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Novel *UBQLN2* mutations linked to amyotrophic lateral sclerosis and atypical hereditary spastic paraplegia phenotype through defective HSP70-mediated proteolysis

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ABSTRACT

Mutations in *UBQLN2* have been associated with rare cases of X-linked juvenile and adult forms of amyotrophic lateral sclerosis (ALS) and ALS linked to frontotemporal dementia (FTD). Here, we report 1 known (c.1489C>T, p.Pro497Ser, P497S) and 3 novel (c.1481C>T, p.Pro494Leu, P494L; c.1498C>T, p.Pro500Ser, P500S; and c.1516C>G, p.Pro506Ala, P506A) missense mutations in the PXX domain of *UBQLN2* in familial motor neuron diseases including ALS and spastic paraplegia (SP). A novel missense mutation (c.1462G>A, p.Ala488Thr, A488T) adjacent to this hotspot UBQLN2 domain was identified in a sporadic case of ALS. These mutations are conserved in mammals, are absent from ExAC and gnomAD browsers, and are predicted to be deleterious by SIFT in silico analysis. Patient lymphoblasts carrying a *UBQLN2* mutation showed absence of ubiquilin-2 accumulation, disrupted binding with HSP70, and impaired autophagic pathway. Our results confirm the role of PXX repeat in ALS pathogenesis, show that *UBQLN2*-linked disease can manifest like a SP phenotype, evidence a highly reduced disease penetrance in females carrying *UBQLN2* mutations, which is important information for genetic counseling, and underline the pivotal role of ubiquilin-2 in proteolysis regulation pathways.

1. Introduction

Mutations in *UBQLN2*, an intronless gene located on the X chromosome which encodes ubiquilin-2, have been identified in families with dominant X-linked juvenile and adult-onset amyotrophic lateral sclerosis (ALS) and/or frontotemporal dementia (FTD) (Deng et al., 2011). Ubiquilin-2 is a component of the ubiquitin inclusions detected in degenerating neurons in ALS patients carrying or not a mutation in the *UBQLN2* gene, which suggests a role for this protein in a final common pathway mediating motor neuron degeneration (Deng et al., 2011). Ubiquilin-2 (also known as PLIC-2 or CHAP1) contains 4 main domains. The N-terminal sequence called the ubiquitin-like domain (UBL) is very similar to ubiquitin and binds to ubiquitin-interacting motifs (UIMs)

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expressed by proteasomes and endocytic receptors degraded by lysosomes (Hofmann and Falquet, 2001; Walters et al., 2002). The central domain contains 4 cochaperone-like regions with homology to STI1 (which binds to Stch, a protein similar to HSP70). There are also the proline/glycine repeats (12 PXX tandem repeats) and a conserved C-terminal domain called the ubiquitin-associated domain (UBA) (Kaye et al., 2000). These protein domains are shared by the 3 other members of ubiquilin family (ubiquilin-1, -3, and -4) except the proline/glycine repeat domain which is unique to ubiquilin-2.

UBQLN2 regulates several protein degradation pathways including the ubiquitin-proteasome system (UPS), the endoplasmic reticulum–associated protein degradation pathway, and macroautophagy. Indeed, UBQLN2 can bind to the polyubiquitinated proteins through its UBA domain and can deliver them to the proteasome through interaction with its UBL domain (Kleijnen et al., 2003; Ko et al., 2004; Walters et al., 2002). UBQLN2 is also thought to address endoplasmic reticulum–associated protein degradation substrates to the proteasome since it was shown to interact with UBXD8 and HERPUD1, both involved in removal of improperly folded newly synthesized proteins (Kim et al., 2008; Xia et al., 2014). UBQLN2 was also recently shown to bind heat shock proteins including HSP70 (encoded by *HSPA1A*) and to deliver ubiquitinated client-bound HSP70 proteins to the proteasome (Hjerpe et al., 2016). Therefore, defects in these processes could contribute to the accumulation of aggregated and/or misfolded proteins in ALS disease. In addition, ubiquilin proteins were reported to regulate macroautophagy in which cytosolic cargo is packaged in a double-membrane structure (autophagosome) that fuses with lysosomes harboring the acid hydrolases involved in protein degradation (N'Diaye et al., 2009). Co-immunoprecipitation studies showed that ubiquilin-1 and -2 were components of a complex with polyubiquitinated proteins and microtubule-associated protein 1 light chain 3 (LC3) involved in the formation of autophagosomes (Rothenberg et al., 2010).

