NEUROPATHOLOGICAL CHARACTERISATION OF A NOVEL TBK1 LOSS OF FUNCTION MUTATION ASSOCIATED WITH AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT

Mutations in TANK binding kinase gene (*TBK1*) have been identified as causative in amyotrophic lateral sclerosis (ALS). Here, we examine the spectrum of *TBK1* mutations in a cohort of ALS patients from Northern England, comparing missense and loss of function mutations with clinical phenotype. Analysis of 290 ALS cases identified seven variants, including one novel in-frame deletion (p.Ile85del). *In silico* analysis and review of the literature suggested that four variants, one nonsense mutation (p.Glu2Ter), two in-frame deletions (p.Ile85del, p.Glu643del) and one missense mutation (p.Gln565Pro) were pathogenic, whilst the remaining three missense mutations were variants of uncertain significance or benign. Post-mortem material was available from the patient with the novel in-frame deletion. Neuropathological examination established this individual had classical ALS pathology, with moderate phosphorylated TDP-43 neuronal and glial cytoplasmic inclusions in the motor cortex, skein-like inclusions in the lower motor neurons and "pre-inclusions" in the medulla. This corresponds to Type B FTLD-TDP pathology and is consistent with previously published literature on TBK1 mutants. In addition to demonstrating no changes in TBK1 staining, we are the first to show there was no differential expression of interferon regulatory factor IRF3, a downstream effector of TBK1 in the innate immunity pathway, in the TBK1-mutant tissue compared to controls. Comparison of clinical and neuropathological data, however, suggests that TBK1-ALS cases show classical ALS pathology but no specific phenotype.

ABBREVIATIONS:

ALS = amyotrophic lateral sclerosis

bv-FTD = behavioural variant FTD

C9ORF72 = chromosome 9 open reading frame 72

EMG = electromyography

FALS = familial ALS

FTD = frontotemporal dementia

FTLD-TDP = frontotemporal lobar degeneration with TDP-43 pathology

IKK = IkB kinase

IRF3/7 = interferon regulator factor 3/7

LoF = loss of function

OPTN = optineurin

PPA = primary progressive aphasia

SALS = sporadic ALS

SOD1 = Cu/Zn superoxide dismutate 1

SQSTM1 = sequestosome/p62

TARDBP = TAR DNA binding protein

TBK1 = TANK binding kinase gene

TDP-43 = 43kDa protein product of TARDBP gene

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult onset, progressive neurodegenerative disease, characterised by the loss of both upper and lower motor neurons. Although the majority of ALS cases are sporadic (SALS), approximately 10% are familial (FALS) and the most common genetic causes are mutations in *C9ORF72*, *SOD1*, *TARDBP* and *FUS* genes (1). However, it is becoming apparent that mutations in the 27 genes currently associated with FALS may also be present in apparently sporadic cases. In addition, there is evidence of mutations in several of these genes being associated with both ALS and frontotemporal dementia (FTD), termed ALS-FTD (1). ALS is a heterogeneous disease with the site of initial clinical presentation, disease progression rates, and involvement of cognitive and behavioural impairment showing variation between individuals (2). A wide range of survival times and age of disease onset are observed, even in individual members of families with the same pathogenic mutation (3).

Mutations in TANK binding kinase gene (*TBK1*) located on chromosome 12 (12q14.2), were first linked to ALS in 2015 (4, 5). To date, multiple loss-of-function (LoF) and missense *TBK1* variants have been reported and the majority of these are associated with either ALS, ALS-FTD or FTD (6-13). However, mutations in *TBK1* have also been identified in cases of progressive bulbar palsy, primary progressive aphasia, monoparesis, progressive supranuclear palsy-like and cerebellar syndromes, as well as in cases of early onset Alzheimer's disease and unspecified dementia (5, 9, 14, 15).

Mutations altering splice-sites, in-frame deletions, frameshifts, and LoF variants show definite or probable pathogenicity, whilst the pathogenicity of missense variants are less certain. However, recently, Cui and colleagues showed that there is a significantly increased risk for ALS/FTD disease in individuals with LoF (Odds Ratio 11.78) and a moderately increased susceptibility for those carrying missense mutations (Odds Ratio 1.62) (16). In addition, de Majo and colleagues demonstrated that missense *TBK1* mutations in functional domains impair the binding and phosphorylation of its target proteins, thereby causing a loss of function similar to that seen with truncation mutations (17).

The TBK1 protein is a ubiquitously expressed serine-threonine kinase belonging to the IbK kinase (IKK) family, which is normally located in the cytoplasm. It is a key regulator of innate immunity signalling pathways. Following activation, TBK1 dimers phosphorylate interferon regulatory factors IRF3 and IRF7, which subsequently form homodimers and translocate to the nucleus, where they act as transcription factors for type 1 interferons (18). TBK1 also plays a role in autophagy and mitophagy, through phosphorylation of adaptor proteins such as optineurin (OPTN), p62/sequestosome (SQSTM)

and nuclear dot protein 25 (NDP25), which act by binding ubiquitinated proteins and mitochondria and enhancing their binding to the autophagosome protein LC3-II (18). Interestingly, mutations in *OPTN* and *SQSTM1* have also been associated with ALS (19, 20).

Correlations between clinical features and *TBK1* mutations have not been fully elucidated. However, one study reported a later age of onset for *TBK1*-mutation carrying FTD patients (7). To explore the relationship between *TBK1* mutations and disease phenotype, the aims of this study were to screen our cohort of ALS patients for mutations in *TBK1* and to correlate LoF and missense variants with clinical and neuropathological features of ALS. Comparison of clinical and neuropathological data, however, did not establish any specific genotype/phenotype correlations within this cohort of TBK1-ALS cases.

MATERIALS AND METHODS

Genomic DNA was extracted from 290 unrelated ALS cases, sourced from post-mortem cerebellum tissue (n=111) or whole blood samples (n=179), using the Promega Maxwell RSC system (Wisconsin, USA), as per manufacturer's instructions. ALS cases all had definite or probable ALS as defined by the El Escorial criteria. The South Sheffield Research Ethics Committee approved the study (12/YH/0330) and informed consent was obtained from those participants who donated a blood sample. The use of post-mortem tissue in the study was reviewed by the Sheffield Brain Tissue Bank (SBTB) Management Board and approval to release tissue was granted under REC 08/MRE00/103.

The *TBK1* gene is comprised of 21 exons, 20 of which are coding, and these were amplified using polymerase chain reaction (PCR) in a thermal cycler (G-Storm, Somerton, UK). Primers for *TBK1* exons 2, 4, 5, 6, 7, 8, 9, 10, 11, 16, 17-18, 19-20, and 21 were those described previously (21). Primers for *TBK1* exons 3, 12, 13, and 14-15 were re-designed using Primer Blast (NCBI; **Supplementary Table 1**). All primers were purchased from Eurofins Genomics (Ebersburg, Germany).

PCR exon amplification was confirmed using gel electrophoresis, and products purified by treatment with Exonuclease 1 (NEB) and Shrimp Alkaline Phosphatase (ThermoFisher). Samples were sequenced using BigDye v3.1 (ThermoFisher) according to standard protocols and run on an Applied Biosystems 3730 DNA analyser at the University of Sheffield Genetics Core Facility. The chromatograms were analysed using Geneious 9.1.5 (22). Sequences were aligned by Multiple Sequence Comparison by Log-Expectation and referenced to the Ensembl transcript ENST00000331710.5 from the Ensembl GRCh37 database. When potential mutations were found following alignment and comparison to the

reference genome, these were confirmed in a second PCR followed by sequencing with both forward and reverse primers.

