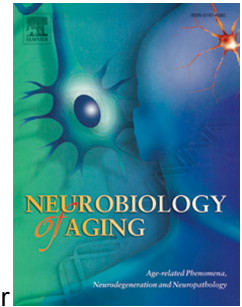


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Novel *TBK1* truncating mutation in a familial amyotrophic lateral sclerosis patient of Chinese origin

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**Abstract**

Missense and frameshift mutations in TANK-binding kinase 1 (*TBK1*) have been reported in European sporadic and familial amyotrophic lateral sclerosis (ALS) cohorts. To assess the role of *TBK1* in ALS patient cohorts of wider ancestry, we have analysed whole exome sequence data from an Australian cohort of familial ALS patients and controls. We identified a novel *TBK1* deletion (c.1197delC) in a familial ALS patient of Chinese origin. This frameshift mutation (p.L399fs) likely results in a truncated protein that lacks functional domains required for adapter protein binding, as well as protein activation and structural integrity. No novel or reported *TBK1* mutations were identified in familial ALS patients of European ancestry. This is the first report of a *TBK1* mutation in an ALS patient of Asian origin and indicates that sequence variations in *TBK1* are a rare cause of familial ALS in Australia.

## 1. Introduction

Amyotrophic lateral sclerosis (ALS, also known as motor neuron disease, MND) is a fatal neurodegenerative disease that is characterised by the rapidly progressive degeneration of the upper and lower motor neurons. The progression of paralysis is profound, rapidly leading to death, usually from respiratory failure within 2 to 5 years of first symptoms. To date the only known cause of ALS are gene mutations, which have been reported in more than 20 genes (ALS Online Database (ALSoD 6.0 at <http://alsod.iop.kcl.ac.uk>) (Abel, et al., 2013) and reviewed in Renton, et al. (2014)). Much of our understanding of disease pathology has stemmed from genetic discoveries in the hereditary form of the disease (familial ALS). Gene discovery in ALS has long comprised of multi-generational pedigree analyses using exome sequencing and/or linkage analysis to identify causative mutations. More recently, gene discovery strategies in ALS have included statistical genetic analyses of large cohorts of probands, including both familial and sporadic cases.

The TANK-binding kinase 1 (*TBK1*) gene was recently reported as an ALS gene through rare variant enrichment analyses (Cirulli, et al., 2015) and familial discovery studies (Freischmidt, et al., 2015). Several ALS-linked mutations have been identified in this gene, predominantly insertions/deletions that result in frameshift mutations and truncated proteins. Cirulli, et al. (2015) used whole exome sequence data from a large cohort of ALS patients (both familial and sporadic cases) and controls to observe for enrichment of genetic-model-specific variants in patients. They used a gene-based collapsing analysis across six genetic models including dominant coding, recessive coding, dominant not benign, recessive not benign, dominant loss of function, and recessive loss of function. Using a discovery dataset of 2843 cases and 4310 controls, 51 genes (including *TBK1*) showed association

with ALS and were taken forwards for replication. Interestingly, *OPTN* was the only previously known ALS gene among the 51 genes. The replication dataset of 1318 cases and 2371 controls confirmed *TBK1* as an ALS gene with significant variant enrichment in cases compared to controls, in both the discovery and replication datasets in the dominant not benign genetic model. In the discovery dataset, *TBK1* was ranked fourth, third and third in dominant coding, dominant not benign and dominant loss of function genetic models respectively. In total, 11 novel loss of function and 21 novel missense variants in *TBK1* were identified in patients.

Freischmidt, et al. (2015) also recently reported rare variant enrichment analysis in 252 familial ALS cases of European descent. They too used a collapsing method combined with multivariate tests (Li and Leal, 2008) to identify significant enrichment of loss of function variants in *TBK1* in familial ALS patients when compared to controls. However, targeted mutation screening of *TBK1* in 1010 sporadic ALS cases and 650 additional controls did not show similar enrichment of loss of function variants. Combined, Freischmidt, et al. (2015) identified 8 different loss of function variants and 9 different missense variants in *TBK1* in ALS patients. Several of the families with *TBK1* loss of function variants described in this study have comorbid ALS and/or frontotemporal dementia (FTD)/cognitive impairment. Further functional analysis suggested that haploinsufficiency is the most likely pathogenic mechanism underlying these loss of function variants. Since Freischmidt, et al. (2015) identified *TBK1* mutations in 4% of genetically unexplained FALS, they suggested that *TBK1* mutations may be a common genetic cause among Caucasians with ALS.

To determine the overall contribution of this particular gene to ALS across multiple ethnicities, mutation screening in extended geographical cohorts is required.

