness 6 years later. A further brother (II.3), who does not carry the p.M337V mutation is healthy at the age of 51, with no evidence of ALS.

FALS2: (Fig 2)

The index case (II.1) developed painless left foot drop when 51 years old. Weakness progressed to the left leg and right arm before becoming generalised involving respiratory muscles. Bulbar function remained relatively intact throughout the disease course. Signs of upper motor neurone involvement were subtle and consisted of retained reflexes in the context of muscle wasting. Respiratory dysfunction necessitated non-invasive ventilation. The individual died from respiratory failure, 36 months from symptom onset.
The younger brother (II.2) developed progressive weakness of the right upper limb at 57 years of age. The weakness progressed bilaterally resembling a flail arm phenotype of MND, before becoming generalised affecting all limbs and respiratory muscles. Upper motor neurone involvement and bulbar dysfunction were again minimal. Thirty-six months after symptom onset the individual died from respiratory failure.
In the preceding generation, the mother (I.2) had died from MND at the age of 65 years. A maternal aunt (1.3) and uncle (1.4) were reported to have died from muscular atrophy in their 40's. No further information was available on these cases.

SALS1: This individual presented at the age of 52 years with a 2-year progressive history of clumsiness of the left hand and left leg. At presentation the individual had mixed upper and lower signs in the left upper and lower limbs with dysarthria and a weak fasciculating tongue. The disease in this individual has run a relatively indolent course and 6 years following symptom onset bulbar symptoms remain mild and there is no evidence of respiratory insufficiency. Limb weakness has progressed with some help required with activities of daily living such as dressing and a wheelchair is necessary for travelling outside the home. There is no known family history of neurological disease.

SALS2: This individual developed progressive weakness and loss of dexterity of the left hand at the age of 38 years. On examination there were mixed upper and lower motor

neurone signs in the left upper limb. EMG demonstrated more widespread denervation consistent with a diagnosis of ALS and investigation, including imaging of the neuroaxis, has not identified an alternative cause. The individual, two years following the onset of symptoms, is experiencing increasing weakness of the left hand and fatigue but no other major problems, particularly no dysarthria, dysphagia or leg weakness. There is no family history of neuromuscular disorder. Her mother died at the age of 44 years and father at 62 years both due to malignancy. Two siblings are healthy at the ages of 37 and 41 years.

It should be noted that the mutations were all found in DNA from the Sheffield MND Blood DNA Bank, rather than the Sheffield Brain Tissue Bank. Therefore no neuropathological investigation has been possible in these cases.

DISCUSSION

Screening of a large cohort of MND cases including 42 FALS and 474 SALS has identified 4 mutations in exon 6 of the *TARDBP* gene, the p.Gly287Ser and p.Ala321Val in two SALS cases and the p.Met337Val and p.Gly348Val in two FALS cases. This suggests a frequency for *TARDBP* mutations in Northern England of 5% for FALS and 0.4% for SALS. Three novel synonymous substitutions were also identified in cases of SALS, p.Leu27, p.Ser104 and p.Lys137. An additional non-synonymous substitution p.Ala90Val, which has previously been found in controls (20, 21), was also found in a SALS case. Although it is predicted to have no effect on protein function, in-vitro studies have shown mis-localisation of this protein (30).

Broad clinical ALS phenotypes are associated with *TARDBP* mutations:

The clinical phenotype encompasses patients with bulbar, limb and respiratory onset disease, and with short and long disease durations. All our patients had clinical features in

keeping with ALS and no mutations were found in patients with a pure lower MN clinical phenotype (PMA). It is noteworthy that despite TDP-43 protein inclusions being originally identified in FTD cases, none of the FALS or SALS cases published to date show any evidence of overt dementia. However, two mutations in TARDBP, p.K263E and c.2076G>A in the 3'UTR, have recently been found to be associated with individuals diagnosed with FTD (31, 32). Further post-mortem studies in cases with *TARDBP* mutations may elucidate differential pathologies in these cases compared to the range of TDP-proteinopathies that show TDP-43 inclusions.

