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Mitochondrial genome analysis in frontotemporal lobar degeneration: *tRNAs* contribution

Análise genómica mitocondrial na degerescência lobar frontotemporal:contribuição dos tRNAs

Isabel Maria Lopes de Matos Oliveira

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Evolução e Biologia Humanas, realizada sob a orientação científica da Professora Doutora Manuela Grazina (Universidade de Coimbra, Faculdade de Medicina) e sob a orientação interna da Professora Doutora Eugénia Cunha (Universidade de Coimbra, Faculdade de Ciências e Tecnologia)

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Resumo

A degenerescência lobar frontotemporal (DLFT) é uma demência neurodegenerativa heterogénea, incluindo nos aspectos clínicos, neuropatológicos e genéticos. É caracterizada por mudanças progressivas no comportamento, disfunção executiva e/ou dificuldades na linguagem, acompanhada por atrofia no lobo frontal e temporal. Alguns doentes apresentam sobreposição clínica e neuropatológica com a doença de Alzheimer, o que sugere semelhanças na fisiopatologia, nomeadamente no envolvimento do DNA mitocondrial (mtDNA).

O objetivo do presente estudo é realizar a sequenciação dos genes no mtDNA que codificam tRNAs, para identificar alterações nos doentes com DLFT, investigando o seu envolvimento na DLFT.

Foram analisadas 70 amostras de DNA provenientes de doentes, 39 mulheres e 31 homens, com diagnóstico provável de DLFT (faixa etária: 38-82 anos, média de 63 ± 11), seguidos na Unidade de Neurologia do Centro Hospitalar e Universitário de Coimbra. O DNA total foi extraído a partir de sangue periférico, e foi efetuada a análise da sequência dos 22 genes de tRNAs mitocondriais, por sequenciação automática. As variantes encontradas foram submetidas a análise *in silico*. Foram encontradas 28 variações diferentes em 32 doentes. Destas, seis variações são provavelmente patogénicas de acordo com a análise *in silico*: a m.4312C>T ocorre em heteroplasmia e apresenta elevada conservação; a m.4435A>G está localizada numa posição potencialmente crítica e é totalmente conservada em todas as espécies analisadas; a variação m.5772G>A está localizada no *T-stem*, levando ao rompimento do emparelhamento da base (CG) Watson-Crick e é 100% conservada; a alteração m.12166T>C está localizada no "anticodon loop" e apresenta alta percentagem de conservação. A variação mais frequente é a m.12308A>G, no *mt-tRNA*^{Leu2},na região variável, e é totalmente conservada em todos os mamíferos estudados. A variação m.15946C>T tem uma elevada taxa de conservação e está localizada no "acceptor stem". São necessários estudos adicionais para compreender melhor a relação entre as alterações do mtDNA identificadas e a FTLD. No entanto, este estudo é original sendo o primeiro a investigar a sequencia dos genes que codificam os tRNA mitocondrias na DLFT.

1. General Introduction

1.1. Dementia

In the last years, life expectancy has increased at a steady rate, which leads to an increment the percentage of elderly in the population (Santana and Cunha, 2005). The decline in mortality and births results in aging of the population in most developed countries and, consequently, the prevalence of age-related diseases, including dementias, are increasing. Dementia syndromes are characterized by progressive impairment in cognitive function and they have become increasingly important in public health (Fratiglioni et al., 1999). Among the various dementias, Alzheimer's disease (AD) and Frontotemporal Lobar Degeneration (FTLD) are the most frequent in the population (Santana and Cunha, 2005).

1.2. FTLD History

In 1892, Arnold Pick described the first clinical case of dementia, in which the patient presented cognitive impairment, progressive aphasia and changes in social behaviour. These manifestations are associated with temporal and frontal lobe atrophy (Kertesz et al., 2005). In 1911, Alois Alzheimer described the histopathology features of these patients, pointing to the presence of argyrophilic neuronal inclusions, later called “Pick bodies” (Pan and Chen, 2013). A century later, research groups of Lund (Sweden) and Manchester (England) published the first clinical and neuropathological criteria set for the diagnosis of frontotemporal dementia (FTD) (Lund and Manchester Groups, 1994).

1.3. FTLD Epidemiology

FTLD is a heterogeneous neurodegenerative dementia in many aspects, including clinical, neuropathological and genetic features, characterized by progressive

changes in behaviour, executive dysfunction and/or language impairment (Seltman and Matthews, 2012) with frontal and temporal lobar atrophy (Pan and Chen, 2013). FTLD occurs most often in the presenile period, with age at onset typically at 45-65 years, and it has an equal distribution among female and male (Galimberti and Scarpini, 2010). There is a wide range in duration of illness (2-20 years) partly reflecting different underlying pathologies (Seelaar et al., 2011).

1.4. Clinical variants of FTLD

Given its heterogeneity, FTLD is classified differently regarding the clinical characteristics. The site of focal cerebral atrophy, frontal and/or temporal, left and/or right determines the clinical presentation (Schlachetzki, 2011). It can be differentiated clinically into three frontotemporal dementia (FTD) syndromes; clinically behavioural variant (bvFTD), characterized by progressive behavioural impairment and a decline in executive function with frontal lobe atrophy; progressive nonfluent aphasia (PNFA) with motor speech deficits and semantic dementia (SD) with loss of object knowledge with anomia (Seltman and Matthews, 2012). Language variants are subsumed under the clinical syndrome of primary progressive aphasia (PPA) and show involvement of the left anterior temporal lobe (Schlachetzki, 2011). Additionally, there is a clinical overlap between FTD with motor neuron disease (FTD-MND or FTD-ALS), as well as the parkinsonian syndromes, progressive supranuclear palsy (PSP) and corticobasal syndrome (CBS) (Pan and Chen, 2013).

1.5. FTLD Neuropathological variants

The neuropathology underlying the FTLD clinical syndromes is also heterogeneous (Boxer et al., 2011). While clinical phenotype, neuropsychology features and brain imaging data provide useful information about the FTLD spectrum pathology, additional information is necessary to define the histopathological abnormality in patients, since a clinical phenotype can be associated with several different pathologies (Grossman, 2011). The major pathological hallmark of FTLD is selective atrophy of the frontal and temporal cortex, with neuronal loss and gliosis (Seelaar et al., 2011). In most cases, it is possible to find an accumulation of abnormal proteins in neurons and glia

(inclusions of aggregates). The identity of the pathological protein is variable (Boxer at al., 2011) and the classification has been changing in the last few years. The currently accepted nomenclature for the various FTLD neuropathological subtypes considers into five groups, FTLD-tau (tau pathology), FTLD-TDP (TAR-DNA binding protein (TDP-43)), FTLD-UPS (ubiquitin-positive and TDP-43-negative histopathology), FTLD-FUS (inclusions of the fused in sarcoma protein) and FTLD-ni (without inclusions) (Mackenzie et al., 2010). It was found that there is a correlation between neuropathological characteristics and genetic mutations (Table I).