We have examined *TBK1* variants in a cohort of Australian familial ALS cases and identified a previously unreported single base pair deletion, resulting in a frameshift mutation, in an Australian patient of Chinese origin. The patient had limb onset and developed cognitive impairment with language disturbance, however they were not formally diagnosed with FTD. As all previous ALS-linked *TBK1* mutations have been identified in European cases, this is the first report of a *TBK1* mutation in an ALS patient of non-European origin. No novel *TBK1* variants were identified in ALS patients of European background.

## 2. Material and methods

### 2.1 Participants

We analysed a total of 127 individuals from 94 Australian ALS families. Freischmidt, et al. (2015) reported segregation of loss of function *TBK1* variant and a pathogenic *FUS* missense variant in a single patient, therefore we included 39 patients who were positive for a mutation in a known ALS gene to prevent omitting possible oligogenic cases (van Blitterswijk, et al., 2012). The remaining 88 patients (from 75 families) were negative for known ALS genes. It should be noted that 89/127 cases, excluding the discovery case described here, are part of the FALS sequencing consortium described in Cirulli, et al. (2015). Samples were recruited from Molecular Medicine Laboratory at Concord Hospital, the Australian MND DNA bank and from the Macquarie University MND Biobank. Patients, family members and unrelated controls were recruited under informed written consent as approved by the institutional review boards of the participating institutions. Patients were diagnosed with definite or probable ALS according to El Escorial criteria (Brooks, et al., 2000). Patients had previously been screened for mutations/expansions in known ALS

genes including *TARDBP*, *SOD1*, *FUS*, *UBQLN2*, *PFN1*, *VCP*, *OPTN* and *C9ORF72*.

## 2.2 Genetic analysis

Patient exomes were captured using the TruSeq Exome Enrichment kit or Agilent SureSelectXT Human All Exon V5+UTR. Paired-end sequencing was performed using the Illumina HiSeq2000 instrument. Read depth of each exon in *TBK1* was calculated using QualiMap v.2.0.2 (Garcia-Alcalde, et al., 2012). *TBK1* variants were identified in exome sequence data using custom bioinformatic scripts. Validation of the identified *TBK1* deletion (c.1197delC) was performed by direct DNA sequencing following PCR amplification of NM\_013254 exon 10 (primers: forward 5'-CGAAACTACATTACAGATTTTGCC-3' and reverse 5'-ACTTGGAAGGGTATCAATTTAAG-3'). PCR products were Sanger sequenced using Big-Dye terminator sequencing and an ABI 3730XL DNA analyser (Applied Biosystems).

The public SNP databases were accessed at: dbSNP Build 141 (<http://www.ncbi.nlm.nih.gov/SNP/>), the 1000 Genomes Project (<http://www.1000genomes.org/>), the Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP, <http://evs.gs.washington.edu/EVS/>) and Exome Aggregation Consortium (ExAC, <http://exac.broadinstitute.org>). Control exome sequence data was obtained (using the same method described above) from 967 neurologically healthy individuals of predominantly Western European descent from the Diamantina Control Cohort (University of Queensland Diamantina Institute, University of Queensland, Australia).

### 3. Results

#### 3.1 Identification of a novel *TBK1* frameshift mutation

We examined whole exome sequence data from familial ALS patients and controls to determine the frequency of *TBK1* mutations in Australian familial ALS. Exome sequencing achieved a mean read depth of >60X across all *TBK1* coding exons. We identified an unreported frameshift mutation in *TBK1* (c.1197delC, p.L399fs) in an Australian ALS patient of Chinese origin (Fig. 1B). The mutation is absent from all public SNP databases including dbSNP141, the 1000 Genomes Project, the NHLBI Exome Sequencing Project (ESP) exome variant server and Exome Aggregation Consortium (ExAC) and the Diamantina Control Cohort. The frameshift deletion is predicted to result in a truncated protein of 407 amino acids (Fig. 1C). No DNA was available from other family members. No tissue was available to assess transcription of the mutant allele. Bioinformatic analysis using MutationTaster (Schwarz, et al., 2014) and SIFT version 5.2.2 (Kumar, et al., 2009) predicted that the mutation would lead to nonsense mediated decay of the mutant transcript.

Two unreported *TBK1* missense variants were identified in the Diamantina Control Cohort in exon 3 (c.A217G, p.I73V; minor allele frequency, MAF = 0.001) and exon 10 (c.A1201G, p.K401E; MAF = 0.0005). These variants are absent from public SNP databases including dbSNP141, the 1000 Genomes Project, and the NHLBI Exome Sequencing Project (ESP) exome variant server, however were present in ExAC at MAF values of 0.00004 and 0.00001 respectively (no further clinical information is available on these control subjects).