The familial p.Met337Val mutation has been identified in three other families with MND (20, 23, 28). In FALS1 (Met337Val) a marked intrafamilial variation in disease duration was observed, ranging from 9 months to 17 years. The average disease duration was 6.9 years compared to 5.5 years in the published detailed pedigree. Age at onset of symptoms is consistent with that previously published as is the classical ALS/MND phenotype observed. It is unclear whether the atypical phenotype manifesting in individual III.1 from this family is as a result of expression of TDP-43 mutation, as genetic testing has not been possible.

The familial p.Gly348Val mutation is a novel substitution, though p.Gly348Cys has been reported in four SALS cases from France, two cases in a family from Germany and in a large multigenerational family from Belgium (21, 22, 26, 27). In FALS2 the phenotype observed was predominantly lower motor neurone, with only subtle upper motor neurone changes consisting of retained reflexes in the context of wasting. In individual II.2 the disease initially resembled the flail arm variant. Bulbar function also remained intact in these individuals. In a previously reported mutation affecting this amino acid it was also commented that there was a lack of bulbar symptoms (22). A paucity of upper motor

neurone signs has been described with other mutations (21). The age at onset and duration were comparable with other published pedigrees.

The p.Gly287Ser mutation has been described previously in 2 SALS cases, one from France, the other from Italy (21, 28). In our SALS1 case, the age of onset was 52 years, beginning in the limb. Whilst the Italian case also had spinal onset, at the age of 70 years, the French case presented with bulbar onset at 65 years. In all three cases disease course has been relatively indolent with the affected individuals still living 5-7 years after symptom onset.

Therefore, taking into account all the published reports of ALS cases with *TARDBP* mutations, there does not appear to be any correlation between the age of onset, site of onset and disease duration of the patients with specific *TARDBP* mutations, taken either individually or as a group. This is a similar scenario to that observed in *SOD1*-related MND, where there is both inter and intra-familial variation with the same mutation. Also in line with *SOD1*, mutations in *TARDBP* are found in both familial and sporadic forms of the disease, with frequencies of *TARDBP* mutations from 0.6 to 6.5% for FALS and 0 to 5% for SALS, depending on the population sampled (20-26, 28, 33). This compares with *SOD1* mutation frequencies of 12-24% for FALS and 0-7% for SALS (4).

Functional effects:

All but one of the mutations identified to date reside in exon 6 of the *TARDBP* gene, which encodes for the end of the second RNA recognition motif (up to amino acid 262) and the entire glycine-rich domain of the TDP-43 protein (aa 274-413) (Swiss-Prot Q13148 TARDBP_HUMAN). This C-terminal region of the protein shows high levels of conservation (Fig 5) and the 4 mutations all affect highly conserved amino acid residues,

which show conservation in mammals as well as chicken and zebrafish. The glycine-rich domain is involved in regulating alternative splicing, as demonstrated with cystic fibrosis transmembrane conductance regulator (*CFTR*) and apolipoprotein A-II (*APOA2*). This domain has also been shown to bind several hnRNP proteins involved in mRNA synthesis (14, 34, 35). The C-terminal of TDP-43 has also been implicated as a transcriptional repressor. Thus mutations in this region could have several deleterious effects on the function of the protein. Sreedharan has demonstrated that expression of the mutant p.M337V TDP-43 in the chick embryo results in the failure of normal limb and tail bud development (20). In addition, both Kabashi and Rutherford show increased aggregation of detergent insoluble mutant TDP-43 (21, 23).

Our bioinformatic analyses suggest that the two FALS and the novel SALS mutation are predicted to alter the function of the protein, whilst the p.Gly287Ser mutation, which has been found in an additional SALS case, is not predicted to have a major effect on the protein. However, QPCR of CDK6, which is repressed by TDP-43, shows an increase in expression in fibroblasts carrying the mutant proteins p.G287S, p.A321V and p.M337V. Therefore, we propose that these 3 mutants are unable to repress CDK6 as efficiently as the control fibroblasts.