All variants were compared to those reported in the literature and in online databases (GnomAD, ExAC, ClinVar, Project Mine Data Browser – all accessed 20 February 2019). Variant effect on protein structure and function were predicted using Mutation Taster (23), which uses a Bayes classifier to predict the effect of a change having been trained on a set of >390,000 known mutations associated with disease and over 6 million non-pathogenic variants. PolyPhen-2 (24) and SIFT (25) were used additionally for amino acid substitution analyses. Mutation Taster results of 'disease causing', SIFT values <0.05 and PolyPhen-2 results of 'probably damaging', or 'possibly damaging' were expected to be pathogenic. Additionally, potential splice site mutations were analysed using Human Splice Finder (26). Finally, PyMol (https://www.pymol.org/Schrödinger) was used to visualise variant effects on protein structure.

Post-mortem material was available from a patient with the p.Ile85del in-frame deletion of *TBK1* (Case 2). Representative blocks of brain, spinal cord and skeletal muscle were taken and fixed in formalin. Blocks were then embedded in paraffin wax, sectioned and stained for haematoxylin and eosin. Immunohistochemistry (IHC) for phosphorylated TDP-43, tau, CD68, TBK1 and IRF3 was performed (**Table 1**) using standard protocols (27, 28). Immunohistochemistry for TBK1 and IRF3 was performed in 3 neurologically normal controls and 3 sporadic MND cases in addition to Case 2.

RESULTS

Mutation analysis

Variant screening of the 20 coding exons of TBK1 identified 22 variants in 290 samples in our cohort of ALS cases (**Table 2; Supplementary Table 2**). Seven variants (one nonsense, two-in-frame deletions, and four missense variants) were predicted to cause amino acid changes in the TBK1 protein. Of these, further analysis predicted four variants to be pathogenic, including a novel mutation in exon 4 (**Table 2; Figure 1**). The remaining 15 variants comprised of 12 non-coding and 3 synonymous variants (**Supplementary Table 2**).

Functional implications of predicted pathogenic mutations

A nonsense mutation was detected in Case 1 within exon 2. The c.4C>T substitution resulted in a stop codon at the second amino acid, p.Glu2Ter. Pathogenicity was predicted by Mutation Taster as "disease causing" and the variant has been reported previously in ALS cases (4, 13, 17).

Two in-frame-deletions were detected in Cases 2 and 3. Case 2 carried a novel 3bp deletion, c.253-255delATT, in exon 4, which results in the deletion of isoleucine at amino acid 85, p.Ile85del (**Figure 2**). This residue is located within the kinase domain and is likely to be pathogenic, as predicted by Mutation Taster as "disease causing". Case 3 carried the second in-frame-deletion c.1928-1930delGAA, found in exon 18, which results in the deletion of a glutamic acid, p.Glu643del, within the coiled coil domain 1, involved in dimerization of TBK1 (**Supplementary Figure 1**). This variant is also predicted to be pathogenic, according to Mutation Taster, (disease causing) and has been reported previously in ALS (4, 5, 13).

In Case 4, a previously reported missense variant was detected at c.1694A>C in exon 15, which causes a glutamine to a proline substitution at amino acid 565, p.Glu565Pro. This mutation is predicted to be "disease causing" by Mutation Taster and as "probably damaging" (0.990) by PolyPhen and "deleterious" by SIFT (0.00). Pymol structural analysis showed a resultant protein change in the coiled coil domain 1, which is responsible for TBK1 protein dimerization (**Supplementary Figure 2**).

As shown in **Table 2**, the remaining three missense variants (p.Lys291Glu, p.His322Tyr, p.Val464Ala) have been found in controls as well as in ALS cases, though the pathogenicity of these is less clear. Mutation Taster predicts they are all "disease causing". The p.Lys291Glu is described in ClinVar as a variant of uncertain significance and it has been found more frequently in ALS than in controls according to the Project MinE Data Browser, based on 4,366 ALS cases and 1,832 controls (29). The p.His322Tyr variant has contradictory evidence, as it has been reported in ExAC and gnomAD and could therefore be considered benign, however, none of these variants have been detected in the controls in Project MinE, therefore labelling these as variants of unknown significance may be more appropriate. In contrast, the p.Val464Ala variant has been reported in both cases and controls in Project MinE and both PolyPhen and ClinVar consider it as benign.

Clinical phenotypes associated with TBK1 mutations

The clinical features of the four cases who carry the predicted pathogenic mutations in *TBK1* are described below and summarised in **Table 3**.

Case 1: The p.Glu2Ter nonsense mutation was detected in a male patient who developed ALS at 46 years of age with unilateral lower limb onset. There was no family history of ALS but a maternal grandmother had an unspecified dementia. Electromyography (EMG) demonstrated reinnervation

and chronic denervation, but no active denervation, and MRI of the head was normal, with spinal MRI showing disc protrusion without cord compromise. Progression manifested as bilateral leg spasticity and arm involvement, followed by a mild depression, which did not require treatment. Mild breathlessness was noted later. There was no evidence of cognitive change and disease progressed relatively slowly with the patient alive 7.75 years after diagnosis.

Case 2: The novel in frame deletion in exon 4, p.Ile85del, was detected in a female with ALS and no family history. She presented at age 59 with lower limb onset disease which progressed to affect her upper limbs and bulbar function, which subsequently led to a gastrostomy. Involvement of her respiratory muscles led to her death, four years and nine months after onset. EMG showed signs of widespread active and chronic denervation and reinnervation. There were no signs of cognitive change, though there was a history of Behçet's disease and depression.

Case 3: The p.Glu643del in frame deletion in exon 18 was found in a male whose father had limb onset ALS. He presented with word finding difficulties and slow speech at 61 years of age. Three years later, bulbar symptoms progressed and upper limb and neck weakness emerged with dysphagia. EMG was consistent with the clinical diagnosis of ALS. The patient also experienced irritability and emotional lability and neuropsychological testing showed cognitive impairment in the form of slowed information processing. Disease-typical deterioration occurred over the following year resulting in death at age 66.

Case 4: The p.Gln565Pro missense variant was found in a female, whose brother had also died of ALS. She presented with limb onset disease at 51 years of age. Disease was characterised as an upper motor neuron predominant/PLS phenotype. Bulbar compromise after a year culminated in the insertion of a gastrostomy tube. EMG revealed widespread but patchy neurogenic changes with spontaneous activity in three muscles, which supported the diagnosis. MRI of the brain showed numerous, non-specific, high-signal foci within the white matter of both hemispheres, in a peripheral distribution, as well as in the pons. The patient also had depression and emotional lability. She had severe spasticity, despite appropriate baclofen prescription, notably in the jaw resulting in oral ulcers. Respiratory failure occurred 3 years after diagnosis resulting in death 4 years after symptom onset.

Neuropathology of the novel TBK1 p.Ile85del mutation

Neuropathological examination of Case 2, carrying the novel p.Ile85del mutation, revealed severe lower motor neuronal loss in the anterior horns of the spinal cord. The lower motor neurons of the

hypoglossal nucleus in the medulla were better preserved. The motor cortex similarly had very little change on conventional histology with rather mild microvacuolation of the underlying white matter.

