ARTICLE

Sequestosome-1 (*SQSTM1*) sequence variants in ALS cases in the UK: prevalence and coexistence of *SQSTM1* mutations in ALS kindred with PDB

Chun T Kwok[1], Alex Morris[1] and Jacqueline S de Belleroche*[1]

Mutations in the *SQSTM1* gene have been reported to be associated with amyotrophic lateral sclerosis (ALS). We sought to determine the frequency of these mutations in a UK familial ALS (FALS) cohort. Sequences of all eight exons of the *SQSTM1* gene were analysed in index cases from 61 different FALS kindred lacking known FALS mutations. Six exonic variants c.463G>A, p.(Glu155Lys), c.822G>C, p.(Glu274Asp), c.888G>T, p.(=), c.954C>T, p.(=), c.1038G>A, p.(=) and c.1175C>T, p.(Pro392Leu) were identified in five FALS index cases, three of which were non-synonymous and three were synonymous. One index case harboured three variants (c.822G>C, c.888G>T and c.954C>T), and a second index case harboured two variants (c.822G>C and c.954C>T). Only the p.(Pro392Leu) and p.(Glu155Lys) mutations were predicted to be pathogenic. In one p.(Pro392Leu) kindred, the carrier developed both ALS and Paget's disease of bone (PDB), and, in the p.(Glu155Lys) kindred, the father of the proband developed PDB. All p.(Pro392Leu) carriers were heterozygous for a previously reported founder haplotype for PDB, where this mutation has an established causal effect. The frequency of the p.(Pro392Leu) mutation in this UK FALS cohort was 2.3% and 0.97% overall including three previously screened FALS cohorts. Our results confirm the presence of the p.(Pro392Leu) *SQSTM1* mutation in FALS. This mutation is the most common *SQSTM1* mutation found in ALS to date, and a likely pathogenicity is supported by having an established causal role in PDB. The occurrence of the same mutation in ALS and PDB is indicative of a common pathogenic pathway that converges on protein homeostasis.

INTRODUCTION

Considerable advances in understanding amyotrophic lateral sclerosis (ALS) pathogenesis have emerged from the identification of new mutations that cause familial ALS (FALS).[1] These include *TARDBP*, *FUS* and *C9ORF72* mutations that are associated with TDP-43-positive poly-ubiquitinated inclusions, the hallmark of most cases of sporadic ALS. These inclusions not only contain TDP-43 or FUS but also p62/sequestosome 1 (p62/*SQSTM1*), optineurin (*OPTN*) and ubiquilin-2 (*UBQLN2*). Indeed, mutations in several components of protein homeostasis, such as *UBQLN2*, charged multivesicular body protein 2B (*CHMP2B*), *OPTN* and valosin containing protein (*VCP*), are also present in less prevalent forms of ALS and ALS/FTD.[2] Interestingly, mutations in *OPTN* and *VCP* are also seen in other allelic forms characterised by TDP-43-positive poly-ubiquitinated inclusions, primary open angle glaucoma (POAG), and inclusion body myopathy with Paget's disease of bone and frontotemporal dementia (IBMPFD), respectively.

Screening of four large ALS cohorts from United States, Europe and Japan for mutations in *SQSTM1* has revealed the presence of a number of novel or rare coding mutations in FALS, FTLD and SALS, some of which are predicted to be pathogenic.[3–6] However, because of lack of DNA in multiple members of FALS kindred, to date it has not been proved that any of these mutations are transmitted with disease in ALS. Nevertheless, among the coding mutations is p.(Pro392Leu), which is common in Paget's disease of bone (PDB),[7,8] where there is

evidence of transmission of the mutation with disease in multiple kindred. In order to further investigate and assess whether they are independently causal for ALS or whether p62/*SQSTM1* DNA variants or modified forms of p62/*SQSTM1* contribute to a significant predisposition to disease, we have examined a further FALS cohort from the United Kingdom.

p62/*SQSTM1* is abundantly expressed in the spinal cord, especially in motor neurons and is present in distinctive p62/*SQSTM1*-positive fibrillar or compact TDP-43-positive inclusions found in ALS and FTD-ALS.[9] In addition, a significant portion of *C9ORF72*-positive FTD/ALS patients have p62 positive, TDP-43-negative inclusions, indicating that multiple substrates are recognised by p62 in these conditions.[10] *SQSTM1* has multiple roles in protein homeostasis,[11] in particular, the interaction of p62/*SQSTM1* with the autophagic marker light chain 3 (LC3) is essential for the generation of autophagosomes necessary for the autophagic degradation of ubiquitinated protein aggregates.[12] Depletion of p62 protein levels inhibits LC3 recruitment to autophagosomes and has been shown to increase cell death induced by mutant huntingtin.[13]