In conclusion, mutations in *TARDBP* are found at a frequency of 5% for non-SOD1 FALS and 0.4% for SALS in a large cohort of MND cases from Northern England. As found previously, these mutations reside in exon 6 of the gene, potentially interfering with the function of the glycine rich domain. Analysis of clinical data suggests variability in the disease phenotype, though it is of note that the MND cases show no overt dementia symptoms.

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FIGURE LEGENDS

Fig 1 a) Chromatograph showing the c.1009A>G nucleotide substitution identified in FALS1 and other affected members of the family. b) Pedigree of FALS1 family. The index case is indicated by a *. WT = wild type, homozygous for M337

Fig 2 a) Chromatograph showing the c.1043G>T nucleotide substitution identified in FALS2 and the other affected family member. b) Pedigree of FALS2 family. The index case is indicated by a *

Fig 3 Chromatograph showing the a) c.859G>A nucleotide change in SALS1 and b) c.962C>T in SALS2

Fig 4 Expression levels of CDK6, relative to ACTB, in mutant TARDBP and control fibroblast cultures. * p=0.015.

Fig 5 Alignment of the TDP-43 protein sequence encoded by exon 6 in human. Protein sequences used in the alignment are Human (Q13148), Chimpanzee (Pan troglodytes:XP_001135199), Orangutan (Pongo abelii:Q5R5W2), Mouse (Mus musculus:Q921F2), Chicken (Gallus gallus:Q5ZLN5), Xenopus (X.tropicalis:Q28F51) and zebrafish (Danio rerio: Q802C7). Location of the mutations identified in Sheffield MND cases are highlighted in green (p.G287S, p.A321V, p.M377V, p.G348V). The locations of the other published mutations in exon 6 are highlighted in blue (p.G290A, p.N267S, p.G294A/V, p.G295S/R, p.G298S, p.M311V, p.A315T, p.Q331K, p.S332N, p.G335D, p.Q343R, p.N345K, p.N352S, p.R361S, p.P363A, p.Y374X, p.S379P/C, p.A383T/P, p.I383V, p.N390S/D and p.S393L) p.G348C has also been reported.

Table 1: PCR primers used to amplify the *TARDBP* gene. Primer sequences, annealing temperature and size of product are given. All PCR reactions were carried out using the following programme: Initial denaturation of 95^{o}C for 5 mins, followed by 35 cycles of 95^{o}C for 30 secs, specific annealing temperature for 30 secs, and 72 ^{o}C for 45 secs, followed by a final elongation step of 72 ^{o}C for 10 mins to complete the programme.

Primer	Sequence	Annealing Temperature	Product Size
TARDBP.1F TARDBP.1R	ggc agc ccg agt ccc tgg gga gag g ctc ggg ccg ccc caa tgc aga aag	61^{o}C	277bp
TARDBP.2F TARDBP.2R	tgg ttt ggg tat tat cat t cca cca aaa gag gct aag a	49 ^{o}C	411bp
TARDBP.3F TARDBP.3R	tag atg tag gag gta gtg ttt tta ata cca ataaat aaa tgc ta	50 ^{o}C	330bp
TARDBP.4F TARDBP.4R	taa gtt taa gcc act gca tcc ag ggc caa aga ctt caa caa gac aa	54 ^{o}C	400bp
TARDBP.5F TARDBP.5R	aag gcg aat gat ttt gtt aaa gtg ctg gga ttg taa g	51 ^{o}C	332bp
TARDBP.6F TARDBP.6R	tat atg aat cag tgg ttt aat ctt caa tat act tac cat gag ttt aga	50 ^{o}C	656bp

Table 2: *TARDBP* nucleotide substitutions identified in FALS and SALS cases. All mutations reported according to the Human Genome Variation Society guidelines. Numbering is taken from the Ensembl transcript ID ENST00000240185. * The ATG resides in exon 2, therefore the first nucleotide of exon 2 is called c.-12.

