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MITOCHONDRIAL DNA VARIANTS IN COMPLEX V CODING GENES CONTRIBUTING TO FRONTOTEMPORAL LOBAR DEGENERATION

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Mitochondrial DNA variants in Complex V coding genes contributing to frontotemporal lobar degeneration

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Abstract

FTLD is the second most common early-onset type of dementia, it has a broad spectrum of clinic and histopathologic manifestations and its pathophysiological mechanisms are not yet fully understood. Over the last decades a growing number of evidence has supported the involvement of the mitochondria in the aetiology of several neurodegenerative diseases. One suggested mechanism is the existence of mitochondrial DNA mutations which render impairment of the mitochondrial respiratory chain. The aim of our study is to add further knowledge to this area. Accordingly, thetwo genes of the mitochondrial DNA coding for complex V subunits, *MT-ATP8* and *MT-ATP6*, were sequenced and analysed, in 70 FTDL patients. The results reveal that29 patients (41.4%) present at least one sequence variation in one of the studied genes, being 38.9% of all the alterations found, synonymous, whereas non-synonymous variations account for 61.1% of total alterations. The latter were submitted to *in silico* analysis. This study disclosed three probably damaging mutations (m.8393C>G,m.8519G>A and m.8945T>C), and others, such asm.8573G>A, m.8839G>A and m.8842A>C, which may cause functional impairment. The majority of the alterations identified in the present study have not been described before in association with any neurodegenerative disease and are barely documented. Even if additional studies are needed, the present study represents a significant contribution to a better understanding of a complex neurodegenerative disease, such as frontotemporal lobar degeneration.

Keywords

Frontotemporal lobar degeneration, mitochondrial DNA, sequence variations, complex V, dementia, mutations.

Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ATP	adenosine triphosphate
bvFTD	behaviour frontotemporal dementia
FTLD	frontotemporal lobar degeneration
FUS	fused in sarcoma
HD	Huntington's disease
KSS	Kearns–Sayre syndrome
LHON	Leber's hereditary optic neuropathy
LS	Leigh Syndrome
LPA	logopenic or phonological variant
MELAS	mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MERRF	myoclonus epilepsy ragged-red fibers
MRC	mitochondrial respiratory chain
mtDNA	mitochondrial DNA
mtDB	Human Mitochondrial Genome Database
NARP	neuropathy ataxia and retinitis pigmentosa
nDNA	nuclear DNA
PD	Parkinson's Disease
PEO	progressive external ophthalmoplegia
PNFA	progressive non-fluent aphasia
SD	semantic dementia
TDP	TAR DNA-bindingprotein
UPS	ubiquitin proteasome system

Introduction

FTLD is the second most common early-onset type of dementia (Grazina *et al.*, 2004; Rollinson *et al.*, 2011; Seelaar *et al.*, 2011), typically manifesting between 45 and 65 years (Neary *et al.*, 2005; Shi *et al.*, 2005; Sleegers *et al.*, 2010), and being diagnosed at youngest ages as 21 or oldest as 85 years old (Neary *et al.*, 2005; Pickering-Brown SM, 2007). No difference has been found between the familial and sporadic forms of the disease concerning the age of onset (Neary *et al.*, 2005).The incidence appears to be equal between both genders (McKhann *et al.*, 2001; Neary *et al.*, 2005; Pickering-Brown SM, 2007; Seelaar *et al.*, 2011). It has a median duration of illness of 6 to 8 years, ranging from 2 to 20 (Neary *et al.*, 2005; Seelaar *et al.*, 2011).

The term FTLD includes a heterogeneous group of clinical presentations which can be divided in behaviour and language variants (McKhann *et al.*, 2001; Seelaar *et al.*, 2011). The first, also called behaviour FTD form, is the most common (McKhann *et al.*, 2001; Pickering-Brown, 2007; Seelaar *et al.*, 2011). Patients who suffer from this variant, show predominantly personality and behaviour changes, becoming either uninhibited, apathetic or displaying stereotypical actions (Neary *et al.*, 2005; Pickering-Brown, 2007; Rollinson *et al.*, 2011). The language component is classically divided in two major syndromes, SD and PNFA (Pickering-Brown, 2007). The first (SD) is characterized by loss of semantic knowledge which impairs word comprehension with a relatively conserved fluency of speech (Sleegers*et al.*, 2010; Rollinson *et al*., 2011), whereas PNFA is roughly the opposite, with affected speech and preserved semantic recognition (Sleegers*et al.*, 2010; Rollinson *et al.*, 2011). Recentevidences support the existence of a third group, the LPA, with slow rate of speech, word finding difficulties and occasional phonemic errors (Seelaar *et al.*, 2011).

As the pathologic process progresses the boundaries of these classifications become blurry, and patients often show a mixed clinical picture (McKhann *et al.*, 2001; Neary *et al.*,

2005; Sleegers *et al.*, 2010; Seelaar *et al.*, 2011). Furthermore, some other conditions can overlap, such as Parkinson syndromes, progressive supra-nuclear palsy, cortico-basal syndromes or motor-neuron disease (Sleegers *et al.*, 2010; Seelaar *et al.*, 2011). The latter, frequently related with the behaviour FTD, specially with the stereotypic variant, is associated with a worst prognosis with a mean survival of 3 years (Neary *et al.*, 2005; Seelaar *et al.*, 2011).

As for the clinical picture, the alterations found in the brain of these patients also present distinct and heterogeneous characteristics. The common finding is the frontal and temporal lobe atrophy (McKhann *et al.*, 2001; Mackenzie *et al.*, 2009; Seelaar *et al.*, 2011), that can be also seen in imaging studies. The clinical presentation usually reflects the affected part of the brain by the disease, whereas the opposite relation is not necessarily valid (Neary *et al.*, 2005; Shi *et al.*, 2005). The histopathological classification allows to distinguishfive variants, according to Mackenzie *et al.* (2010): FTDL-tau, characterized by insoluble forms of tau proteins in form of neurofibrillary tangle-like structures or in Prick body type;FTDL-TDP,in which the accumulation of TDP-43 protein is detected; FTDL-FUS, as consequence of the accumulation of FUS protein; FTDL-UPS for tau, TDP-43 and FUS negative histology test that are positive for the immunohistochemistry of the UPS proteins, and FTDL-ni for cases in which inclusions are not detected by the current tests. FTDL-tau and FTDL-TDP are the most common forms (Mackenzie *et al.*, 2010).

The aetiology of FTDL has not yet been fully understood. To date, there are some known nDNA mutations which lead to the histopathologic changes described. According to Seelaar *et al.* (2011), the most frequents are mutations in *MAPT* gene (locus 17q21.1) that lead to the accumulation of tau protein (Mackenzie et al., 2009, 2010; Seelaar *et al.*, 2011), and in *GRN* gene (locus 17q21.32) leading to the formation of TDP-43 positive inclusions (Mackenzie et al., 2009, 2010; Rollinson *et al.*, 2011; Seelaar *et al.*, 2011).

Additionally,although less frequently, mutations in *TARDBP*(locus 1p36.22),*VCP* (locus 9p13.3) and*C9orf72* (locus 9p21.2)genes have also been described, all associated with TDP-43 inclusions. Other mutations have been assigned to *FUS* gene (locus 16p11.2) with FUS protein accumulation, andto*CHMP2B* gene (locus 3p11.2), which is one known cause of FTDL-UPS (Mackenzie *et al.*, 2010).

Despite all the causative mutations identified, most of FTDL cases, either sporadic or familial, cannot be explained by these alterations. Therefore, other pathogenic mechanisms must be implicated in the genesis of the disease (Rollinson *et al.*, 2011; Seelaar *et al.*, 2011).Some patients present an overlapbetween Alzheimer's disease (AD) and FTD both in neuropathologicaland clinical aspects. This may suggest a similar overlap in physiopathology,namely an involvement of mitochondrial DNA (mtDNA) inFTD, as it has been associated to AD (Grazina *et al.*, 2004). For review on genes involved in FTDL see Cruts *et al.* (2012).

