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Investigating the role of ALS genes *CHCHD10* and *TUBA4A* in Belgian FTD-ALS spectrum patients

Federica Perrone [a,b,1], Hung Phuoc Nguyen [a,b,1], Sara Van Mossevelde [a,b], Matthieu Moisse [c,d], Anne Sieben [a,b,e], Patrick Santens [e], Jan De Bleecker [e], Mathieu Vandenbulcke [c,f], Sebastiaan Engelborghs [b,g], Jonathan Baets [b,h,i], Patrick Cras [b,i], Rik Vandenberghe [c,j], Peter De Jonghe [b,h,i], Peter P. De Deyn [b,g], Jean-Jacques Martin [b], Philip Van Damme [c,d,j], Christine Van Broeckhoven [a,b,*], Julie van der Zee [a,b,**], on behalf of the Belgian Neurology consortium[2]

[a] *Neurodegenerative Brain Diseases Group, Center for Molecular Neurology, VIB, Antwerp, Belgium*
[b] *Laboratory of Neurogenetics, Institute Born-Bunge, University of Antwerp, Antwerp, Belgium*
[c] *Department of Neurosciences, Faculty of Medicine, KU Leuven, Leuven, Belgium*
[d] *Laboratory of Neurobiology, Vesalius Research Center, VIB, Leuven, Belgium*
[e] *Department of Neurology, University Hospital Ghent and University of Ghent, Ghent, Belgium*
[f] *Department of Old Age Psychiatry and Memory Clinic, University of Leuven, Leuven, Belgium*
[g] *Department of Neurology and Memory Clinic, Hospital Network Antwerp Middelheim and Hoge Beuken, Antwerp, Belgium*
[h] *Neurogenetics Group, Department of Molecular Genetics, VIB, Antwerp, Belgium*
[i] *Department of Neurology, Antwerp University Hospital, Antwerp, Belgium*
[j] *Department of Neurology, University Hospitals Leuven, Leuven, Belgium*

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ABSTRACT

Mutation screening and phenotypic profiling of 2 amyotrophic lateral sclerosis–(ALS) and frontotemporal dementia–(FTD) associated genes, *CHCHD10* and *TUBA4A*, were performed in a Belgian cohort of 459 FTD, 28 FTD-ALS, and 429 ALS patients. In *CHCHD10*, we identified a novel nonsense mutation (p.Gln108*) in a patient with atypical clinical FTD and pathology-confirmed Parkinson's disease (1/459, 0.22%) leading to loss of transcript. We further observed 3 previously described missense variants (p.Pro34Ser, p.Pro80Leu, and p.Pro96Thr) that were also present in the matched control series. In *TUBA4A*, we detected a novel frameshift mutation (p.Arg64Glyfs*90) leading to a truncated protein in 1 FTD patient (1/459 of 0.22%) with family history of Parkinson's disease and cognitive impairment, and a novel missense mutation (p.Thr381Met) in 2 sibs with familial ALS and memory problems (1 index patient/429, 0.23%) in whom we previously identified a pathogenic Chromosome 9 open reading frame 72 repeat expansion mutation. The present study confirms the role of *CHCHD10* and *TUBA4A* in the FTD-ALS spectrum, although genetic variations in these 2 genes are extremely rare in the Belgian population and often associated with symptomatology of related neurodegenerative diseases including Parkinson's disease and Alzheimer's disease.

* Corresponding author at: Neurodegenerative Brain Diseases Group, VIB Center for Molecular Neurology, University of Antwerp - CDE, Universiteitsplein 1, 2610 Antwerp, Belgium. Tel.: +32 3 265 1101; fax: +32 3 265 1113.
** Corresponding author at: Neurodegenerative Brain Diseases Group, VIB Center for Molecular Neurology, University of Antwerp - CDE, Universiteitsplein 1, 2610 Antwerp, Belgium. Tel.: +32 3 265 1030; fax: +32 3 265 1113.
E-mail addresses: christine.vanbroeckhoven@molgen.vib-ua.be (C. Van Broeckhoven), julie.vanderzee@molgen.vib-ua.be (J. van der Zee).

[1] Shared first authors have contributed equally.

[2] Belgian Neurology (BELNEU) consortium side author list: the following members of the BELNEU consortium have contributed to the clinical and pathological phenotyping and follow-up of the Belgian patient cohorts: Dirk Nuytten (Hospital Network Antwerp, Antwerp); Katrien Smets (Antwerp University Hospital, Edegem); Jan Versijpt and Alex Michotte (University Hospital Brussels, Brussels); Adrian Ivanoiu (Saint-Luc University Hospital, Brussels); Olivier Deryck and Bruno Bergmans (General Hospital Sint-Jan Brugge, Bruges); Jean Delbeck (General Hospital Sint-Maria, Halle); Marc Bruyland (General Hospital Glorieux Ronse); Christiana Willems (Jessa Hospital, Hasselt); and Eric Salmon (University of Liège and Memory Clinic, CHU Liège, Liège).