#### 3.2 Clinical presentation of the *TBK1* frameshift mutation family



The proband (III:1; Fig. 1A) is from a three-generation family of Chinese origin in which the proband and his mother had ALS and two maternal uncles and maternal grandfather have historical diagnoses of Parkinson's Disease (Fig. 1A). The proband had a long standing history of bronchiectasis secondary to pneumonia as a child. He also had a six-year history of mild depression, and a two-year history of complex partial seizures controlled with carbamazepine. At age 60 he developed progressive right arm weakness and, after two years, was referred to a neurologist. At presentation he had global weakness in the right arm associated with cramp on muscle activation. There was no weakness in the left arm or legs, although he had lost five kilograms in weight over the previous 12 months. There was no cranial nerve or cognitive involvement at presentation. Physical examination revealed widespread muscle atrophy, generalised fasciculations and focal weakness in the right upper limb, with normal tone and deep tendon reflexes. There were no other upper motor neuron signs, and no involvement of the left upper limb or legs. Neurophysiology confirmed widespread denervation and re-innervation consistent with a motor neuronopathy. Magnetic resonance imaging of the brain and spinal cord was normal. Biochemistry including creatine phosphokinase was normal.

The proband progressed with weakness in the upper limbs and subsequently the lower limbs over the next three years. He developed progressive respiratory failure due to a combination of his pre-existing bronchiectasis and respiratory muscle weakness. He began non-invasive ventilation at night that increased to 24-hour use over the next year. He developed cognitive problems with impairment of short-term memory and recall, as well as subsequent language disturbance with word finding difficulty, speech arrest and errors in syntax and grammar. Formal cognitive assessment was not performed. With progressive respiratory failure and limb

weakness, he died at age 66, 44 months after diagnosis and 68 months after symptom onset.

The proband's mother, II:1; Fig. 1A, initially had symptoms of spastic weakness in her right leg at age 73. Six months later, her left leg also developed a progressive spastic weakness. Sensory examination was normal. Eighteen months after first symptoms, she had slight right foot drop with evidence of bilateral spasticity. Deep tendon reflexes were overall increased in the legs, more so in the right leg. Plantar responses were flexor, but sustained clonus was noted on the right and unsustained on the left. In her upper limbs, power, tone and coordination was normal and there was no evidence of fasciculations. Lower motor neuron signs were absent at this stage.

Over the next 6 months, she had progressive difficulty in using her hands and fingers, with fasciculations present in all four limbs. Speech and swallowing was normal at this stage. Other lower motor neuron signs were still absent, but the combination of fasciculations, weakness and upper motor neurone signs resulted in a diagnosis of amyotrophic lateral sclerosis. No further clinical information was available for the final 5 years of her disease progression. She died at age 82, six years after diagnosis and eight years after symptom onset.

The proband's maternal grandfather (I:1; Fig. 1A) was reported to have had walking difficulties and Parkinson's disease because of a tremor. Similarly, the proband's maternal uncle, II:3, was reported to have shaking in the limbs, walking difficulties and Parkinson's disease.

#### **4. Discussion**

Here we describe a novel *TBK1* truncating mutation in an Australian familial ALS patient of Chinese origin. The normal TBK1 protein comprises 729 amino acids and this frameshift mutation truncates the protein by 44% to 407 amino acids. The truncated protein retains the N-terminal kinase domain (KD) and the ubiquitin-like domain (ULD) however lacks the entire  $\alpha$ -helical scaffold and dimerization domain (SDD) and the C-terminal domain (Fig. 1C) (Larabi, et al., 2013, Tu, et al., 2013). As such, structural integrity of the protein and its adapter protein binding may be hindered.

Normal TBK1 functions as a dimer through extensive hydrophobic contact between the ULD and SDD (Larabi, et al., 2013, Tu, et al., 2013). However since the truncated protein does not contain the SDD, it is unlikely to form this native dimer conformation and would thereby remain inactive.

Activation of TBK1 requires polyubiquitination of amino acids K30 and K401 (Tu, et al., 2013). Interestingly, one of the missense variants (p.K401E) identified in the Diamantina control cohort is located at one of these polyubiquitination sites, with unknown consequence. This rare variant was also present in a public SNP database.

A major functional consequence of the described frameshift mutation would be impairment of TBK1 binding to the adapter proteins TANK, NAP1, SINTBAD, and optineurin (coded by *OPTN*) (Larabi, et al., 2013). The C-terminal domain structurally extends away from the KD-ULD-SDD dimer complex (Larabi, et al., 2013) and binds to the adapter proteins (Goncalves, et al., 2011). Immunoprecipitation and GST pull-down assay experiments performed by Freischmidt, et al. (2015) showed that mutations in the C-terminal domain render TBK1 unable to bind optineurin.