Mitochondria have been implicated in the pathophysiology of several neurodegenerative diseases such as PD (Autere *et al.*, 2004; Mawrin *et al.*, 2004; DiMauro and Schon, 2008; Lee *et al.*, 2009; Federico *et al.*, 2012), AD (Emerit *et al*., 2004;Grazina *et al.*, 2005; Grazina *et al.*, 2006; Onyango *et al.*, 2006; DiMauro and Schon, 2008), HD (DiMauro and Schon, 2008; Lee *et al*., 2009) and ALS (Mawrin *et al.*, 2004; DiMauro and Schon, 2008; Lee *et al.*, 2009). In fact, the role that the dysfunction of this organelle plays in the human pathology has begun to be better understood over the last decades, being one of the major contributors not only to neurodegeneration, but also to several encephalomyopathic diseases like LS, LHON, PEO, KSS, MELAS or MERRF (Leonard and Schapira, 2000; Emerit *et al*., 2004;DiMauro and Schon, 2008; Lee, 2009; Federico *et al.*, 2012; Schapira, 2012).

The pathologies that arise from mitochondrial dysfunction affect essentially high energy demanding tissues, such as liver, skeletal or cardiac muscle and nervous system. In fact, these tissues withelevated metabolic needs, relying mainly on the ATP produced via aerobic metabolism in the mitochondria (Pieczenik and Neustadt , 2007; Lee *et al.*, 2009), compared to other tissues (Emerit *et al.*, 2004; Lee *et al.*, 2009; Greaves *et al.*, 2012). In spite of being the powerhouse of the eukaryotic cells, mitochondria is also promoter of other key functions of the cell like apoptosis-signalling pathway (Pieczenik and Neustadt, 2007; Greaves *et al.*, 2012), cytosolic calcium concentration and iron-sulfur cluster biogenesis (Greaves *et al.*, 2012), moreover its involvement in ageing and carcinogenesis processes is only beginning to be unveiled (Greaves et al., 2012; Schapira, 2012).

The process by which mitochondrial dysfunction leads to neurodegeneration is relatively well established: it is a consequence of the impairment of the MRC activity, which by its turn decreases ATP production, increasing the ROS generation and the concentration of intracellular calcium (Emerit *et al.*, 2004; Grazina *et al.*, 2005; Federico *et al.*, 2012; Greaves *et al.*, 2012; Schapira AHV, 2012). This process ultimately ends in the activation of the intrinsic mitochondrial pathway resulting in apoptosis and consequent neuronal cell loss (Emerit *et* al., 2004; Grazina *et* al., 2006; DiMauro and Schon, 2008; *Lee et al.*, 2009).

There are several mechanisms that can trigger this events, among them are the presence of environmental stressors such as diet deficits or toxics (Pieczenik and Neustadt, 2007) that inhibit the MRC enzymes, inflammatory mediators such as TNF-α (Pieczenik and Neustadt, 2007), proteasome malfunctions and protein misfoldings, or DNA mutations. Such mutations can occur in the nDNA, affecting the MRC proteins, the mtDNA maintenance and expression, the salvage nucleotide synthesis and transport, or the mitochondrial dynamics. They can also occur directly over the mtDNA in which case they affect the MRC proteins, the tRNA's or the rRNA's. To date almost 600 mtDNA mutations have been reported associated

to disease (http://www.mitomap.org). Some of those mtDNA polymorphisms have been associated with several neurodegenerative disorders like PD (Federico *et al.*, 2012), AD (Grazina *et al.*, 2005, 2006) or FTDL (Grazina *et al.*, 2004). This relation with neurodegenerative diseases is not always easy to establish as these mutations also can occur with normal ageing resulting from the ROS aggression (Mawrin *et al*., 2004; Grazina *et al*., 2006). Currently, two theories seek to explain this relation. The first one proposes a vicious cycle occurring between ROS production and mtDNA mutations in which one leads to another (Pieczenik and Neustadt, 2007; Greaves et al., 2012), however a more likely explanation is the occurrence of mtDNA mutations throughout life, either resulting from ROS damage, errors in mtDNA replication or repair mechanisms, that clonally expand as the mitochondria divides independently from the cell (Greaves *et al*., 2012).

The aim of this study is therefore to search and access the implication of mtDNA variations in patients suffering from FTLD. The screening included the analysis of*MTATP6*and *MTATP8*genes (coding for ATP synthase F0 subunits 6 and 8, respectively), the only two genes of mtDNA that encode Complex V subunits, as part of a larger project whose objective is to analyse the whole mtDNA in FTDL patients. ATP synthase is the last complex of the MRC, essential for the oxidative phosphorylation process, where the majority of the ATP molecules are produced. Therefore, its dysfunction could lead to a major impairment in the ATP generation with consequent neuronal degeneration. Currently, two diseases are associated with point mutations in the *MTATP6* gene, NARP and LS (Greaves *et al*., 2012).Mutations in Complex V nDNA coding genes *ATPAF2* and *ATP5E* had been also related with encephalopathy (Schapira, 2012). So far, there is no evidence of association between mutations in *MTATP8* gene and disease.

To our knowledge, this is the first study investigating the genetic sequence of *MTATP6*and *MTATP8* genesin FTLD patients.

Patients and Methods

The samples included on this study belong to70 patients (31 males and 39 females; mean age:63 years, range: 38 to 82 years)with a probable diagnosis of FTLD according to the standard criteria of DSM-IV (Brun *et al.*, 1994; McKhann *et al.*, 2001), recruited at the Neurological Unit of the "Centro Hospitalar e Universitário de Coimbra". The mean age of onset was60 years, ranging from 34 to 79 years. Concerning clinical forms,60 patients (85.7%) presented bvFTD, while 4 (5.7%) had bvFTD with CBS, other 4 (5.7%) PNFA and 2 (2.9%) showed SD. All patients gave written informed consent in order to participate in the study, which has been approved by the localEthical Committee. A list of all patients' data can be found in appendix 1.

Total cellular DNA was extracted from peripheral blood leukocytes after erythrocytes lysis, following standard phenol-chloroform method. Automated sequencing analysis was performed using 3130 ABI Prism sequencing system and BigDye® Terminator Ready Reaction Mix 3.1 (Applied Biosystems) with specific primers for target genes in order to study both *MTATP6* and *MTATP8*coding genes. The sequences obtained were compared with the human mithochondrial DNA revised Cambridge reference sequence (Andrews *et al.*, 1999), obtained from GenBank, using Sequencing Analysis® v.5.4 and SeqScape® v.2.5 software (Applied Biosystems)to search for mutations, polymorphisms or novel sequence variations. All sequence variations were classified using the MITOMAP database (http:\\www.mitomap.org) and frequency was obtained in the Human Mitochondrial Genome Database (Ingman *et al.*, 2006)toestimate its frequency in general population.

For non-synonymous sequence variations an *in silico*analysis was performed using a set of softwares: PolyPhen v. 2.2.2, which takes into account sequence, phylogenetic and structural information, predicting protein functional outcomes of sequence variations by two models, HumVar and HumDiv; Mutation Assessor v. 2, that predicts the impact of a variation

by aligning multiple sequences, clustering them into subfamilies and scoring a variation by global and sub-family specific conservation patterns; Provean, that allows a similar prediction approachoffered by Mutation Assessor, but calculates the mutation score by the average of each sub-family average score; and SIFT, which predicts the mutation score by aligning user defined sequences. For this purpose,we have selected 10 different species (*Homo Sapiens*, *Pan troglodytes*, *Gorilla gorilla*, *Pongo abelii*, *Macaca mulatta*, *Mus musculus*, *Rattus norvegicus*, *Bos taurus*, *Sus scrofa* and *Canis familiaris*). Clustal Omega softwarewas used in order to visualize the alignment of the referred sequences as shown in figure 3 and appendix 2. The sequences were obtained through the UniProt sequence databases, being the majority of them from the European Nucleotide Archive database.

Statistic analysis was performed to infer if variations in the studied locus correlates with different variables of the patients. Accordingly,t-test or Mann-Whitney test (if data did not follow a Gaussian distribution), were performed to compareage, age of onset, CDR staging or the MMSE score of the patients, according to the presence of genetic variations; contingency tables, with Fisher's exact test, were used to investigate gender, age of onset, MMSE class and clinical outcome, in association to genetic variants.

Results

Sequence variants inthe mtDNA genes under study were found in 29 patients (41.4%). From these,8 (11.4%) presented one *MTATP8* variation, 14 (20.0%) one *MTATP6*alteration, 1 (1.4%) one variation in both genes, and 6 (8.6%) presented two *MTATP6*alterations. The sequence variations found in *MTATP6*and *MTATP8* genes are listed in Table 1 and Table 2, respectively.

