Mutations in *SQSTM1* encoding p62 in Amyotrophic Lateral Sclerosis: genetics and neuropathology

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Disclosure statement

The authors declare that they have no conflict of interest.

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Abstract

Mutations in *SQSTM1* encoding the sequestosome 1/p62 protein have recently been identified in families with and sporadic cases of amyotrophic lateral sclerosis (ALS). p62 is a component of the ubiquitin inclusions detected in degenerating neurons in ALS patients. We sequenced *SQSTM1* in 90 French patients with familial ALS (FALS) and 74 autopsied ALS cases with sporadic ALS (SALS). We identified, at the heterozygote state, one missense c.1175C>T, p.Pro392Leu (exon 8) in one of our FALS and one substitution in intron 7 (the c.1165+1G>A, previously called IVS7+1 G-A, A390X) affecting the exon 7 splicing site in one SALS. These mutations that are located in the ubiquitin-associated domain (UBA domain) of the p62 protein have already been described in Paget's disease and ALS patients carrying these mutations had both concomitant Paget's disease. However, we also identified two novel missense mutations in two SALS: the c.259A>G, p.Met87Val in exon 2 and the c.304A>G, p.Lys102Glu in exon 3. These mutations that were not detected in 360 control subjects are possibly pathogenic. Neuropathology analysis of three patients carrying *SQSTM1* variants revealed the presence of large round p62 inclusions in motor neurons and immunoblot analysis showed an increased p62 and TDP-43 protein level in the spinal cord. Our results confirm that *SQSTM1* gene mutations could be the cause or genetic susceptibility factor of ALS in some patients.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is the most common adult onset motor neuron disease. Pathological hallmarks detected in spinal motor neurons of most ALS patients include skein like inclusions, which are positive for ubiquitin, ubiquitin-binding protein p62 (also called sequestosome 1, [38]) and TDP-43 (TAR DNA-binding protein 43, [39]), and cystatin C-positive Bunina bodies [40]. Recently, *SQSTM1* encoding sequestosome 1/p62 protein (440 amino acids) was screened as a candidate gene in a large cohort of ALS patients. Ten different mutations (9 heterozygous missense and one deletion) were identified in 6/340 FALS (1.8%) and 9/206 SALS (4.4%) [14]. Following, two reports screened *SQSTM1* gene in different populations. Out of 124 SALS and 170 frontotemporal lobar degeneration (FTLD) patients of Italian origin around 3% carried the mutations and this gene was also responsible for 2/54 SALS (3.7%) of Japanese origin [23, 44]. Although expanded hexanucleotide repeats in *C9ORF72* occurring in 23-46% of FALS and 4-8% of SALS became the major genetic defects in ALS [12, 43], mutations in *SQSTM1* could account for a substantial part of ALS causing genes making p62 a potential player in ALS. The aim of this study was to confirm the contribution of *SQSTM1* to ALS by analyzing 164 French ALS patients (90 FALS and 74 autopsied SALS) in whom all the other ALS genes had been excluded and to analyze the neuropathological features in patients carrying *SQSTM1* variants.

Methods

Genotyping. The eight coding exons with exon-intron boundaries of *SQSTM1* (ensembl reference sequence: ENSG00000161011) were sequenced in 90 French FALS with mean age of onset of 60 years (SE 1, range 33 to 80 years), mean disease duration of 46 months (SE 5, range 8 to 260 months, including 13 censored data) and 74 SALS French autopsied cases (30 DNA were from blood and 50 DNA were extracted from Liver slices) with mean age of onset of 61 years (SE 1, range 35 to 81 years), mean disease duration of 49 months (SE 5, range 8 to 206 months) (Table 1). Data were censored at the last date of patient visit. Control samples were obtained from sex and age-matched healthy Caucasian individuals of French background.

Initial ALS populations comprised 270 FALS with probable or definite ALS [6] including 10 autopsied cases and 80 more autopsied SALS cases (30 DNA were from blood and 50 DNA were extracted from Liver slices). Analyses of *C9ORF72*, *SOD1*, *TARDBP*, *FUS*, *ANG*, *VAPB*, *DAO*, *OPTN* and *UBQLN2* genes had previously been performed [33-37]. Mutation frequencies of *C9ORF72*, *SOD1*, *TARDBP* and *FUS* have been described

elsewhere for FALS [34]. Autopsied SALS included 3 patients with *C9ORF72* expansion, 2 with *SOD1* and one with *TARDBP* mutation.

Sequence of the primers and PCR conditions are available upon request.

Immunoblotting. Transversal sections of spinal cords were homogenized in 50 mM Tris-HCl pH 8, 150 mM NaCl, protease inhibitors (Complete mini tablets, Roche) and 0.5 U/μl Benzonase endonuclease (Merck). The samples were incubated at 37°C during 30 minutes. SDS was then added at a final concentration of 2%. Tissue extracts were centrifuged at 13,000 rpm for 10 min and protein concentration of supernatants was estimated by the bichinonic acid assay (Sigma Aldrich). Proteins (20 μg) were separated on NuPAGE™ 4-12% Bis-Tris Gel (Invitrogen) and electrophoretically transferred to nitrocellulose membranes. Membranes were incubated overnight with primary antibodies in PBS/ 5% milk/ 0.1% Tween 20, followed by one hour of incubation with appropriate peroxidase-conjugated antibodies. Signals were detected using ECL plus Western Blotting Detection System (GE Healthcare). Densitometric analysis was performed using Multi Gauge V3.0 (Fujifilm) software using tubulin as internal standard. For each patient, 5 measures (from 5 independent western blot analyses) were recorded for p62 and TDP-43. p62 and TDP-43 levels were compared between patients with *SQSTM1* mutation and control SALS patients (with no mutation in the screened genes) using Mann Whitney test (GraphPad Prism 4 software). The significance level for all comparisons was set at 0.05.

Primary antibodies included mouse monoclonal anti-p62 antibody raised against aminoacids 257-437 of the human protein (610833, BD Biosciences), rabbit anti-TARDBP raised against TDP-43 protein (12892-1-AP, ProteinTech) and mouse anti-β-tubulin B-5-1-2 (Sigma-Aldrich). Peroxidase-conjugated AffiniPure Goat Anti-Rabbit and Anti-Mouse (all with minimal cross-reaction to human proteins) were from Jackson immunoResearch Laboratories.

RT-PCR. Total RNAs were extracted from whole transverse sections (including anterior and posterior horn) of spinal cords using the Trizol reagent (Invitrogen). First-Strand cDNA synthesis was performed using ThermoScript RT-PCR system (Invitrogen) according to the manufacturer's instructions. PCR was performed with Green Taq DNA polymerase (Fermentas) and the following primers: 5'-ACGTTGGGGAGAGTGTGGCAG-3' (forward primer in *SQSTM1* exon 5) and 5'-CCTTCATCAGAGAAGCCCATGGA-3' (reverse primer in *SQSTM1* exon 8). The PCR conditions were: 95°C,

3 min and 25 cycles (95°C, 30s, 62°C, 30s, 72°C, 30s). Amplified fragments were sequenced using standard procedure.