SAMPLE	EXON	DNA	PROTEIN	COMMENTS
SALS1	6	c.859G>A	p.Gly287Ser	Previously seen in SALS (21, 28)
SALS2	6	c.962C>T	p.Ala321Val	Novel mutation
FALS1	6	c.1009A>G	p.Met337Val	Previously seen in FALS (20, 23, 28)
FALS2	6	c.1043G>T	p.Gly348Val	Novel mutation (p.Gly348Cys previously reported) (21, 22, 26, 27)
SALS3 and CONTROL1	3	c.269C>T	p.Ala90Val	Previously reported in control samples (18, 20, 21)
SALS4	1	c.-69C>T	5'UTR	Novel polymorphism
SALS5	1	c.-66G>T	5'UTR	Novel polymorphism
SALS6	Intron 1	c.-12-54G>A*		Novel polymorphism
FALS3	2	c.81G>A	p.Leu27	Novel polymorphism, synonymous change
SALS7 and SALS8	2	c.198T>C	p.Ala66	rs61730366, synonymous change (18, 21, 23, 26, 27)
SALS9	3	c.312C>T	p.Ser104	Novel polymorphism synonymous change
SALS10	4	c.411A>G	p.Lys137	Novel polymorphism, synonymous change
SALS11	Intron 3	c.403-80G>A		Novel polymorphism
SALS12	Intron 4	c.543+112C>A		Novel polymorphism

Table 3: Summary of bioinformatic analyses to determine the functional effects of the non-synonymous changes identified in our samples. G348C is also included for comparison.

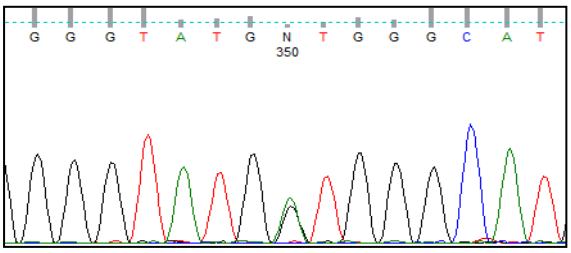
Mutation	**I-Mutant**	**NetPhos**	**PolyPhen**	**PMut**
A90V	Decreased stability	No change	Benign substitution	Neutral
G287S	Increased stability	Serine not phosphorylated	Benign substitution	Neutral
A321V	Decreased stability	No change	Benign	Pathological
M337V	Decreased stability	No change	Possibly damaging	Pathological
G348V	Increased stability	No change	Probably damaging	Pathological
G348C	Increased stability	No change	Probably damaging	Pathological

Table 4: Summary of clinical details for the *TARDBP*-related ALS cases. n/a = not applicable, * = index case, [a] = individual alive at time of writing. WT = wild type, homozygous for M337

	MND Status	**Mutation**	**Age of Onset**	**Site of Onset**	**Duration**	**Dementia**
FALS1						
I:2	Affected	Not tested	55	Cervical/Bulbar	6 yrs	
II:1*	Affected	M337V	57	Bulbar	>4 yrs[a]	No
II:2	Affected	M337V	42	Cervical	17 yrs	No
II:3	Unaffected	WT	n/a	n/a	n/a	n/a
II:4	Affected	Not tested	32	Cervical	9 mths	
III:1	Unclear	Not tested	2.5	n/a	>15 yrs[a]	Intellectual impairment
FALS2						
I:2	Affected	Not tested	Unknown	Unknown	Died at 65yrs	Unknown
II:1*	Affected	G348V	57	Lumbar	3 yrs	No
II:2	Affected	G348V	52	Cervical	1yr 9 mths	No
SALS1	Affected	Gly287Ser	52	Cervical/Lumbar	>6 yrs[a]	No
SALS2	Affected	Ala321Val	38	Cervical	>2 yrs[a]	No

Fig 1

a)


G G G T A T G N T G G G C A T
350

b)