PATIENTS AND METHODS

Sample collection

The FALS cohort used in the initial screen of *SQSTM1* mutations consisted of 61 FALS kindred that are known to lack mutations in *SOD1*, *TARDBP*, *FUS*, *VAPB*, *DAO*, *VCP* and *C9ORF72*. This is a subset of a larger cohort in which

[1]Neurogenetics Group, Division of Brain Sciences, Faculty of Medicine, Imperial College London, Hammersmith Hospital Campus, London, UK
*Correspondence: Professor JS de Belleroche, Neurogenetics Group, Division of Brain Sciences, Faculty of Medicine, Imperial College London, Hammersmith Hospital Campus, London W12 0NN, UK. Tel: +44 207 5946649; Fax: +44 207 59465448; E-mail: j.belleroche@imperial.ac.uk

known mutations have been previously characterised and has the following distribution of FALS mutations, *C9ORF72* (30.2%), *SOD1* (18.0%), *TARDBP* (4.3%), *FUS* (2.2%), *DAO* (0.72%) and *VAPB* (0.72%).[14–19] This study employed ALS cases, presenting with motor neuron symptoms and diagnosed as ALS according to EL-Escorial criteria, from the United Kingdom (Imperial College Healthcare NHS trust). All ALS patients were positive for a familial history (FALS), and each patient was an index case from a separate kindred. Research governance regulations of the college were satisfied with appropriate informed consent from subjects. Controls included consented UK cases obtained within the Trust and genotype data from samples from the EUR subgroup of 1000 genome project (http://www.1000genomes.org) and the European American subgroup of NHLBI EVS (http://evs.gs.washington.edu/EVS/). DNA was extracted from whole blood or the buffy coat layer using a DNA extraction kit (Qiagen, Manchester, UK). In addition, a further group of 26 C9ORF72-positive FALS cases were also screened.

Genotyping

All eight exons of the *SQSTM1* gene that encode the major 440 amino-acid isoform (SQSTM1-001, NM_003900.4) were amplified using polymerase chain reaction (PCR). Primers flanking at least 50bp of these exons were designed using a Primer 3 program (http://frodo.wi.mit.edu/primer3) (Supplementary Information) and were purchased from Invitrogen (Paisley, UK). PCR products were purified using Sureclean (Bioline, London, UK), and sequenced using an ABI Prism BigDye terminator kit (Applied Biosystems, Warrington, Cheshire, UK). The presence of the p.(Pro392Leu) mutation was confirmed by RFLP analysis using the restriction enzyme *Bst*U1 (New England Biolabs, Hitchin, UK). DNA fragments were separated on a 2% agarose gel (Electran, VWR) and stained with 0.5 μg/ml ethidium bromide for 1h at 11 V/cm. φX-174-HaeIII (New England Biolabs) was used as DNA marker. Gel profiles were visualized and analysed using a GelDoc system (Bio-Rad, Hertfordshire, UK). Novel variants have been submitted to DbSNP, (http://www.ncbi.nlm.nih.gov/SNP/) (JDB_2013 NCBI ss 0825679008-10).

Data analysis

Mutation pathogenicity was evaluated using SIFT (http://sift.jcvi.org), Polyphen 2 (http://genetics.bwh.harvard.edu/pph2/) and Mutation taster (http://www.mutationtaster.org). The effects of intronic variants on splicing were analysed using a Human splicing finder program (http://www.umd.be/HSF/). Genotype and allelic distributions of variants were compared with controls using 2×2 and 2×3 Fisher's tests and subjected to Benjamini–Hochberg correction. To examine genetic background, haplotypes were reconstructed using the Expectation–Maximization algorithm, implemented in PLINK. Linkage disequilibrium was measured by r^2 values calculated using Haploview (http://www.broadinstitute.org/haploview). We also analysed the age of onset and survival time of FALS using the Kaplan–Meier curve and log-rank test. The combined effect of pathogenic variants was calculated using the Mantel–Haenszel test.