Table 1:mtDNA sequence variations found in *MTATP6* gene.

Sequence Variation	Aminoacid Change	Reported in MITOMAP	Frequency (n)	Frequency in European population
m.8573G>A	G16D	Polymorphism	1.43% (1)	0.07%
m.8577A>G	Synonymous	Polymorphism	1.43% (1)	0.07%
m.8584G>A	A20T	Polymorphism	1.43% (1)	4.77%
m.8697G>A	Synonymous	Polymorphism; Thyroid tumor cells	2.86% (2)	4.73%
m.8701A>G	T59A	Polymorphism; Thyroid tumor cells	2.86% (2)	34.5%
m.8839G>A	A105T	Polymorphism	1.43% (1)	0.22%
m.8841C>T	Synonymous	Polymorphism	1.43% (1)	0.07%
m.8842A>C	I106L	Polymorphism	1.43% (1)	0.04%
m.8857G>A	G111S	Polymorphism	1.43% (1)	0.04%
m.8865G>A	Synonymous	Polymorphism	1.43% (1)	0.15%
m.8869A>G	M115V	Polymorphism	1.43% (1)	0.59%
m.8870T>C	M115T	Polymorphism	1.43% (1)	0.11%
m.8945T>C	M140T	Polymorphism	1.43% (1)	NR
m.8950G>A	V142I	Polymorphism; Leber Optic Atrophy and Dystonia	1.43% (1)	0.04%
m.8994G>A	Synonymous	Polymorphism	4.29% (3)	2.37%
m.9055G>A	A177T	Polymorphism; PD protective factor	5.71% (4)	4.96%
m.9064G>A	A180T	Polymorphism	1.43% (1)	0.11%
m.9070T>G	S182A	Polymorphism; Pancreatic cancer cell line	2.86% (2)	0.11%
m.9123G>A	Synonymous	Polymorphism	1.43% (1)	2.66%

NR: notreported in mtDBdatabase.

In *MTATP6* gene, a total of 19 different sequence variations were found: 6 synonymous and 13 non-synonymous. All variations were reported in MITOMAP database as polymorphisms and 4 were also reported in different diseases, and 1 as a protective factor of PD. These alterations were detected predominantly in only one patient each, except for variations m.8697G>A, m.8701A>G and m.9070T>G, detected in two patients each, m.8994G>A detected in three, and for m.9055G>A, that was detected in four different patients.

Table 2: mtDNA sequence variations found in *MTATP8* gene.

Sequence Variation	Aminoacid Change	Reference in MITOMAP	Frequency (n)	Frequency in European population
m.8393C>T	P10S	Polymorphism; Reversible Brain Pseudoatrophy	2.86% (2)	0.53%
m.8440A>G	Synonymous	Polymorphism	1.43% (1)	0.15%
m.8473T>C	Synonymous	Polymorphism	1.43% (1)	2.18%
m.8478C>T	S38L	Polymorphism	1.43% (1)	0.07%
m.8485G>A	Synonymous	Polymorphism	1.43% (1)	0.44%
m.8512A>G	Synonymous	Polymorphism	2.86% (2)	0.04%
m.8519G>A	E52K	Polymorphism	1.43% (1)	0.04%

In *MTATP8* gene, 7 different sequence variations were found: 4 synonymous and 3 non-synonymous alterations. All variations were reported in MITOMAP database as polymorphisms, being m.8393C>Talso reported in reversible brain pseudoatrophy (Galimberti *et al.*, 2006). The alterations m.8393C>T and m.8512A>G were detected in two patients each, whereas the others were identified in one patient only.

Overall, a total of 36 sequence variations were found in 70 FTLD patients, 38.9% of which aresynonymous, whereas non-synonymous variations account for 61.1% (Figure 1). All variations were found in homoplasmy.

The *in silico* analysis performed for the non-synonymous variations is shown on Table 3. Evolutionary genetic conservation is presented is Figures 2 and 3.

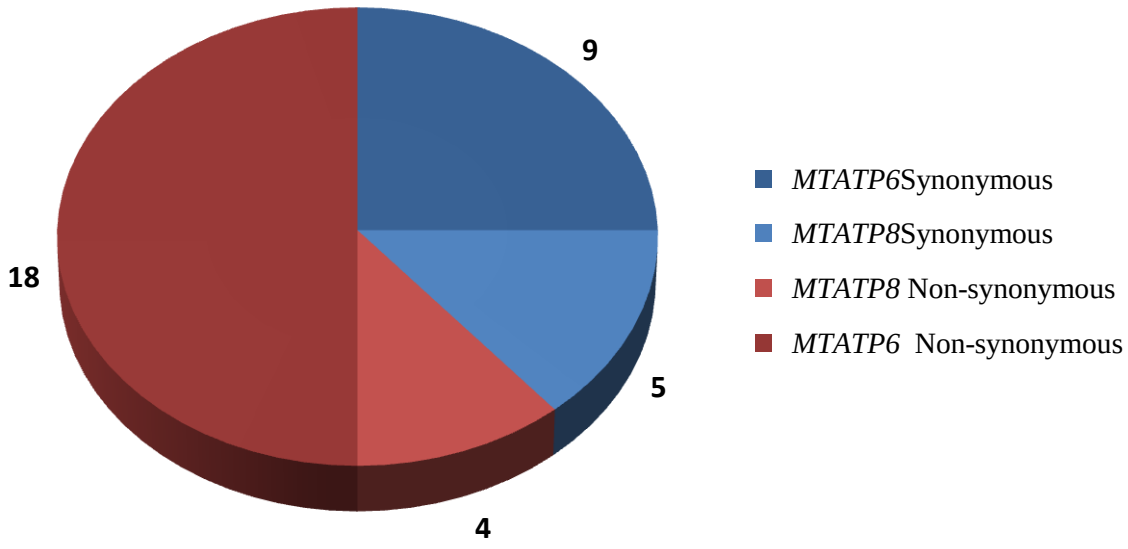

Figure 1: Graphicalrepresentationofthe total numberofvariationsfound in each gene, according to type.

Table 3:*In silico* analysis performed to the non synonymous variations found.

GENE	Nucleotide Change	Amino acid change	Poly-Phen 2.2.2		Mutation Assessor		Provean		SIFT		Conser-vation*
			Score	Prediction	Score	Prediction	Score	Prediction	Score	Prediction	
MTATP6	m.8573G>A	G16D	0.050	Benign	3.67	High damage	-5.425	Deleterious	0.07	Tolerated	90%
	m.8584G>A	A20T	0.004	Benign	0.06	Neutral	-0.404	Neutral	0.34	Tolerated	40%
	m.8701A>G	T59A	0.002	Benign	0.365	Neutral	-0.935	Neutral	0.70	Tolerated	40%
	m.8839G>A	A105T	0.999	Probably damaging	3.62	High Damage	-3.644	Deleterious	0.00[#]	Affected protein function	100%
	m.8842A>C	I106L	0.059	Benign	-0.54	Neutral	-1.592	Neutral	0.00[#]	Affected protein function	100%
	m.8857G>A	G111S	0.185	Benign	0.66	Neutral	-2.097	Neutral	0.20	Tolerated	80%
	m.8869A>G	M115V	0.002	Benign	0.38	Neutral	0.293	Neutral	1.00	Tolerated	30%
	m.8870T>C	M115T	0.000	Benign	-0.57	Neutral	0.150	Neutral	0.54	Tolerated	30%
	m.8945T>C	M140T	0.949	Possibly damaging	1.845	Lowdamage	-3.553	Deleterious	0.00[#]	Affected protein function	100%
	m.8950G>A	V142I	0.000	Benign	0.08	Neutral	0.118	Neutral	0.54	Tolerated	70%
	m.9055G>A	A177T	0.845	Possibly damaging[†]	1.315	Low damage	-2.606	Deleterious	0.26	Tolerated	80%
	m.9064G>A	A180T	0.011	Benign	0.03	Neutral	-0.536	Neutral	0.05	Tolerated	80%
	m.9070T>G	S182A	0.225	Benign	0.685	Neutral	-0.122	Neutral	0.11	Tolerated	40%
MTATP8	m.8393C>T	P10S	0.993	Probably damaging	1.17	Low damage	-1.257	Neutral	0.08	Tolerated	40%
	m.8478C>T	S38L	0.000	Benign	1.285	Low damage	-2.708	Deleterious	0.43	Tolerated	40%
	m.8519G>A	E52K	0.955	Possibly damaging	2.185	Medium damage	-3.078	Deleterious	0.10	Tolerated	80%

[*]Percentage of sequences that present the same reference nucleotide as *Homo sapiens*.
[#]Low confidence in the prediction.
[†]Benign score by the HumVar model.
Test scores that predict an altered protein function are highlighted.