Cirulli, et al. (2015) and Freischmidt, et al. (2015) identified multiple truncation and missense mutations scattered throughout the entire TBK1 protein, in all four

functional domains. Cirulli, et al. (2015) identified 11 loss of function mutations in ALS patients, with most falling within the SDD, and the remainder upstream of the SDD. Seven of the eight *TBK1* loss of function mutations identified in familial ALS patients by Freischmidt, et al. (2015) also lie within or upstream of the SDD. Similarly, the p.L399fs frameshift mutation described here lies upstream of the SDD, providing additional evidence for its pathogenic role.

Haploinsufficiency of *TBK1* has been shown to lead to ALS and FTD (Cirulli, et al., 2015, Freischmidt, et al., 2015), while duplication of *TBK1* results in overexpression of TBK1 leading to normal tension glaucoma, a neurodegenerative disorder involving the optic nerve (Kawase, et al., 2012). This suggests that *TBK1* gene dosage is critical for normal function. Previously reported data from patient cell lines showed no expression of the mutant *TBK1* alleles at the mRNA or protein level for all loss of function mutations that fall within or upstream of the SDD (Freischmidt, et al., 2015). As such, the mutation described here would likely also lead to dosage effects through the generation of a non-functional protein or via nonsense mediated decay.

Normal tension glaucoma caused by *TBK1* duplication (Shields, 2008) is part of a continuum of optic nerve neurodegenerative disorders with primary open-angle glaucoma (Shields, 2008) caused by mutations in *OPTN* (Rezaie, et al., 2002). Missense and nonsense mutations in *OPTN* have also been identified in familial and sporadic ALS cases (Maruyama, et al., 2010).

The discovery family in this study presented with ALS and Parkinson's disease (Fig. 1A). The proband had limb-onset ALS with cognitive impairment and 68 month disease duration. The proband's mother also had limb-onset ALS with predominant upper motor neuron involvement and a long disease duration (>96

months). The two maternal uncles and maternal grandfather have historical diagnoses of Parkinson's disease and should therefore be taken with caution. Unfortunately no DNA or tissue was available from any of these patients to determine whether they also harboured the *TBK1* p.L399fs mutation. The two neurodegenerative phenotypes seen in this family, coupled with *TBK1* duplications found in normal tension glaucoma (Kawase, et al., 2012) and *TBK1* mutations in FTD patients (Freischmidt, et al., 2015) broadens the phenotype of *TBK1*-associated neurodegenerative disease. However, further assessment of *TBK1* is required in other neurodegenerative disorders, including Parkinson's disease.

No novel or reported *TBK1* mutations were found in patients of European ancestry within the Australian ALS cohort. Our discovery of a *TBK1* mutation in an ALS case of Asian origin suggests that a screen of *TBK1* is warranted in ALS cohorts of non-European ancestry.

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## Disclosure statement

The authors declare no conflicts of interest.