RESULTS

Identification of *SQSTM1* sequence variants in a UK-FALS cohort

We screened all eight coding exons of the *SQSTM1* gene (NM_003900.4) (Figure 1a) in a cohort of 61 Familial ALS patients. All subjects lacked mutations in *SOD1*, *TARDBP*, *FUS* (exons 14 and 15, which harbour most known mutations found in ALS), *VAPB*, *DAO*, *VCP* and *C9ORF72* genes, and each individual was an index case from unrelated families. Six exonic variants c.463G>A, p.(Glu155Lys) (Figure 1b), c.822G>C, p.(Glu274Asp), c.888G>T, p.(=), c.954C>T, p.(=), c.1038G>A, p.(=) and c.1175C>T, p.(Pro392Leu) (Figure 1c) were identified in five FALS index cases, three of which were non-synonymous and three were synonymous. One index case harboured three variants (c.822G>C, c.888G>T and c.954C>T) and a second index case harboured two variants (c.822G>C, c.954C>T).

The c.822G>C (rs55793208, p.(Glu274Asp)) substitution has been previously reported in ALS by Rubino *et al.*[4] and results in a

Figure 1 Sequence variants in *SQSTM1* in ALS and PDB. (**a**) Summarizes *SQSTM1* mutations previously reported in ALS and PDB. Probable pathogenic variants are in red and variants in the present study are outlined in blue. Domains are annotated as summarized by Geetha *et al.*[11] SH2 (src homology 2 binding domain), AID(acidic interaction domain), ZZ (ZZ finger), TRAF6 (binding site for ring-finger protein tumour necrosis factor), PEST (PEST sequence) and UBA (Ubiquitin-associated domain). (**b–d**) Representative chromatographs showing the heterozygous c.463G>A, p.(Glu155Lys), c.1175C>T, p.(Pro392Leu), c.205+12G>T, c.205+21C>T variants and control sequences. The protein sequences are conserved in mammals. (**e**) The p.(Pro392Leu) mutation was confirmed by RFLP. Upper band showing the mutation only appeared in lane 2, whereas samples with normal sequences (other lanes) were completely digested.

glutamate to aspartate change in the PEST domain of *SQSTM1*. All three synonymous variants have been previously reported, but not in ALS. The p.(Glu274Asp) and other synonymous variants were predicted to be tolerated, using SIFT, Polyphen2 and Mutation taster programs. However, while such programs can predict pathogenicity for recessive mutations, their predictions may not always apply to dominant mutations that may have subtle effects on the protein structure or splicing.[20,21] There were no significant differences in allele frequency between cases and controls for the p.(Glu274Asp) and other synonymous variants using a 2×2 Fisher's test.

However, the substitution c.1175C>T, p.(Pro392Leu) disrupts a highly conserved sequence in the UBA domain[11] and may abolish the ubiquitin-binding ability of the protein. Using SIFT, Polyphen2 and Mutation taster programs, this mutation was predicted to be pathogenic by all three programs. The proband was diagnosed with ALS at the age of 53 years, and disease duration was 6 years (Figure 2a and Supplementary Information). The patient had previously been diagnosed with PDB. To confirm the presence of this mutation, we digested the PCR product with *Bst*U1, which selectively cuts the major allele leaving the mutated allele uncut (Figure 1e). We also screened for the p.(Pro392Leu) mutation in sporadic ALS cases ($n = 86$), controls ($n = 78$) and FALS cases with hexanucleotide expansions in *C9ORF72* ($n = 26$). Cases with *C9ORF72* expansions were included at this stage as they are known exist together with other FALS mutations.[22] One further p.(Pro392Leu) mutation was detected in these FALS cases and confirmed by sequencing, but the mutation was absent from all controls and SALS cases that were screened. This FALS case showed typical ALS features with prominent bulbar features including marked tongue spasticity (Figure 2b). Disease onset occurred at 63 years of age, and disease duration was 27 months. There was no record of PDB in this kindred. Further information is available in Supplementary Information.

The overall prevalence of the p.(Pro392Leu) substitution is 2.3% in our UK-FALS cohort, which is significantly higher than the general population ($P = 0.0455$, Supplementary Figure 1).

c.463G>A, p.(Glu155Lys) is a novel non-synonymous variant found in the ZZ-type zinc-finger domain, which is responsible for protein–protein interactions and interacts with RIP in the TNFα/NFkB

pathway.[11] The mutation is absent from all available databases and was predicted to be pathogenic by all three programs. The proband was diagnosed with ALS at 71 years and survived for 21 months and was heterozygous for the variant. There was no record of PDB in this case. The father of the proband developed PDB but not ALS, while the brother of the proband was diagnosed with ALS at 69 years. He survived for 12 months, and there was no record of PDB in this case (Figure 2c). No DNA was available for these samples.