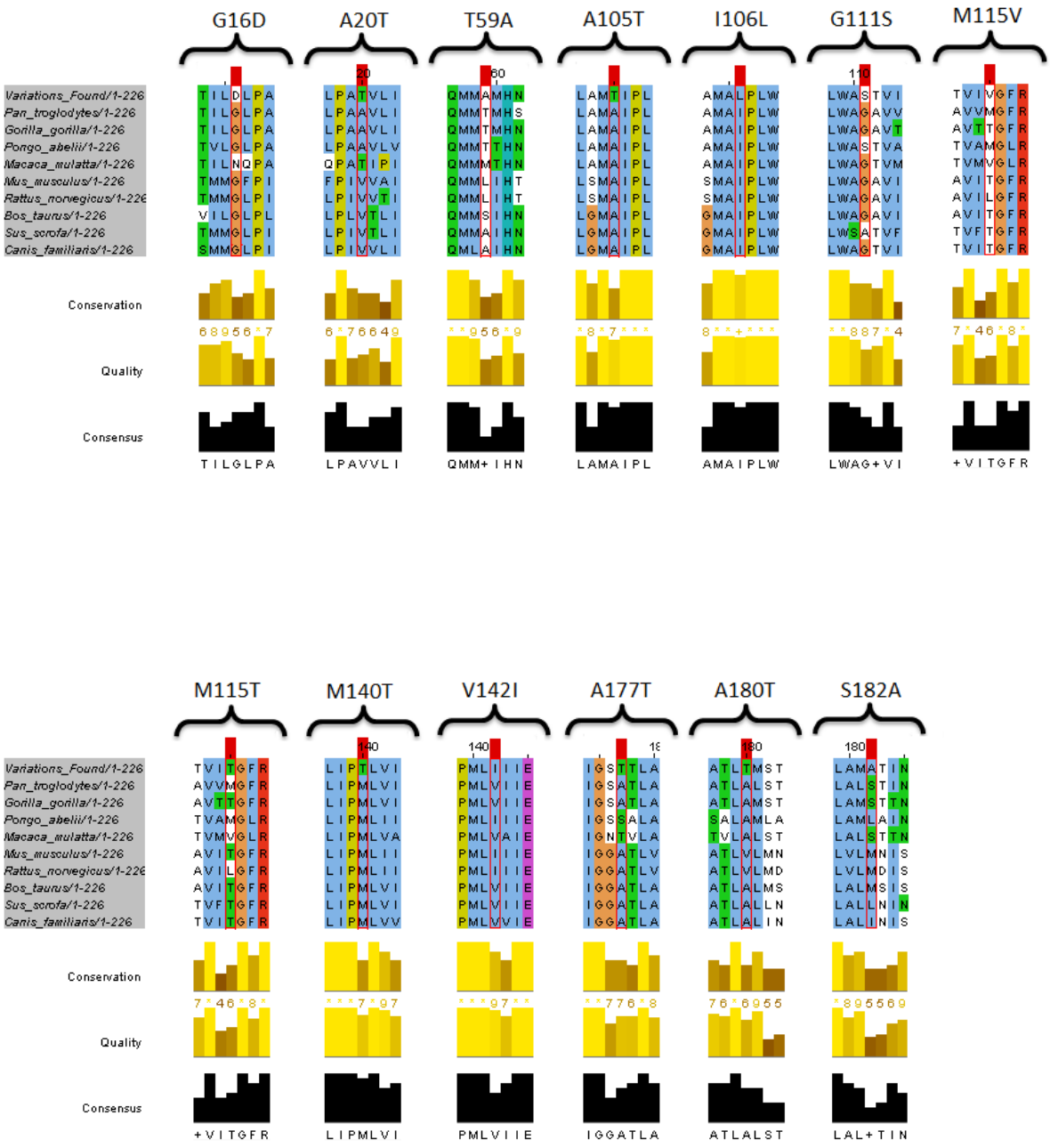

Figure 2: Alignedsequences for the12 non-synonymousvariationsfound in*MTATP6* gene.Nucleotideofinterestishighlightedwith a redsquare.

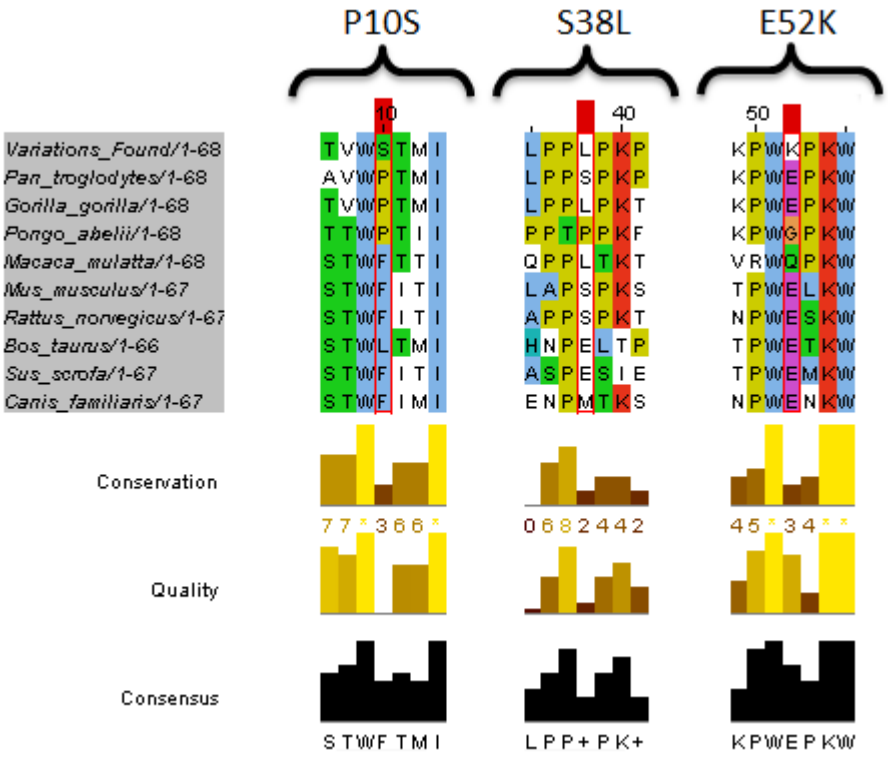

Figure 3: Alignedsequences for the3 non-synonymousvariationsfound in*MTATP8* gene.Nucleotideofinterestishighlightedwith a redsquare.

Tables 4 and 5 present the data of the statistic analysis. Table 4shows the t-test *p*values for the variables age, age of onset, CDR stage and MMSE score for patients harbouring variations in *MTATP6*and *MTATP8*genes, compared to the absence of alterations. Table 5 shows the *p* values of the Fisher's test for the variables gender, age of onset, MMSE class and clinical outcome for patients in which variations were identified *versus* patients without alterations in the genes under study.

Table 4: Statistic analysis using t-test to evaluate if variations in *MTATP6*, *MTATP8* or in *MTATP6 +MTATP8* significantly affect the age of the patients, age of onset of disease, CDR staging or the MMSE Score.

	p value		
	MTATP6	*MTATP8*	*MTATP6 +MTATP8*
Age	0.8986	0.2060	0.5631
Age of onset	0.6633	0.1421	0.7101
CDR stage	0.9944*	0.5353*	0.7904*
MMSE Score	0.7552	0.1995	0.6018

*Results refering to Mann-Whitney test because the data did not followed a normalized distribution.

Table 5: Contingency analysis sorting the patients by presence or absence of mtDNA variations in *MTATP6,8* genes variations, and by gender, age of onset, MMSE class and clinical outcome, according to Fisher's exact test.

	p value
Gender	1.0000
Age of Onset*	1.0000
MMSE Class#	0.7948
Clinical Outcome†	0.3974§

*Cut-off 65years old.