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1. Introduction

There is increasing evidence that frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) share a common molecular etiology and represent a disease continuum rather than 2 separate disease entities (Van Langenhove et al., 2012). Approximately, 15% of FTD patients develop motor neuron disease (MND) at some stage in the disease course and conversely up to 50% of MND patients show some signs of cognitive impairment, meeting diagnostic criteria of FTD in about 5% (Cruts et al., 2013). Moreover, common genetic factors underlying FTD and ALS, that is, the genes Chromosome 9 open reading frame 72 (*C9orf72*) (DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Renton et al., 2011), TANK-binding kinase 1 (*TBK1*) (Cirulli et al., 2015; Freischmidt et al., 2015; Gijselinck et al., 2015; Pottier et al., 2015), Valosin-containing protein (*VCP*) (Johnson et al., 2010; Watts et al., 2004), TAR DNA-binding protein (*TARDBP*) (Borroni et al., 2009; Gelpi et al., 2014; Kabashi et al., 2008; Sreedharan et al., 2008), and Fused in sarcoma RNA-binding protein (*FUS*) (Kwiatkowski et al., 2009; Van Langenhove et al., 2010), are key pathologic genes in both diseases.

Recently 2 new genes, the coiled-coil-helix-coiled-coil-helix domain containing 10 gene (*CHCHD10*) on chromosome 22q11.23, and tubulin alpha 4a (*TUBA4A*) gene on chromosome 2q35, have been associated with FTD and ALS (Bannwarth et al., 2014; Smith et al., 2014). Interestingly, the same *CHCHD10* missense mutation was found in a family with mixed phenotype of MND, cognitive decline resembling FTD, cerebellar ataxia, and myopathy, resulting from mitochondrial DNA breakage syndrome and an unrelated patient with pathology confirmed FTD-ALS with parkinsonism (Bannwarth et al., 2014). Furthermore, a double missense mutation was identified in a large multigenerational kindred with isolated mitochondrial myopathy (Ajroud-Driss et al., 2015), further underlining the heterogeneous clinical presentation associated with *CHCHD10* mutations. So far, 12 patient-specific missense and 1 nonsense mutation have been reported (Ajroud-Driss et al., 2015; Bannwarth et al., 2014; Chaussenot et al., 2014; Dols-Icardo et al., 2015; Jiao et al., 2016; Johnson et al., 2014; Kurzwelly et al., 2015; Muller et al., 2014; Ronchi et al., 2015; Zhang et al., 2015).

In *TUBA4A*, 10 missense, 1 nonsense, and 1 splice donor site mutation have been identified in both sporadic and familial ALS patients, with some also presenting with FTD (Pensato et al., 2015; Smith et al., 2014).

Despite the results of the previously listed studies, a large-scale exome-sequencing study of 3000 ALS patients and over 6000 controls did not observe association for either *CHCHD10* or *TUBA4A* (Cirulli et al., 2015). Here, we aimed to further investigate the genetic contribution of these genes and their associated clinical phenotypes in a Belgian cohort of FTD and ALS patients.

2. Materials and methods

2.1. Study population

Mutation screening of *CHCHD10* and *TUBA4A* was performed in a Belgian study population of FTD (n = 459), FTD-ALS patients (n = 28), and ALS (n = 429) patients. Demographic and clinical characteristics of the patients are shown in Table 1. Research participants were ascertained in Belgium through an ongoing multicenter collaboration of neurology departments and memory clinics partnering in the Belgian Neurology consortium and in frame of Project MinE. Index patients were evaluated using a standard protocol, which included a detailed clinical and family history, neurologic examination, neuropsychological testing, biochemical analyses, and neuroimaging. The diagnosis of FTD was made according to the international consensus criteria (Rascovsky et al., 2011). Diagnosis of FTD-ALS was made in patients that also met the criteria for clinical possible ALS according to the revised El Escorial criteria (Brooks et al., 2000). Likewise, diagnosis of ALS followed the revised El Escorial criteria (Brooks et al., 2000) and Awaji criteria (de Carvalho et al., 2011; Schrooten et al., 2011).

Table 1
Descriptives of the Belgian patient and control cohorts

Phenotype	n	Age, y[a]	STDEV, y	Male, n (%)	Familial, n (%)
FTD	459	62.4	10.1	246 (53.6)	129 (28.1)
FTD-ALS	28	62.9	10.6	16 (57.1)	8 (28.6)
ALS	429	60.8	12.1	264 (61.5)	59 (13.8)
Controls	703	68.7	10.1	286 (40.7)	n.a.

Key: ALS, amyotrophic lateral sclerosis; Familial, positive family history; FTD, frontotemporal dementia; n.a., not applicable; STDEV, standard deviation.
[a] Mean disease onset is given for patients and age at inclusion for control individuals.