Table I: Neuropathological characterization of FTLD and associated genes (adapted from Mackenzie et al., 2010)

FTLD-tau	**FTLD with tau-negative and ubiquitin-positive**			**FTLD-ni**
	FTLD-TDP	**FTLD-FUS**	**FTLD-UPS**	
Mutation in the gene MAPT	Mutation in genes *GRN* *VCP* *TARDBP* *C9ORF72*	Mutation in the gene *FUS*	Mutation in the *CHMP2B*	No known mutations

About 40% of FTLD cases are FTLD-tau, including all cases of FTLD with *MAPT* mutations. The most frequent FTLD pathology cases are characterized by tau-negative and ubiquitin-positive inclusions the FTLD-TDP, patients have mutations in *GRN*, *TARDBP*, *VCP* or *C9ORF72* genes; CHMP2B cases show ubiquitin-positive inclusions; TDP-43 negative histopathology (FTLD-UPS) and many of the ubiquitin-positive, TDP-43- negative cases have been show to have FTLD-FUS. However, the majority of these patients do not harbour mutations in *FUS* gene (Goldman et al., 2011).

1.6. Genetic variants of FTLD

Positive family history was observed in 40-50% of the FTLD patients (Sieben, et al., 2012). The autosomal dominant mode of inheritance has been described in 10-27% of all FTLD patients with mutations identified. The familial cases are more common as bvFTD and less frequent in patients with SD and PNFA. The genetic heterogeneity of FTLD is reflected by the identification of mutations in several nuclear genes. The most common mutations occur in the *GRN* and MAPT genes in approximately 50% of the familial cases, while more rare mutations occur in the *CHMP2B*, *FUS*, *VCP* and *TARDBP* genes (Seelaar et al., 2011). Recently, a gene responsible for FTLD has been discovered on chromosome 9p (*C9ORF72*). More than 40 pathogenic *MAPT* mutations have been described in 134 families and 69 different mutations in *GRN* gene have been described in 231 families (Galimberti e Scarpini, 2012) (Table II).

Table II: Genetic characterization of FTLD (adapted from Schlachetzki, 2011)

Gene	Chromosome	Protein
MAPT	17q21.1	Microtubule associated protein tau
GRN	17q21.31	Progranulin
VCP	9p13.3	Valosin-containing protein
TARDBP	1p36.2	TAR DNA-binding protein TDP-43
CHMP2B	3p11.2	Charged multivesicular body protein 2B
FUS	16p11.2	Fused in sarcoma protein
C9ORF72	9p13.2-21.3	Not determined

1.7. Etiological mechanisms of FTLD

Some FTLD patients present a clinical and neuropathological overlap with AD. This event may suggest similarities in pathophysiology, including the involvement of mitochondrial DNA (mtDNA) in FTLD (Grazina et al., 2004), as it has been observed

in AD (Onyango et al., 2006). There are several studies that have identified mtDNA mutations in AD patients, suggesting the existence of causal factors related to mtDNA. This points to the involvement and contribution of mitochondrial genome to dementia (revision Grazina et al., 2006). Accordingly, it is important to study the role of mtDNA in FTLD (Grazina et al,. 2004).

1.8. Mitochondrial DNA study in FTLD

A number of essential cellular functions take place in the mitochondria. However, the major mitochondrial event is the production of adenosine -5`-triphosphate (ATP), the key energy source of the cell (Morán et al., 2012). Mitochondrial ATP is generated via oxidative phosphorylation (OXPHOS), that occurs in the mitochondrial respiratory chain (MRC), located within the inner mitochondrial membrane (Reddy, 2008). The process of OXPHOS system comprises five multiprotein complexes. Each OXPHOS complex consists of polypeptide subunits encoded by nuclear and mitochondrial DNA, except complex II, which is exclusively encoded by the nuclear genome (Grazina, 2004). The correct biosynthesis of the OXPHOS complexes is a highly intricate regulated process that requires the concerted action of the two cellular genomes (Morán et al., 2012). Human mtDNA consists of a 16,568 nucleotides organized in a double stranded, circular DNA molecule, containing 37 genes, encoding for 12S and 16S rRNA, 22 tRNAs and 13 polypeptides , essential components of the MRC (Reddy, 2008). The 2 rRNA, 14 tRNAs and 12 proteins are encoded on the heavy strand (HS), while 8 tRNAs and 1 protein are encoded on the light strand (LS) (Clayton, 1984) (Figure I). The remaining mitochondrial factors are encoded by nuclear genome and are subsequently transported into the mitochondria (Nicholls et al., 2013), including the proteins involved in mt-RNA metabolism.

The mitochondrial genome has its own genetic code, is regulated semi-autonomously, which depends on the replication and transcripton factors of nuclear origin, being the essential regulatory sequences located at the D-loop (non-coding region of the mitochondrial genome). DNA replication is bidirectional, starts from the origin of replication of HS (O_H), located in the D-loop going clockwise, to allow the synthesis of HS. After, the replication of LS begins, in the opposite direction of the HS replication. Moreover, mtDNA transcription results in an asymmetrical way, as the two

chains promoters, heavy strand promoter (PH) and light strand promoter (PL) are both located on the D-loop. The two promoters work in opposite directions, and HS is transcribed in the opposite to clockwise direction (Grazina, 2004).