#Defined by MMSE score below or equal 22 or above 22.

†bvDFT versus language variants.

§A 95% confidence interval could not be calculated due to the low number of language variant cases.

Discussion

The total number of sequence variations found washigher for*MTATP6*gene (n=27) than for*MTATP8* (n=9). This fact may be due to the length of each gene, being the first one larger (681 bp) than *MTATP8* (207 bp). Apparently, the alterations are evenly distributed.Concerning the alteration type, a different scenario was observed: the non-synonymous to synonymous ratio in *MTATP8*gene is 1:0.8; however in the *MTATP6*locus that ratio drops to 1:2. This couldindicate that non-synonymous variations in the *MTATP8* locus are less tolerated than in the *MTATP6*, possibly leading to cell apoptosis and thus being less prevalent. In fact, the scarcity of described deleterious mutations in the final MRC complexes, specially in the *MTATP8* locus,suggests that either those are rare or incompatible with life (DiMauro and Schon, 2008). However,*Canis lupus familiaris* (dog), *sus scrofa* (pig), *mus musculus* (mouse) and *rattus norvegicus* (rat) ATP8 protein lacked 1 aminoacid compared to the other species, and thus their mtDNA was 3 nucleotides shorter. *Bos taurus* (bovine) lacked even one more aminoacid (and 6 nucleotides). It could mean that this protein structure is not highly conserved among species, and thus allowing some alterations without loss of function. Despite that, all amino acid variations found in *MTATP8* were represented in the 10 species analysed.

All the variations found in the present work are already reported in MITOMAP database (http://www.mitomap.org), although only a few are reported in association with pathologies.The majority of the mtDNA variations found (60%) in the patients studied are non-synonymous variations. Concerning the other 40% synonymous variations, it is not expected that these represent a major cause of protein malfunction. Interestingly, some of the synonymous variations here reported are fairly rare, such asm.8512A>G,identified in two FTLD patients, but only in one of the 2704 sequences in the mtDB database. Moreover,m.8577A>G and m.8841C>Tboth reported in two different patients of this study,

wereonlyeach one reported in 2 of the 2704 mtDB sequences.Although synonymous alterations do not change protein structure, they can still be involved in pathologic process (Rollins*et al.*, 2009). It is possible that different arrays of polymorphisms coding for MRC coding components may result in different MRC functional outcomes (Grazina *et al.*, 2005; DiMauro and Schon, 2008), and so, albeit mtDNA polymorphisms are not pathological by themselves, they can create susceptibility, or protection, for certain diseases (Autere, 2004; DiMauro and Schon, 2008), as result of DNA expression modulation.

The m.8573G>A changes the non polar glycine at position 16 of the ATP6 protein,which is preserved in 9 of the 10 sequences analysed (Figure 2). The sorroundingaminoacids are also preserved among higher primates. As a result, Mutation Assessor and Provean predicts that this variation is harmful, being the SIFT score close enough to the cut-off point so that it can be considered that this variation is possibly pathogenic, although the change to an acidic polar aspartic acid is not enough to raise the PolyPhen score.

The m.8697G>A, a synonymous variation,and m.8701A>G wereidentifiedby Máximo *et al.*, (2002) in the thyroid tumor cells of three different patients. Thesecond variation is also a haplogroup marker for the macro-haplogroup N, that includes the European-specific haplogroups.Therefore,it could be involved in population adaptation or neoplastic transformation (Brandon *et al.*, 2006). In the present study,this variation was found in a notably less proportion, when compared to the one found in mtDB datatbase. Therefore, it could be postulated that this variation would represent a protective factor regarding FTDL development.However, this could also be a consequence of the prevalence of the haplogroups in the populations, being diminished in the group of patients analysed. Studies in groups of patients from different world regions should be carried out to clarify this point. As for the *in silico* analysis, a non functional impairment was predicted by all the softwares used. The same

resultsmay apply tom.8584G>A, m.8857G>A, m.8869A>G, m.8870T>C and m.9064G>A. Nevertheless, as discussed earlier for synonymous variations, this does not mean that these variations could not have a role in the modulation of pathophysiological process. Being so, it is difficult to demonstrate given the present scientific knowledge.

For the m.8839G>A variation, the in silico analysis gave similar results for each of the 4 softwares, predicting thatchanges of a nonpolar alanine to a polar threonine at position 105, significantly affectsthe ATP6 proteinfunction. Additionally, this amino acidand the the others surrounding this position, are conserved in all of the sequences aligned (Figure 2). Thus, this variation is probably harmful.

A change of a non polar isoleucine to a nonpolar leucine conditioned by the m.8842A>C alteration should not have a major impact in the functional outcome of the ATP6 protein, as predicted by PolyPhen, Mutation Assessor and Provean. Regardless of that, this variation occurs in the vicinity of the previously mentioned one, in a highly conserved region of the protein among species.Adding to that, its frequency in mtDB database is 0.04%, so although it might not have a direct impact on the protein function, could have a pathologic relevance.

The m.8945T>C is predicted to alter protein function in every software, except forMutation assessor. Additionally, this variation is not reported in mtDB database, meaning that it has a frequency of less than 0.04% in the population. One possible explanation is that this variation, which changes a nonpolar methionine to a polar threonine on a highly conserved site of the protein (position 140), renders a severe dysfunction in the MRC and the cell.

Despite thefact that m.8950G>A variation has been pointed out to be involved in LHONand Dystonia, according to MITOMAP database, the references do not seem to support

such hypothesis. It is apparently a tolerated mutation that occurs in a less conserved site of the protein.

The most frequent variation, found,m.9055G>A(identified in 4 patients) is reported to decrease PD risk in women and it is also a haplogroup K marker (van der Walt *et al.*, 2003). Could it be a protective factor for PD and a risk factor for FTDL? Apparently no, due to the fact that its frequency in FTLD patientsof the present study, is similar to the one reported in mtDB. This variation promotes a switch from a nonpolar alanine to a polar threonine in the amino acid 177, a conserved position in 8 of the 10 sequences (Figure 2), which is enough to have a deleterious score in Provean and in one model of the PolyPhen analysis. It appears that this variation could have some functional effect in the ATP6 protein that is notclear yet.

The variation m.9070T>Ghas been reported in associationto a pancreatic cancer cell line byJones *et al.*(2001).However, according to the present analysis, it is not expected that it could lead to functional disruption of the ATP6 protein.

The m.8393C>G alteration was reported by Galimberti*et al.* (2006) as being associated with brain pseudoatrophy and mental regression. This causes a change of a nonpolar proline to a polar serine at position 10 of the ATP8 protein; therefore, the PolyPhen score is fairly high, although all the other *in silico*tests predict that it would be a tolerated change. This could be explained by the analysis of the sequences alignment (Figure 3), where the amino acid 10 is not globally well conserved. Even if the surrounding amino acids and the closest phylogenetic species are taken into account, it is clear that this change occur in a highly conserved region of the protein among higher primates. In fact, the SIFT score approaches the cut-off value of the test. And so it is possible that the m.8393C>G variation is not only associated with the brain pseudoatrophy and mental regression, but also with FTDL.

The m.8478C>T variation is classified as deleterious by Provean software. However, the change of a polar serine to a non polar leucine in a poorly conserved aminoacid is not

enough to alter the protein function outcome in any other software used. Accordingly, itseems unlikely that this alteration could be pathogenic. A fact that could make us think otherwise is the low frequency in which is reported in mtDB database and the absence of literature concerning this variation.

The last non-synonymous mutation found in the *MTATP8* gene, m.8519G>A, seems to be pathogenic. The shift of an acidic polar glutamic acid for a basic polar lysine at the amino acid position 52, conserved in 8 of the 10 sequences, takes place in the middle of a highly conserved region of the protein (Figure 3). Theseresults reveal a deleterious effect prediction by PolyPhen, Mutation Assessor and Provean softwares. This result, in addition to the fact that the frequency of the m.8519G>A variation in 2704 reported mtDB sequences is only 0.07%, suggest that it as a probably deleterious mutation.

Although the *in silico* analysis presented showed interesting and important results, it is important to complement this study with functional studies, such as determination of the MRC activity, particularly complex V, in the patients presenting such alterations. Additionally, future functional genomics analyses will allow clarifying the pathogenic role of the mtDNA sequence variations identified in the present study.