The patients from the Belgian Neurology consortium were genetically profiled using a massive parallel sequencing gene panel based on the multiplex amplification of specific targets for resequencing technology (Multiplicom, Niel, Belgium; http://www.multiplicom.com) for the exonic resequencing of the major genes associated with FTD and ALS (*GRN, MAPT, VCP, TARDBP, FUS*, *SOD1*, and *TBK1*), as well as Mendelian genes for Alzheimer's disease (AD: *APP*, *PSEN1*, and *PSEN2*), prion disease (*PRNP*), and Parkinson's Disease (PD: *LRRK2*, *PARK2*, and *SNCA*). The *C9orf72* repeat was typed by repeat-primed polymerase chain reaction (PCR) and fragment-length analysis, as described (Gijselinck et al., 2012). Patients and controls of project MinE underwent whole-genome sequencing (Illumina's FastTrack services [San Diego, CA, USA] using PCR free library preparation and paired-end [100 bp] sequencing on the HiSeq platform [Illumina, San Diego], yielding 35X coverage at minimum). Read alignment and base calling was performed by Illumina using the Isaac pipeline (Raczy et al., 2013) followed by base quality control.

In addition, a Belgian control cohort of 703 individuals was included in the study (Table 1). The control individuals were primarily community dwelling volunteers or spouses of patients. Subjective memory complaints, neurologic or psychiatric antecedents, and a familial history of neurodegeneration were ruled out by means of an interview. Cognitive screening was performed using the mini-mental state examination (cutoff score $\geq$26) (Folstein et al., 1975) and the Montreal Cognitive Assessment test (cutoff score >25) (Nasreddine et al., 2005). The spouses of patients were examined at the Memory Clinic of ZNA Middelheim and Hoge Beuken in Antwerp, Belgium and the Memory Clinic at the University Hospitals of Leuven, Gasthuisberg in Leuven, Belgium.

Genomic DNA from all participants was extracted from lymphocytes using standard laboratory procedures.

All research participants or their legal guardian provided written informed consent for participation in genetic and clinical studies. Clinical study protocols and informed consent forms for patient ascertainment were approved by the local medical ethics committees of the collaborating medical centers in Belgium. Genetic study protocols and informed consent forms were approved by the ethics committees of the University Hospital of Antwerp and the University of Antwerp, Belgium.

2.2. CHCHD10 *and* TUBA4A *exonic resequencing*

The 4 coding exons and exon-intron boundaries of *CHCHD10* (NM_213720.2) were amplified by PCR using the KAPA HiFi HotStart

kit (kapabiosystems). Primers were designed using Primer3 (http://bioinfoutee/primer3-040/). PCR products were purified with ExoSAP-IT (Affymetrix) and subsequently sequenced in both directions using the BigDye Terminator Cycle Sequencing kit v31 (Applied Biosystems) and analyzed on an AB3730 DNA Analyzer (Life technologies, Foster City, CA, USA) in both directions. All sequences were analyzed by NovoSNP (Weckx et al., 2005) and visually inspected.

For *TUBA4A* (NM_006000.2), a multiamplicon panel for multiplex PCR amplification of the 4 exons was designed using the Multiplex Amplification of Specific Targets for Resequencing technology (Multiplicom, Niel, Belgium). Primers for multiplex PCR were designed using the multiplex PCR primer design tool (Multiplicom, Niel, Belgium) (Goossens et al., 2009). Specific target regions were amplified using multiplex PCR, followed by purification of the equimolar pooled amplicons using Agencourt AMPureXP beads (Beckman Coulter, CA, USA). Individual barcodes (Illumina Nextera XT) were incorporated in a universal PCR step before sample pooling. Bridge amplification and sequencing of barcoded samples was performed using an Illumina MiSeq platform, with the Illumina v3 reagent kit, generating 300 bp paired-end reads. Alignment and mapping of the reads against the whole genome (hg19) were performed with the Burrows-Wheeler Aligner (Li and Durbin, 2009). Variant calling and annotation were performed using GATK [version 2.2 (McKenna et al., 2010)] in combination with GenomeComb software (Reumers et al., 2012; http://genomecomb.sourceforge.net/). Raw reads of rare variants were manually checked using the integrative genomics viewer (IGV; Broad Institute, Cambridge, MA, USA). Identified rare variants with minor allele frequencies $\leq$1% and predicted to affect protein function or structure were validated by direct Sanger sequencing on genomic DNA, and CADD_Phred scores for prediction of deleterious effects were computed (Kircher et al., 2014; http://cadd.gs.washington.edu).

ALS patients (n = 272) and controls (n = 129) from Project MinE were profiled for *CHCHD10* and *TUBA4A* by whole-genome sequencing as previously described.