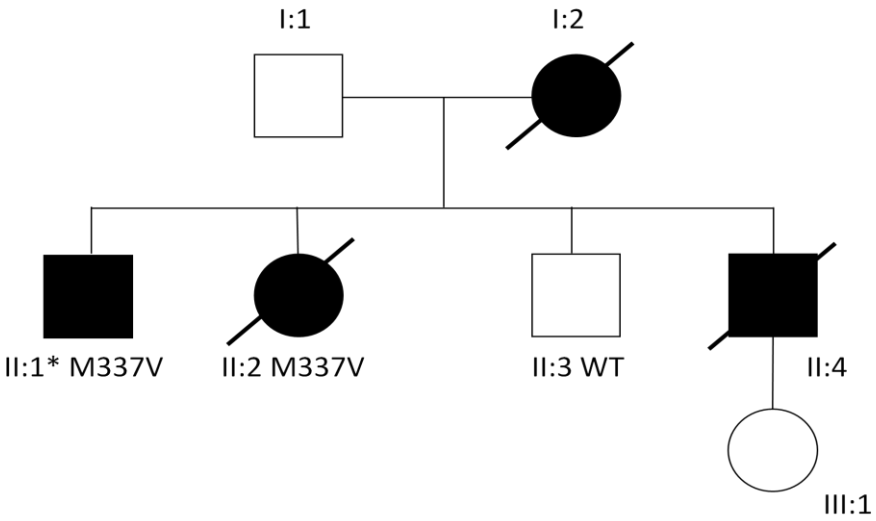
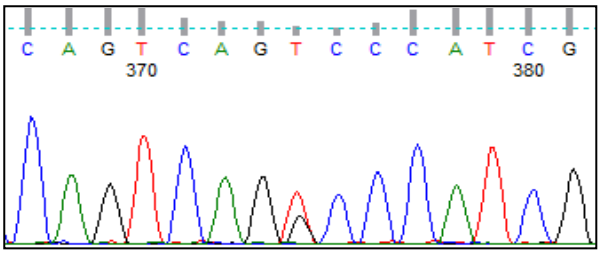

I:1
I:2
II:1* M337V
II:2 M337V
II:3 WT
II:4
III:1

Fig 2

a)



b)

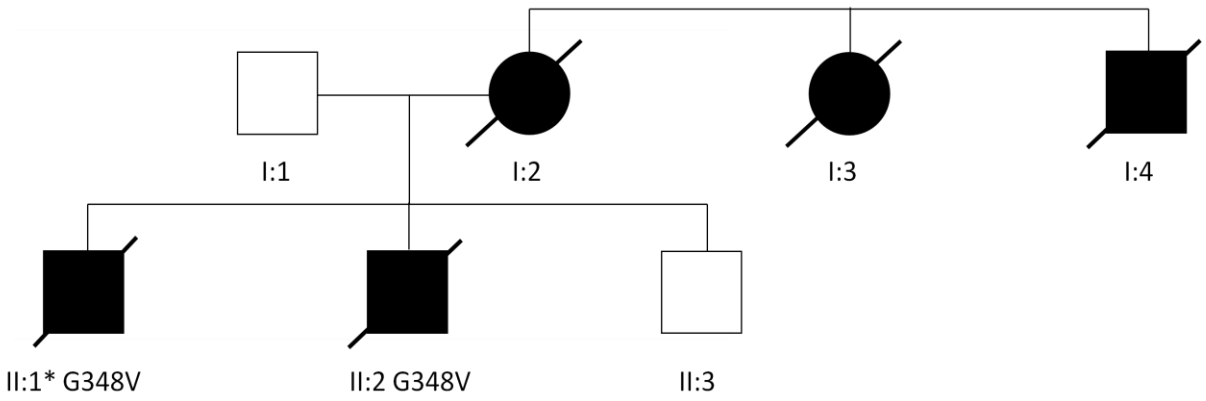


Fig 3

a) b)