Neuropathology.

ALS patients were enrolled in a brain donation program declared to the Ministry of Research and Universities, as requested by French Law. An explicit consent was signed by the patient himself, or by the next of kin, in the name of the patient, in accordance with the French Bioethical Laws. For each autopsied patient, the brain was weighted before fixation. Neuropathologic examination was done on the formalin-fixed half brain, the other half being kept frozen in the brain bank. On the fixed hemi-brain, 16 samples were embedded in paraffin for microscopic examination: midfrontal gyrus, motor cortex, first temporal gyrus, hippocampus, ventral amygdala, primary visual cortex, parietal cortex, orbital frontal cortex, head of the caudate nucleus, lenticular nucleus, thalamus, mesencephalon, pons, medulla oblongata, cerebellum, three levels of spinal cord (cervical, thoracic, lumbar). Slides were stained with hematoxylin-eosin or, for the spinal cord with Bodian silver impregnation associated with Luxol fast blue.

Immunostainings for p62, ubiquitin and TDP-43 were performed on three levels of the spinal cord (cervical thoracic and lumbar) and on the medulla oblongata, frontal isocortex, hippocampus and cerebellum. Immunohistochemistry for cystatin C was, in addition, performed on spinal cord and medulla oblongata. After deparaffinization, the 5 µm thick sections were immunolabeled with the antibodies listed in Table 2. For most antibodies, the immunostaining was performed by an automatic slide stainer (Benchmark® XT Ventana® staining system), the slides being pre-treated at 95°C in either CC1 (pH=8) or CC2 (pH=6) proprietary retrieval buffers (Ventana Medical Systems®) (Table 2). The biotinylated secondary antibody was included in the detection kit (Ventana Medical Systems Basic DAB Detection Kit 250-001®). The streptavidin-biotin-peroxidase complex was revealed by diamino-benzidine. For ubiquitin immunohistochemistry, the slides were pre-treated by formic acid (FA, 80 %), for 5 to 10 minutes (Table 2). For p62 staining, the slides were microwave pretreated (450W) during 2x10 min in citrate buffer pH 6 (Table 2).

The semi quantitative analysis of the lesions focused on anterior horn motor neurons of medulla oblongata and spinal cord in 45 autopsied ALS patients including patients with *SQSTM1* (n=3), *C9ORF72* (n=8), *SOD1* (n=4), *TARDBP* (n=2) mutations and other patients with no mutation in any of these genes (n=28).

A consensual definition based on the shape and localization of ubiquitinated inclusions (filamentous or skein-like, large round and granular) was established by five of us (DS, TT, VL, BD, SM). The semi-quantitative

assessment for p62 and TDP-43 in cervical, thoracic and lumbar spinal cord and in medulla oblongata was performed by three of us (TT, VL, BD) who were blinded to the genetic data, following a 4-point ordinal scale (0, none; 1, rare; 2, moderate; 3, severe), a similar scoring approach had been used effectively in several similar comparative pathology studies [4, 5, 16, 17, 21]. The score means obtained for filamentous, large round, and granular inclusions were compared between patients carrying *SQSTM1*, *C9ORF72*, *SOD1* mutations and other patients with no mutation in any of these genes using ANOVA (SPSS 11.0 software). If the difference was significant ($p < 0.05$), the groups were compared by pairs using a Tukey's post hoc test analysis. The patient group with mutation in *TARDBP* (n=2 patients only) was not included in this analysis.

Results

Genetic analysis

In FALS we identified, at the heterozygous state, one missense mutation in exon 8 (the c.1175C>T, p.Pro392Leu) in one patient (case 1) that presented with distal lower limb disease onset at 54 years and deceased after a 133 months disease duration (Fig. 1a, 1c). No DNA was available for other affected family members and the segregation could not be further studied (Fig. 1d). In SALS the c.1165+1G>A substitution in intron 7 presumably affecting the exon 7 splicing site (Fig. 2a) was identified in one woman who had a distal lower limb disease onset at 75 years and disease duration of 42 months (case 2). These two patients had concomitant Paget's disease (Table 3).

We also identified, at the heterozygote state, two missense mutations in two SALS patients: the c.259A>G, p.Met87Val in exon 2 (carried by case 3) and the c.304A>G, p.Lys102Glu in exon 3 (carried by case 4, Fig. 1a). The first patient (case 3) carrying a missense variant showed bulbar onset at 68 and disease duration of 18 months and the second one (case 4) started the disease at 81 years of age which lasted for 10 months (Table 3). History of Paget's disease was not recorded for these patients. These variants affect aminoacids that are highly conserved in mammals (Fig. 1b). They were not identified in the dbSNP Short Genetic Variations, the 1000 Genomes Project and the NHLBI ESP Exome Variant Server databases and in our analysis of 360 control Caucasian individuals. The sequencing of exon 7 and 8 in this control population identified one single variant, not reported previously: the c.1313C>T, p.Pro438Leu in one 60 year-old man.

To further analyse the effect of the c.1165+1G>A in the intron 7 splicing site variant (carried by case 2), we amplified the p62 cDNA sequence spanning from exons 5 to 8 (Fig. 2c) and sequenced the corresponding fragments. We found that this substitution led to the deletion of part of the exon 7 (r.1052_1165del, ΔEx7) in the

mRNA sequence (Fig. 2b-c) which probably results in the production of a truncated p.Gly351_Pro388del protein of 402 aminoacids as suggested by the additional band present on the western blot analysis (Fig. 2d, case 2).

Quantitative analysis of p62 (for case 2, p62 level is the sum of the two bands) and TDP-43 levels on immunoblots showed that p62 and TDP-43 protein levels were increased in the group of patients with *SQSTM1* mutations compared to the control SALS group (Fig. 2e) with a significant difference reached for both stainings ($p=0.0129$ for p62 and $p<0.0001$ for TDP-43).

Neuropathology analysis

Autopsy had been performed for 3 of the patients carrying mutations in *SQSTM1* (cases 2-4).

The brain of the woman carrying the c.1165+1G>A (p.Gly351_Pro388del) mutation (case 2) weighted 1180g. Atrophy of the frontal lobe including the precentral gyrus, associated with atrophy of the anterior spinal roots was noticed. Microscopic examination of the spinal cord and medulla oblongata showed mild neuronal loss, cystatin C positive Bunina bodies, TDP-43 and p62 positive skein like and rounded inclusions and TDP-43 positive diffuse granular staining. Pronounced neuronal loss and gliosis were observed in the substantia nigra, without Lewy bodies. In the midfrontal and precentral gyri, fibrillary gliosis was associated with superficial spongiosis. Immunohistochemistry showed numerous TDP-43 positive filamentous inclusions in layer 2, associated to thin neurites (Fig. 4a), resembling Type A pathology according to Mackenzie classification [29]. p62 immunohistochemistry showed a lower number of paranuclear inclusions, and no neurites (Fig. 4c). Despite these neuropathological observations on cortical regions, this patient had no clinical FTD (Table 3).