2.3. Nonsense-mediated messenger RNA (mRNA) decay experiments

Lymphoblast cell lines of the *CHCHD10* p.Glu108* and the *TUBA4A* p.Arg64Glyfs*90 were incubated with 150-μg/mL cycloheximide (Sigma, St Louis, MO, USA) at 37 °C for 4 hours to inhibit nonsense-mediated mRNA decay (NMD). As a negative control, cells were treated with an equal volume of dimethyl sulfoxide. Total RNA was extracted from cells using the RiboPurekit (Ambion, Life Technologies, Carlsbad, CA, USA) followed by a DNase treatment (TURBODNase Kit). First-strand complementary DNA was synthesized using the SuperScript III First-Strand Synthesis System (Life Technologies) using both onligo(dT) and random hexamer primers. For *CHCHD10,* exon 3 was amplified with flanking PCR primers in exon 2 and exon 4. For *TUBA4A,* the flanking sequence around the mutation in exon 2 was amplified with PCR primers in exon 2 and exon 3. PCR products were purified with ExoSAP-IT (Affymetrix) and subsequently sequenced in both directions using the BigDye Terminator Cycle Sequencing kit v31 (Applied Biosystems) and analyzed on an AB3730 DNA Analyzer (Life technologies) in both directions. Sequences were analyzed by NovoSNP (Weckx et al., 2005) and visually inspected.

3. Results

Here, we report on a systematic mutation screen of *CHCHD10* and *TUBA4A* in 459 FTD patients, 28 FTD-ALS, and 429 ALS patients.

3.1. Mutation screen in CHCHD10

In the *CHCHD10* gene, we identified a total of 4 nonsynonymous mutations, comprising 3 missense substitutions located in exon 2 and 1 nonsense mutation in exon 3 (Fig. 1A).

The nonsense mutation, c.322C>T (p.Gln108*) was a previously undescribed mutation, present in 1/459 patients of the FTD cohort (0.22%) and absent from 703 control individuals. The nonsense mutation was also absent from online databases of human polymorphisms including dbSNP (build 142, http://www.ncbi.nlm.nih.gov/SNP/), the 1000 Genome (1 KG) Project Consortium phase 2; (Abecasis et al., 2012), Exome Variant Server (http://evs.gs.washington.edu/EVS/), and the more than 60,000 cases of the Exome Aggregation Consortium (ExAC, http://exacbroadinstituteorg/). Previous mutation screens excluded mutations in the genes *C9orf72*, *GRN*, *MAPT*, *VCP*, *FUS*, *TARDBP*, *SOD1*, *TBK1*, *ATXN2*, *UBQLN2*, *SQSTM1*, and *TREM2* in this patient. The generated premature termination codon (PTC) locates to the CHCH domain (Fig. 1A). The biological relevance of this region is not yet fully understood, but it may affect the stability of the protein or its ability to interact with other proteins. However, because of the position of the mutation, 88 nucleotides before the exon 3-exon 4 junction, the mutant transcript is expected to be degraded by NMD, as we confirmed on complementary DNA (Fig. 2A).

Furthermore, a heterozygous missense variant, c.100C>T (p.Pro34Ser; rs551521196; CADD score: 14.20) was identified in 3/459 FTD patients (0.65%) and 5/429 ALS patients (1.17%). Interestingly, this variant had been reported before in FTD and ALS patients of multiple studies (Bannwarth et al., 2014; Chaussenot et al., 2014; Dols-Icardo et al., 2015; Johnson et al., 2014; Muller et al., 2014; Ronchi et al., 2015; Zhang et al., 2015). However, we also observed p.Pro34Ser in 9/703 control individuals (1.28%), in line with other reported frequencies of p.Pro34Ser in control individuals ranging from 0.63% to 1.12% by Dobston-Stone et al. (2015), Dols-Icardo et al. (2015) and Zhang et al., 2015, indicating that it is likely a benign polymorphism.

The 2 other missense variants identified in the Belgian cohort were the c.286C>A (p.Pro96Thr; rs111677724; CADD score: 10.15) variant present in 1/459 FTD (0.22%) and 1/429 ALS patients (0.23%) and the c.239C>T (p.Pro80Leu; CADD score: 12.08) variant also present in 1/459 FTD (0.22%) and 1/429 ALS patients (0.23%). Both were observed in 1 control individual each 1/703 (0.14%). The p.Pro96Thr variant had been reported before to be present in both patients and control individuals (Chio et al., 2015). Moreover, in the study by Dols-Icardo et al., this variant was also found in homozygous state in 2 ALS patients (Dols-Icardo et al., 2015). Two studies found the p.Pro80Leu variant in ALS patients only and absent from 147 and 497 control individuals, respectively (Ronchi et al., 2015; Zhang et al., 2015).

3.2. Mutation screen in TUBA4A

For *TUBA4A*, 1 frameshift mutation in exon 2 and 1 missense mutation in exon 4 were identified (Fig. 1B). The frameshift mutation c.187del (p.Arg64Glyfs*90) was identified in a patient with FTD (1/459 or 0.22%) and was absent from control individuals and public databases of human polymorphism EVS, dbSNP142, 1 KG, and ExAC. Also here, previous mutation screens excluded mutations in the genes *C9orf72*, *GRN*, *MAPT*, *VCP*, *FUS*, *TARDBP*, *SOD1*, *TBK1*, *ATXN2*, *UBQLN2*, *SQSTM1*, and *TREM2* in this patient. The indel occurs in exon 2, 40 nucleotides upstream of the exon-exon junction, and results in a frameshift encoding PTC after 90 amino acids in the final exon 4. In contrast to what we have observed in *CHCHD10*, the *TUBA4A* mutant mRNA is not degraded by NMD and leads to the translation of a truncated protein (Fig. 2B).