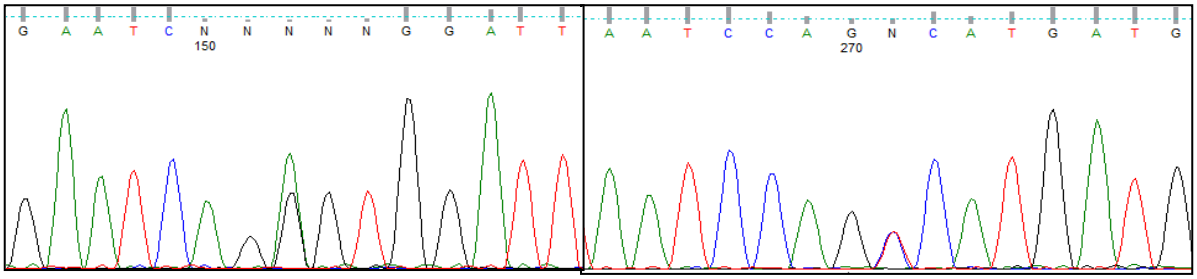


Fig 4

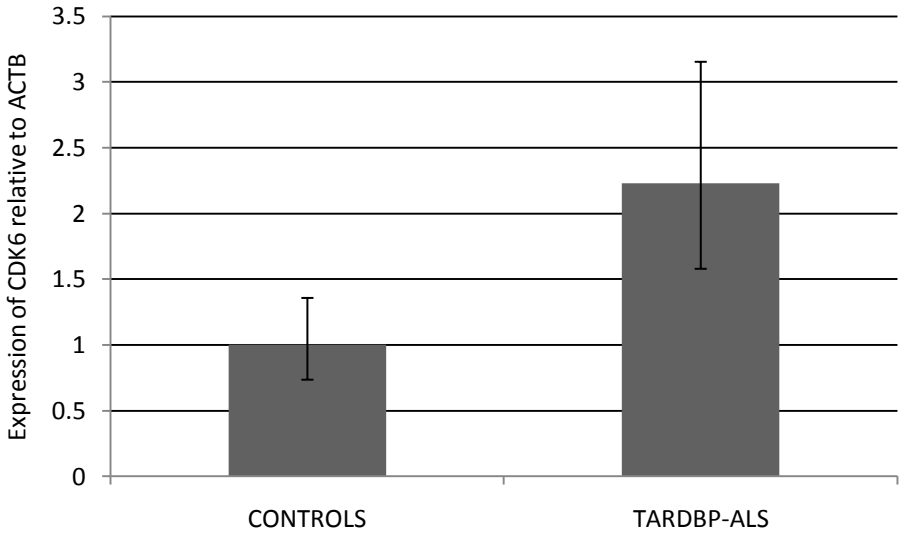
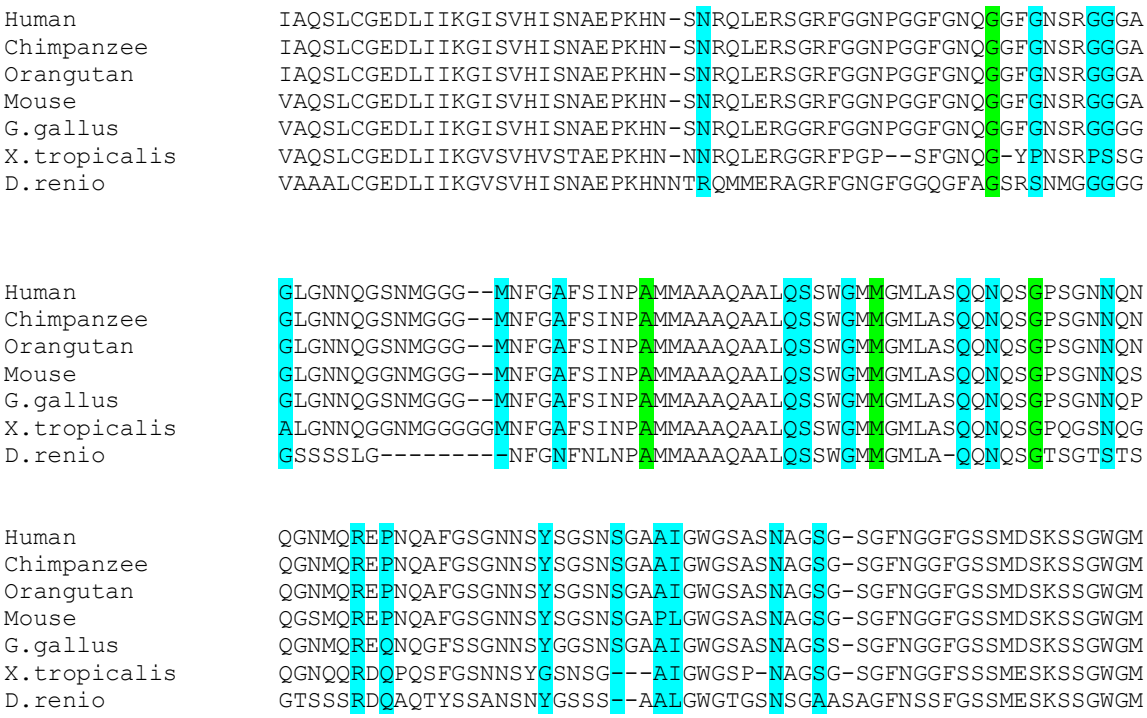