Conventional histology of the brainstem and midbrain was normal including a well-populated substantia nigra without evidence of neuronal loss. There was no cerebellar atrophy and the Purkinje cell layer was well preserved. Conventional histopathology of the extra motor neocortices, hippocampus, deep grey structures and cerebellum was normal for the age of the patient.

Immunohistochemistry for phosphorylated TDP-43 in the spinal cord highlighted skein-like inclusions in approximately 9% of lower motor neurons in the cervical and lumbar enlargements and granular, so-called "pre-inclusions" in the medulla. Occasional glial cytoplasmic inclusions were also observed in the anterior horns and corticospinal tracts (**Figure 3**). In all layers of the motor cortex, there was a moderate number of neuronal cytoplasmic inclusions together with a very small number of neurites (approximately 1 per spinal cord section). Glial cytoplasmic inclusions were quite numerous. This pattern best corresponds to FTLD-TDP type B pathology (30). Neuronal intranuclear inclusions were not present and there was no significant accentuation of TDP-43 pathology at the interface between the motor cortex and the underlying white matter. TDP-43 pathology was not present in extramotor regions (middle frontal gyrus, superior and middle temporal gyri and hippocampus).

Immunohistochemistry for CD68 revealed a moderate microglial reaction with both ramified and amoeboid forms in the corticospinal tracts at all levels examined (spinal cord, medullary pyramids, cerebral peduncles, and white matter underlying the precentral gyrus) as well as the grey matter of the spinal cord anterior horns, medulla and motor cortex.

Immunohistochemistry for hyper phosphorylated tau revealed low-level pathology in the form of neurofibrillary tangles and neuropil threads in the entorhinal cortex of moderate density in the inner and outer layers of the remnants of the entorhinal region, corresponding to Braak stage II by BrainNet Europe protocols (31). Tau pathology was otherwise absent from the motor cortex, occipitotemporal gyrus, superior and middle temporal gyri, motor cortex and spinal cord.

Immunohistochemistry for TBK1 was performed in spinal cord to determine if there was any distinctive pathology associated with this TBK1 variant. In controls, there was moderate, diffuse staining of the neuropil that was greater in grey than white matter. The degree of motor neuron TBK1 expression ranged from strong to similar levels to that of the surrounding neuropil. There was no staining of glial nuclei or cell bodies. No differences were observed in the spinal cord of the mutant *TBK1* case compared to the SALS and neurologically normal controls (**Figure 4, a&b**).

Immunohistochemistry for IRF3 was also performed on spinal cord, to see if there were changes in expression and/or location of this protein which acts downstream of TBK1 in the innate immune response. Since TBK1 phosphorylates IRF3 resulting in translocation of IRF3 to the nucleus, we hypothesised that any loss of function TBK1 would result in less phosphorylation of IRF3 and less nuclear localisation. In controls, expression of IRF3 was variable. The majority of the protein was in the grey matter neuropil, with virtually no neuronal staining. A number of glia in both grey and white matter labelled strongly for IRF3, with straining primarily in the glial cytoplasm, as most nuclei were negative for IRF3. However, the degree of staining was variable, such that other glia did not seem to express IRF3. Again, no differences were observed between mutant TBK1*,* SALS and control spinal cord (**Figure 4, c&d**). This suggests that the TBK1 loss of function mutation did not have any negative effect on IRF3 expression or localisation.

DISCUSSION

The mutation screening of *TBK1* in 290 ALS cases identified 4 variants (one nonsense, two in frame deletions and one missense) that were predicted to be pathogenic and associated with the disease, providing a frequency for *TBK1* mutations in our cohort of 1.38. One of the in-frame deletions, p.Ile85del, is a novel mutation, not previously reported in the literature or in online databases. A further three missense variants were identified, though the pathogenicity of two of these (p.Lys291Glu, p.His322Tyr) is uncertain. Unfortunately, no further biosamples are available from these patients to investigate functional implications of these variants. Fifteen additional variants which caused synonymous substitutions or occurred in non-coding regions were also identified, though these were not considered to be pathogenic.

Pathogenic Variants

Of the four TBK1-ALS cases, the age of onset ranged from 46 to 61 years of age. In the three deceased patients, the range of disease duration was two to five years, though one patient with a nonsense mutation is alive after having the disease for over 7 years. This variability is consistent with the clinical heterogeneity of cohorts within other studies of *TBK1* mutation carriers. In one large study, loss of function mutations were associated with a range of onset from 48-78 years and a disease duration of 6-136 months, whilst perhaps surprisingly, functional missense mutations were associated with an earlier onset of 34-57 years (6). Our case with a predicted functional missense mutation, p.Gln565Pro, had an age of onset of 51 years, though the youngest onset in our cohort was at 46 years, with the loss of function mutation p.Glu2Ter, which is younger than the range cited in the earlier study.

There does not appear to be a clear phenotype associated with *TBK1* mutations when compared to other genetic and sporadic ALS cases. Equally, there is no clear pattern with regard to specific mutations being associated with clinical features or disease. In the case of the p.Glu2Ter, this mutation has been reported in three other studies (**Supplementary Table 3**) (4, 13, 17). Whilst limited information was available in one paper - the patient had ALS as did our Case 1 – in a second report the patient had sporadic behavioural variant FTD (bvFTD), with an onset at 56 years and a disease duration of 4 years. The third case reported was also of an ALS patient, with limb-onset disease at 60 years. Only two other cases of Gln565Pro have been reported (4, 17), both of which were ALS. Again, limited information is available on one, however, the other was very similar to our Case 4, with limb onset familial ALS and a disease duration of 3 years.

The pGlu643del mutation has previously been found in 8 independent cases with either ALS or dementia (4, 5, 13) as well as within a large family from Belgium, presenting with both ALS and dementia (13). Interestingly, where the type of dementia has been documented, this is predominantly bvFTD, though one case with primary progressive aphasia (PPA) has also been described, which correlates with Case 3 presenting with word finding difficulties prior to motor symptoms. Thus, mutations in TBK1 appear to underlie a range of dementia phenotypes as well as ALS, with variability in age and site of onset.

Neuropathology

The pathology found in the novel p.Ile85del patient is representative of classical ALS-type TDP-43 proteinopathy in addition to Type B FTLD-TDP proteinopathy. The FTLD pathology is consistent with the majority of neuropathology reports so far describing *TBK1* mutation carriers with either FTD-MND or pure FTD phenotypes (**Table 4**) (5-7, 9, 13). The exception to this is the neuropathology found by Pottier and colleagues in a study of FTD cases, who have described Type A FTLD-TDP pathology in an individual with FTLD carrying a *TBK1/OPTN* double mutant and an second FTLD individual with a *TBK1* missense mutation (9). In the same study, two *TBK1* missense mutation carriers with a pathological diagnosis of FTLD-MND showed Type B FTLD-TDP pathology, whilst an FTLD case with a double OPTN mutation displayed Type A, suggestive that the OPTN mutation could be driving the Type A pathology in the double mutant.

The degree of motor system pathology of cases reported so far is variable. Thus the two cases of Pottier and colleagues lack brainstem motor neuron pathology (the motor cortex was not commented on and spinal cord tissue was unavailable) (9). ALS patient DR1124 (p.Ser518Leufs*32) reported by

Gijselinck and colleagues had TDP-43 proteinopathy of brainstem and spinal cord lower motor neurons and 2 out of the 3 patients with TBK1 mutations (13) and autopsy data in the van der Zee study showed the TBK1 mutants had motor TDP-43 proteinopathy (6).