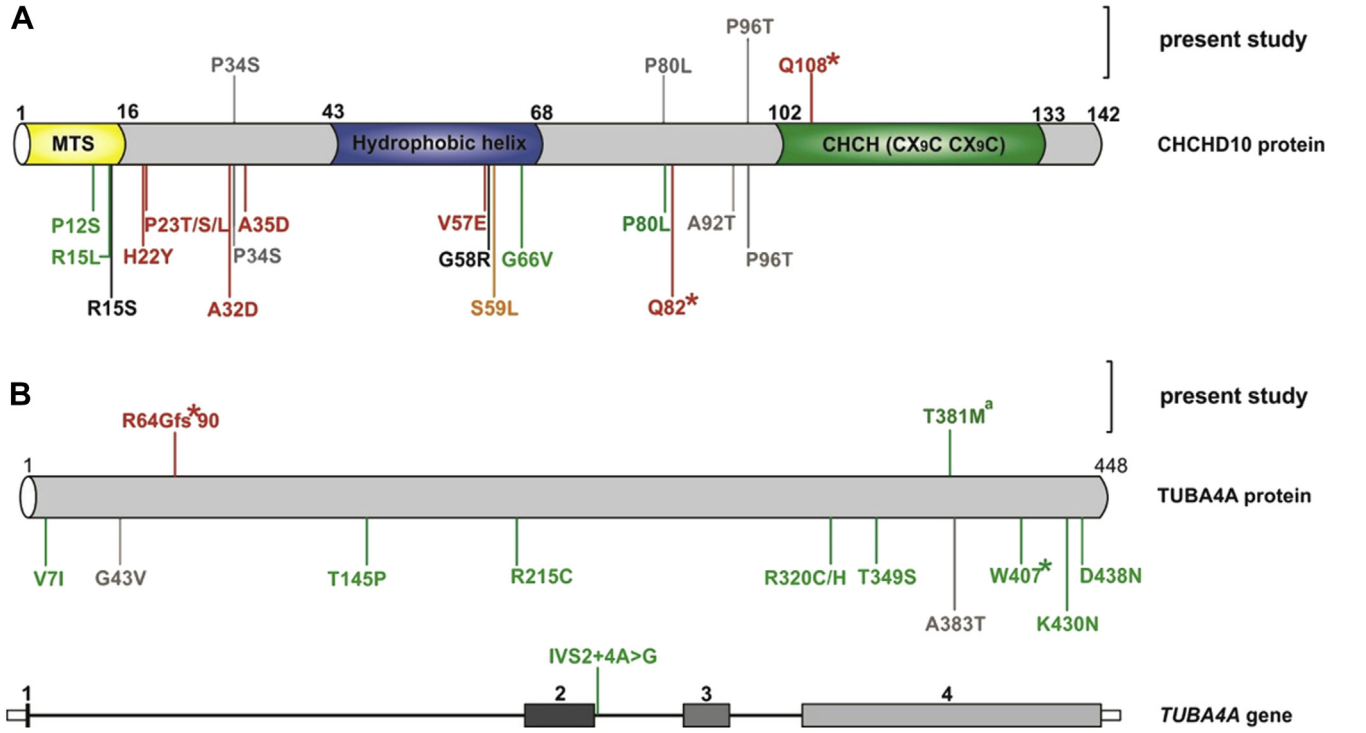

Fig. 1. Schematic representation of the CHCHD10 and TUBA4A protein structure with identified and reported mutations. (A) Predicted structural and functional domains of CHCHD10 with identified coding variations (NM_213720.2) in the present study (top) and reported variations (bottom). Mitochondrial targeting sequence (1–16), hydrophobic helix (43–68), coiled helix coiled helix (CHCH) domain (twin CX_9C; 102–133) (Cozzolino et al., 2015) are indicated. Coding variants in FTD patients are in red, in ALS patients in green, in FTD/ALS patients in orange, and in mitochondrial myopathy patients in black. Variants that were found in both patients and control individuals are in gray. Listed coding variants in *CHCHD10* are reported by the present study and by Ajroud-Driss et al. (2015), Bannwarth et al. (2014), Chaussenot et al. (2014), Dols-Icardo et al. (2015), Jiao et al. (2016), Johnson et al. (2014), Kurzwelly et al. (2015), Muller et al. (2014), Ronchi et al. (2015), Teyssou et al. (2016) and Zhang et al. (2015). (B) The TUBA4A protein with identified coding variations (NM_006000.2) in the present study (top) and reported variations (bottom, Pensato et al., 2015; Smith et al., 2014). The color scheme of the variants is similar to that of panel A. [a]This variant was found in an index patient with a *C9orf72* repeat expansion mutation.