Concerning the statistical analysis, no significant positive correlation was found between patients with variations in*MTATP6*,*MTATP8,* or both genesand their age, age of onset, CDR staging or the MMSE score. The same observation was achieved relating to clinical outcome. However, it is worth to mention that the number of patients with the language variants is insufficient to calculate a 95% confidence interval.

The existence of an association between the variations found and one of these variables would give more strength to the assumption that such variations are a contributing factor to the development of a particular clinical form of FTDL. However the absence of statistically significant association means that the variations found are not involved in the

pathophysiology of a particular form of the disease, but their effect is related to the neurodegeneration instead. On the other hand, even the majority of patients present alteration on the 2 genes investigated, *MTATP6* and *MTATP8,* they are not present in every patient and not all the patients harbour the same alterations, meaning that different alterations could lead to the same effect of impairing, even slightly, MRC function, and that there are other factors which contribute to the modulation of this complex disease. Extending the analysis to anage matched healthy control group, could add some clarification, but the comparison with the frequency of the variations from mtDB database was very helpful to address important points about the alterations found.

On the other hand, the present findings report to peripheral blood mtDNA variations. The fact that all these variations are found in homoplasmy is a good indicator that they may be present in other tissues, including the brain. Nonetheless, somatic mtDNA mutations may occur in brain cells, and thus not being present in peripheral blood (Grazina*et al.*, 2003). Testing other tissues, such as muscle or skin fibroblasts, or post-mortem brain,wouldadd more evidence to this association.

Although we are aware that it is impossible for the majority of cases, the study of maternal lineage would also be a significant contribution to the present study.

On the other hand, further studies are needed in order to replicate these findings in patients' cohorts from other world populations.

Conclusion

Our results report new data regarding the presence of mtDNA variations in patients with FTLD. None of the variations described had been associated to any neurodegenerative disorder in the references available in literature.

It is reported here that alterations m.8393C>G and m.8519G>A, both located to *ATP8* locus, a gene rarely found to be involved in human pathology, areprobably damaging mutations contributing to the development of FTLD. We also describe two variations in *MTATP6*gene, m.8839G>A and m.8945T>C, which are expected to have deleterious effectson the MRC function. In the same gene, we also reportm.8573G>A, m.8839G>A and m.8842A>C that have potential to be functionally impairing. Other variations here reported either are synonymous or the *in silico* analysis classified them as benign. However, they can also play a role in the pathophysiological mechanisms leading to complex pathologies such like FTLD. Data from literature point toboth directions: one stating that mtDNA mutations aremore likely to be a by-product, secondary to other pathologicalfeatures during the development of the disease; the otherbeing consistent with the 'mitochondrial cascade hypothesis',claiming that mtDNA modifications (both pathogenicand/or polymorphic) are a cause of the disease (Grazina *et al.*, 2006). In particular, it is possiblethat distinct combinations of nonmutated electrontransport chain components do not function identically;being so, nonpathogenic mtDNA variations could contribute to FTLD risk, similarly to what has been suggested for AD (Grazina *et al.*, 2005; Onyango *et al.*, 2006).

The mtDNA mutations might modify age ofonset, contributing to the neurodegenerative process,probably due to an impairment of MRC and/or translationmechanisms (Grazina *et al.*, 2006). Additionally, mtDNA mutations may have a

cumulative effect, increasingthe probability to develop an energy failure (Grazina *et al.*, 2004).

The report and identification of mtDNA mutations is not only a valuable contribution towards the better understanding of the FTDL etiology, but also a way to improve diagnosis protocols of this pathology in a near future, and certainly a way to develop new treatment strategies in the long term (Grazina *et al.*, 2006).

The present work is a valuable and original contribution to the knowledge of genetic factors involved in FTLD.

The more we understand about FTDL, the more we unveil its complexity, walking towards better ways to diagnose, manage and treat this neurodegenerative and fatal disease.

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Appendix 1 – Patients characterization and data from genetic analysis.

Patient	Sex	Age	Diagnostic	Age of Onset	CDR Stage	MMSE Score	mtDNA variations
1	M	73	bvFTD	73	1	28	
2	F	77	bvFTD	73	3	10	m.8994G>A
3	F	69	bvFTD	69	1	19	
4	F	68	bvFTD	66	2	15	
5	M	64	bvFTD	62	0,5	28	
6	M	45	bvFTD	40	3	7	
7	M	75	bvFTD	64	1	27	
8	F	49	PNFA	48	1	30	m.9055G>A
9	F	78	bvFTD	78	1	28	m.8869A>G m.8870T>C
10	M	58	bvFTD	44	3	6	
11	M	43	bvFTD + CBS	42	1	22	
12	M	65	bvFTD	63	0.5	29	
13	M	64	bvFTD	60	1	22	m.9055G>A
14	M	71	bvFTD	68	0.5	30	
15	M	70	bvFTD	67	1	25	
16	F	81	bvFTD	75	2	13	
17	F	77	bvFTD	64	1	21	m.8865G>A
18	M	43	bvFTD	43	2	16	
19	F	53	bvFTD	52	3	0	m.8857G>A
20	F	65	bvFTD	63	3	1	
21	F	54	bvFTD	53	2	18	
22	M	54	SD	52	1	21	
23	F	71	bvFTD	67	3	14	
24	M	51	bvFTD	51	1	25	
25	M	60	PNFA	58	3	9	
26	M	69	bvFTD	54	0.5	28	
27	F	64	bvFTD	63	1	25	
28	F	65	bvFTD	64	3	11	
29	M	46	SD	41	3	10	m.8994G>A
30	M	65	bvFTD	63	1	29	m.8485G>A
31	M	61	bvFTD	56	2	12	
32	M	67	bvFTD	59	1	27	m.8945T>C
33	M	72	bvFTD	69	2	13	m.8701A>G m.8841C>T
34	M	66	bvFTD	63	1	29	m.8512A>G
35	M	68	bvFTD	67	1	27	
36	M	70	bvFTD	69	1	20	m.8473T>C m.8842A>C
37	F	82	bvFTD	78	1	20	
38	F	69	PNFA	56	1	13	m.9055G>A m.9064G>A

Appendix 1 – Patients characterization and data from genetic analysis (continuation).

Patient	Sex	Age	Diagnostic	Age of Onset	CDR Stage	MMSE Score	mtDNA variations
39	F	62	bvFTD	59	2	17	
40	F	74	bvFTD	72	1	15	m.8584G>A m.9070T>G
41	F	48	bvFTD	47	3	0	
42	M	64	bvFTD	61	1	24	m.8697G>A
43	M	74	bvFTD	73	2	16	m.8440A>G
44	F	59	bvFTD	53	3	0	
45	F	59	bvFTD	55	3	0	
46	F	59	bvFTD	57	1	18	m.8393C>T
47	F	60	bvFTD	56	1	15	m.8839G>A
48	F	65	PNFA	62	3	4	m.9070T>G
49	F	71	bvFTD	68	2	12	m.8393C>T
50	F	58	bvFTD + CBS	58	1	18	
51	F	59	bvFTD	58	2	16	
52	F	54	bvFTD	54	1	26	
53	F	55	bvFTD	54	1	22	
54	F	54	bvFTD	50	2	19	m.8478C>T
55	F	74	bvFTD	73	1	17	
56	F	75	bvFTD	72	1	20	
57	F	62	bvFTD + CBS	60	1	20	m.8573G>A
58	F	49	bvFTD	48	0,5	30	m.8950G>A
59	F	50	bvFTD	50	2	17	m.9055G>A
60	F	74	bvFTD	69	2	15	
61	F	75	bvFTD	74	1	27	
62	F	46	bvFTD	43	1	22	
63	F	81	bvFTD	79	1	21	
64	F	66	bvFTD	60	3	16	m.8994G>A
65	M	48	bvFTD	45	2	13	m.8697G>A m.8701A>G
66	M	38	bvFTD	34	1	25	
67	M	54	bvFTD + CBS	53	3	0	
68	M	70	bvFTD	68	1	22	m.8512A>G
69	M	76	bvFTD	70	1	28	m.8519G>A
70	M	59	bvFTD	56	1	21	m.8577A>G m.9123G>A

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Revised July 2012

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CONFLICT OF INTEREST DISCLOSURE

Wiley-Blackwell requires that all authors disclose any potential sources of conflict of interest. Any interest or relationship, financial or otherwise, that might be perceived as influencing an author's objectivity is considered a potential source of conflict of interest. These must be disclosed when directly relevant or indirectly related to the work that the authors describe in their manuscript. Potential sources of conflict of interest include but are not limited to patent

or stock ownership, membership of a company board of directors, membership of an advisory board or committee for a company, and consultancy for or receipt of speaker's fees from a company. The existence of a conflict of interest does not preclude publication in this journal.