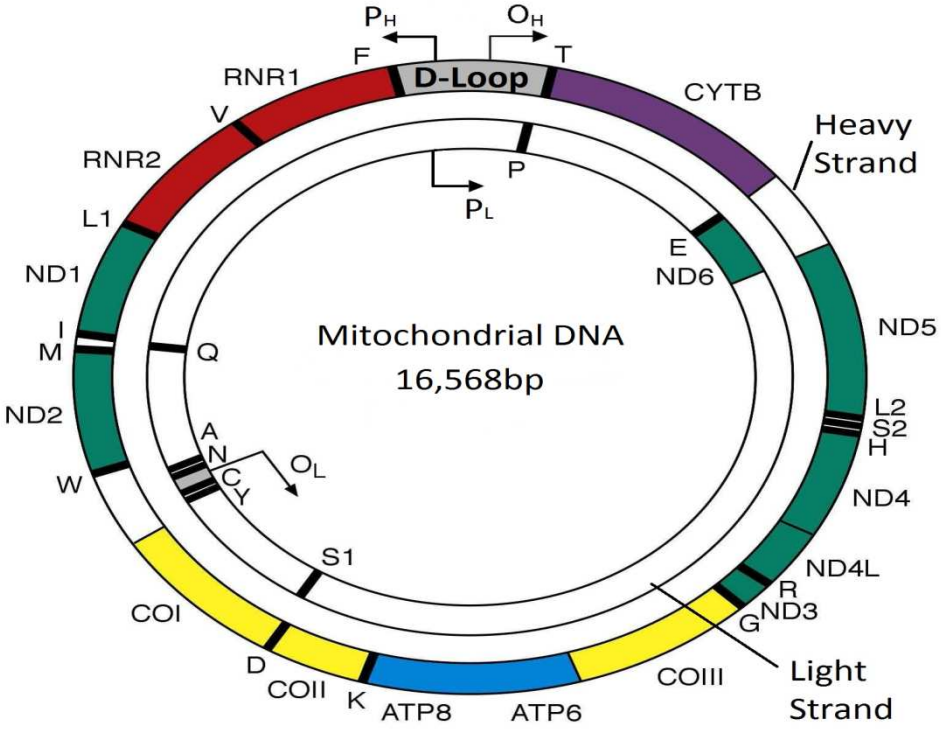

Figure I: **Human mitochondrial DNA** (adapted from Greaves et al., 2012). ND1 – NADH dehydrogenase, subunit 1; ND2 – NADH dehydrogenase, subunit 2; ND3 – NADH dehydrogenase, subunit 3; ND4 – NADH dehydrogenase, subunit 4; ND4L – NADH dehydrogenase, subunit 4L; ND5 – NADH dehydrogenase, subunit 5; ND6 – NADH dehydrogenase, subunit 6; CYTB – Cythocrome B; COI – cytochrome c oxidase I; COII – cytochrome c oxidase II; COIII – cytochrome c oxidase III; ATP6 – ATP synthase F0 subunit 6; ATP8 – ATP synthase F0 subunit 8; RNR1 – Mitochondrially encoded 12S RNA; RNR2 – Mitochondrially encoded 16S RNA; T - Mitochondrially encoded tRNA threonine; P - Mitochondrially encoded tRNA proline; E - Mitochondrially encoded tRNA glutamic acid; L2 - Mitochondrially encoded tRNA leucine 2; S2- Mitochondrially encoded tRNA serine 2; H- Mitochondrially encoded tRNA histidine; R- Mitochondrially encoded tRNA arginine ; G- Mitochondrially encoded tRNA glycine; K- Mitochondrially encoded tRNA lysine; D- Mitochondrially encoded tRNA aspartic acid; S1- Mitochondrially encoded tRNA serine 1; Y- Mitochondrially encoded tRNA tyrosine **;** C-Mitochondrially encoded tRNA cysteine; N-Mitochondrially encoded tRNA asparagines; A- Mitochondrially encoded tRNA alanine; W- Mitochondrially encoded tRNA tryptophan; M- Mitochondrially encoded tRNA methionine: I- Mitochondrially encoded tRNA isoleucine; Q- Mitochondrially encoded tRNA glutamine; L1- Mitochondrially encoded tRNA leucine 1; V- Mitochondrially encoded tRNA valine; F- Mitochondrially encoded tRNA phenylalanine.

Most human cells contain hundreds of mitochondria and thousands of mtDNA copies. This genome is transmitted by maternal inheritance. Due to this fact, maternal

and paternal mtDNAs are rarely mixed in the same cytoplasm, not having recombination between the two types of mtDNA. The only way that mtDNA sequence may change is through the accumulation of mutations along the maternal lineage. The high mutation rate of mtDNA results from the lack of protective histones, and inefficient mtDNA repair systems (Wallace, 1994). In the OXPHOS process, besides ATP synthesis, there is also the reactive oxygen species (ROS) production and mtDNA is located close to the main source of ROS formation, being vulnerable to damage (Reddy and Reddy et al., 2011). The first mtDNA pathogenic mutations were identified in the late 1980s. Since then, more than 200 mutations in mtDNA were found (Chinnery, 2006). When there is a mtDNA mutation, a mixture of wild and mutant molecules could coexist and this situation is called heteroplasmy; on the other hand, the presence of pure wild or pure mutant molecules are called homoplasmy. In case of heteroplasmy, as the percentage of mutant molecules increases, oxidative phosphorylation enzyme activities decrease. When the energy threshold is reached, the probability of disease manifestation becomes higher (Wallace, 1994). The percentage of mutated DNA may vary in different patients, from organ to organ and even between cells within of the same tissue (Chinnery, 2006).

Mitochondrial changes, including MRC dysfunction due to enzymatic defects, increases ROS production. Morphological changes in the mitochondrial network and cell death are common features of neurodegenerative diseases of different genetic origins. Mutations in genes encoding proteins involved in mitochondrial dynamics were identified in neurodegenerative diseases (Móran et al., 2012).

1.9. Human Mitochondrial tRNAs

Mitochondrial genome encodes 22 tRNAs ($tRNA^{Phe}$, $tRNA^{Val}$, $tRNA^{Leu1}$, $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Ser1}$, $tRNA^{Asp}$, $tRNA^{Lys}$, $tRNA^{Gly}$, $tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Ser\ 2}$, $tRNA^{Leu\ 2}$, $tRNA^{Gln}$, $tRNA^{Thr}$ and $tRNA^{Pro}$), essential to intramitochondrial protein synthesis. Amino acids are added to the protein during translation, by transfer RNAs. Each tRNA molecule is encoded by a different gene and its transcriptional nucleotide sequence results in a pre-tRNA that is organized into a characteristic secondary structure. This structure, common to the pre-tRNA and tRNA, is clover-shaped due to the hydrogen bonds established between

complementary bases (Cooper and Hausman, 2007). The tRNA is composed by acceptor stem, D-stem, D-loop, anticodon stem, anticodon loop, variable region, T-loop and T-stem (Suzuki et al., 2011), as presented in Figure II.