Second, a novel heterozygous missense variant of unknown significance c.1142G>A (p.Thr381Met, CADD score 12.73) was detected in 1 patient with familial ALS (1/429 ALS patients or 0.238%) as well as in her affected brother. Also, this missense mutation was absent in control individuals but was reported as a rare variant in the ExAC data set with minor allele frequency of 0.002%. Mutation screen of *C9orf72*, *GRN*, *MAPT*, *VCP*, *FUS*, *TARDBP*, *SOD1*, *TBK1*, *ATXN2*, *UBQLN2*, *SQSTM1*, and *TREM2* previously revealed a *C9orf72* repeat expansion mutation in both sibs. The p.Thr381Met substitution changes a conserved threonine (a polar amino acid) into methionine (a nonpolar amino acid). This missense mutation is located in exon 4 where it may exert a disease-modifying effect by affecting interaction with β-tubulin, similar to the other predicted pathogenic missense mutations reported in previous studies (Smith et al., 2014).

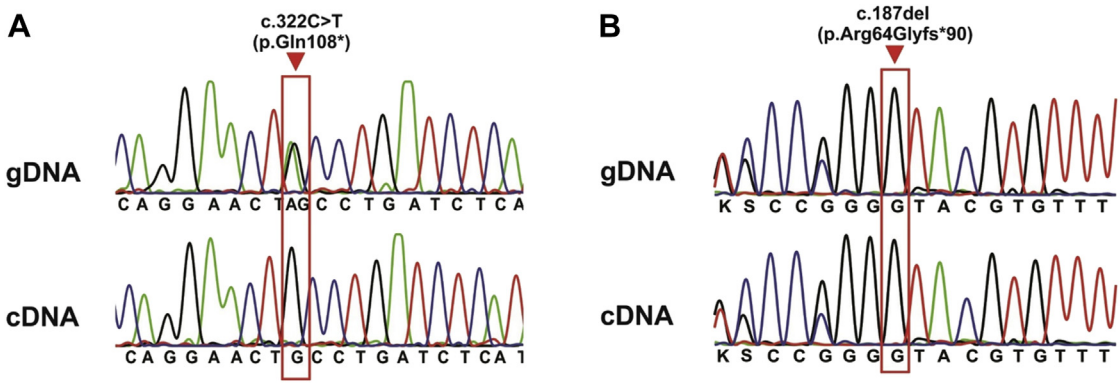

Fig. 2. Expression of the mutant transcripts in lymphoblast cell lines of PTC mutation carriers. Presence or absence of the mutation on gDNA extracted from blood and cDNA prepared from lymphoblast cell lines of the carrier of the *CHCHD10* c.322C>T (p.Gln108*) mutation (A) and of the *TUBA4A* c.187del (p.Arg64Glyfs*90) mutation (B) showing loss of the mutant transcript for the *CHCHD10* p.Gln108* mutation predicting a null allele and presence of the mutated frameshift transcript for the p.Arg64Glyfs*90 mutation predicting translation of a truncated protein. The *CHCHD10* and *TUBA4A* genes are both transcribed from the antisense strand, as such the G>A substitution as shown on the trace file is complementary to the *CHCHD10* c.322C>T substitution on the sense strand of the reference genome (Human GRCh38/hg38). Abbreviations: cDNA, complementary DNA; gDNA, genomic DNA; PTC, premature termination codon.

3.3. *Case report* CHCD10 *p.Q108* carrier*

The patient presented symptoms of parkinsonism at the age of 48 years, a diagnosis of idiopathic PD was suggested. After a couple of years, his clinical picture became rather atypical and dominated by general bradykinesia with frequent episodes of gait disturbances and falling. Clinical neurological examination at the age of 55 years was characterized by a remarkable bradykinesia, axial as well as in the limbs. The patient exhibited a poker face with important anterocollis and axial rigidity. Discrete cogwheel rigidity was present in the right wrist, and a tremor was observed in the chin as well as in the left leg. The levodopa response, which was initially mild to moderate, became unnoticeable. Furthermore, extreme bradypsychism was seen, although neuropsychological examination was negative for dementia with a score of 27/30 on mini-mental state examination. He exhibited intermittent confusion and was apathetic with the impression of unwillingness to speak. More behavioral disturbances appeared with verbal aggressiveness and changed eating habits. An magnetic resonance imaging of the brain at the age of 55 years demonstrated mild and aspecific cortical atrophy. A DaT-scan was performed, which displayed severely decreased (123) I-N-fluoropropyl-2b-carbomethoxy-3b-(4-iodophenyl) nortropane binding bilaterally in the basal ganglia, a bit more pronounced on the left side. This image was interpreted as being in accordance with advanced idiopathic PD or multiple system atrophy. However, because of the evolution to a clinical picture of atypical parkinsonism not characteristic for idiopathic PD, and important behavioral features, the clinical diagnosis of bvFTD was considered instead of idiopathic PD.

A familial history was present. His sister presented with parkinsonism at the age of 50 years, and a cousin was diagnosed with PD. Several other relatives were reported (hearsay) to have exhibited symptoms of dementia.

The patient died at the age of 60 after a recurrent catatonic presentation with pronounced rigidity and repetitive volvulus. Postmortem pathologic examination was performed. Observations were compatible with mild PD with Lewy bodies in the substantia nigra and absence of FTLD-like pathology.