Several previous reports have performed immunohistochemistry with TBK1 and found variable cytoplasmic neuronal staining, consistent with our findings. To determine whether loss of TBK1 function had an effect on the downstream proteins in the immune pathway, we also stained for IRF3. However, no changes in expression or localisation of this protein were seen associated with the p.Ile85del TBK1 mutation compared to controls, suggesting that disruption of the IRF3-related innate immunity signalling pathway is not effected by the TBK1 loss of function mutation.

In summary, screening of the TBK1 gene in a cohort of patients from the North of England identified four pathogenic variants, accounting for a frequency for TBK1 mutations of 1.38% of this ALS population. We identified a novel loss of function mutation, p.Ile85del, and neuropathological characterisation showed this mutation was associated with classical ALS and Type B FTLD-TDP pathology. No changes in expression or localisation of TBK1 or IRF3, a downstream effector of TBK1, were detected between the p.Ile85del case and other SALS or control cases. Thus, within this cohort, classical ALS pathology has been seen in the context of mutant TBK1.

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Conflicts of Interest

The Editors of *Neuropathology and Applied Neurobiology* are committed to peer-review integrity and upholding the highest standards of review. As such, this article was peer-reviewed by independent, anonymous expert referees and the authors (including JRH and JK) had no role in either the editorial decision or the handling of the paper.

FIGURE LEGENDS

Figure 1: Predicted pathogenic *TBK1* variants identified in the cohort of ALS patients from Northern England.

Figure 2: Novel *TBK1* exon 4 p.Ile85del in frame deletion **a.** Representative reverse primer chromatogram **b.** Predicted effect of exon 4 p.Ile85Del (blue) on TBK1 protein structure (red/yellow) and **c**. in depth, showing changes in polar bonds.

Figure 3: Neuropathology of case 2 with a novel p.Ile85del. Immunohistochemistry for phosphorylated TDP-43 in the ventral horns of the spinal cord reveals neuronal (a, arrow, bar = 10µm) and glial (b, arrow, bar = 10µm) cytoplasmic inclusions. Immunohistochemistry for the microglial marker reveals a mild reaction that is more prominent in the corticospinal tracts and ventral horns (c, high magnification, arrow highlights an amoeboid microglial cell, bar = 25µm; d, low magnification, dotted outline, bar = 1µm). Immunohistochemistry for phosphorylated TDP-43 in the motor cortex reveals both neuronal (e, arrow) and glial (f, arrow) cytoplasmic inclusions but a lack of TDP-43 positive neurites (bar = 10µm)

Figure 4: Immunohistochemistry for TBK1 and IRF3 protein. Staining of TBK1 in a representative control (a) and patient with TBK1 mutation (b) in the spinal cord anterior horn and adjacent white matter shows neuropil expression is greater in the grey matter (GM) than white matter (WM). Neuronal cytoplasmic staining (arrow, a & b) is greater than neuropil staining. Bar = 250µm. Immunohistochemistry for IRF3 protein in a representative control (c) and patient with TBK1 mutation (d) in the spinal cord anterior horn shows both nuclear (open arrow) and cytoplasmic (closed arrow) glial expression. Expression is variable, such that some glia are positive, while others are negative (grey arrows, c & d) for IRF3. The blue arrow denotes artefactual lipofuscin labelling in neurons. Bar = 50µm.

TABLES

Table 1: Antibodies used for Neuropathological Examination

Antibody	Supplier	Antigen retrieval	Primary antigen concentration
Phosphorylated TDP-43	CAC-TIP-PTD-MO1 Cosmo Bio/2B scientific	Pressure cooker pH6	1:4000
Phosphorylated tau	AT8 (ThermoFisher)	Pressure cooker pH6	1:200
CD68	Abcam, PGM1, ab783	Pressure cooker pH6	1:100
TBK1	Abcam ab109735	Pressure cooker pH9	1:3000
IRF3	Abcam ab76409	Pressure cooker pH9	1:100

Table 2: *TBK1* coding variants identified and bioinformatic analyses performed to determine functional effects of non-synonymous coding changes. Shaded grey = predicted pathogenic variants. Bold = novel mutation. All = frequency in all populations; Eur = European (non-Finnish). N/A = not applicable; NR = not reported in database
gnomAD = https://gnomad.broadinstitute.org/ ; ExAC = http://exac.broadinstitute.org/ ; Project MinE Data Browser = http://databrowser.projectmine.com/ ; ClinVar = https://www.ncbi.nlm.nih.gov/clinvar/

Case	Exon	Type	Mutation (cDNA)	Amino acid change	Mutation Taster	PolyPhen-2 (HumVar)	SIFT	Allele Frequencies			ClinVar	References
								gnomAD All / Eur Nº Alleles	ExAC All/Eur Nº Alleles	Project MinE Cases/ Controls		
1	2	Nonsense	c.4C>T	p.Glu2Ter	Disease causing	N/A	N/A	NR	NR	NR	NR	(4, 13, 17)
2	**4**	**In-frame deletion**	**c.253 255delATT**	**p.Ile85del**	**Disease causing**	**N/A**	**N/A**	**NR**	**NR**	**NR**	**NR**	**NOVEL MUTATION**
3	18	In-frame deletion	c.1928 30delGAA	p.Glu643del	Disease causing	N/A	N/A	0.00001102/ 0.00002389 3 alleles	NR	NR	NR	(4, 5, 13)
	8	Missense	c.871A>G	p.Lys291Glu	Disease causing	Probably damaging 0.992	Deleterious 0.03	0.0001394/ 0.0002653 39 alleles	0.0001733/ 0.0002852 21 alleles	0.00126/ 0.000819	Uncertain significance	Reported in ALS & controls
	8	Missense	c.964C>T	p.His322Tyr	Disease causing	Possibly damaging 0.870	Tolerated 0.06	0.0004286/ 0.0002960 116 alleles	0.0005226/ 0.0003516 62 alleles	0.000916/ 0	Uncertain significance / Likely benign	Reported in ALS & controls
	12	Missense	c.1391T>C	p.Val464Ala	Disease causing	Benign 0.062	Tolerated 0.52	0.01358/ 0.01990 3718 alleles	0.01621/ 0.02416 1561 alleles	0.0318/ 0.0240	Benign	Reported in ALS & controls
4	15	Missense	c.1694A>C	p.Gln565Pro	Disease causing	Probably damaging 0.969	Deleterious 0.00	0.000004343/ 0.000009585 1 allele	NR	0.000229/ 0	NR	(4, 17)

Table 3: Clinical features of ALS patients with pathogenic *TBK1* mutations.
*Maternal grandmother had a form of dementia; **Patient alive 7.75 years after symptom onset; [#]Cognitive impairment began at 61 years with word finding difficulties, whilst ALS symptoms began aged 64 years.