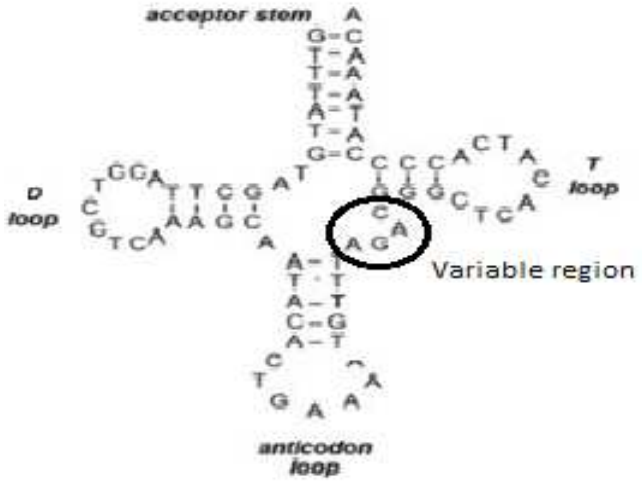

Figure II: **Structure of a *mt-tRNA*** (adapted from Yarham et al., 2011).

The pre-tRNA processing leads to their maturation into RNA and its activation. The processing of mitochondrial tRNAs requires precise endonucleolytic cleavage at both 5` and 3` ends. Excision of tRNAs is catalyzed by two specialized enzymes (Levinger et al., 2004), RNase P (the tRNA 5´ end processing endonuclease) and tRNase Z (the tRNA 3´ end processing endonuclease). After excision, pre-tRNA 3` ends are subsequently completed by the addition of the "CCA" triplet through an ATP (CTP) – tRNA-specific nucleotidyltransferase activity (Rossmanith et al.,1995) necessary for amino acid attachment and proper tRNA positioning at the ribosome (Nicholls et al., 2013)**.** Once the tRNA is synthesized, the fidelity of protein synthesis depends on the specific attachment of amino acid to its specific tRNA. This process is catalyzed by aminoacyl-tRNA synthetases (aaRS) (Suzuki et al., 2011). The human genome encodes one enzyme per amino acid. The tRNAs are subsequently carried to the ribosome by translation factor elongation factor (EF)-Tu, allowing protein synthesis (Levinger et al., 2004), as presented in Figure III.

The encoding system in mitochondrial genome is different from the universal genetic code because of the use of "AUA" for Met, "UGA" for Trp and "AGR" R = A

and G) as stop signal (Suzuki et al., 2011). The genetic mtDNA code has sixty codons for the 22 mt-tRNAs (Suzuki et al., 2011), as presented in (Table III).

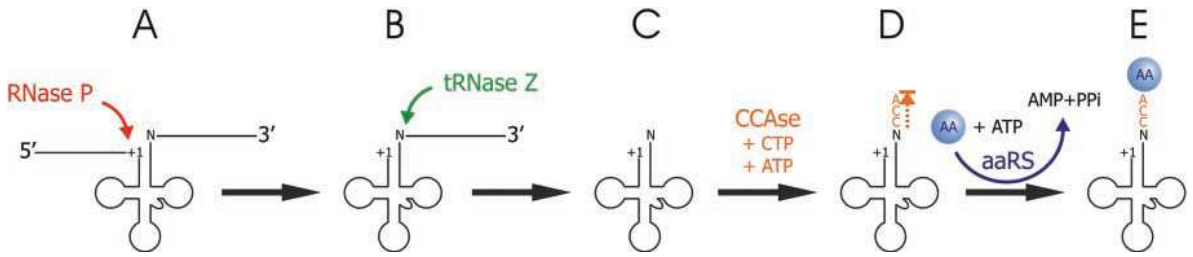

Figure III: The tRNA end processing pathway followed by aminoacylation (adapted from Levinger et al., 2004).

Table III: Human mitochondrial genetic code (from Suzuki et al., 2011).

Codon	Amino acid (anticodon)	Codon	Amino acid (anticodon)	Codon	Amino acid (anticodon)	Codon	Amino acid (anticodon)
UUU	Phe	UCU		UAU	Tyr	UGU	Cys
UUC	(GAA)	UCC	Ser	UAC	(QUA)	UGC	(GCA)
UUA	Leu	UCA	(UGA)	UAA	stop	**UGA**	Trp
UUG	(τm^5UAA)	UCG		UAG		UGG	(τm^5UCA)
CUU		CCU		CAU	His	CGU	
CUC	Leu	CCC	Pro	CAC	(QUG)	CGC	Arg
CUA	(UAG)	CCA	(UGG)	CAA	Gln	CGA	(UCG)
CUG		CCG		CAG	($\tau m^5 s^2$UUG)	CGG	
AUU	Ile	ACU		AAU	Asn	AGU	Ser
AUC	(GAU)	ACC	Thr	AAC	(QUU)	AGC	(GCU)
AUA	Met	ACA	(UGU)	AAA	Lys	**AGA**	stop
AUG	(f^5CAU)	ACG		AAG	($\tau m^5 s^2$UUU)	**AGG**	
GUU		GCU		GAU	Asp	GGU	
GUC	Val	GCC	Ala	GAC	(QUC)	GGC	Gly
GUA	(UAC)	GCA	(UGC)	GAA	Glu	GGA	(UCC)
GUG		GCG		GAG	($\tau m^5 s^2$UUC)	GGG	

From the approximately 400 mutations with possibly pathogenic character found in mtDNA, about the half are located in mt-tRNA genes (MITOMAP, 2013). These genes are highly susceptible to point mutations, which are one of the main causes of mitochondrial dysfunction associated with a variety of diseases (Levinger et al., 2004). Mutations in mt-tRNAs can affect a large variety of mechanisms, including

transcription, maturation, post-transcriptional modification, structure, stability, aminoacylation, capability of binding to elongation factor EF-Tu and codon reading (Zhang et al., 2011).

Due to the central role in protein synthesis, the malfunction of mutant tRNAs can play a key role in mitochondrial diseases. Disorders related to mitochondrial tRNAs have been associated with several clinical phenotypes, including encephalopathies, myopathies, myoclonus, ophthalmoplegia, heart disease, deafness and others (Levinger et al., 2004). Changes in mitochondrial genes encoding tRNAs have also been described in neurodegenerative diseases such as AD and Parkinson's disease (Egensperger et al., 1997; Hutchin et al., 1997; Grasbon-Frodl et al., 1999; Edland et al., 2002). Since there is no previous investigation of the involvement of mt-tRNAs encoding genes in FTLD, it is important to study their contribution to this pathology.

