Striking phenotypic variation in a family with the P506S *UBQLN2* mutation including amyotrophic lateral sclerosis, spastic paraplegia and frontotemporal dementia.

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Abstract

Analysis of 226 exome-sequenced UK cases of familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) identified two individuals that harboured a P497H and P506S *UBQLN2* mutation respectively (n=0.9%). The P506S index case presented with behavioral variant frontotemporal dementia (FTD) at the age of 54, then progressed to ALS surviving three years. Three sons presented with a) slowly progressive pure spastic paraplegia with an onset at 25 years and b) ALS with disease onset of 25yrs and survival of two years respectively and c) ALS presenting symptoms at 26yrs of age. Analysis of post mortem tissue from the index case revealed frequent TDP-43 positive neuronal cytoplasmic inclusions in the frontal and temporal cortex and granular cell layer of the dentate gyrus of the hippocampus. The presentation of multiple intrafamilial phenotypes suggests P506S to be pleiotropic. Furthermore, a comprehensive analysis of published *UBQLN2* mutations demonstrated that only proline-rich domain mutations contribute to a significantly earlier age of onset in male patients (p=0.0026).

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive, neurodegenerative disease with an average age at onset of ~60 years and mean survival of ~3-5 years. Most ALS+/-FTD cases are apparently sporadic ALS (SALS) but ~10% of patients have a family history indicating incompletely penetrant autosomal dominant inheritance. Mutations in superoxide dismutase 1 (*SOD1*), TAR DNA-binding protein (*TARDBP*), fused in sarcoma (*FUS*), and the G4C2 expansion on chromosome 9p (*C9ORF72*) account for approximately 50% of European FALS cases (Smith et al., 2013). Mutations in *UBQLN2* were originally identified in X-linked kindreds with ALS and FTD accounting for a modest proportion of FALS cases (2.7% in Deng et al., 2011). A recent large-scale burden analysis of exome sequencing data has independently verified the significance of *UBQLN2* mutations in FALS (Kenna et al., 2016). Interestingly, *UBQLN2* mutations are associated with a wide range of neurodegenerative phenotypes (ALS+/-FTD, FTD, Primary Lateral Sclerosis (PLS) and Hereditary Spastic Paraplegia (HSP)), (Vengoechea et al., 2013). Here we present further evidence of this phenomenon by the presentation of striking intra-familial phenotypic variation in a family carrying a p.P506S *UBQLN2* mutation supported by patient specific neuropathology demonstrating UBQLN2 positive aggregates.

1. Methods

1.1 Participants

The DNA of 226 FALS patients was exome sequenced as part of an existing novel ALS gene hunting project. All cases were from the UK, and sourced from local ALS clinics and the MNDA DNA Bank. All patients were diagnosed with ALS according to El Escorial criteria (Brooks et al., 2000) and full consent was obtained for research purposes. All cases were devoid of mutations in *TARDBP*, *SOD1*, *FUS*, *OPTN*, *VCP*, *TBK1*, *MATR3*, *PFN1*, *TUBA4A*, *ANXA11*, *CHCHD10*, *NEK1*, *CCNF*, *C21ORF2*, *KIF5A*, and the *C9ORF72* intronic *GGGGCC* expansion. DNA from 500 UK SALS cases were screened by Sanger sequencing for mutations in the proline rich encoding region (residues 491 to 526) of *UBQLN2* (NM_013444), which is a mutation hotspot.

1.2 Genetic analysis

The exomes of the 226 FALS cases were captured using the Roche Nimblegen seqcap EZ exome probeset v3.0 and sequenced with a HiSeq2000 sequencing system (Illumina, Guy's campus, King's College London, UK). Exome sequencing paired-end FASTQ files were aligned to the hg19 human reference using NovoCraft Novoalign, and variants called with samtools v1.1 mpileup. Validation and direct sequencing of *UBQLN2* was performed by amplification of the entire single exon gene with 1 PCR reaction and Sanger sequencing with nested internal primers. PCR products were Sanger sequenced using Big-Dye V1.1 and products run on an ABI3130 Genetic Analyzer (Applied Biosystems Pty Ltd, Warrington). Mutations were identified using Sequencher Version 5.1. Primer sequences and amplification conditions are available upon request. Conservation of *UBQLN2* was examined by ClustalW alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/).

1.3 Immunohistochemistry

In brief, sections of 7µm thickness were cut from paraffin-embedded tissue blocks, then deparaffinised in xylene. Endogenous peroxidases were blocked by immersion in 2.5% H_2O_2 in methanol and immunohistochemistry performed. To enhance antigen retrieval sections were kept in citrate buffer (pH 6) for 10 minutes following microwave treatment. After blocking in normal rabbit serum (1:10, DAKO, UK) in Tris-buffered saline (pH 7.6), anti-Ubiquilin2 antibody (1:500, Abnova) was applied overnight at 4^{o}C. Following washes, sections were incubated with biotinylated secondary antibody (rabbit anti-mouse, 1:100, DAKO, UK), followed by avidin:biotinylated enzyme complex (Vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK). Finally, sections were incubated for 10-15 min with 0.5 mg/mL 3,3′-diaminobenzidine chromogen (Sigma-Aldrich Company Ltd, Dorset, UK) in TBS containing 0.05% H_2O_2. Sections were counterstained with Harris' haematoxylin and immunostaining was analysed using a light microscope (Leica, Wetzlar, Germany).