3.4. *Case report* TUBA4A *p.Arg64Glyfs*90 carrier*

The patient was diagnosed with semantic dementia (SD). The first symptoms appeared at the age of 49 years and consisted mainly of difficulties in word retrieval, word comprehension, and prosopagnosia. In addition, he also developed behavioral changes such as severe obsessive compulsive behavior, increased appetite with loss of table manners, discrete disinhibition, loss of decorum, and emotional indifference. Neurologically, this patient exhibited an inexhaustible glabellar reflex and discrete stooped posture with decreased arm swing. There was no extrapyramidal rigidity. The diagnosis of SD was confirmed on magnetic resonance imaging and fluorodeoxyglucose positron emission tomography of the brain, which displayed bilateral, right more than left sided, anterior temporal atrophy as well as hypometabolism.

There was a familial history of PD. The father of the patient suffered from PD with cognitive problems. His disease onset was situated around 63 years of age. The paternal grandmother also suffered from PD since the age of 50.

3.5. *Case report of sibs with* TUBA4A *p.Thr381Met*

Since the age of 47, the patient exhibited complaints of a drop foot on the right with difficulties to climb stairs and to change position from lying down to sitting. Neurologic examination one and a half year after disease onset showed dysarthria, clinical observable fasciculations in the upper limbs without loss of muscle strength, decreased abduction of the right leg with decreased dorsiflexion of the right foot, and decreased muscle strength of the right muscle quadriceps. Brisk biceps and patellar reflexes were present in contrast with weak ankle jerk reflexes. Furthermore, a Babinski sign was present on the left side. A diagnosis of ALS was suspected. Three years after disease onset, the patient presented with progressive loss of muscle strength in both hands and both legs with frequent falls and decreased handiness. By that time, she had developed bilateral foot drop (more severe on the right side), fasciculations in the forelimbs with presence of pyramidal reflexes in the left leg, brisk reflexes in both arms, and frontal release signs. Electromyography was compatible with MND and was in line with the diagnosis of ALS. Chronic as well as discrete active axonal denervation signs and reinnervation signs were present in all limbs in combination with rare fasciculations and some giant potentials more pronounced in the left leg. Progressive muscle weakness, with respiratory muscle involvement led to the death of the patient at the age of 57 years.

Familial history was positive. The mother died of reported AD, and memory problems were also reported in a maternal aunt since the age of 50 and maternal grandmother since the age of 60 years. Her brother was diagnosed with ALS with cognitive problems. At the age of 52, he developed muscle weakness in the right arm, with fasciculations and muscle cramps. Mild cognitive problems, such as loss of initiative and mild memory problems were reported. On neurologic examination, muscle weakness with fasciculations and muscle atrophy were noted in the upper limbs, most pronounced on the right side. The reflexes were reduced in the upper limbs, but brisk in the lower limbs. There was no muscle weakness in the bulbar muscles or in the lower limbs. Neuropsychologic examination revealed frontal executive dysfunction, without evidence of memory loss. The criteria for FTD were not met. Fluorodeoxyglucose positron emission tomography showed reduced uptake in the motor cortex, the anterior temporal lobes, and the thalamus. The patient died at the age of 60 years. Both sibs were found to have a *C9orf72* repeat expansion.

4. Discussion

We investigated the genetic contribution of 2 recently reported genes to the FTD-ALS spectrum, the *CHCHD10* gene on chromosome 22q11.23 and the *TUBA4A* gene on chromosome 2q35. We performed in-depth exonic resequencing of these 2 genes in, respectively, 459 FTD, 28 FTLD-ALS, and 429 ALS patients as well as 703 control individuals from Belgian origin.

CHCHD10 is a nuclear gene encoding a 142 amino acid protein that locates to the intermembrane space of mitochondria where it is likely involved in mitochondrial genome stability, cristae integrity, and mitochondrial fusion. Mutations in *CHCHD10* are associated with high clinical heterogeneity encompassing ALS, FTD, cerebellar ataxia, parkinsonism, and mitochondrial myopathy (Ajroud-Driss et al., 2015; Bannwarth et al., 2014) and point toward a role for mitochondrial disease in the FTD-ALS spectrum.

In the present study, we identified a novel nonsense mutation (p.Gln108*) in exon 3 of *CHCHD10*. The mutation p.Gln108* introduces a PTC, and we showed that the resulting mRNAs are unstable and degraded by NMD. In addition to 14 patient-specific missense mutations, this is the second PTC mutation reported in *CHCHD10* (Dols-Icardo et al., 2015). The observation of recurring heterozygous loss-of-functions mutations suggests the disease mechanism of *CHCHD10* in the FTD-ALS spectrum, is through a loss of protein function and that the pathogenic missense mutations must somehow also reduce CHCHD10 functionality.