Mitochondrial genome analysis in frontotemporal lobar degeneration: *tRNAs* contribution

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Abbreviations

AD	Alzheimer's disease
bvFTD	Behavioural variant of FTD
CBS	Corticobasal syndrome
CDR	Clinical Dementia Rating
FTD-ALS	FTD with Amyotrophic lateral sclerosis
FTD-MND	FTD with Motor neurone disease
FTLD	Frontotemporal lobar degeneration
FTLD-FUS	FTLD with inclusions of the fused in sarcoma protein
FTLD-ni	FTLD without inclusions
FTLD-tau	FTLD with tau pathology
FTLD-TDP	FTLD with TAR-DNA binding protein (TDP-43)
FTLD-UPS	FTLD with ubiquitin-positive and TDP-43-negative histopathology
MCR	Mitochondrial Chain Respiratory
OXPHOS	Oxidative Phosphorilation
ROS	Reactive Oxygen Species
MMSE	Mini–mental state examination
mtDNA	Mitochondrial DNA
mt-rRNA	Mitochondrial ribosomal RNA
mt-tRNA	Mitochondrial transfer RNA
PNFA	Progressive nonfluent aphasia
PPA	Primary progressive aphasia
PSP	Progressive supranuclear palsy
SD	Semantic dementia

Abstract

FTLD is a heterogeneous neurodegenerative dementia in many aspects, including clinical, neuropathological and genetic features, characterized by progressive changes in behaviour, executive dysfunction and/or language impairment. It is characterized by progressive changes in behaviour, executive dysfunction and/or language impairment with frontal and temporal lobar atrophy. Some patients present clinical and neuropathological overlap with Alzheimer's disease, suggesting similarities in pathophysiology, including mitochondrial DNA (mtDNA) involvement.

The aim of the study includes sequencing the 22 tRNAs genes encoded in mtDNA for identifying variations in FTLD patients, ascertaining their involvement in FTLD.

We investigated 70 patients, 39 females and 31 males with probable diagnosis of FTLD (age range: 38-82 years, mean 63 ± 11), recruited at Neurology Unit of the Centro Hospitalar e Universitário de Coimbra. Total DNA was extracted from peripheral blood and analysis of 22 *tRNA* mtDNA encoded genes sequences was performed by automated DNA Sanger sequencing and variants were submitted to *in silico* analysis. A total of 28 different sequence variations were identified in 32 patients (46%). From these, 6 variations are probably pathogenic, according to the *in silico* analysis, all causing structure and binding minimum free energy changes: m.4312C>T is heteroplasmic and presents and high percentage of conservation; m.4435A>G is in a critical position and is totally conserved in all species studied; The m.5772G>A is located in T-stem and leads to the disruption of Watson–Crick base pairing (C-G), being 100% conserved in all species; m.12166T>C alteration is in anticodon loop and has high percentage of conservation. The most frequent variation found is m.12308A>G, in the variable region of *mt-tRNA*^{Leu2} and it is totally conserved in all mammals tested; m.15946C>T variation has a high rate of conservation and it is located in the acceptor stem. Further investigation is needed to better understand the relationship between mtDNA alteration found and FTLD, considering also the involvement of nuclear genes in this disorder. However, this is an original study, being the first to investigate the sequence of the tRNA genes encoded by mtDNA in FTLD.

Keywords: mitochondrial DNA; FTLD; *mt-tRNA* genes; sequence variations.

Introduction

Frontotemporal lobar degeneration (FTLD) is a heterogeneous neurodegenerative dementia, in many aspects, including clinical, neuropathological and genetic features. It is characterized by progressive changes in behaviour, executive dysfunction and/or language impairment (Seltman and Matthews, 2012) with frontal and temporal lobar atrophy (Pan and Chen, 2013). FTLD occurs most often in the presenile period and the age of onset is typically at 45-65 years, and it has an equal distribution among men and women (Galimberti and Scarpini, 2010). There is a wide range in durations of illness (2-20 years), partly reflecting different underlying pathologies (Seelaar et al., 2011). The FTLD clinical spectrum encompasses three distinct syndromes: the behavioural variant (bvFTD) characterized by progressive behavioural impairment and a decline in executive function; progressive nonfluent aphasia (PNFA), with motor speech deficits; and semantic dementia (SD), with loss of object knowledge with anomia (Seltman and Matthews, 2012). Additionally, there is a significant clinical overlap between FTD with motor neuron disease (FTD-MND or FTD-ALS), as well as the parkinsonian syndromes, progressive supranuclear palsy (PSP) and corticobasal syndrome (CBS) (Pan and Chen, 2013). Neuropathology classification of includes five groups: FTLD-tau (tau patology); FTLD-TDP (TAR-DNA binding protein, TDP-43), FTLD-UPS (ubiquitin-positive and TDP-43-negative histopathology); FTLD-FUS (inclusions of the “fused in sarcoma protein”) and FTLD-ni (without inclusions) (Mackenzie et al., 2010). Positive family history is observed in 40-50% of the FTLD patients (Sieben, et al., 2012). The autosomal dominant mode of inheritance is found in 10-27% of all FTLD patients. The genetic heterogeneity of FTLD is reflected by the identification of mutations in several nuclear genes, associated with different neuropathological subjects. The most common mutations occur in *GRN* and *MAPT* genes, in approximately 50% of familial cases, while more rare mutations occur in *CHMP2B*, *FUS*, *VCP*, *TARDBP* and *C9ORF72* genes (Seelaar et al., 2011). Some FTLD patients present a clinical and neuropathological overlap with AD. This event may suggest similarities in pathophysiology, including the involvement of mitochondrial DNA (mtDNA) in FTLD (Grazina et al., 2004; Grazina et al., 2006), as it has been observed in AD (Onyango et al., 2006). For this reason, it is important to study the role of mtDNA in FTD. Mitochondrial changes, including mitochondrial chain