Case	Amino acid change	Phenotype	Age at Onset (years)	Duration of illness (months)	Cognitive change
1	p.Glu2Ter	(F)ALS*	46	92**	No
2	p.85delIle	SALS	59	57	No
3	p.Glu643del	FALS	61 (64)[#]	24	Slow information processing, irritability and emotional lability
4	p.Gln565Pro	FALS	51	48	Emotional lability

Table 4: Neuropathological features of *TBK1* mutation carriers. The OPTN double mutant published with TBK1 mutant associated pathology by Pottier et al, 2015 is included for completeness. Both clinical diagnosis (and pathological diagnosis in parentheses) are provided where available. OPTN mutations in italics. AD = Alzheimer's disease; FTD = frontotemporal dementia; bvFTD = behavioural variant FTD; FTLD = frontotemporal lobar dementia; agPPA = progressive non fluent/agrammatic variant of primary progressive aplasia.

TBK1 Mutation	Clinical Diagnosis (Pathological Diagnosis)	Age at death	FTLD-TDP Type	Comments	Reference
p.690-713del	ALS-FTD (not available)	66	Type B	TDP-43 and p62 positive perinuclear neuronal inclusions temporal lobe. Spinal cord unavailable.	(5)
p.Gly272-Thr331 del	bvFTD (FTLD)	50	Type B	Moderate TDP-43 and p62 positive neuronal cytoplasmic inclusions (NCI), short dystrophic neurites, no intraneuronal intranuclear inclusions. Motor system not described.	(13)
p.Ser518Leufs*32	Bulbar onset ALS (ALS)	64	Type B	Upper and lower motor neuron loss, TDP-43 in motor neurons, variable cytoplasmic neuronal staining with TBK1, no co-localisation with TDP-43 NCIs	(13)
p.Thr79del	Bulbar onset ALS + bvFTD (FTLD-ALS)	58	Type B	Neuronal loss, reactive astrogliosis and microglial activation, TDP-43 positive NCI, isolated dystrophic neurites, CD68 positive macrophages; also argyrophilic grain pathology (AgD) characterised by hyperphosphorylated tau (AT8). Involvement of both motor (anterior horn and brainstem motor nuclei) as well as extra motor deep grey, neocortical and mesial temporal structures.	(6)
p.Val97Phefs*2	ALS (MND)	63		TDP-43 positive inclusions.	(6)
p.Ala417*	bvFTD (FTLD)	71	Type B	Mild TDP-43 proteinopathy in affected brain regions.	(6)
OPTN p.Gln235 p.Ala481Val*	AD (FTLD)	70	Type A	Mild cortical atrophy, p62 and TDP-43 pathology in cortical grey matter, no OPTN or TBK1 pathology, no neuronal loss. Brainstem motor nuclei unaffected.	(9)
*OPTN p.Gly538Glufs*27* TBK1 p.Arg117*	agPPA (FTLD)	72	Type A	Severe focal cortical atrophy in frontal lobe, many neuronal and glial cytoplasmic and intranuclear inlcusions, no neuronal loss in motor cortex or brain stem. Brainstem motor nuclei unaffected.	(9)
p.Glu696Lys	AD (FTLD-MND)	84	Type B	No further information available	(9)
p.Leu306Ile	FTD-ALS (FTLD-MND)	72	Type B	No further information available	(9)
p.Lys401Glu	AD (FTLD)	90	Type A	No further information available	(9)

Figure 1: Predicted pathogenic *TBK1* variants identified in the cohort of ALS patients from Northern England.

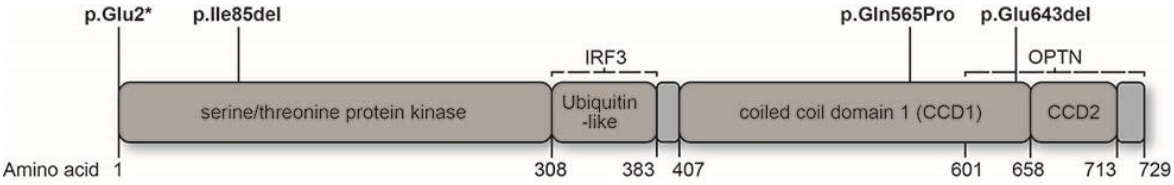


Figure 2: Novel *TBK1* exon 4 p.Ile85del in frame deletion **a.** Representative reverse primer chromatogram **b.** Predicted effect of exon 4 p.Ile85Del (blue) on TBK1 protein structure (red/yellow) and **c**. in depth, showing changes in polar bonds.

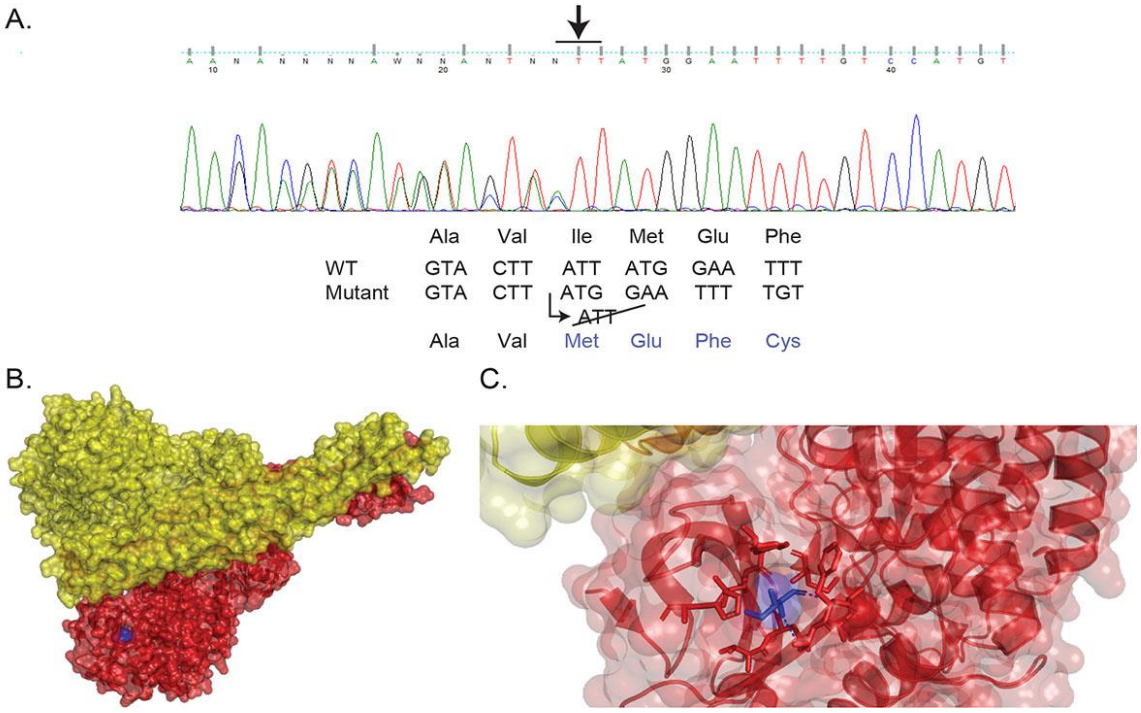


Figure 3: Neuropathology of case 2 with a novel p.Ile85del. Immunohistochemistry for phosphorylated TDP-43 in the ventral horns of the spinal cord reveals neuronal (a, arrow, bar = 10µm) and glial (b, arrow, bar = 10µm) cytoplasmic inclusions. Immunohistochemistry for the microglial marker reveals a mild reaction that is more prominent in the corticospinal tracts and ventral horns (c, high magnification, arrow highlights an amoeboid microglial cell, bar = 25µm; d, low magnification, dotted outline, bar = 1µm). Immunohistochemistry for phosphorylated TDP-43 in the motor cortex reveals both neuronal (e, arrow) and glial (f, arrow) cytoplasmic inclusions but a lack of TDP-43 positive neurites (bar = 10µm).