1.4 Statistics

The significance between the means for the ages of females and males in this study was calculated using a two-tailed t-test on Graph-pad online calculator (https://www.graphpad.com/quickcalcs/ttest1/).

2. Results

We performed exome sequencing in 226 FALS and identified two *UBQLN2* mutations; P497H and P506S in two FALS (0.88% frequency), both located in the proline rich region and previously described (Gellera et al. 2013, Vengoechea et al. 2013, Deng et. al., 2011, Teyssou et.al 2017) (Fig 1A, 1B). Both mutations were absent from 1000 local UK controls and online exome control databases (1000 Genomes, Exome Variant Server n=6503 and ExAC n=60,706). The male index patient harbouring the P497H mutation presented at 39 years of age with symptoms in the fingers of the right hand, slowly progressing to involve both arms, which demonstrated fasciculations and profound wasting. There were no bulbar features. He died 8 years after disease onset with no evidence of FTD (Fig 1A, III.5). In contrast, his mother (II.8) presented with bulbar symptoms at 69 years of age and survived only two years following disease onset. The proband's two maternal aunts (II.4, II.6) were also affected and died in their forties. An affected uncle (II.3) presented at 24 years of age, and a male affected cousin (III.1) presented at 40 years. No additional clinical details were available to further characterize this kindred and no DNA was available from other affected/unaffected family members for segregation testing.

The proband of the family harbouring the P506S mutation (Fig 1B, II.2) was diagnosed with FTD at age 54 with gradual behavioural and personality changes over a period of two years. A year after her initial diagnosis, she clearly showed signs of ALS with muscle wasting, fasciculation and exaggerated reflexes in the cranial nerve, upper limb territories and lower limbs alone being the first symptoms. The duration of disease was three years. Two of her sons presented with distinct motor syndromes. III.2 was first diagnosed with HSP at age 25 and is still alive. III.3 was diagnosed with early onset ALS and died at the age of 27 years. Their half-brother, III.1, was also diagnosed with pure ALS at 26 years of age. All affected members of the kindred carry the P506S change. Both the P497H and P506S mutations are fully conserved within mammals (Fig 1D).

Post mortem tissue was available from the P506S ALS/FTD proband (Fig 1B, Individual II.2). Staining with a UBQLN2 antibody showed frequent neuronal cytoplasmic UBQLN2 positive

inclusions in the dentate gyrus (Fig 2A) and scattered neuronal cytoplasmic inclusions and neurites in the neocortex. Unfortunately, spinal cord tissue was not available to confirm presence of motor neurone damage. Thirteen cases of ALS, FTLD-ALS, and FTLD-TDP devoid of known ALS/FTD mutations were also stained with the UBQLN2 antibody. The density and distribution of the staining was markedly variable (data not shown), as has been described previously (Brettschneider et al 2012). Some cases had more, and some less extensive staining than the P506S ALS/FTD proband (Fig 2B). No UBQLN2 inclusions were seen in the cerebral tissue from the control cases (Fig 2C). Histological examination of the P506S proband also showed frequent TDP-43 positive neuronal cytoplasmic inclusions (NCIs) in the frontal and temporal cortices and granular cell layer of the dentate gyrus of the hippocampus (Fig 2D-E). TDP-43 positive neuronal cytoplasmic inclusions were also observed in the XIIth cranial nerve nucleus (Fig 2F). Deposition of TDP-43 was also noticed in the putamen, thalamus and amygdala. The motor cortex revealed only occasional TDP-43 positive NCIs. This case of TDP-43 pathology is classified as most closely matching histological type B as there were frequent granular intraneuronal cytoplasmic TDP-43 inclusions but only occasional neurites, which are frequently observed in cases of FTD and ALS.

As two mutations were identified in the proline rich domain, we investigated this hotspot further by Sanger Sequencing this domain in 500 UK SALS cases. However, no mutations were detected. Interestingly, the male UBQLN2 carriers from our P497H and P506S kindreds (Fig 2A and B) had a significantly earlier age of onset compared to females (females=54,69, males= 25,25,26,39,40 t=4.3294, p=0.0075). This suggests that due to the lack of a wild-type allele (*UBQLN2* is located on the X chromosome), male *UBQLN2* mutation carriers may have elevated disease penetrance and therefore manifest ALS earlier than female heterozygous *UBQLN2* mutations carriers. This has been previously observed in several *UBQLN2* screening studies (Deng et al., 2011; Vengoechea et al., 2013, Teyssou et.al 2017). With these observations, we therefore surveyed all published ALS+/-FTD cases harbouring *UBQLN2* mutations to address this question. Supplementary Table 1 lists all index cases with *UBQLN2* mutations in ALS+/-FTD cases published to date. Ten of the 28 different amino acid changes observed reside in the PXX domain and most of the index cases are Caucasian. 56% (24/43) of the cases were female and demonstrated a significantly later age of onset than males (females: mean = 51.75 years, males: mean = 38.5 years, t = 2.6110, $p = 0.0126$). In particular, when cases harbouring proline