If the authors have no conflict of interest to declare, they must also state this at submission.It is the responsibility of the corresponding author to review this policy with all authors and to collectively list in the cover letter to the Editor-in-Chief, in the manuscript (under the Acknowledgments section), and in the online submission system ALL pertinent commercial and other relationships.

EDITORIAL FEATURES

Research Articles

Describe functional/structural consequences of mutations in proteins.

Examine important genotype/phenotype relationships, especially in context of multiple mutations, SNPs, and/or novel gene(s) in single-gene disorders or complex diseases.

Apply large-scale mutation and polymorphism detection or screening techniques for clinical diagnosis and counseling.

Highlight identification and use of mutations in forensics, disease diagnosis, tissue typing, and cancer.

Describe a significant number of related novel mutations in expressed genes.

Describe in detail mutations in other species that have relevance to human disease.

Describe in-depth functional studies of novel single mutations in newly cloned genes or unique cases, when of demonstrated significance.

Brief Reports

Are concise (~2-4 page), high-impact observations relevant to medical and molecular genetics. Evaluation is highly selective and only a few will be published per issue. Brief Reports describe:

Original discoveries of novel disease-causing genes of broad interest, with phenotypic data but with little functional analysis;

Descriptions of one or a few novel disease mutations with basic functional studies that the editors feel provide key insights into the biological basis of inherited Mendelian or common disease or phenotype;

Other observations relevant to medical and molecular genetics, at the editors' discretion.

The editors welcome author inquiries regarding potential content. Please contact the Managing Editor with questions (humu@wiley.com).

Rapid Communications

Report exceptionally timely new research results. Contact the Managing Editor at humu@wiley.com before submission for advice or appropriateness.

Methods Articles

Detail novel methods for mutation detection, analysis, and use in screening.

Report in-depth comparative analysis of different detection methods; should include cost/benefit analysis and/or discuss quality control.

Describe progress useful in mutation collection, documentation, databasing and distribution.

Mutation Updates

Briefly review and summarize all mutations and polymorphisms in specific genes, including functional and clinical significance.

Synthesize the spectrum of mutations in genes with particular research or medical interests.

Highlight relevant animal models.

Include sections discussing genotype-phenotype correlation, diagnostic relevance, clinical implications, and future prospects.

In cases where a significant number of novel mutations/polymorphisms accompanied with detailed functional analysis are to be reported, the Editors may consider co-review and co-publication of a separate Research Article presenting such variants.

Please write the Reviews Coordinator, Rania Horaitis (r.horaitis@hfi.unimelb.edu.au), to suggest a topic and to receive the journal's Mutation Update author guidelines (updated 2009).

Review Articles

Summarize information on gene families or regions and their function and clinical significance.

Analyze strategies in the clinical investigation of mutations.

Survey methodologies or diagnostic strategies relevant to the field of mutation/SNP detection, clinical diagnosis, etc.

Survey mutation databases or programs used for databases.

Please write the Reviews Coordinator, Rania Horaitis (r.horaitis@hfi.unimelb.edu.au), to suggest a topic.

Informatics

Describe software tools, platforms, etc., for bioinformatic analysis.

Database Articles

Describe individual locus-specific (LSDB) or central mutation databases and related software or bioinformatic technologies.

Include data analysis that provides novel biologic information gained from the database.

Discuss current issues of importance regarding genome variation databases.

At the editors' discretion, Database articles that are notable, concise, descriptive, but that do not provide significant information or insight regarding the biology of mutations, may be published as a Database in Brief (DIB), an online-only article type.

Simple descriptions of LSDBs or other databases that in the editors' view do not add sufficiently to the published literature will not be considered.

Special Articles

Cover a wide range of topics pertinent to the field, including nomenclature, genetic testing, bioinformatics, and ethical issues.

Usually invited. Suggestions are welcome. Please contact the Managing Editor at (humu@wiley.com).

Letters to the Editors

Peer-reviewed general correspondence. Letters commenting on and/or calling into question research published in Human Mutation will be sent to the original author for rebuttal if they pass peer and editorial review.

CRITERIA FOR ACCEPTANCE

Ethical Compliance

Authors are responsible for confirming that research performed on human subjects complies with standards established by an appropriate ethics review committee (IRB in the United States) and the granting agency. If the manuscript includes data or description of patients, the authors should provide (1) a statement in the manuscript that the research was prospectively reviewed and approved by a duly constituted ethics committee OR (2) a statement in a cover letter to the editors that the manuscript is a retrospective case report that does not require ethics committee approval at that institution. For Database articles: it should be stated that patient clinical data have been obtained in a manner conforming with IRB and/or granting agency ethical guidelines. Any other situations not covered here should be discussed with the editorial staff.

Patient Anonymity and Pedigrees

To protect patient confidentiality, authors must not use actual patient names or initials in the manuscript text, tables, or pedigrees. Please use a coded designation instead. Patient pedigrees should only be used when absolutely necessary, and the editors may request their omission. Pedigree nomenclature guidelines should be as stipulated in Bennett et al. AJHG 56:745–52, 1995. At the editor's discretion, patient photos can be included if proper consent is provided.

Animal Research Data

Animal experiments should be in keeping with ARRIVE guidelines (published in PLoS Biology http://dx.doi.org/10.1371/journal.pbio.1000412; for more information see http://www.nc3rs.org.uk/ARRIVE). These guidelines describe the minimum information necessary to ensure that the reporting of animal experiments is transparent and comprehensive.

Validation of Mutations (approved by the HUGO Mutation Database Initiative/Human Genome Variation Society)

Identified mutations resulting in disease should be confirmed on a second sample. Mutations detected by PCR methods must be confirmed on a second PCR product.

The complete coding sequence should have been scanned or sequenced to eliminate the possibility of the presence of other mutations.

For disease-causing mutations, it must be established by at least two methods (in absence of expression studies, and with the exceptions of generated stop codons and deletions) that the mutation likely causes disease. Possible methods include linkage to disease in a family,

concurrent appearance of the phenotype with a de novo mutation, or determination that the mutation is absent among at least 50 normal individuals (100 alleles) or involves a highly conserved amino acid.

Submission of Data to Genetic Databases

Human Mutation supports the recommendations of the Human Variome Project (Cotton RGH et al. 2007. Nat Genet. 39: 433 http://www.nature.com/ng/journal/v39/n4/full/ng2024.html). Consequently, authors are required to submit all variants included in an article to the respective Locus Specific Database (LSDB) prior to acceptance. (Submission to LSDB is a requirement for Mutation Updates if such an LSDB is available.) In the case of dbSNP, the identification numbers should be used to describe the SNPs in the manuscript. Authors must confirm the status of database submission in their cover letter. In addition, authors should note in the manuscript (e.g., in the methods section) the LSDB(s) to which they have submitted their variants and provide the URL. The Editors also encourage the use of widely accessible genetics databases as repositories for human gene mapping information, including loci (genes, fragile sites, DNA segments), and probes. Further information and updates on this policy, including links to Locus-Specific Databases, can be obtained from the Human Genome Variation Society (HGVS) web site http://www.hgvs.org/dblist/dblist.html.

Microarray data should be MIAME compliant (for guidelines see http://www.mged.org/Workgroups/MIAME/miame.html).

Conventions and Nomenclature

MUTATIONS AND POLYMORPHISMS

Because of the importance of the issue and the overall consensus on the rules, Human Mutation is adopting an editorial policy that requires absolute compliance with the rules to describe sequence variants before manuscripts will be accepted and published.

The most current guidelines are summarized on the Mutation Nomenclature Homepage at the HGVS website (http://www.hgvs.org/mutnomen/). Examples of acceptable nomenclature are also provided. Important considerations include:

Variants should be described in the text and tables using both DNA and protein designations whenever appropriate.