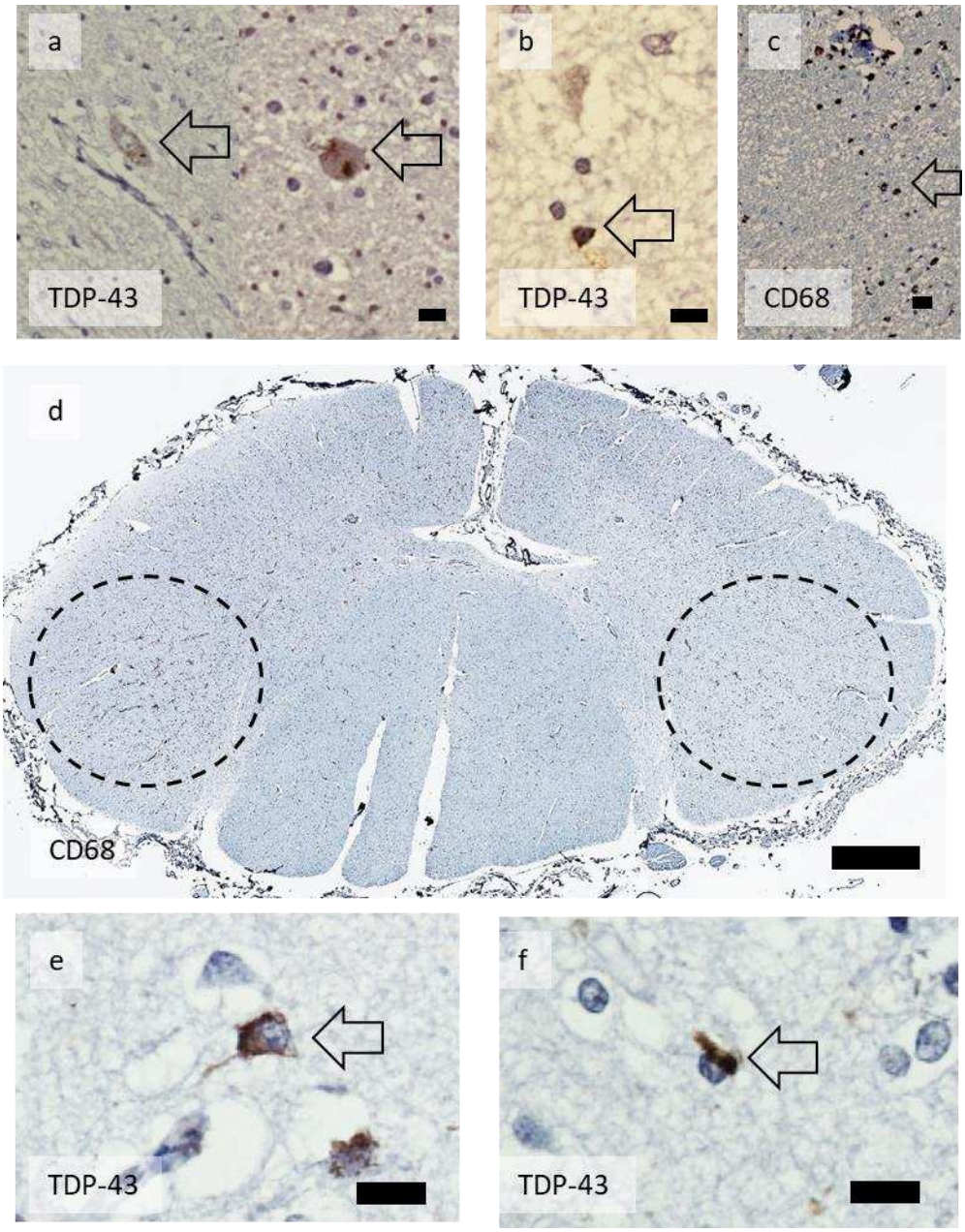
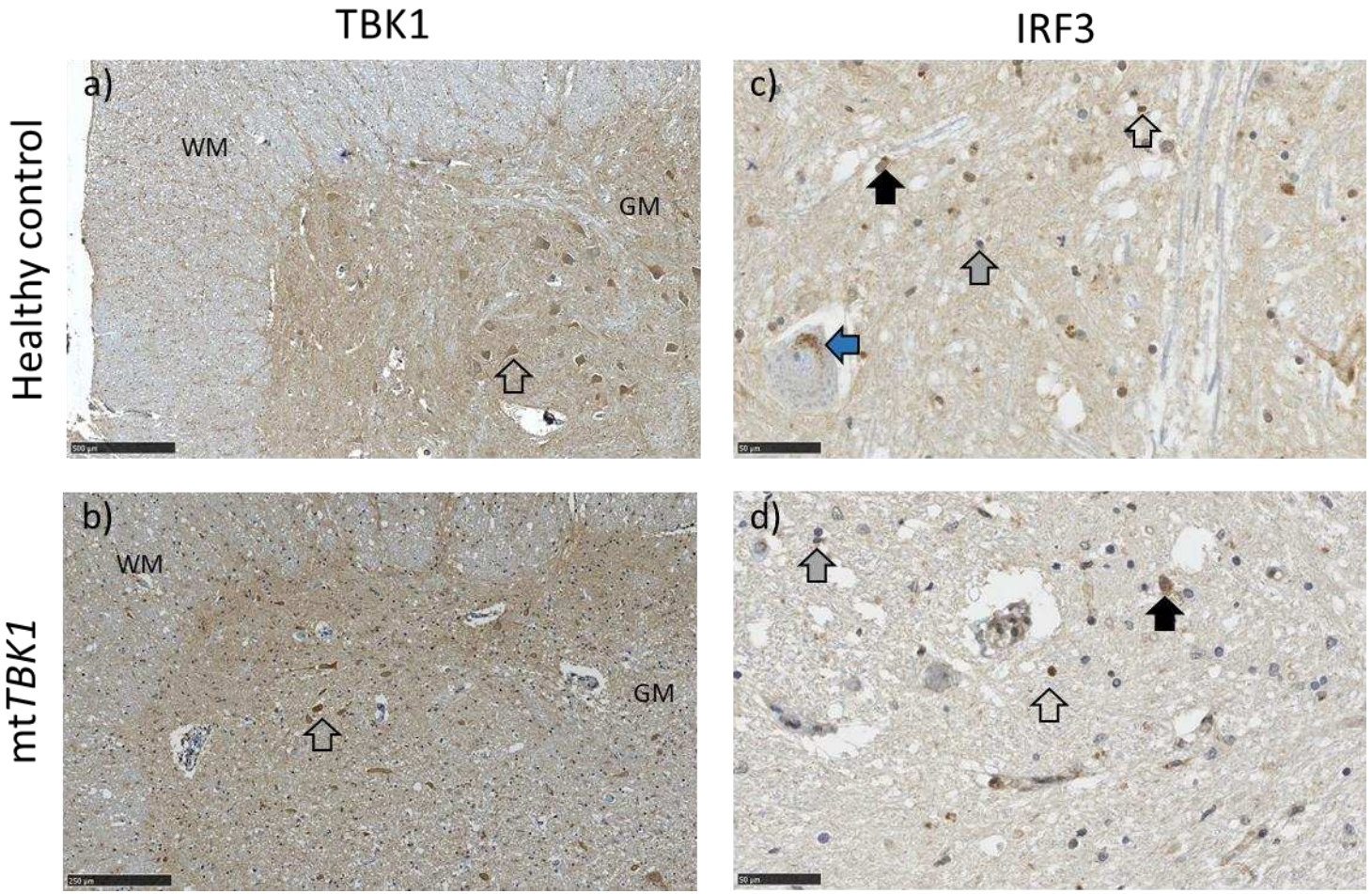