respiratory (MRC) dysfunction due to enzymatic defects, increased ROS production. Morphological changes in the mitochondrial network and cell death are common features of neurodegenerative diseases of different genetic origins. Mutations in genes encoding proteins involved in mitochondrial dynamics were identified in neurodegenerative diseases (Móran, 2012). Human mtDNA consists of a 16,568 nucleotides organized in a double stranded, circular DNA molecule, containing 37 genes, encoding for 12S and 16S rRNA, 22 tRNAs and 13 polypeptides, essential components of the MRC (Reddy, 2008). The present work includes the study of the 22 tRNAs encoded by mtDNA, essential for intramitochondrial protein synthesis. From the 400 mutations with possibly pathogenic character found in mtDNA, approximately are belongs to mt-tRNA genes (MITOMAP, 2013). These genes highly susceptible to point mutations, which are one of the main causes of dysfunction mitochondrial associated with a variety of diseases. Due to the central role in protein synthesis, the malfunction of mutant tRNAs could be important in the pathologies occurring in mitochondria. Disorders related to mt-tRNA have been associated with several clinical phenotypes, including encephalopathies, myopathies, myoclonus, ophthalmoplegia, heart disease, deafness, and others (Levinger et al., 2004). Changes in mitochondrial genes encoding tRNAs have also been described in neurodegenerative diseases such as AD and Parkinson`s disease (Egensperger et al., 1997; Hutchin et al., 1997; Grasbon-Frodl et al., 1999; Edland et al., 2002). Since there is no previous investigation of the involvement of mt-tRNAs encoding genes in FTLD, it is important to study their contribution to this pathology.

Objectives

This study aims to investigate the sequence of 22 mtDNA encoded tRNA genes for identifying variations in FTLD patients, ascertaining their involvement in FTLD.

Patients and Methods

Samples

DNA samples of 70 patients (39 females and 31 males; age range: 38-82 years, mean 63 ± 11) with probable diagnosis of FTLD according to the standard criteria of DSM-IV (Brun et al., 1994; McKhann et al.,2001) recruited at Neurology Unit of the Centro Hospitalar e Universitário de Coimbra were investigated.

Total DNA was extracted from peripheral blood by standard phenol chloroform method (Treco, 1999).

The scaling of the dementia was obtained by scoring, the CDR (Clinical Dementia Rating) and MMSE (Mini–mental state examination). In CDR, the scale is between 0 and 3 and the higher values correspond to higher degree of dementia. In MMSE, the scale is between 0 and 30 and lower values correspond to higher degree of dementia (Folstein et al., 1975)

PCR amplification

Amplification of the 22 mitochondrial tRNA enconding genes was performed by Polymerase Chain Reaction (PCR). This technique allows obtaining multiple copies of a particular DNA fragment. The amplification conditions included initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 45 s, 50-60ºC for 45 s, 72°C for 60 s, and a final extension step at 72°C for 5 min using a master mix containing 2-10 ng of DNA, 10X buffer, dNTP (2mM), primer forward (2,5µM), primer reverse (2,5µM), Taq DNA polymerase, H_2O milli Q and $MgCl_2$ (Landsverk et al., 2012).

Agarose gel electrophoresis

PCR procedure was followed by agarose gel electrophoresis for the separation of DNA fragments, in order to verify the success of amplification. PCR products were mixed with loading dye (1:1) and then applied to 1% agarose gel for 1 hour, at 100 Volts, using weight marker. After migration, ethidium bromide labeled DNA molecules were visualized under ultraviolet irradiation (Landsverk et al., 2012).

DNA Sanger Sequencing

After PCR product analysis, samples were purified with ExoSAP-IT®, consisting of exonuclease I (exo I) and alkaline phosphatase (SAP) to degrade the excess of primers and nucleotides, which are the main factors interfering with PCR sequencing (Werle et al., 1994).

The sequencing PCR involves the synthesis of single stranded DNA using the DNA previously amplified in PCR as template. Synthesized chains are terminated prematurely with various possible sizes. The synthesis begins at the primer binding site and ending with the incorporation of a terminator nucleotide that lack the hydroxyl group at the 3' position of the deoxyribose, preventing the establishment of connections phospho-diester and the incorporation of new nucleotides to DNA strand. When a terminator nucleotide is incorporated, the synthesis of new chain ends (Buitrago and Jimenez, 2001). The amplification conditions were an initial denaturation at 96°C for 2 min followed by 45 cycles at 96°C for 10 s, 55ºC for 5 s, 60°C for 4 min, using BigDye® Terminator Ready Reaction Mix v3.1, 5X sequencing buffer, 2,5µM of primer forward or reverse and H2O milli Q (Landsverk et al., 2012).

After sequencing PCR, samples were submitted to standard Sephadex® purification (gel filtration), which removes substances which have not been added during the reaction. After purification, the samples were loaded in the sequencer ABI Prism® 3130 (Applied Biosystems).

This is a fast and automated process that allows the determination of nucleotide sequence comprising the DNA fragment to be studied (Buitrago and Jimenez, 2001). Automated DNA Sequencing is based on electrophoretic procedures using polymer gels. Applied Biosystems DNA sequencers detect fluorescence from four different dyes that are used to identify the A, C, G and T terminators. Each dye has a fluorescence wavelenght when excited by argon ion laser, allowing detection and distinction of all four bases.

After the automated sequencing, samples sequences were analysed using *Sequencing Analysis v5.4*® and *SeqScape v2.5*® software, which allow to compare the obtained data with the reference sequence. Thus, it is possible to detect any variation in the sequence under study.

In silico analysis

After the analysis of all sequences, an *in silico* study of the detected changes was performed, using different databases such as MITOMAP where the variations are reported (MITOMAP, 2013), RNAfold (Hofacker et al., 1994) that predict the RNA secondary structure based on minimum energy requirements and pair probabilities (Mezghani et al., 2011). The localization of the sequence variations in *mt-tRNA* and sequences from the species were obtained from the Mamit-tRNA database (Putz et al., 2007). Evolutionary conservation was performed for all alterations (12 *mt-tRNAs)* in 10 different species (*Homo sapiens*, *Pan paniscus*, *Pan troglodytes*, *Gorilla gorilla*, *Pongo pygmaeus*, *Macaca mulatta*, *Bos taurus*, *Canis familiaris*, *Mus musculus*, *Rattus norvegicus*). The sequence alignment of the *mt-tRNA*s was performed using the ClustalW® software (Sievers et al., 2011). In 2004, the first criteria applied to particular the mutations in mt-tRNA have been created (MacFarland et al., 2004). Subsequently, a comparison of various forms of evaluation criteria scoring systems are use to increase the accuracy of assignment of pathogenicity. (Yarham et al., 2011).