All coding mutations in *SQSTM1* detected in ALS (SALS and FALS) in this study and in previous studies are presented in Table 1 together with their prevalence in ALS and control populations and pathogenicity predictions. Analysis of all *SQSTM1* variants that are predicted as being pathogenic in published cohorts,[3–6] and the current study demonstrated that pathogenic *SQSTM1* variants are significantly associated with FALS with a combined odds ratio of 3.91 ($P_{MH} = 0.0002$, Supplementary Table 1). No significant association was found for SALS cases. In combination with previous studies, it can be concluded that p.(Pro392Leu), which has been identified in five out of 517 FALS families, is the most common *SQSTM1* mutation in FALS and PDB to date with a mutation frequency of 2.3% in our UK-FALS cohort and 0.97% in pooled FALS populations published to date (Table 1; Supplementary Figure 1).

We also identified two novel intronic variants, c.205+12G>T and c.205+21C>T, which are located at 12 bps and 21 bps from the exon 1/intron 1 junction, respectively. These variants were present in the same FALS individual, who was heterozygous for the first variant and homozygous for the second (Figure 1d). Their effects on splicing were analysed using the Human splicing finder program, which showed that the mutant allele of c.205+21C>T may give rise to a novel donor site with a consensus value of 82.06 (+48.59% variation compared to wildtype) and elongate exon 1 by 16 bps.

Founder haplotype of P392L *SQSTM1* kindred

A four-SNP founder haplotype, H_2, has been previously described to carry most P392L mutations in PDB patients of British descent.[23] We carried out haplotype analysis to investigate whether this was also the case in FALS. We first reconstructed the haplotypes in the control population (1000 genome EUR group) where all four SNPs are in high LD ($r^2 > 0.8$) (Supplementary Figure 2A), showing that H_1
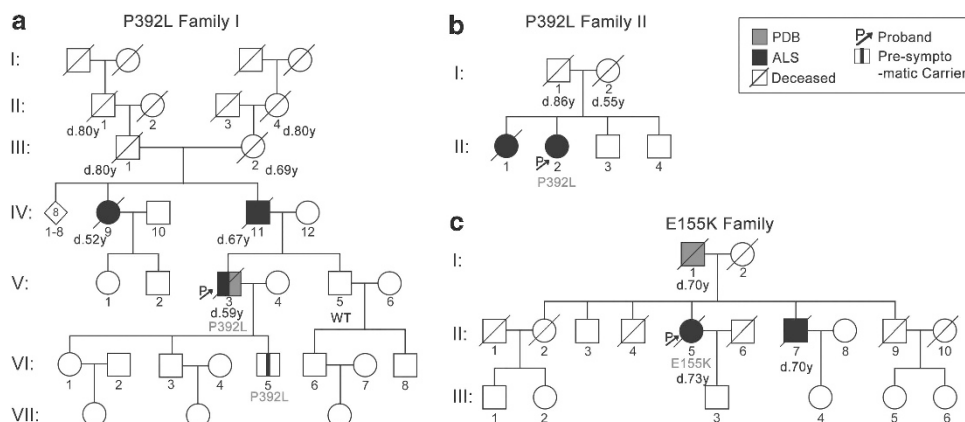

Figure 2 Pedigrees of the FALS index cases carrying p.(Pro392Leu) and p.(Glu155Lys) *SQSTM1* mutations. Age of death and genotypes are indicated where available. (**a**) In the p.(Pro392Leu) Family I, individual II:1 and II:4 died of senility and III:1 and III:2 died of arteriosclerosis and heart disease, respectively. DNA was available for three individuals. The index case developed both PDB and ALS, and no disease phenotypes were present in generations VI and VII at the time of sampling. (**b**) In Family II, individual I:2 died of nephritis, and no DNA was available for other family members. (**c**) In the p.(Glu155Lys) Family, the father of the proband developed Paget's disease and died at 70 years.