Expression of CDK6 relative to ACTB
3.5
3
2.5
2
1.5
1
0.5
0
CONTROLS
TARDBP-ALS

Fig 5


Human IAQSLCGEDLIIKGISVHISNAEPKHN-SNRQLERSGRFGGNPGGFGNQGGFGNSRGGGA
Chimpanzee IAQSLCGEDLIIKGISVHISNAEPKHN-SNRQLERSGRFGGNPGGFGNQGGFGNSRGGGA
Orangutan IAQSLCGEDLIIKGISVHISNAEPKHN-SNRQLERSGRFGGNPGGFGNQGGFGNSRGGGA
Mouse VAQSLCGEDLIIKGISVHISNAEPKHN-SNRQLERSGRFGGNPGGFGNQGGFGNSRGGGA
G.gallus VAQSLCGEDLIIKGISVHISNAEPKHN-SNRQLERGGRFGGNPGGFGNQGGFGNSRGGGG
X.tropicalis VAQSLCGEDLIIKGVSVHVSTAEPKHN-NNRQLERGGRFPGP--SFGNQG-YPNSRPSSG
D.renio VAAALCGEDLIIKGVSVHISNAEPKHNNTRQMMERAGRFGNGFGGQGFAGSRSNMGGGG
Human GLGNNQGSNMGGG--MNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQN
Chimpanzee GLGNNQGSNMGGG--MNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQN
Orangutan GLGNNQGSNMGGG--MNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQN
Mouse GLGNNQGGNMGGG--MNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQS
G.gallus GLGNNQGSNMGGG--MNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQP
X.tropicalis ALGNNQGGNMGGGGGMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPQGSNQG
D.renio GSSSSLG---------NFGNFNLNPAMMAAAQAALQSSWGMMGMLA-QQNQSGTSGTSTS
Human QGNMQREPNQAFGSGNNSYSGSNSGAAIGWGSASNAGSG-SGFNGGFGSSMDSKSSGWGM
Chimpanzee QGNMQREPNQAFGSGNNSYSGSNSGAAIGWGSASNAGSG-SGFNGGFGSSMDSKSSGWGM
Orangutan QGNMQREPNQAFGSGNNSYSGSNSGAAIGWGSASNAGSG-SGFNGGFGSSMDSKSSGWGM
Mouse QGSMQREPNQAFGSGNNSYSGSNSGAPLGWGSASNAGSG-SGFNGGFGSSMDSKSSGWGM
G.gallus QGNMQREQNQGFSSGNNSYGGSNSGAAIGWGSASNAGSS-SGFNGGFGSSMDSKSSGWGM
X.tropicalis QGNQQRDQPQSFGSNNSYGSNSG---AIGWGSP-NAGSG-SGFNGGFSSSMESKSSGWGM
D.renio GTSSSRDQAQTYSSANSNYGSSS--AALGWGTGSNSGAASAGFNSSFGSSMESKSSGWGM

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