Figure 4: Immunohistochemistry for TBK1 and IRF3 protein. Staining of TBK1 in a representative control (a) and patient with TBK1 mutation (b) in the spinal cord anterior horn and adjacent white matter shows neuropil expression is greater in the grey matter (GM) than white matter (WM). Neuronal cytoplasmic staining (arrow, a & b) is greater than neuropil staining. Bar = 250µm. Immunohistochemistry for IRF3 protein in a representative control (c) and patient with TBK1 mutation (d) in the spinal cord anterior horn shows both nuclear (open arrow) and cytoplasmic (closed arrow) glial expression. Expression is variable, such that some glia are positive, while others are negative (grey arrows, c & d) for IRF3. The blue arrow denotes artefactual lipofuscin labelling in neurons. Bar = 50µm.



Supplementary Table 1: PCR primers used to amplify *TBK1* exons. All PCR reactions were performed as follows: initial denaturation of 95°C for 5min, followed by 35 cycles of 95°C for 30sec, annealing at 60°C for 30sec, and 72°C for 45sec, followed by a final elongation step at 72°C for 10mins.

Exon	Forward primer sequence	Reverse Primer Sequence	Length (bp)	Source
2	CAGATCCGTCTCTCCAGAGGAA	ACCTGTGCCACCACTGGGTA	565	(21)
3	TCCCTGTGCCTAAAAGATGC	AGGTTCTATTTCTTGACTCGTAACT	327	Primer blast
4	CACCCGGCCGGAAGATATTA	TGGGCTGTCTTTTCACTTACCTG	525	(21)
5	TCAATGAATTTGAGACATGCACAC	TTGGCTGACCAATTACCCAATTA	334	(21)
6	TTTGAGGAGGGAGGGGAAGA	TGGGCCAAAGACTGAATACCA	564	(21)
7	TGGATCCTGTGAGCATCAATCA	AAGGTCCTGAGCATGATCCCTA	543	(21)
8	GCCCCAGAGTTTGAGACTGC	TGGTGAAGCTGAGGCATCTTT	580	(21)
9	GAAATGGATGTTGTTGCACTCATT	CAGGGTCCCACACTGTCTCC	532	(21)
10	AAAGAGAAGCATTGGACTCATTGTG	TTTGGTGCTAATTGTGGTATAGGG	692	(21)
11	TCTCTCCATTGAGATAGTGCATGTT	TCAATGATGGGGCAAGGTCT	361	(21)
12	ATACATCAGGATCACAGAAATGCT	ATGCCATATTGTGCCAAGGAT	370	Primer blast
13	ACAATCTGCTGGCTTATAGACTTTG	AGTGCCAGCAAAGGAATAGGT	283	Primer blast
14-15	GACTTTGTTGGGACTGTGAGT	AGCTACCCTTACAGATATACCAAAT	459	Primer blast
16	TTGCCCAATCTTCCCATTTC	GGGAGCAGATGTGTGAACCTC	486	(21)
17-18	GCCACAACAATCATTATAGGAAAGA	TTGGTGGGTGGAACTGAATG	532	(21)
19-20	TTAAAAGCTGTAACACTTGATGTCAGA	TGACCCTTTACCACTGCTGAAT	597	(21)
21	CAGGCCGGTCTCAAACTTGT	ACATCTTCACAGCAGCCAAAAA	598	(21)

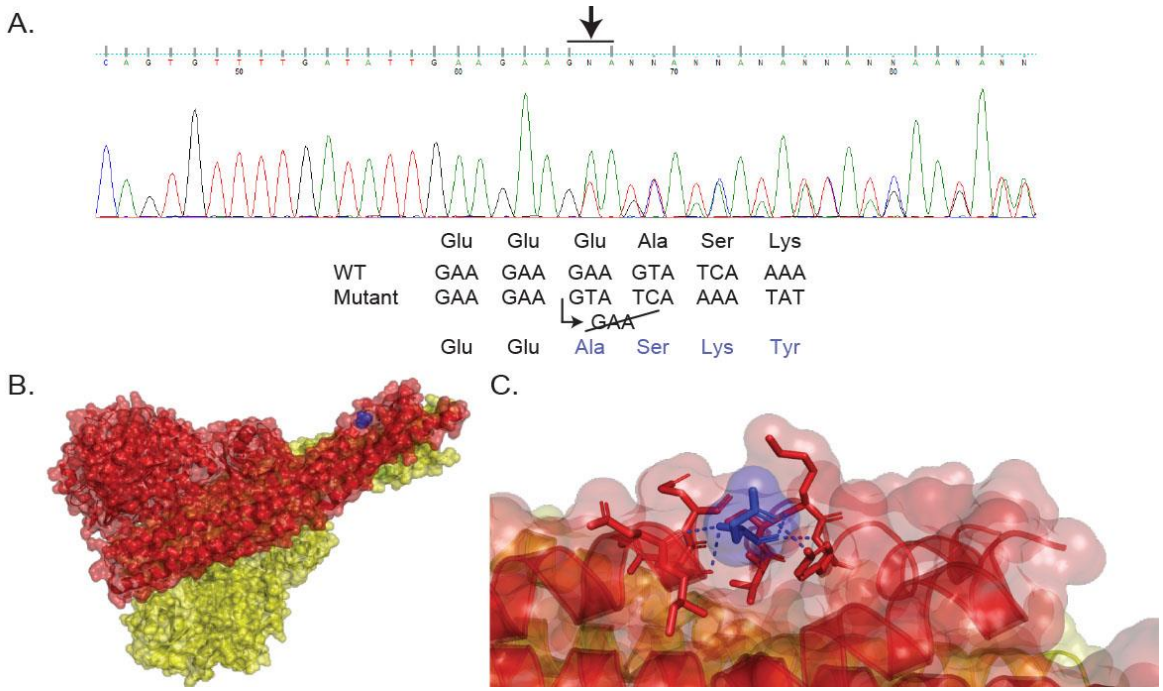
Supplementary Table 2: Predicted non-pathogenic *TBK1* variants identified in this study. Allele frequencies as **accessed 3 April 2019**