rich domain mutations were analysed in isolation, the age of onset between the two genders was even more significant (females: mean = 53.56, males: mean = 34.38, t = 3.6053, *p = 0.0026*). Interestingly, within the proline rich domain, with the exception of variants P509S and P525S that are heterozygous in ExAC in approximately 1:10,000 and 1:1,000 Europeans respectively, all other ALS linked proline rich domain variants are novel. Moreover, in gnomAD, while P509S is again found 1:10,000 Europeans, the P525S variant is frequently present in Ashkenazi Jews, with around 1% of the population carrying the common variant.

Discussion

We have identified two *UBQLN2* mutations, P497H and P506S that are absent from ExAC and local UK controls in a cohort of 226 ALS patients (0.88% frequency). Although previously identified, this report confirms that both variants are common, recurrent *UBQLN2* mutations that occur in ALS patients from distinct populations, emphasizing the requirement for routine diagnostic screening (Deng et al., 2011; Vengoechea et al., 2013; Gellera et al., 2013; Özoğuz et al., 2015). Significantly though, we demonstrate here that the P506S mutation results in UBQLN2 positive aggregates in post-mortem tissue. This is the first neuropathological report of a patient harbouring a *UBQLN2* P506S mutation that adds to the characterized list of proline rich mutations in ALS/FTD tissue (Deng et al., 2011; Williams et al., 2012; Scotter et al., 2016). Furthermore, this provides additional evidence of aggregation prone mutations located in the proline rich domain of Ubiquilin 2; highlighting the significance of this region. Furthermore, we observed striking intra-familial clinical phenotypic heterogeneity of individuals possessing the P506S mutation. Firstly the proband P506S mother presenting initially with FTD and then subsequently with ALS, then her two sons demonstrating rapidly progressive, young onset ALS in contrast to another son with early onset HSP. This intra-familial phenotypic difference due to P506S has previously been reported (Vengoechea et al., 2013, Teyssou et.al 2017). Interestingly, the P506 residue in particular when mutated, is highly prone to manifesting multiple phenotypes as Teyssou et.al also reported a P506A carrying kindred with a predominant upper motor neuron phenotype that progressed, in time, to ALS. In light of this observed reduction in age of onset, a comprehensive analysis of published data, suggests that the proline-rich domain *UBQLN2* mutations in particular account for the significant earlier disease onset in males. However, why

male carriers with the same P506S mutation would present with either ALS or HSP is currently unexplainable. One possibility is that UBQLN2 may be variably expressed in different sets of neurons, or potentially X chromosome inactivation due to somatic mosaicism may skew or influence either penetrance or neurodegenerative phenotype as previously postulated (Vengoechea et al., 2013, Teyssou et.al 2017). This is fuel for further investigation in UBQLN2 mediated ALS/FTD. However, our study highlights the importance of proline-rich domain UBQLN2 mutations firstly as a rare contributor to ALS pathology and also to neurodegenerative pleiotropy.

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Figure Legends

Figure 1. Family pedigrees for affected individuals harbouring either the *UBQLN2* P497H (A) or P506S (B) mutations. Probands are indicated by a block diagonal arrow. Affected individuals of the P497H family had a pure ALS phenotype in comparison to the P506S kindred whereby affected individuals manifested either FTD/ALS, ALS or HSP as indicated. (C) Both the P497H and P506S mutations are located in the proline rich domain of UBQLN (residues 491-526). No mutations were found in the UBL=ubiquitin-like domain (33-103), or STI1=four heat-shock-chaperonin-binding motifs (382-426), or the UBA=ubiquitin-associated domain (581-620). (D) Both the P497H and P506S UBQLN2 mutations are fully conserved in mammals and rodents.

Figure 2. Immunohistochemistry of cellular inclusions in post-mortem tissue from the ALS/FTD patient carrying the *UBQLN2* P506S mutation. Staining for Ubiquilin 2 revealed neuronal cytoplasmic inclusions positive for Ubiquilin 2 in the cerebral cortex and here in the dentate gyrus of the proband (A) whilst neuronal cytoplasmic inclusions positive for Ubiquilin 2 were found in cases of sporadic FTLD-TDP, FTLD-ALS and here in an SALS case (B). No

staining for Ubiquilin 2 was found in the dentate gyrus of control cases (C). Also, staining for pTDP-43 revealed neuronal cytoplasmic inclusions positive for pTDP-43 in the granular cell layer of dentate gyrus of hippocampus and upper layers of the frontal and temporal cortex of the proband (D-E). pTDP-43 positive neuronal cytoplasmic inclusions were also observed in the XIIth nerve nucleus (F). Scale bar (A-E)- 20µm, F-30 µm.

Supplementary Table 1. A summary of all published *UBQLN2* mutations to date. All mutations located within the PXX domain are bolded in red.

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Disclosure statement

The authors declare no conflicts of interests to disclose.