Intriguingly, 7 different *UBQLN2* mutations identified in ALS patients involved a proline residue of the unique 12 PXX tandem repeat domain of the protein (Deng et al., 2011; Fahed et al., 2014; Gellera et al., 2013; Ozoguz et al., 2015; Vengoechea et al., 2013) and other mutations were identified on adjacent residues (Gellera et al., 2013; Williams et al., 2012) underlying the relevance of this domain for ALS pathogenesis. The whole exome sequencing analyses conducted on familial ALS (FALS) index cases and controls (1022 FALS and 7315 controls) revealed that *UBQLN2* has a study-wide significant enrichment of rare variants in FALS cases compared with controls, ranking this gene third after *SOD1* and *TARDBP*, when excluding FALS cases harboring a repeat expansion in the *C9orf72* gene (Kenna et al., 2016). These data underlined that *UBQLN2* contributes to a significant proportion of FALS.

We previously reported the genetic analyses of a population of 130 French FALS patients and 240 sporadic ALS (SALS) without finding any causative *UBQLN2* mutation (Millecamps et al., 2012a). In the present study, we analyzed 850 additional ALS patients (285 FALS and 565 SALS) and identified 5 *UBQLN2* mutations, including 4 novel ones, for which we explored the possible consequences on protein degrading pathways.

2. Material and methods

2.1. Genetic analyses

All participants signed a consent form for the genetic research and protocols were approved by the Medical Research Ethics Committee of "Assistance Publique-Hôpitaux de Paris". The diagnosis of ALS, spastic paraplegia (SP), and FTD were based on the published criteria (Brooks et al., 2000; Gasser et al., 2010; Rascovsky et al., 2011).

We systematically sequenced by Sanger analysis a region of 390 bp spanning the PXX repeat domain of *UBQLN2* in 415 FALS and 805 SALS to find possible novel *UBQLN2* mutations causing ALS and/or concurrent *UBQLN2* variants in patients carrying a mutation in the other ALS genes using a procedure described previously (Millecamps et al., 2012a). The entire *UBQLN2* gene sequence was further analyzed in patients with an identified *UBQLN2* mutation in this region to ensure that positive patients carry only 1 mutation in this gene. All these ALS patients were also systematically screened for *C9orf72* expansion and *SOD1*, *TARDBP*, and *FUS* mutations (Millecamps et al., 2012b).

For patients presenting SP, we used a panel covering 210,363 bases corresponding to 1001 regions of 70 genes (Table S1) known to be responsible for dominant and recessive hereditary SP forms (Tesson et al., 2015). The procedure consisted in a customized ROCHE/Nimblegen capture followed by massive parallel 75 bp sequencing on MiSeq apparatus (Illumina).

Before sequencing cDNA, total RNAs were extracted from lymphoblasts using the Trizol reagent (Life Technologies), subjected to RNase-free DNAse treatment (Qiagen), and purified using RNeasy columns (Qiagen). First-strand cDNA synthesis was performed using ThermoScript RT-PCR system (Life Technologies) according to the manufacturer's instructions.

2.2. Lymphoblast cultures

Lymphoblastoid cell lines from the ALS patients carrying the P494L, the P497S, or the P506A mutations were established by Epstein-Barr virus transformation of the peripheral blood mononuclear cells. Lymphoblasts from age-matched healthy men were used as controls. Lymphoblasts were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin (Life Technologies) renewed twice a week. Lymphoblasts (5.10^6 cells) were treated with 5 mM of NH_4Cl (Sigma-Aldrich) during 1 hour at 37 °C for lysosomal degradation inactivation and incubated at 42 °C during 2 hours for heat shock induction. For pellets, they were centrifuged 5 minutes at 3000 rpm, rinsed in phosphate-buffered saline solution, and frozen at −80 °C.

2.3. Antibodies

Primary antibodies against ubiquilin-2 (NBP2-25164, Novus Biologicals), HSP70 (MAB3516, Merck Millipore), p62 (MABC32, Merck Millipore), and nuclei clone 235-1 (MAB1281, Merck Millipore) were from mouse and those against LC3B (NB100-2220, Novus Biologicals) and GAPDH (D16H11, Cell Signaling Technology) were from rabbit. Peroxidase-conjugated secondary antibodies were goat antirabbit or antimouse with minimal cross-reaction to human serum proteins (Jackson ImmunoResearch Laboratories).

2.4. Western blot analysis

For immunoblots, cell pellets were homogenized in 50 mM Tris-HCl pH8, 150 mM NaCl, 1 mM $MgCl_2$, complete Mini EDTA-free protease inhibitor cocktail, and PhosSTOP phosphatase inhibitors and incubated at 37 °C for 30 minutes with 0.5 U/μL Benzonase Nuclease (all from Sigma-Aldrich). Sodium dodecyl sulfate (SDS) was added at a final concentration of 2% and cells were homogenized again. Protein extracts were centrifuged at 13,000 rpm for 10 minutes. The protein concentration of supernatants was estimated by the bicinchoninic acid assay (Sigma-Aldrich). Proteins (15 μg) were separated on NuPAGE 4%–12% Bis-Tris Gel