Location	Type	Genome change (GRCh37)	Mutation (cDNA)	Amino acid change	Rs number	Cohort alleles	Allele frequency	
							All (gnomAD)	EUR (gnomAD)
Exon 8	Missense	g.64875680A>G	c.871A>G	p.Lys291Glu	rs34774243	1	0.0001394	0.0002653
		g.64875773C>T	c.964C>T	p.His322Tyr	rs145905497	1	0.0004286	0.0002960
Exon 12		g.64882317T>C	c.1391T>C	p.Val464Ala	rs35635889	13	0.01358	0.01990
Exon 2	Synonymous	g.64849716T>C	c.964C>T	p.Asp22Asp	rs41292019	38	0.03246	0.04653
Exon 8		g.64875787T>A	c.978T>A	p.Ile326Ile	rs7486100	223	0.4730	0.5559
Exon 9		g.64878200C>T	c.1110C>T	p.Phe370Phe	rs143590388	1	0.001464	0.002371
Intron 7-8	Non coding	g.64874102A>C		-	rs7303577	59	0.1474	0.1196
		g.64875468C>G	-	-	-	1	-	-
Intron 8-9		g.64878021A>C	-	-	-	2	-	-
Intron 9-10		g.64879165T>C	-	-	rs11175411	34	0.1475	0.1093
Intron 10-11		g.64879436G>T	-	-	rs41292023	40	0.1643	0.1201
Intron 11-12		g.64882257delT		-	rs201728462	2	0.006802	0.008970
Intron 12-13		g.64883761C>A	-	-	rs10878177	161	0.2240	0.3039
Intron 14-15		g.64889417T>C	-	-	rs149784987	2	0.00184	0.00319
Intron 15-16		g.64889915A>C	-	-	rs117570362	3	0.0092	0.0133
Intron 18-19		g.64891093G>A	-	-	rs746426213	1	0.0001	0.0001
Intron 20-21		g.64894932dup	-	-	rs773423523	1	0.0002	0.0003
		g.64895099G>A	-	-	rs41292027	6	0.01363	0.00937

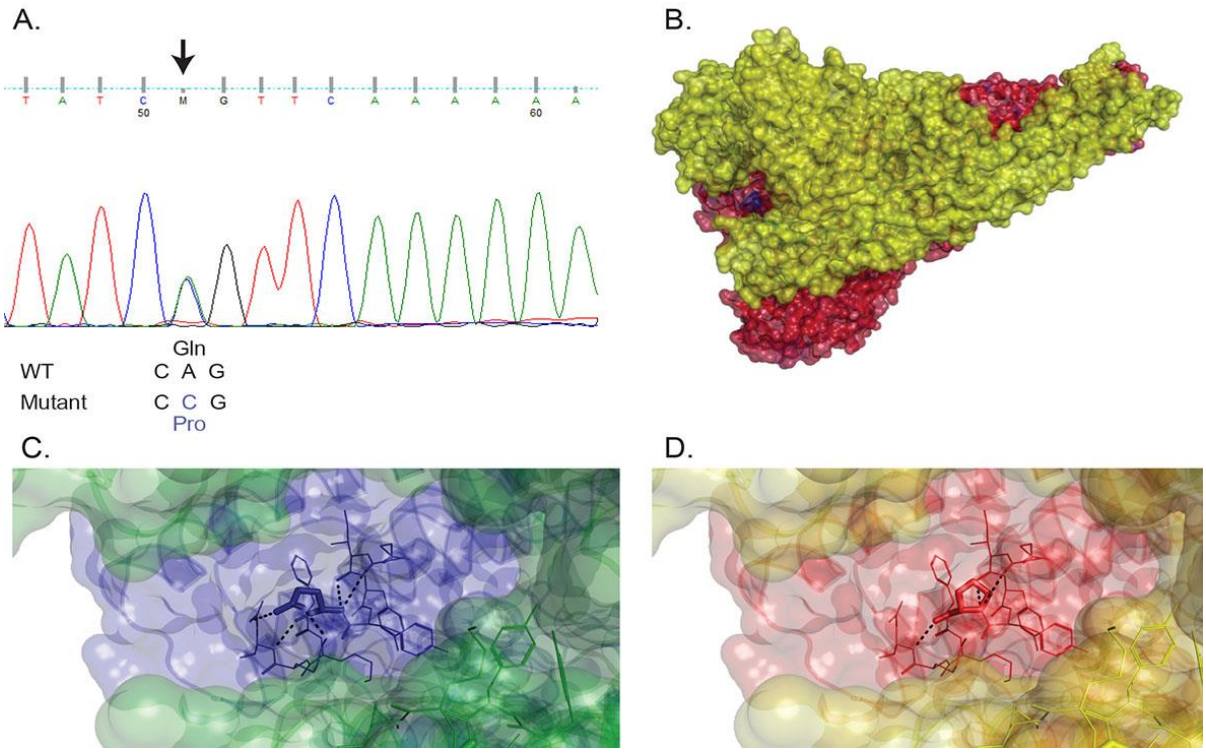
Supplementary Table 3: Literature summary of clinical data of patients with the 3 previously reported pathogenic TBK1 mutations. Ages and survival time in years. *Individuals all members of same family

Reference	Disease	Inheritance	Gender	Onset site	Age at onset	Age at death	Survival time	Cognitive change
p.Glu2Ter								
Case 1	***ALS***	***(F)ALS***	***M***	***Limb***	***46***	***-***	***8+***	***No***
(4)	ALS	-	-	-	-	-	-	-
(13)	FTD	Sporadic	F	-	56	60	4	bvFTD
(17)	ALS	Familial	F	Spinal	60	-	-	-
p.Glu643del								
Case 3	***ALS***	***Familial***	***M***	***Limb***	***61***	***66***	***5***	***Word finding difficulties, emotional lability***
(5)	ALS	Sporadic	M	-	-	-	-	-
(5)	ALS	Sporadic	M	Spinal	-	-	-	-
(13)	Dementia	Familial*	F	-	71	81	10	Dementia
(13)	Dementia	Familial*	F	-	86	90	4	Dementia
(13)	ALS	Familial*	M	Spinal	69	72	3	-
(13)	FTD	Familial*	M	-	69	75	6	bvFTD
(13)	Dementia	Familial*	M	-	70	73	3	Dementia
(13)	FTD	Familial*	F	-	61	74	13	bvFTD
(13)	Dementia	Familial*	F	-	63	69	6	Dementia
(13)	FTD-ALS	Familial*	F	Spinal	62	74	11	bvFTD
(13)	Dementia	Familial*	F	-	73	84	11	Dementia
(13)	FTD	Familial	F	-	64	-	>9	bvFTD
(13)	FTD	Familial	M	-	70	-	>6	PPA
(13)	FTD	Sporadic	F	-	69	-	>7	bvFTD
(13)	ALS	Sporadic	M	-	63	66	3	-
(13)	ALS	Familial	M	Bulbar	41	41	<1	-
(4)	ALS	-	-	-	-	-	-	-
p.Gln565Pro								
Case 4	***ALS***	***Familial***	***F***	***Limb***	***51***	***55***	***4***	***Emotionally labile***
(4)	ALS	-	-	-	-	-	-	-
(17)	ALS	Familial	F	Limb	50	53	3	-

Supplementary Figure 1: *TBK1* exon 18 p.Glu643del in frame deletion **a.** Representative forward primer chromatogram highlighting area of deletion **b.** Predicted effect of exon 18 p.Glu643del variant (blue) on overall TBK1 protein structure (red/yellow) and **c**. in depth, showing changes in polar bonds.



Supplementary Figure 2: *TBK1* exon 15 p.Gln565Pro missense mutation **a.** Representative forward primer chromatogram **b.** Predicted TBK1 whole protein structure (red/yellow) with area of missense mutation (blue) **c.** In depth reference TBK1 protein structure (blue/green) with polar bonds, compared to **d**. in depth missense mutation TBK1 protein structure (red/yellow) with polar bonds.



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