In the male patient with M87V mutation (case 3), the brain weighted 1345 g before fixation. No cerebral atrophy was noticed, macroscopic abnormalities being limited to atrophy of the anterior spinal roots. At microscopic examination, neuronal loss was severe in the anterior horns of the spinal cord. Immunohistochemistry showed TDP-43 and p62 positive skein like and rounded inclusions, numerous TDP-43 positive granules, and cystatin C positive Bunina bodies. A mild neuronal loss was observed in the substantia nigra, without Lewy bodies. No lesion was found at supratentorial level, except diffuse A beta deposits.

In the female patient with K102E mutation (case 4), the brain weight was 1066g. No noticeable focal cerebral atrophy was found at macroscopic examination, contrasting with atrophy of anterior spinal roots. At microscopic examination, neuronal loss was mild in the spinal anterior horns and in the XIIth nucleus, although Bunina bodies

were quite numerous. Immunohistochemistry showed ubiquitin, TDP-43 and p62 positive rounded inclusions (Fig. 3d-f, 3g-i, supplementary Fig. 1a) and cystatin C positive Bunina bodies. In addition numerous motor neurons contained intracytoplasmic small ubiquitin and TDP-43 positive granules (Fig. 3a, 3c). This pattern was not observed with p62 immunohistochemistry (supplementary Fig. 1c). In some rare neurons bigger “seed-like”, p62 positive aggregates were observed (Fig. 3b). These seed-like inclusions were also seen with TDP-43 staining (supplementary Fig. 1b). A mild neuronal loss was observed in the substantia nigra without Lewy bodies. There was neither evidence of major neuronal loss in the central motor cortex nor severe degeneration of the cortico-spinal tracts. A mild superficial spongiosis was observed in the second layer in the midfrontal gyrus. Immunohistochemistry showed some TDP-43 positive inclusions of various shapes, either round or filamentous, spread throughout the cortex, with only a few positive neurites (Fig. 4b), resembling Type B pathology according to Mackenzie classification [29]. p62 immunohistochemistry showed a lower number of inclusions, generally rounded, and no neurites (Fig. 4d). p62 positive glial inclusions were found in both cortex and spinal cord.

The three cases showed numerous p62 and TDP-43 glial positive inclusions in spinal cord. No p62 or TDP-43 inclusion was found in the granular cells of the cerebellum for the three patients.

To determine whether there were differences in ubiquitinated inclusion pathology in cases with or without *SQSTM1* variants, we examined spinal cord tissues from 45 ALS patients including the 3 SALS patients carrying *SQSTM1* variants (p.Gly351_Pro388del, p.Met87Val and p.Lys102Glu corresponding to cases 2, 3 and 4, respectively), 8 patients with *C9ORF72* repeat expansion, 4 patients with *SOD1* mutations, 2 patients with *TARDBP* mutations and 28 other patients devoid of mutation in any of these genes. Several types of ubiquitinated inclusions (stained with p62 and TDP-43) were quantified for these patients (Supplementary Table 1): skein like filamentous inclusions (Fig. 3e-f), large round cytoplasmic inclusions (Fig. 3h-i) and intracytoplasmic diffuse granular aggregates (Fig. 3c). The score means obtained for large round inclusions were compared between patients carrying *SQSTM1*, *C9ORF72*, *SOD1* mutations and other patients with no mutation in any of these genes using ANOVA. The patient group with mutation in *TARDBP* was not included in this analysis since we only had n=2 patients. One way ANOVA analyses between the 4 groups of patients revealed that the p62 round inclusion pathology scores were different ($p=0.041$). Tukey’s post hoc test analysis for comparison of the 4 groups by pairs showed that p62 positive rounded inclusions were more numerous in ALS patients with *SQSTM1* variant than in patients with no mutation in the screened genes (Tukey’s post hoc test, $p=0.03$) but were not different between *SQSTM1* mutation carriers and patients with mutation in another gene

(*SOD1* or *C9ORF72*). The lack of statistically significant difference between *SQSTM1* mutation carriers and patients with *C9ORF72* and *SOD1* mutation could be due to too small sample sizes. Indeed, there was a significant difference (Mann Whitney's test, $p=0.045$) between patients with *SQSTM1* patients and all the other patients pooled together (devoid of mutation in any of the screened genes and carrying a mutation in *SOD1* or *C9ORF72*). The number of rounded inclusions stained for TDP-43 was not statistically different between the 4 groups of patients ($p=0.08$). These semiquantitative differences in the numbers of p62 versus TDP-43-positive inclusions may simply reflect differences in the sensitivity of the antibodies employed. Occurrence of filamentous inclusions positive for p62 ($p=0.006$) or TDP-43 ($p=0.01$) differed between the 4 groups of patients. Less TDP-43 positive filamentous inclusion was detected in the *SOD1* group than in *C9ORF72* ($p=0.02$) or *SQSTM1* ($p=0.02$) patients which is in accordance with the absence of TDP-43 immunoreactivity previously reported for patients with *SOD1* mutation [28]. There was no difference in the incidence of granular inclusions stained by p62 or TDP-43 antibodies for the four groups of patients.

Discussion

Our *SQSTM1* genetic analysis identified 4 variants corresponding to a frequency of 1.1% in FALS and 4% in SALS which is similar to the frequencies previously reported in FALS (1.7%) and SALS (3-4.4%) [14, 23, 44]. We identified two novel *SQSTM1* mutations, p.Met87Val and p.Lys102Glu, which are possibly pathogenic. The mutations that we identified in intron 7 and exon 8 have already been described in patients with ALS [14] or Paget's disease of bones (PDB) [8, 31, 32]. PDB is a chronic disease of the skeleton due to focal increased bone turnover affecting 2-3% of individuals older than 55 and 6-7% of individuals over 85 years of age in Caucasian populations [9]. Manifestations of the disease include bone pain, enlargement, and deformities at the pagetic sites, susceptibility to fractures, deafness and neurologic complications [20]. Recent studies have confirmed that both genetic and environmental factors are implicated in its etiology [19]. Seven loci (PDB1 to 7) have been identified and mutations in *SQSTM1* gene, mostly located in the c terminus region of the protein encoded by exon 7 and 8, containing the highly conserved ubiquitin binding domain (Fig. 1c), are responsible for the phenotype linked to chromosome 5q35 (PDB3). These UBA domain mutations impair the ability of p62 to bind to ubiquitin [7], probably resulting in aberrant NF- κ B signaling [19].

In France, *SQSTM1* mutations represent 13% of PDB patients [8] corresponding to an estimated frequency of 0.4% for the overall French population. This estimation is in accordance with the single variant we found

(c.1313C>T, p.Pro1438Leu) in one out of 360 control patients (mutation frequency of 0.3%) after sequencing *SQSTM1* exon 7 and 8. However, as shown in both previous studies that have analyzed patients of Caucasian origin [14, 44] and now in our study, the frequency of this mutation in *SQSTM1* is 10 fold higher in ALS patients therefore arguing in favor of a role of *SQSTM1* as a causative or a susceptibility gene in ALS.