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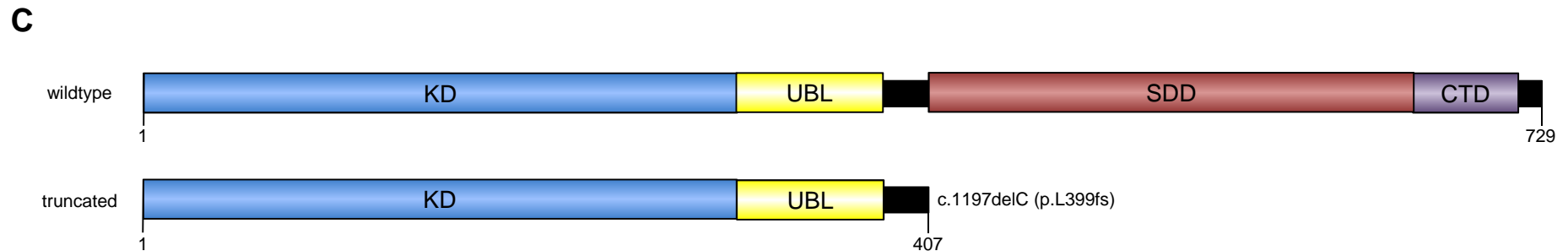
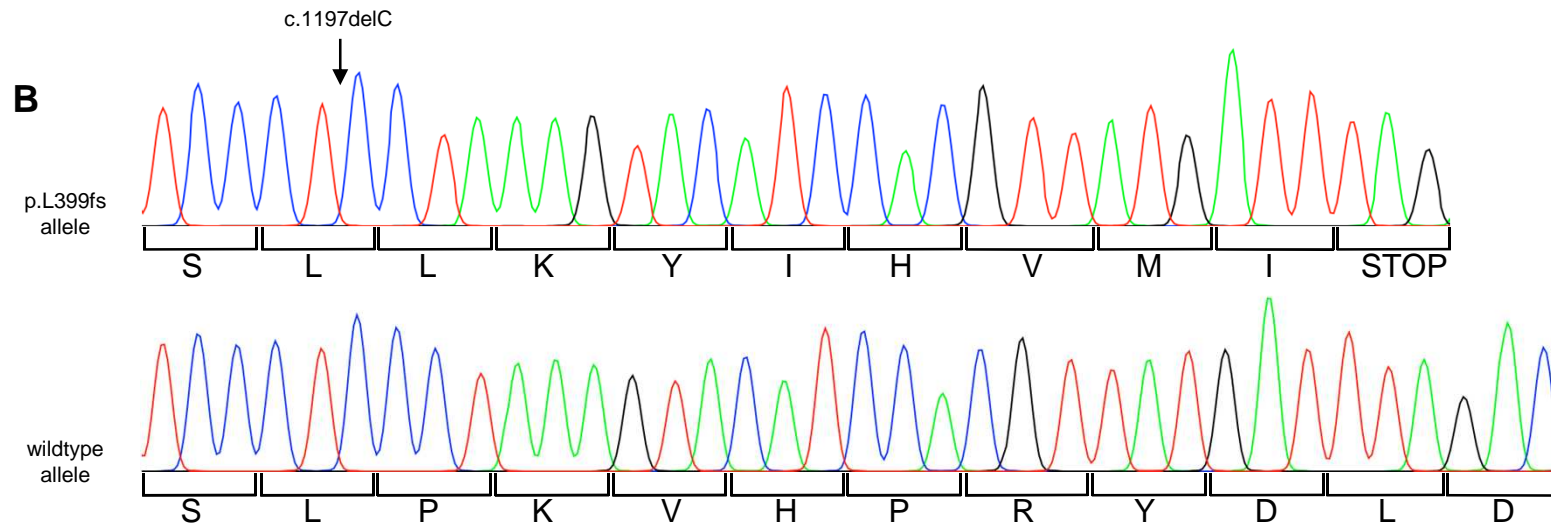
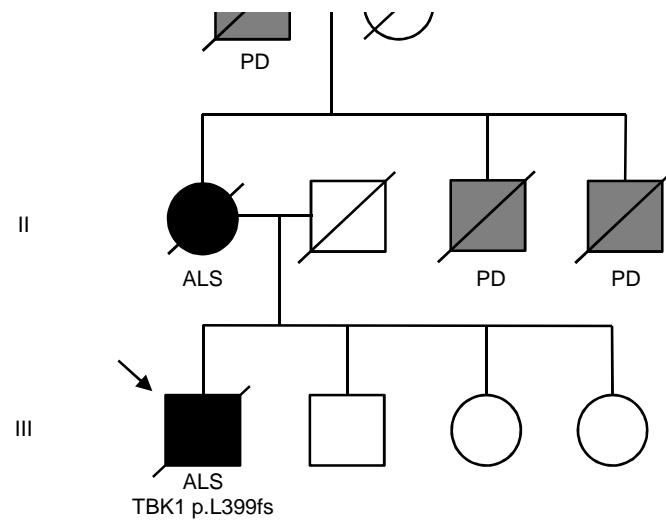
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**Figure legend**

**Figure 1.** TBK1 deletion mutation in an Australian ALS family. **A.** Pedigree of the 3-generation discovery family. The arrow indicates the proband. Males are shown by squares, females by circles. Individuals with a diagonal line are deceased. Symbols filled with black represent ALS, filled with grey represent Parkinson's disease. **B.** Sequence chromatograms showing the two alleles, the resultant amino acids for this section of protein and the premature stop codon in the mutant allele. **C.** Diagrammatic representation of wildtype TBK1 and the predicted truncated protein. Functional domains include N-terminal kinase domain (KD), the ubiquitin-like domain (ULD),  $\alpha$ -helical scaffold and dimerization domain (SDD) and the C-terminal domain (CTD) (Larabi, et al., 2013, Tu, et al., 2013).



**Highlights**

- A novel *TBK1* truncating mutation was identified in an Australian familial ALS patient
- This is the first report of a *TBK1* mutation in an ALS patient of Asian origin
- *TBK1* mutations are a rare cause of ALS in Australia