Table 1: The Pathogenicity Scoring System (from Yarham et al., 2011)

Scoring criteria	Score/20	
More than one independent report	Yes	2
	No	0
Evolutionary conservation of the base or base-pair	One change	2
	Two changes	1
	Multiple changes	0
Variant heteroplasmy	Yes	2
	No	0
Segregation of the mutation with disease	Yes	2
	No	0
Histochemical evidence of mitochondrial disease	Strong evidence	2
	Weak evidence	1
	No evidence	0
Biochemical defect in complexes I, III, or IV	Yes	2
	No	0
Evidence of mutation segregation with biochemical defect from single-fiber studies	Yes	3
	No	0
Mutant mt-tRNA steady-state level studies Or Evidence of pathogenicity in *trans*-mitochondrial cybrid studies	Yes	5
	No	0

Thresholds for the new, adjusted scoring system:
≤6 points—neutral polymorphisms
7–10 points—possibly pathogenic
11–13 points—probably pathogenic

≥11 points—definitely pathogenic

Results

A total of 70 patients (39 females and 31 males) with FTLD were included in this study.

From these, 32 (17 females and 15 males) present 28 different genetic variations in *mt-tRNA* genes. We have found 22 patients with only one variation, 9 patients with 2 variations and 3 patients with 4 variations. Patients 5, 43 and 51 have the higher number of variations (Table 2).

Concerning the CDR, 15 patients presented the maximum degree of dementia but only 5 of these have mtDNA variations. The remaining patients presented moderate or mild dementia. For MMSE, 10 patients exhibited maximum degree of dementia but only 4 of these present variations (Table 3).

From the 28 different genetic variations, there are 19 variations that were identified only in 1 patient, 4 variations twice, 3 variations three times, 1 variation four times and other variation tenfold. Only 12 *mt-tRNAs* have variations identified ($tRNA^{Ala}$, $tRNA^{Ile}$, $tRNA^{Met}$, $tRNA^{His}$, $tRNA^{Thr}$, $tRNA^{Gln}$, $tRNA^{Cys}$, $tRNA^{Gly}$, $tRNA^{Leu2}$, $tRNA^{Val}$, $tRNA^{Arg}$ *and* $tRNA^{Asp}$*)*, as presented in Figure 1.

The variations identified were found in MITOMAP database: 14 have been described as “polymorphism”, 8 as “polymorphism” and “mutation”; 3 variations as “polymorphism” and “somatic mutation”; 1 as “mutation”; 1 variation as “polymorphism” and “mutation”; and 1 “novel” (m.7567C>A). The minimum free energy was changed in 22 of the 28 found sequence variations but 5 (m.5775T>C, m.7521G>A, m.10410T>C, m.12192G>A and m.15927G>A) of these are not predicted to alter the tRNA structure. Concerning the location of the variations in *mt-tRNAs* structure, 14 were found in the stems, 10 in the loops and 4 in the variable region (Table 3).

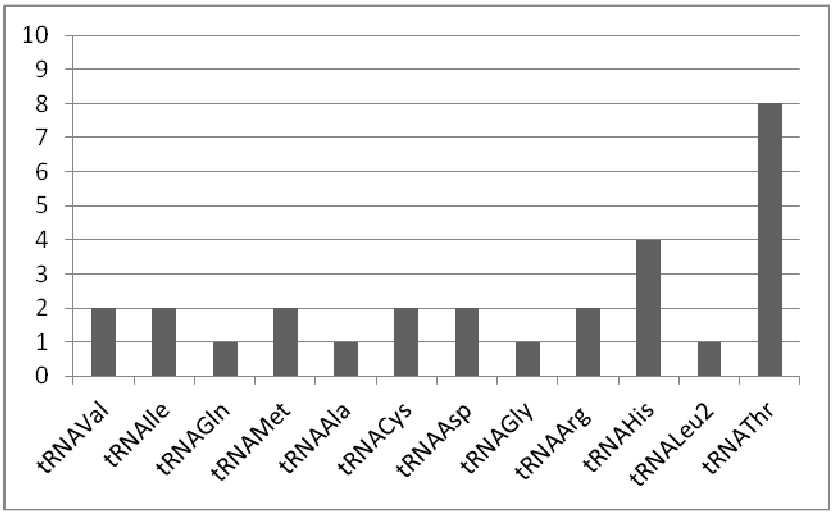


Figure 1: Number of alteration per gene in which they were identified.

There are 6 variations probably pathogenic, according to the *in silico* analysis, all causing structure and binding minimum free energy changes: m.4312C>T is heteroplasmic and presents and high percentage of conservation (Figure 2); m.4435A>G is in a critical position and is totally conserved in all species studied (Figure 3) ; The m.5772G>A is located in T-stem and leads to the disruption of Watson–Crick base pairing (C-G), being 100% conserved in all species (Figure 4); m.12166T>C alteration is in anticodon loop and has high percentage of conservation (Figure 5). The most frequent variation found is m.12308A>G, in the variable region of *mt-tRNA*^{Leu2} and it is totally conserved in all mammals tested (Figure 6); m.15946C>T variation has a high rate of conservation and it is located in the acceptor stem (Figure 7).

Table 2: Patients characterization and data from mtDNA sequence variations.

Patient	Clinical Variant	Gender	Age	Age of Onset	CDR	MMSE	mtDNA Sequence variation	*Gene*
1	bvFTD	F	53	52	3	0	m.5633C>T	*tRNA^{Ala}*
2	bvFTD	F	65	64	3	11		
3	bvFTD	F	74	73	1	17		
4	CBS	M	54	53	3	0		
5	bvFTD	M	67	59	1	27	m.4312C>T(Het) m.4318 C>T(Het) m.4456C>T(Het) m.12184A>G	*tRNA^{Ile}* *tRNA^{Met}* *tRNA^{His}*
6	CBS	F	58	58	1	18		
7	bvFTD	M	45	40	3	7		
8	bvFTD	M	43	43	2	16		
9	bvFTD	M	58	44	3	6		
10	bvFTD	F	54	53	2	18		
11	bvFTD	F	69	69	1	19		
12	bvFTD	F	59	53	3	0		
13	bvFTD	F	75	72	1	20		
14	bvFTD	F	71	68	2	12	m.15927G>A	*tRNA^{Thr}*
15	SD	M	46	41	3	10	m.12179A>G	*tRNA^{His}*
16	bvFTD	F	64	63	1	25	m.15904C>T	*tRNA^{Thr}*
17	bvFTD	F	59	55	3	0	m.4336T>C	*tRNA^{Gln}*
18	bvFTD	F	75	74	1	27		
19	PPA	M	60	58	3	9		
20	bvDFT	F	55	54	1	22	m.12308A>G	*tRNA^{Leu2}*
21	bvFTD	F	77	73	3	10		
22	bvFTD	F	46	43	1	22		
23	bvFTD	F	81	79	1	21	m.5772G>A m.15904C>T	*tRNA^{Cys}* *tRNA^{Thr}*
24	bvFTD	M	61	56	2	12	m.10034T>C m.15924A>G	*tRNA^{Gly}* *tRNA^{Thr}*
25	bvFTD	M	66	63	1	29	m.15930G>A	*tRNA^{Thr}*
26	bvFTD	M	75	64	1	27		
27	bvFTD	F	62	59	2	17		
28	bvFTD	F	74	69	2	15		
29	bvFTD	F	74	72	1	15	m.12308A>G	*tRNA^{Leu2}*
30	PPA	F	49	48	1	30	m.12308A>G	*tRNA^{Leu2}*
31	bvFTD	M	64	62	0.5	28		
32	bvFTD	F	50	50	2	17	m.12308A>G m.15924A>G	*tRNA^{Leu2}* *tRNA^{Thr}*
33	bvFTD	F	60	56	1	15		
34	bvFTD	M	64	60	1	22	m.12308A>G	*tRNA^{Leu2}*
35	bvDFT	F	71	67	3	14		
36	SD	M	54	52	1	21		
37	bvFTD	F	65	63	3	1	m.15905T>C	*tRNA^{Thr}*