The c.1175C>T, p.Pro392Leu has been previously detected in 2/340 FALS and 1/206 SALS but 0/737 control patients [14]. It is the recurrent mutation identified in Paget's disease patients (PDB), responsible for 46% of familial and 16% of sporadic cases of PDB in the French Canadian population [27]. In addition, mice carrying a proline to leucine mutation at codon 394 of mouse *sqstm1* (P394L), corresponding to the P392L *SQSTM1* mutation in humans, develop a disease similar to human PDB with focal increases in bone turnover and disruption of the normal bone architecture [11]. No obvious sign of motor neuron disease as impaired motor function or muscular atrophy has been described [11] although no study has assessed motor neuron numbers and shape in the spinal cord of this Paget's disease mouse model.

The c.1165+1G>A substitution in intron 7 affecting the exon 7 splicing was previously identified in a family of Australian descent with Paget's disease (it was called IVS7 + 1 G-A, A390X since it was thought to lead to the production of a truncated protein of 390 amino acids terminating at an inframe stop codon at position +6–9 within intron 7) [24]. Our results show that this mutation led to the deletion of part of exon 7 in the mRNA sequence (r.1052_1165del) and the production of a truncated p.Gly351_Pro388del protein of 402 aminoacids in the spinal cord. Both pPro392Leu and p.Gly351_Pro388del mutations have been repeatedly identified in French patients with Paget's disease [32]. In contrast to the previously reported ALS patients with *SQSTM1* mutations [14], both ALS patients with mutations in ubiquitin binding domain of *SQSTM1* described here (i.e intron 7 for one SALS and exon 8 for one FALS) had concomitant PDB. However coexistence of these two disorders could be underestimated as PDB is often asymptomatic [20] and its diagnosis is based on radiographic findings. Coexistence of these two diseases has already been described in some patients with mutations in valosin containing protein (VCP) [25]. This gene has already been linked to Inclusion Body Myopathy with early-onset Paget's disease of the bone and Frontotemporal Dementia (IBMPFD) [47]. In addition, *OPTN*, another ALS causing gene [30] is also known as a susceptibility gene for PDB [2]. Interestingly *SQSTM1*, *VCP* and *OPTN* all encode proteins that bind to ubiquitin [10, 18, 46]. These three genes that share common functions in protein degradation pathways could confer susceptibility to Paget's disease and motor neuron disease when mutated. The clinical evaluation of bone integrity and the neuropathological assessment of motor neuron should be explored in ALS and PDB patients, respectively. In the present study, the observation of p62/TDP-43 positive

inclusions in spinal motor neurons of case 2 (with c.1165+1G>A, r.1052_1165del, p.Gly351_Pro388del mutation) coupled to the accumulation of p62/TDP-43 proteins that could be detected in the spinal cord of this patient by immunoblot analysis strongly argues in favor of a participating role of this “PDB mutation” in ALS phenotype.

p62 positive inclusions are common in many degenerative diseases including tauopathies and synucleopathies [15, 26] and a key features in neuropathological diagnosis and classification of ALS and FTLT, both sporadic and familial [38, 41]. The cortical pathology observed in the present study in ALS patients with *SQSTM1* mutations (cases 2 and 4) confirms again the overlap between motor neuron disease and fronto-temporal lobar degeneration.

In contrast to the *SQSTM1* Paget’s disease linked mutations, those reported to date in ALS patients are distributed throughout the protein (Fig. 1c) suggesting that different domains of the p62 protein endowed with other functions, beside its ubiquitin binding one, could be impaired in ALS. Considering the implication of TDP-43 in ALS and since p62 has been shown to bind to TDP-43 (and to be potentially involved in its degradation), another p62 function that could be impaired by the ALS causing mutations would involve disruption in TDP-43 - p62 interaction, previously suggested to participate in the pathogenesis of TDP-43 proteinopathy [45]. Additional evidence have shown that overexpression of p62/SQSTM1 reduces TDP-43 aggregation in an autophagy and proteasome-dependent manner [3]. Our immunoblot findings showing concomitant accumulation of p62 and TDP-43 proteins in spinal cord of *SQSTM1* mutation carriers are in line with a shared pathological effect of these two proteins.

Data from p62 deleted mice showed that p62 loss of function results in increased ubiquitin staining in hippocampus and cortex together with accumulation of insoluble ubiquitinated proteins, of hyperphosphorylated tau and neurofibrillary tangles resulting in neurodegeneration and Alzheimer-like phenotype [42]. It is therefore tempting to hypothesize that a loss of a specific function of p62 protein due to mutations in a specific domain of *SQSTM1* could also result in ALS phenotypes.

Previous studies have described that the profile of p62 neuropathology can predict the underlying genetic defect in ALS and FTLT [5]. However the number of p62 positive inclusions is not necessarily correlated to the number of TDP-43 aggregates (Al Sarraj et al., 2011). In FTLT-ALS with *C9ORF72* mutations, neuronal inclusions are both TDP-43 and p62 positive, except in the cerebellum where inclusions are only p62 positive [1]. In patients with *SQSTM1* mutations reported here, the granular deposit of TDP-43 and ubiquitin is not positive

for p62. These results underline that the relationship between p62 and other accumulating proteins remains largely unknown.

Our study described two novel *SQSTM1* mutations identified in ALS patients which are possibly pathogenic and provided neuropathological examination of three mutation carriers. The presence of large round p62 inclusions was consistently observed in these *SQSTM1* patients. In mean, there was a higher number of these round p62 inclusions in spinal cord of *SQSTM1* carriers compared to the other patients although they were rare in two of the *SQSTM1* cases examined. Moreover increased protein levels of p62 and TDP-43 were detected in the spinal cord extracts of *SQSTM1* patients. These observations support that these *SQSTM1* mutant probably contribute to the ALS phenotype. Overall our study confirmed that *SQSTM1* gene mutations could be the cause or a genetic susceptibility factor of the disease in some ALS patients.

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Legends of the figures

Figure 1. *SQSTM1* missense mutations identified in ALS patients.

(a) Part of chromatograms showing the normal sequence (control), the c.259A>G, p.Met87Val variant in exon 2, the c.304A>G, p.Lys102Glu in exon 3 and the c.1175C>T, p.Pro392Leu variant in exon 8 pointed by an arrow and the corresponding normal sequences (control).