The p.Gln108* was identified in a male patient, who was included in a cohort of FTD index patients. On identification of the mutation,

the medical history of this patient was carefully reviewed and indicated that the patient received a clinical diagnosis of FTD but that early disease presentation around the age of 48 had been suggestive of idiopathic PD. Furthermore, this patient had multiple affected relatives with parkinsonism, PD and/or cognitive deterioration, including a sister and first cousin. The patient died at the age of 60 years, and neuropathological investigation eventually confirmed the diagnosis of PD. Since our *CHCHD10* (p.Gln108*) carrier turned out to have PD, it is of interest that missense mutations in *CHCHD2*, another member of the CHCHD mitochondrial protein family, have been linked to autosomal dominant PD in a Japanese population (Funayama et al., 2015). Protein alignment by Pairwise Sequence Alignment (https://www.ebi.ac.uk/Tools/psa/emboss_water/) indicated that the homology in amino acid sequence between the 3 functional domains, the mitochondrial targeting sequence, hydrophobic helix, and coiled helix coiled helix (CHCH) domain of CHCHD10 and CHCHD2, is about 85% (63/74), suggesting that these genes may have comparable function. In another study, Zhang et al. sequenced 153 PD and 141 AD patients for *CHCHD10* mutations, yet failed to identify possible pathogenic mutations (Zhang et al., 2015). Additional genetic studies in PD patients and families are needed to elucidate if the implication of *CHCHD10* in neurodegenerative diseases spans beyond the FTD-ALS spectrum and may also involve PD or other related disorders such as AD.

In addition to the patient-specific nonsense mutation, we identified 3 missense substitutions of unclear significance in patients that were also observed in the control cohort. This included the known missense variant *CHCHD10* p.Pro34Ser (rs551521196) present in 3 FTD and 5 ALS patients but also in 9 control individuals. In contrast, p.Pro80Leu and p.Pro96Thr (rs111677724) were observed in just 1 control individual. The p.Pro80Leu variant was identified in a female FTD patient with disease onset of 65 years devoid of mutations in known FTD/ALS genes and an unrelated male ALS patient with disease onset of 60 years. Moreover, p.Pro80Leu was recently reported in 2 ALS patients with common disease haplotype (Zhang et al., 2015) and 2 Italian ALS patients with muscle mitochondrial pathology (Ronchi et al., 2015). Although we detected p.Pro80Leu also in 1/703 controls, this unaffected individual was just 57 years at inclusion in the study and may therefore still develop disease at a later age. Taken together, ours and other's findings in patients suggest that p.Pro80Leu may be a pathogenic mutation, possibly with reduced penetrance. In contrast, p.Pro96Thr (rs111677724) was also reported by other studies in healthy individuals, and is more suggestive for a benign polymorphism.

Also *TUBA4A* was recently identified as a novel causal gene in ALS. *TUBA4A* encodes 1 of 8 human α-tubulins (448 amino acids), which polymerize with β-tubulins to form the microtubule cytoskeleton, implicating the neuroskeletal architecture. *TUBA4A* mutations have primarily been associated with ALS, although some patients also had cognitive involvement ranging from mild cognitive impairment to FTD (Pensato et al., 2015; Smith et al., 2014). In the present study, we identified a novel *TUBA4A* frameshift mutation (p.Arg64Glyfs*90) and confirmed the formation of a truncated protein with loss of the C-terminal half of the last exon 4. We observed this truncating mutation in a 49-y-old SD patient with no signs of MND. To our knowledge, this is the first report of a *TUBA4A* mutation carrier with pure FTD. Truncating mutations in *TUBA4A* have been reported before. Smith et al., identified a nonsense mutation (p.Trp407*), removing the last 41 amino acids of the protein (Smith et al., 2014). This C-terminal region is important for the polymerization with β-tubulins to from the microtubule cytoskeleton. In line with this, Smith et al., were able to demonstrate that this particular *TUBA4A* mutant failed to incorporate into microtubules in transfected primary motor neurons (Smith et al., 2014). Furthermore, we found a novel patient-specific missense substitution (p.Thr381Met) in a familial ALS patient with disease onset at age 47. The presence of p.Thr381Met was also confirmed in her affected brother with ALS and memory problems. Notably, they also carried a *C9orf72* repeat expansion.

In conclusion, we observed a small number of disease-causing mutations in *CHCHD10* (in 1 PD patient and 1 FTD patient) and *TUBA4A* (in 1 FTD and 1 ALS). The low mutation frequency of $<1\%$, complex clinical heterogeneity, and the observation of comparable variants in unaffected carriers limit the clinical relevance of *CHCHD10* and *TUBUA4A* gene testing for molecular diagnostics. However, at least for *CHCHD10*, clinical presentation of neurodegenerative disease (including FTD, ALS, PD, or AD) in combination with myopathy and other symptoms that could be related to mitochondrial disease, may warrant specific genetic testing of *CHCHD10*. Regardless of its limited clinical impact, the major impact of genetic screening studies of rare genes in extended study populations of different related phenotypes, is rather in the relevance of understanding the disease biology, in this case, of clinical FTD-ALS and exploring the implications and potentials for disease remediation of mitochondrial DNA instability (CHCHD10) and neuroskeletal defects (TUBA4A) herein.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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