Table 1 Summary of coding variants in *SQSTM1* in FALS, SALS and FTD from different studies

Protein	Position	rs	Changes	FALS	Population prevalence SALS	FTD	Control	Prediction SIFT	PP	MT
A33V	179248034	rs200396166	c.98C>T	1/491[a]	2/456[a]		6/4418[a,b]	+	−	+
A53T	179248093		c.157G>A		1/456[c]		0/4550[c,b]	−	−	−
M87V	179250011		c.259A>G		1/456[d]		0/4660[d,b]	+	−	+
K102E	179250056		c.304A>G		1/456[d]		0/4660[d,b]	+	−	+
V153I	179251013	rs145056421	c.457G>A		2/456[a]		9/5024[a,b]	−	−	−
E155K	**179251019**		**c.463G>A**	**1/491[e]**			0/4300[e,b]	+	+	+
P228L	179252155	rs151191977	c.683C>T		1/456[a]		4/5024[a,b]	+	−	+
V234V	179252174		c.702G>A	1/491[a]			0/5024[a,b]	−	−	−
K238E	179252184	rs11548633	c.712A>G		1/456[f]		32/4824[f,b,g]	+	+	+
K238del	179252186-8		c.714-716delGAA		1/456[a]		0/724[a]			+
V259L	179260052		c.775G>C			1/170[f]	0/4445[f,b]	+	−	+
H261H	179260060	rs145001811	c.783C>T		1/456[a]		1/5038[a,b]	−	−	−
E274D	**179260099**	rs55793208	**c.822G>C**	**2/491[f,e]**	11/456[f]	5/170[f]	236/4824[f,b,g]	−	−	−
P296P	**179260165**	rs148984239	**c.888G>T**	**1/491[e]**			1/4679[b,g]	−	−	−
S318P	179260229		c.952T>C	1/491[a]			0/5038[a,b]	−	−	−
S318S	**179260231**	rs56092424	**c.954C>T**	**2/491[e]**			199/4674[b,g]	−	−	−
E319K	179260232	rs61748794	c955G>A			1/170[f]	2/4817[f,b,g]	−	−	−
R321C	179260238	rs140226523	c.961C>T		1/456[a]		5/5407[a,b,g]	+	−	−
K344E	179260647		c.1032A>G			1/170[f]	0/4805[f,d,b]	+	+	+
V346V	**179260655**	rs150470670	**c.1038G>A**	**1/491[e]**			4/4300[b]	−		+
P348L	179260660		c.1044C>T		1/456[f]		0/4805[f,d,b]	+	+	+
S370P	179260725	rs143956614	c.1108T>C	1/491[a]			0/5393[a,f,b]	−	−	+
A390X	179260783		c.1165+1G>A		1/456		−			+
P392L	**179263445**	rs104893941	**c.1175C>T**	**5/517[a,d,e]**	1/542[a,e]		22/5999[a,f,d,e,b,g]	+	+	+
G411S	179263501	rs143511494	c.1231G>A	1/491[a]			0/5397[a,d,b]	+	+	+
G425R	179263543	CM041449	c.1273G>A	1/491[a]			0/5397[a,d,b]	+	+	+
P438L	179263586		c.1313C>T		1/456[f]		1/4805[f,d,b]	+	+	+
P439L	179263676	rs199854262	c.1316C>T		1/456[c]		0/4910[c,d,b]	+	+	+

Mutations identified in this study are in bold, and the frequencies comprise heterozygous and homozygous minor genotypes. All changes shown are exonic except that p.Ala390*, also known as IVS7+1 G>A, occurred at the splice site of intron 7. Data from the EUR subgroup of 1000 Genome Project and EA subgroup of NHLBI Exome sequencing project and 'in house' controls published in previous studies were combined as reference controls. The effects of mutations were predicted using SIFT, Polyphen 2(PP) and Mutation taster (MT): '+'=probably damaging; '−'=tolerated.
[a]Fecto (FALS, $n=340$; SALS, $n=206$; control, $n=724$).
[b]Exome variant server, NHLBI GO exome sequencing project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) (April, 2013).
[c]Hirano (SALS, $n=52$; control, $n=250$).
[d]Teyssou (FALS, $n=90$; SALS, $n=74$; control, $n=360$).
[e]Current study (FALS, $n=61+26$, SALS, $n=86$, control, $n=78$).
[f]Rubino (SALS, $n=124$; control=145).
[g]1000 genomes (EUR, $n=379$).

(TACT) and H_2 (CGTG) accounted for 95% of haplotypes, as previously reported[23] (Supplementary Table 2). All p.(Pro392Leu) mutation carriers in this study were heterozygous for H_2 and $H_{2\alpha}$ (CGCG) haplotypes, and the p.(Glu155Lys) carrier was heterozygous for H_1 and H_2 haplotypes. In the FALS cohort as a whole, there were no significant differences in the frequency of a two-SNP haplotype between cases and controls (Supplementary Table 2). Survival data were available for 16 samples, and there were no significant differences between haplotypes (Supplementary Figure 2B).