(b) Sequence alignment of part of the *SQSTM1* amino acids from diverse species using MultAlin website. The position of the Met87, Lys102 and Pro392 (pointed by an arrow) are in red. Sequences used include *Homo sapiens* (NP_003891.1), *Pan troglodytes* (XP_001153075.1), *Bos taurus* (NP_788814.1), *Rattus norvegicus* (NP_787037.2), *Mus musculus* (NP_035148.1), *Gallus gallus* (XP_001233249.2) and *Xenopus laevis* (NP_001079920.1). (c) Representation of p62 protein domains with the position of the aminoacid substitutions previously reported in Paget's disease (green), ALS (black) and FTLD (orange). Mutations reported in the present study are indicated in red. Data are compiled from [13, 14, 19, 23, 31, 44]. SH2, src homology 2 domain;

AID, acidic interaction domain; ZZ, zinc finger region; TRAF6, tumor necrosis factor receptor-associated factor 6 binding domain; PEST, Proline, Glutamic acid, Serine, Threonine rich region; UBA, ubiquitin-associated domain. (d) Pedigree of the patient carrying the c.1175C>T, p.Pro392Leu mutation (case 1). DNA was available for the index cases only (indicated by an arrow). The age at onset, site of onset (in brackets) and age at death (in brackets) are indicated below the patients when the information was available.

Figure 2. Molecular analysis of the effect of the c.1165+1G>A mutation on *SQSTM1* splicing and p62 protein expression.

Part of chromatograms showing the position of the c.1165+1G>A variant pointed by an arrow in intron 7 (a) leading to the r.1052_1165del in cDNA sequence (b) identified in one autopsied ALS case (Δ Ex7, case 2). Amplification of the cDNA product with primers localized in exon 5 and 8 of the *SQSTM1* gene resulting in a single fragment of 514 bp in the M87V carrier (case 3), the K102E carrier (case 4) and SALS individuals and in two fragments of 514 and 410 bp in the c.1165+1G>A carrier (Δ Ex7, case 2) (c). Translation of this mRNA product results in a protein that lacks 38 aminoacids encoded by exon 7 (p.Gly351_Pro388del) that could be visualized on immunoblots (lane Δ EX7, case 2, d). For this patient, expression levels of the full length p62 protein are decreased by half. Protein extracts from transversal sections of spinal cord were prepared from patients carrying the c.1165+1G>A (Δ Ex7, case 2), the c.259A>G, p.Met87Val (M87V, case 3) and the c.304A>G, p.Lys102Glu (K102E, case 4) variants and three SALS with no genetic defect in *SQSTM1*. Protein levels of p62 and TDP-43 were compared in these patients. Samples were standardized using tubulin as internal control. Densitometric analyses of p62 (black) and TDP-43 (grey) protein levels. Data are means $\pm$ standard error of the means (s.e.m.) of 5 values (from 5 independent western blot analyses) for each patient. Values for control SALS patients were pooled in a control group. For case 2 with c.1165+1G>A variant (Δ Ex7), p62 level is the sum of the two bands.

Figure 3. Ubiquitinated inclusions in the spinal cord of patients with mutation in *SQSTM1*.

Spinal cord sections were stained with anti-ubiquitin (a, d, g), anti-p62 (b, e, h) and anti-TDP-43 (c, f, i) antibodies. Skein like filamentous inclusions (d-f) and large round cytoplasmic inclusions (g-i) were detected with all three antibodies. Intracytoplasmic diffuse small granular aggregates were observed with ubiquitin (a) and TDP-43 (c) immunochemistry. This pattern was not found with p62 immunohistochemistry, in which bigger “seed-like”, p62 positive aggregates were observed (b). Scale Bar: 10 μ m.

Figure 4. Cortical pathology in patients with *SQSTM1* mutations.

In the midfrontal cortex of patients with p.Gly351_Pro388del (Δ Ex7, case 2) and with K102E (case 4) *SQSTM1* variants, immunohistochemistry showed TDP-43 (a-b) and p62 (c-d) positive intracytoplasmic inclusions. TDP-43 positive inclusions, either round or filamentous (black arrows), spread throughout the cortex with numerous thin neurites in case 2 (a, arrowheads) and only a few positive short neurites in case 4 (b). TDP-43 cortical pathology in case 2 and 4 resembles Type A and B pathology, respectively, according to Mackenzie classification [29]. In both patients, p62 immunohistochemistry showed a lower number of inclusions, generally round with no neurites (c-d). Scale Bar: 10 μ m.

Supplementary Figure 1. Colocalisation of p62 and TDP-43 in the inclusions.

Double immunofluorescence analyses using p62 (in green) and TDP-43 (in red) antibodies were performed after deparaffinization using a procedure that was previously described [22]. Antibodies were diluted at 1:100 (p62) and 1:250 (TDP-43). Round (a) and seed-like (b) inclusions were positive for p62 and TDP-43 (arrows). Granular inclusions were positive for TDP-43 only (c, arrow heads). Presence of some lipofuscin is visible in the cell body of the motoneuron in (c). Scale bars: 10 μ m.

Table 1. Clinical features of studied populations

	M:F ratio	Age at onset (years)*	Site of disease onset	Disease duration (months)*
FALS n=90	1.9:1 (n=90)	60 +/- 1.3 (n=86)	43% upper limbs 34% lower limbs 23% bulbar (n=79)	46 +/- 5 (n=64)**
SALS n=74	1.5:1 (n=74)	61 +/- 1 (n=72)	27% upper limbs 42% lower limbs 31% bulbar (n=71)	49 +/- 5 (n=72)

*data are mean +/- standard error.

**disease duration data included 13 censored data for FALS.

n=number of patients for whom the information was available.

Table 2. Immunohistochemical methods

Antigen	Species	Producer	Immunogen	Clone	Pretreatment	Dilution	Incubation time
CST3 (cystatin-C)	Polyclonal (rabbit)	Sigma®	Cystatin-C precursor recombinant protein epitope signature tag (PrEST)		CC2® 36 min at 95°C	1:50	32 min +4°C
p62 lck ligand	Monoclonal (mouse)	BD Biosciences®	Human p62 lck ligand aa. 257-437	257-437	Citrate Buffer pH=6 microwave	1:1000	Overnight +4°C
TARDBP (TDP-43)	Polyclonal (rabbit)	Protein Tech Group®	Human recombinant protein		CC1® 60 min at 95°C	1:1000	80 min +20°C
Ubiquitin	Polyclonal (rabbit)	Dako®	Ubiquitin isolated from cow erythrocytes		CC1® at 95°C 30 min + FA 10 min	1:500	32 min +4°C

Table 3. Clinical characteristics of patients with *SQSTM1* variants

Patients	Type	Mutations	Gender	Age onset (y)	Site of disease onset	Disease (months)	duration	FTD	Paget's disease of bone
Case 1	FALS	P392L	M	54	lower limbs	133		no	yes
Case 2	SALS	ΔEX7	F	75	lower limbs	42		no	yes
Case 3	SALS	M87V	M	68	bulbar	18		NA	no
Case 4	SALS	K102E	F	81	bulbar	10		NA	no

NA: information was not available.

Supplementary Table 1. Semi-quantitative assessment for the presence of filamentous, round and granular inclusions stained by TDP-43 and p62.