If alternative nomenclature schemes are commonly found in the literature, they may also be used in addition to approved nomenclature, but they must be defined clearly.

Variants may be described using dbSNP identifiers (e.g., rs123456:A>G).

Authors should always include the GenBank Accession Number of the relevant wild-type gene sequence(s), with version number (e.g.: RefSeq NM_123456.3 or GenBank U654321.1), in the Materials and Methods section and as a footnote in tables listing mutations.

Acceptance and/or publication may be delayed if authors are unable to follow the guidelines properly. Authors are advised to check sequence variant descriptions using the Mutalyzer program (http://www.LOVD.nl/mutalyzer/). Using batch mode, all variants can be analyzed at once. Recently, an update to the mutation nomenclature was published regarding complex rearrangements (Taschner and den Dunnen, Hum Mutat 32:507-511, 2011). In addition, please see the article Wildeman et al., Hum Mutat 29:6-13, 2008. Authors should also refer to

den Dunnen and Antonarakis (Hum Mutat 15:7–12, 2000) and den Dunnen and Paalman (Hum Mutat 22:181-182, 2003) for additional information. Visit www.hgvs.org/mutnomen/ for the latest nomenclature updates or if you have further questions.

GENE SYMBOLS

All manuscripts must include (when available) HGNC-approved gene symbols and OMIM database reference numbers (visit their new website at http://www.omim.org) for genes and/or disorders. Approved human gene symbols should be obtained prior to submission from the HUGO Gene Nomenclature Committee (HGNC), at www.genenames.org or by email to hgnc@genenames.org. In addition, commonly used alternative gene and disease symbols may also be used in the abstract (180–200 words) and key words. Note: OMIM entries now clearly indicate the most current HGNC-approved gene symbol, but it may not be listed in the main title.

CONSIDERATIONS FOR REVIEW AND ACCEPTANCE

Priority scores given by reviewers and the Editorial Board, and available space, will be used to determine acceptance or rejection. In the interest of space, page limits will be strictly enforced at the discretion of the Editors. Online use of supporting information may be requested.

MANUSCRIPT SUBMISSION

Authors should submit their manuscript directly to the journal via the online submission system, available at http://mc.manuscriptcentral.com/humu/. A cover letter should include a list of 2-3 Communicating Editors whom the author would consider appropriate to administer the paper. (The most up-to-date list of Communicating Editors can be found on the journal home page.) The Editorial Office will make every effort to accommodate these requests. If the manuscript is considered by the Editorial Board as a candidate for peer review, the Managing Editor will work with the Communicating Editors and/or Co-Editors to coordinate peer review and will receive recommendations regarding publication or rejection. Please note that Communicating Editors cannot communicate work from their own laboratories and are discouraged from communicating work from colleagues at their own institutions.

IMPORTANT: If the manuscript was invited, please indicate so, and by whom, in a cover letter.

Redundant material such as a repeat of often-described background or previously defined methodology should be avoided by referring to reviews or recent papers.

Authors who have questions about the appropriateness of their work for Human Mutation or questions about submissions should contact the Managing Editor, Dr. Mark H. Paalman, at the Editorial Office (humu@wiley.com).

Delayed revisions policy: Following review, any manuscript for which revision has been requested must be returned within three months or it will be considered withdrawn. A revised manuscript sent after three months may be treated as a new submission, subject to any new editorial policies.

All Manuscripts

Formatting of manuscripts: Manuscripts must be formatted as double-spaced, 8½ x 11 inch or A4 documents with 1" margins all around. A 12-point font (preferably Times New Roman) should be used, but common Greek letters, symbols, or special characters are allowed.

Color figures policy: Color figures are welcome but they are published in the print journal at cost to the author; quotes will be provided from the publisher upon acceptance. All color figures will be reproduced in full color in the online edition of the journal at no cost to authors. Authors are encouraged to submit color illustrations that convey essential scientific information. For best reproduction, bright, clear colors should be used. Dark colors against a dark background do not reproduce well; please place your color images against a white background wherever possible. There are no page charges for publication, other than for color reproduction. Please contact Production Editor Tom O'Brien at humuprod@wiley.com for further information.

Article types:

Research Articles, Methods, and Rapid Communications must contain sections in the following order: Title Page, Abstract (200 words max), Key Words, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, References, and Figure Legends. Tables and figures must be submitted as separate files. Long tables and excessive figures may be published as online Supporting Information. Use generic names of drugs and give manufacturer of all trademarked equipment mentioned in the text. Research Article page limits are normally 20–30 double-spaced manuscript pages, including references. Methods

articles should not exceed 25 manuscript pages with references. Rapid Communications should not exceed 20 manuscript pages with references and may include 1–2 tables and 2 figures as needed. To facilitate rapid review of Rapid Communications, kindly contact the Managing Editor prior to submission of the manuscript for advice on appropriateness of subject.

Brief Reports are limited to 12 double-spaced manuscript pages max with references and a maximum of 2 figures/short tables. Abbreviated methods and minimal references are allowed. A title page and an abstract of 150 words are required. The main text should then be continuous, not subdivided with headings. Authors are encouraged to supplement Brief Reports with online supporting information as necessary, in order to maintain the brief format.

Informatics and Database Articles must include an Abstract (200 words max), but otherwise can contain headings appropriate to the subject discussed. Figures and tables are encouraged. Length should be approximately 18–25 double-spaced manuscript pages with references. Extra data can be published online. If the editors suggest instead a Database in Brief (DIB) format, further instructions will be provided by the Editorial Office.

Mutation Updates must include an Abstract (180–200 words), Key Words, and specific headings such as Mutations and Polymorphisms Defined, Genotype-Phenotype Correlation, Biological Significance, Clinical Significance, Diagnostic Strategies, and Future Prospects. They normally range from 15–20 double-spaced pages of text without references. Clear tables with the correct mutation nomenclature are required and figures are encouraged. Mutation Updates are usually invited but the Editors welcome proposals and suggestions for topics. Authors may use Mutation Updates to publish some novel mutations/polymorphisms for the

first time only if accompanied by verification of variant sequence and proof of causation (see Validation of Mutations, above). Very long tables and/or extra data can be published online as Supporting Information.

Important: Please write Rania Horaitis (r.horaitis@hfi.unimelb.edu.au), to suggest a topic and to receive the latest author guidelines (new as of April 2008).

Review Articles cover a specific topic through an appropriate literature survey. An Abstract (180-200 words) and Key Words are required. Although there are no restrictions on the number of pages or figures, the Review should be as concise as possible. Review Articles are usually invited. The Editors welcome proposals and suggestions for topics.

REFERENCE STYLE

The accuracy of references is the responsibility of the authors. Only published papers and those in press may be included in the reference list. Unpublished data and submitted manuscripts must be cited parenthetically within the text. Personal communications should also be cited within the text; permission in writing from the communicator is required. References cited only in Supporting Information should still be added to the main Reference list (confirm if this is the case in a cover letter).

Citations in the text should be made by author name followed by year of publication, arranged chronologically and then alphabetically (e.g., Hershkovitz and Leipe, 1998; Jones, 2000). For three or more authors, use the first author's name and "et al." in citation. When references are made to more than one paper by the same author, published in the same year, they are to be

designated as a, b, etc. in alphabetical order of the second author (e.g., Smith et al., 1998, 2000a, 2000b).

In the reference list, references are to be arranged alphabetically by author and then by year of publication. List only the first 12 authors, followed by "et al.". Journal titles should be abbreviated according to Index Medicus; the titles of unindexed journals should be spelled out in full. Examples:

Journal article

Pollin TI, Dobyns WB, Crowe CA, Ledbetter DH, Bailey-Wilson JE, Smith ACM. 1999. Risk of abnormal pregnancy outcome in carriers of balanced reciprocal translocations involving the Miller-Dieker Syndrome (MDS) Critical Region in Chromosome 17p13.3. Am J Med Genet 85:369–375.

Book

Reece RJ. 2004. Analysis of Genes and Genomes. New York: Wiley-Liss. 469 p.

Chapter in a Book

Hunter AGW. 2005. Down syndrome. In: Cassidy SB, Allanson JE, editors. Management of Genetic Syndromes, 2e. New York: Wiley-Liss, p 191–210.

References to government publications should include the department, bureau or office, title, location of the publisher, year, pages cited, and the publication series, report, or monograph.