The frequencies of common non-coding variants detected in this study are summarised in Supplementary Table 3; the allele frequencies between cases and controls were compared using 2×2 Fisher's tests and corrected for multiple testing. No marked changes were detected.

DISCUSSION

In this study, we screened all exons of the *SQSTM1* gene and confirmed the presence of *SQSTM1* mutations in FALS cases of UK descent. We identified six candidate exonic variants, including a novel p.(Glu155Lys) mutation, and two novel intronic variants in FALS index cases. There were positive familial histories of Paget's disease of bone in two families carrying either the p.(Pro392Leu) or the p.(Glu155Lys) mutation, which were predicted to be pathogenic.

The p.(Glu274Asp) and other synonymous exonic variants detected in this study were not predicted to be pathogenic. In contrast to the findings of Rubino *et al.*[4] we found no significant differences in allelic and genotypic frequencies of the p.(Glu274Asp) or other synonymous variants between our ALS cohorts and controls derived from a UK control cohort and publically available databases. In contrast, the properties of the p.(Pro392Leu) and p.(Glu155Lys) mutations are more likely to contribute pathogenic effects. First, these mutations are located in highly conserved domains that are required for the basic functions of p62/*SQSTM1*. The p.(Pro392Leu) mutation occurs in the UBA domain, which is required for the binding of ubiquitin,[11] and modifies the structure of this domain by extending the N-terminus of helix 1.[24] This results in a reduced affinity for mono-ubiquitin and shortened polyubiquitin chains.[25] The ZZ domain, which accommodates the p.(Glu155Lys) mutation, is a conserved structural component in different ZIP/p62 homologues and has an

important role in the formation of complexes that participate in the NFkB signalling pathway.[26] Second, the coexistence of both PDB and ALS in the two families reported here and in other studies[5] is indicative that common pathogenic mechanisms underlie both these diseases. P62/SQSTM1 has an established causal role in PDB, and the p.(Pro392Leu) mutation is, in fact, the most common cause of PDB.[7,8] The p.(Pro392Leu) mutation segregates with PDB, and, although the binding of polyubiquitin remains intact, it causes a phenotype that is indistinguishable from those caused by mutations that truncate the entire domain.[27] Third, multiple studies have confirmed the existence of the p.(Pro392Leu) mutation in ALS from different populations,[3,5] and, in combination with the data presented here, it can be concluded that it is the most common mutation in the gene in ALS to date (Table 1). The coexistence of the p.(Pro392Leu) mutation in a family with a C9ORF72 expansion is not unexpected as C9ORF72 has also been found in FALS cases harbouring SOD1, FUS or TARDBP mutations.[22] The SQSTM1 p.(Pro392Leu) mutation index case with the C9ORF72 mutation (Family II) showed classical features of ALS with no evidence of cognitive impairment and similar survival (age at death: 62–65 years) compared with Family I that lacked a C9ORF72 expansion (age at death: 52–67 years). It is possible that the severity of the disease in the proband may have been affected by the coexistence of both mutations, but whether the effect of the SQSTM1 mutation is potentiated by the expansion remains to be established.

The fundamental role of p62 in autophagy is well established and relevant to multiple tissues. However, more recently considerable evidence has accumulated for the specific involvement of p62 in ALS and ALS with fronto-temporal lobar degeneration (ALS/FTLD), where p62 is associated with both TDP-43-positive and TDP-43-negative inclusions in the spinal cord and cerebral cortex. Further, similar inclusions have been demonstrated in ALS cases harbouring SQSTM1 mutations.[5]

In spite of the phenotypic heterogeneity found in ALS, ALS/FTLD and PDB and related disorders such as IBMPFD and POAG, the discovery of mutations in overlapping candidate genes, such as VCP, UBQLN2, CHMP2B and OPTN, points to a common pathogenic mechanism affecting the formation and clearance of misfolded proteins.[2] We previously reported that the genotypes of protein disulphide isomerase (P4HB), a redox enzyme that reduces formation of aberrant disulphide bonds, were associated with survival time of FALS,[28] supporting the hypothesis that factors interfering with proteostasis may modify disease progression and can be considered as therapeutic targets for ALS. The investigation of p62/SQSTM1 mutations in multiple cohorts provides further support for their contribution to a significant predisposition to disease in ALS.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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