Patients	Type	Genetic Groups	IHC	Filamentous	Round	Granular
Case 2	SALS	<i>SQSTM1</i> (ΔEX7)	p62 TDP-43	1 2	1 1	0 1
Case 3	SALS	<i>SQSTM1</i> (M87V)	p62 TDP-43	1 2	1 2	0 3
Case 4	SALS	<i>SQSTM1</i> (K102E)	p62 TDP-43	1 3	2 2	0 3
Case 5	FALS	<i>C9ORF72</i>	p62 TDP-43	0 0	0 1	0 0
Case 6	SALS	<i>C9ORF72</i>	p62 TDP-43	2 1	2 1	0 1
Case 7	FALS	<i>C9ORF72</i>	p62 TDP-43	0 3	0 1	1 2
Case 8	SALS	<i>C9ORF72</i>	p62 TDP-43	1 2	1 1	0 0
Case 9	FALS	<i>C9ORF72</i>	p62 TDP-43	3 3	1 1	2 2
Case 10	SALS	<i>C9ORF72</i>	p62 TDP-43	2 1	0 1	1 1
Case 11	FALS	<i>C9ORF72</i>	p62 TDP-43	2 2	0 0	0 1
Case 12	FALS	<i>C9ORF72</i>	p62 TDP-43	2 3	1 0	1 1
Case 13	FALS	<i>SOD1</i>	p62 TDP-43	0 0	0 0	0 0
Case 14	FALS	<i>SOD1</i>	p62 TDP-43	0 0	1 0	3 0
Case 15	SALS	<i>SOD1</i>	p62 TDP-43	0 0	1 1	1 1
Case 16	SALS	<i>SOD1</i>	p62 TDP-43	0 0	0 0	0 0
Case 17	FALS	<i>TARDBP</i>	p62 TDP-43	1 3	0 1	0 1
Case 18	SALS	<i>TARDBP</i>	p62 TDP-43	0 1	0 0	0 0
Case 19	SALS	-	p62 TDP-43	0 1	0 2	1 1
Case 20	SALS	-	p62 TDP-43	0 1	0 2	0 0
Case 21	SALS	-	p62 TDP-43	1 2	1 2	0 0
Case 22	SALS	-	p62 TDP-43	1 0	1 2	0 0
Case 23	SALS	-	p62 TDP-43	1 2	0 1	0 0

Case 24	SALS	-	p62 TDP-43	0 0	0 0	0 1
Case 25	SALS	-	p62 TDP-43	0 3	1 2	0 2
Case 26	SALS	-	p62 TDP-43	0 1	0 0	0 0
Case 27	SALS	-	p62 TDP-43	0 1	0 0	1 1
Case 28	SALS	-	p62 TDP-43	2 3	0 0	1 1
Case 29	SALS	-	p62 TDP-43	0 0	1 1	0 2
Case 30	SALS	-	p62 TDP-43	2 2	1 0	0 1
Case 31	SALS	-	p62 TDP-43	1 1	0 0	2 0
Case 32	SALS	-	p62 TDP-43	0 0	1 1	0 2
Case 33	SALS	-	p62 TDP-43	1 1	0 1	1 1
Case 34	SALS	-	p62 TDP-43	0 0	0 1	0 2
Case 35	SALS	-	p62 TDP-43	0 0	0 2	0 1
Case 36	SALS	-	p62 TDP-43	0 2	0 0	0 0
Case 37	SALS	-	p62 TDP-43	1 1	1 1	0 0
Case 38	SALS	-	p62 TDP-43	1 2	1 1	0 3
Case 39	SALS	-	p62 TDP-43	0 1	0 1	0 3
Case 40	SALS	-	p62 TDP-43	0 0	0 1	0 0
Case 41	SALS	-	p62 TDP-43	0 0	0 0	0 0
Case 42	SALS	-	p62 TDP-43	1 3	0 0	1 2
Case 43	SALS	-	p62 TDP-43	1 3	0 1	1 3
Case 44	SALS	-	p62 TDP-43	2 2	1 2	1 3
Case 45	SALS	-	p62 TDP-43	0 2	0 1	1 1
Case 46	SALS	-	p62 TDP-43	1 2	1 2	0 2

0, none; 1, rare; 2, moderate; 3, severe
-, no mutation in the screened genes.

References

1. Al-Sarraj S, King A, Troakes C, Smith B, Maekawa S, Bodi I, Rogelj B, Al-Chalabi A, Hortobagyi T, Shaw CE (2011) p62 positive, TDP-43 negative, neuronal cytoplasmic and intranuclear inclusions in the cerebellum and hippocampus define the pathology of C9orf72-linked FTL and MND/ALS. *Acta Neuropathol* 122:691-702
2. Albagha OM, Visconti MR, Alonso N, Langston AL, Cundy T, Dargie R, Dunlop MG, Fraser WD, Hooper MJ, Isaia G, Nicholson GC, del Pino Montes J, Gonzalez-Sarmiento R, di Stefano M, Tenesa A, Walsh JP, Ralston SH (2010) Genome-wide association study identifies variants at CSF1, OPTN and TNFRSF11A as genetic risk factors for Paget's disease of bone. *Nat Genet* 42:520-524
3. Brady OA, Meng P, Zheng Y, Mao Y, Hu F (2011) Regulation of TDP-43 aggregation by phosphorylation and p62/SQSTM1. *J Neurochem* 116:248-259
4. Brettschneider J, Libon DJ, Toledo JB, Xie SX, McCluskey L, Elman L, Geser F, Lee VM, Grossman M, Trojanowski JQ (2012) Microglial activation and TDP-43 pathology correlate with executive dysfunction in amyotrophic lateral sclerosis. *Acta Neuropathol* 123:395-407

5. Brettschneider J, Van Deerlin VM, Robinson JL, Kwong L, Lee EB, Ali YO, Safren N, Monteiro MJ, Toledo JB, Elman L, McCluskey L, Irwin DJ, Grossman M, Molina-Porcel L, Lee VM, Trojanowski JQ (2012) Pattern of ubiquitin pathology in ALS and FTLT indicates presence of C9ORF72 hexanucleotide expansion. *Acta Neuropathol* 123:825-839
6. Brooks BR, Miller RG, Swash M, Munsat TL (2000) El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord* 1:293-299
7. Cavey JR, Ralston SH, Sheppard PW, Ciani B, Gallagher TR, Long JE, Searle MS, Layfield R (2006) Loss of ubiquitin binding is a unifying mechanism by which mutations of SQSTM1 cause Paget's disease of bone. *Calcif Tissue Int* 78:271-277
8. Collet C, Michou L, Audran M, Chasseigneaux S, Hilliquin P, Bardin T, Lemaire I, Cornelis F, Launay JM, Orcel P, Laplanche JL (2007) Paget's disease of bone in the French population: novel SQSTM1 mutations, functional analysis, and genotype-phenotype correlations. *J Bone Miner Res* 22:310-317
9. Cooper C, Schafheutle K, Dennison E, Kellingray S, Guyer P, Barker D (1999) The epidemiology of Paget's disease in Britain: is the prevalence decreasing? *J Bone Miner Res* 14:192-197
10. Dai RM, Li CC (2001) Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. *Nat Cell Biol* 3:740-744
11. Daroszewska A, van 't Hof RJ, Rojas JA, Layfield R, Landao-Basonga E, Rose L, Rose K, Ralston SH (2011) A point mutation in the ubiquitin-associated domain of SQSTM1 is sufficient to cause a Paget's disease-like disorder in mice. *Hum Mol Genet* 20:2734-2744
12. DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, Nicholson AM, Finch NA, Flynn H, Adamson J, Kouri N, Wojtas A, Sengdy P, Hsiung GY, Karydas A, Seeley WW, Josephs KA, Coppola G, Geschwind DH, Wszolek ZK, Feldman H, Knopman DS, Petersen RC, Miller BL, Dickson DW, Boylan KB, Graff-Radford NR, Rademakers R (2011) Expanded GGGGCC Hexanucleotide Repeat in Noncoding Region of C9ORF72 Causes Chromosome 9p-Linked FTD and ALS. *Neuron* 72:245-256
13. Falchetti A, Di Stefano M, Marini F, Ortolani S, Olivieri MF, Bergui S, Masi L, Cepollaro C, Benucci M, Di Munno O, Rossini M, Adami S, Del Puente A, Isaia G, Torricelli F, Brandi ML (2009) Genetic epidemiology of Paget's disease of bone in Italy: sequestosome1/p62 gene mutational test and haplotype analysis at 5q35 in a large representative series of sporadic and familial Italian cases of Paget's disease of bone. *Calcif Tissue Int* 84:20-37
14. Fecto F, Yan J, Vemula SP, Liu E, Yang Y, Chen W, Zheng JG, Shi Y, Siddique N, Arrat H, Donkervoort S, Ajroud-Driss S, Sufit RL, Heller SL, Deng HX, Siddique T (2011) SQSTM1 mutations in familial and sporadic amyotrophic lateral sclerosis. *Arch Neurol* 68:1440-1446
15. Geetha T, Vishwaprakash N, Sycheva M, Babu JR (2012) Sequestosome 1/p62: across diseases. *Biomarkers* 17:99-103
16. Geser F, Robinson JL, Malunda JA, Xie SX, Clark CM, Kwong LK, Moberg PJ, Moore EM, Van Deerlin VM, Lee VM, Arnold SE, Trojanowski JQ (2010) Pathological 43-kDa transactivation response DNA-binding protein in older adults with and without severe mental illness. *Arch Neurol* 67:1238-1250
17. Geser F, Stein B, Partain M, Elman LB, McCluskey LF, Xie SX, Van Deerlin VM, Kwong LK, Lee VM, Trojanowski JQ (2011) Motor neuron disease clinically limited to the lower motor neuron is a diffuse TDP-43 proteinopathy. *Acta Neuropathol* 121:509-517
18. Gleason CE, Ordureau A, Gurlay R, Arthur JS, Cohen P (2011) Polyubiquitin binding to optineurin is required for optimal activation of TANK-binding kinase 1 and production of interferon beta. *J Biol Chem* 286:35663-35674
19. Goode A, Layfield R (2010) Recent advances in understanding the molecular basis of Paget disease of bone. *J Clin Pathol* 63:199-203
20. Hamdy RC (1995) Clinical features and pharmacologic treatment of Paget's disease. *Endocrinol Metab Clin North Am* 24:421-436
21. Hart MP, Brettschneider J, Lee VM, Trojanowski JQ, Gitler AD (2012) Distinct TDP-43 pathology in ALS patients with ataxin 2 intermediate-length polyQ expansions. *Acta Neuropathol* 124:221-230
22. Hernandez Lain A, Millecamps S, Dubourg O, Salachas F, Bruneteau G, Lacomblez L, LeGuern E, Seilhean D, Duyckaerts C, Meininger V, Mallet J, Pradat PF (2011) Abnormal TDP-43 and FUS proteins in muscles of sporadic IBM: similarities in a TARDBP-linked ALS patient. *J Neurol Neurosurg Psychiatry* 82:1414-1416
23. Hirano M, Nakamura Y, Saigoh K, Sakamoto H, Ueno S, Isono C, Miyamoto K, Akamatsu M, Mitsui Y, Kusunoki S (2013) Mutations in the gene encoding p62 in Japanese patients with amyotrophic lateral sclerosis. *Neurology* doi: 10.1212/WNL.0b013e31827f0fe5

24. Hocking LJ, Lucas GJ, Daroszewska A, Mangion J, Olavesen M, Cundy T, Nicholson GC, Ward L, Bennett ST, Wuyts W, Van Hul W, Ralston SH (2002) Domain-specific mutations in sequestosome 1 (SQSTM1) cause familial and sporadic Paget's disease. *Hum Mol Genet* 11:2735-2739
25. Johnson JO, Mandrioli J, Benatar M, Abramzon Y, Van Deerlin VM, Trojanowski JQ, Gibbs JR, Brunetti M, Gronka S, Wu J, Ding J, McCluskey L, Martinez-Lage M, Falcone D, Hernandez DG, Arepalli S, Chong S, Schymick JC, Rothstein J, Landi F, Wang YD, Calvo A, Mora G, Sabatelli M, Monsurro MR, Battistini S, Salvi F, Spataro R, Sola P, Borghero G, Galassi G, Scholz SW, Taylor JP, Restagno G, Chio A, Traynor BJ (2010) Exome sequencing reveals VCP mutations as a cause of familial ALS. *Neuron* 68:857-864
26. Kuusisto E, Salminen A, Alafuzoff I (2001) Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies. *Neuroreport* 12:2085-2090
27. Laurin N, Brown JP, Morissette J, Raymond V (2002) Recurrent mutation of the gene encoding sequestosome 1 (SQSTM1/p62) in Paget disease of bone. *Am J Hum Genet* 70:1582-1588
28. Mackenzie IR, Bigio EH, Ince PG, Geser F, Neumann M, Cairns NJ, Kwong LK, Forman MS, Ravits J, Stewart H, Eisen A, McCluskey L, Kretzschmar HA, Monoranu CM, Highley JR, Kirby J, Siddique T, Shaw PJ, Lee VM, Trojanowski JQ (2007) Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. *Ann Neurol* 61:427-434
29. Mackenzie IR, Neumann M, Baborie A, Sampathu DM, Du Plessis D, Jaros E, Perry RH, Trojanowski JQ, Mann DM, Lee VM (2011) A harmonized classification system for FTLTDP pathology. *Acta Neuropathol* 122:111-113
30. Maruyama H, Morino H, Ito H, Izumi Y, Kato H, Watanabe Y, Kinoshita Y, Kamada M, Nodera H, Suzuki H, Komure O, Matsuura S, Kobatake K, Morimoto N, Abe K, Suzuki N, Aoki M, Kawata A, Hirai T, Kato T, Ogasawara K, Hirano A, Takumi T, Kusaka H, Hagiwara K, Kaji R, Kawakami H (2010) Mutations of optineurin in amyotrophic lateral sclerosis. *Nature* 465:223-226
31. Michou L, Collet C, Laplanche JL, Orcel P, Cornelis F (2006) Genetics of Paget's disease of bone. *Joint Bone Spine* 73:243-248
32. Michou L, Collet C, Morissette J, Audran M, Thomas T, Gagnon E, Launay JM, Laplanche JL, Brown JP, Orcel P (2012) Epidemiogenetic study of French families with Paget's disease of bone. *Joint Bone Spine* 79:393-398
33. Millicamps S, Boillee S, Chabrol E, Camu W, Cazeneuve C, Salachas F, Pradat PF, Danel-Brunaud V, Vandenberghe N, Corcia P, Le Forestier N, Lacomblez L, Bruneteau G, Seilhean D, Brice A, Feingold J, Meininger V, LeGuern E (2011) Screening of OPTN in French familial amyotrophic lateral sclerosis. *Neurobiol Aging* 32:557 e511-553
34. Millicamps S, Boillee S, Le Ber I, Seilhean D, Teyssou E, Giraudeau M, Moigneu C, Vandenberghe N, Danel-Brunaud V, Corcia P, Pradat PF, Le Forestier N, Lacomblez L, Bruneteau G, Camu W, Brice A, Cazeneuve C, Leguern E, Meininger V, Salachas F (2012) Phenotype difference between ALS patients with expanded repeats in C9ORF72 and patients with mutations in other ALS-related genes. *J Med Genet* 49:258-263
35. Millicamps S, Corcia P, Cazeneuve C, Boillee S, Seilhean D, Danel-Brunaud V, Vandenberghe N, Pradat PF, Le Forestier N, Lacomblez L, Bruneteau G, Camu W, Brice A, Meininger V, LeGuern E, Salachas F (2012) Mutations in UBQLN2 are rare in French amyotrophic lateral sclerosis. *Neurobiol Aging* 33:839 e831-833
36. Millicamps S, Da Barroca S, Cazeneuve C, Salachas F, Pradat PF, Danel-Brunaud V, Vandenberghe N, Lacomblez L, Le Forestier N, Bruneteau G, Camu W, Brice A, Meininger V, LeGuern E (2010) Questioning on the role of D amino acid oxidase in familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 107:E107; author reply E108
37. Millicamps S, Salachas F, Cazeneuve C, Gordon P, Bricka B, Camuzat A, Guillot-Noel L, Russaouen O, Bruneteau G, Pradat PF, Le Forestier N, Vandenberghe N, Danel-Brunaud V, Guy N, Thauvin-Robinet C, Lacomblez L, Couratier P, Hannequin D, Seilhean D, Le Ber I, Corcia P, Camu W, Brice A, Rouleau G, Leguern E, Meininger V (2010) SOD1, ANG, VAPB, TARDBP, and FUS mutations in familial amyotrophic lateral sclerosis: genotype-phenotype correlations. *J Med Genet* 47:554-560
38. Mizuno Y, Amari M, Takatama M, Aizawa H, Mihara B, Okamoto K (2006) Immunoreactivities of p62, an ubiquitin-binding protein, in the spinal anterior horn cells of patients with amyotrophic lateral sclerosis. *J Neurol Sci* 249:13-18
39. Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, Bruce J, Schuck T, Grossman M, Clark CM, McCluskey LF, Miller BL, Masliah E, Mackenzie IR, Feldman H, Feiden W, Kretzschmar HA, Trojanowski JQ, Lee VM (2006) Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 314:130-133

40. Okamoto K, Hirai S, Amari M, Watanabe M, Sakurai A (1993) Bunina bodies in amyotrophic lateral sclerosis immunostained with rabbit anti-cystatin C serum. *Neurosci Lett* 162:125-128
41. Pikkarainen M, Hartikainen P, Alafuzoff I (2008) Neuropathologic features of frontotemporal lobar degeneration with ubiquitin-positive inclusions visualized with ubiquitin-binding protein p62 immunohistochemistry. *J Neuropathol Exp Neurol* 67:280-298
42. Ramesh Babu J, Lamar Seibenhener M, Peng J, Strom AL, Kemppainen R, Cox N, Zhu H, Wooten MC, Diaz-Meco MT, Moscat J, Wooten MW (2008) Genetic inactivation of p62 leads to accumulation of hyperphosphorylated tau and neurodegeneration. *J Neurochem* 106:107-120
43. Renton AE, Majounie E, Waite A, Simon-Sanchez J, Rollinson S, Gibbs JR, Schymick JC, Laaksovirta H, van Swieten JC, Myllykangas L, Kalimo H, Paetau A, Abramzon Y, Remes AM, Kaganovich A, Scholz SW, Duckworth J, Ding J, Harmer DW, Hernandez DG, Johnson JO, Mok K, Ryten M, Trabzuni D, Guerreiro RJ, Orrell RW, Neal J, Murray A, Pearson J, Jansen IE, Sondervan D, Seelaar H, Blake D, Young K, Halliwell N, Callister JB, Toulson G, Richardson A, Gerhard A, Snowden J, Mann D, Neary D, Nalls MA, Peuralinna T, Jansson L, Isovita VM, Kaivorinne AL, Holtta-Vuori M, Ikonen E, Sulkava R, Benatar M, Wu J, Chio A, Restagno G, Borghero G, Sabatelli M, Heckerman D, Rogaeva E, Zinman L, Rothstein JD, Sendtner M, Drepper C, Eichler EE, Alkan C, Abdullaev Z, Pack SD, Dutra A, Pak E, Hardy J, Singleton A, Williams NM, Heutink P, Pickering-Brown S, Morris HR, Tienari PJ, Traynor BJ (2011) A Hexanucleotide Repeat Expansion in C9ORF72 Is the Cause of Chromosome 9p21-Linked ALS-FTD. *Neuron* 72:257-268
44. Rubino E, Rainero I, Chio A, Rogaeva E, Galimberti D, Fenoglio P, Grinberg Y, Isaia G, Calvo A, Gentile S, Bruni AC, St George-Hyslop PH, Scarpini E, Gallone S, Pinessi L (2012) SQSTM1 mutations in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Neurology* 79:1556-1562
45. Tanji K, Zhang HX, Mori F, Kakita A, Takahashi H, Wakabayashi K (2012) p62/sequestosome 1 binds to TDP-43 in brains with frontotemporal lobar degeneration with TDP-43 inclusions. *J Neurosci Res* 90:2034-2042
46. Vadlamudi RK, Joong I, Strominger JL, Shin J (1996) p62, a phosphotyrosine-independent ligand of the SH2 domain of p56lck, belongs to a new class of ubiquitin-binding proteins. *J Biol Chem* 271:20235-20237
47. Watts GD, Wymer J, Kovach MJ, Mehta SG, Mumm S, Darvish D, Pestronk A, Whyte MP, Kimonis VE (2004) Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. *Nat Genet* 36:377-381

Fig. 1

Fig. 2

Fig 3.

Fig. 4