Table 2: Patients characterization and data from mtDNA sequence variations (cont).

38	bvFTD	F	49	48	0.5	30		
39	bvFTD	F	59	57	1	18	m.12166T>C m.15927G>A	$tRNA^{His}$ $tRNA^{Thr}$
40	bvFTD	F	54	50	2	19		
41	bvFTD	M	51	51	1	25	m.4435A>G	$tRNA^{Met}$
42	bvFTD	M	68	67	1	27	m.5633C>T	$tRNA^{Ala}$
43	bvFTD	M	76	70	1	28	m.1640A>G m.1659T>C m.10034T>C m.15928G>A	$tRNA^{Val}$ $tRNA^{Val}$ $tRNA^{Gly}$ $tRNA^{Thr}$
44	bvFTD	M	64	61	1	24	m.15924A>G	$tRNA^{Thr}$
45	bvFTD	F	68	66	2	15		
46	bvFTD	M	70	69	1	20	m.10463T>C m.15908T>C	$tRNA^{Arg}$ $tRNA^{Thr}$
47	bvFTD	F	66	60	3	16		
48	bvFTD	M	59	56	1	21		
49	bvFTD	M	74	73	2	16	m.15904C>T	$tRNA^{Thr}$
50	bvFTD	F	54	54	1	26	m.5775T>C	$tRNA^{Cys}$
51	bvFTD	F	59	58	2	16	m.7567C>A m.10034T>C m.10410T>C m.12192G>A	$tRNA^{Asp}$ $tRNA^{Gly}$ $tRNA^{Arg}$ $tRNA^{His}$
52	bvDFT	F	78	78	1	28	m.15904C>T	$tRNA^{Thr}$
53	bvFTD	F	48	47	3	0		
54	bvFTD	F	82	78	1	20		
55	bvFTD	M	73	73	1	28		
56	CBS	M	43	42	1	22	m.12308A>G	$tRNA^{Leu2}$
57	bvFTD	M	38	34	1	25		
58	bvFTD	M	72	69	2	13	m.7521G>A	$tRNA^{Asp}$
59	bvFTD	F	81	75	2	13		
60	PPA	F	69	56	1	13	m.12308A>G m.14956C>T	$tRNA^{Leu2}$ $tRNA^{Thr}$
61	PPA	F	65	62	3	4	m.12308A>G	$tRNA^{Leu2}$
62	bvFTD	M	65	63	0.5	29		
63	bvFTD	M	71	68	0.5	30	m.12308A>G m.15905C>T	$tRNA^{Leu2}$ $tRNA^{Thr}$
64	bvFTD	M	65	63	1	29		
65	bvFTD	F	77	64	1	21		
66	bvFTD	M	70	67	1	25		
67	bvFTD	M	70	68	1	22		
68	bvFTD	M	48	45	2	13	m.10463T>C m.15928C>A	$tRNA^{Arg}$ $tRNA^{Thr}$
69	CBS	F	62	60	1	20	m.12308A>G m.15905C>T	$tRNA^{Leu2}$ $tRNA^{Thr}$
70	bvFTD	M	69	54	0.5	28		

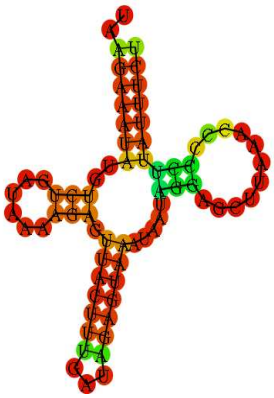
Table 3: *In silico* analysis of the sequence variations found in mt-tRNA enconding genes.

Change of nucleotide	Locus (tRNA)	Reported in MITOMAP	Frequency in the sample (n)	Minimum free energy (kcal/mol)		Structure	Localization in tRNA structure	Evolutionary conservation
				Normal	Changed			
m.1640A>G	$tRNA^{Val}$	Polymorphism	1.429%(1)	-12.5	-17.6	*Changed*	Anticodon stem	90%
m.1659T>C	$tRNA^{Val}$	Mutation (Movement Disorder)	1.429%(1)	-12.5	-10.7	*Changed*	T-stem	60%
m.4312 C>T	$tRNA^{Ile}$	Polymorphism Somatic Mutation	1.429%(1)	-7.8	-8.5	*Changed*	T-loop	80%
m.4318C>T	$tRNA^{Ile}$	Polymorphism	1.429%(1)	-7.8	-9.6	*Changed*	T-loop	60%
m.4336T>C	$tRNA^{Gln}$	Polymorphism Mutation (ADPD/Hearing Loss & Migraine)	1.429%(1)	-19.5	-19.5	Normal	Acceptor stem	70%
m.4435A>G	$tRNA^{Met}$	Polymorphism Mutation (LHON/hypertension)	1.429%(1)	-12.8	-12.9	*Changed*	Anticodon loop	100%
m.4456C>T	$tRNA^{Met}$	Polymorphism Mutation (Poss. hypertension factor)	1.429%(1)	-12.8	-12.4	*Changed*	T-loop	60%
m.5633C>T	$tRNA^{Ala}$	Polymorphism Somatic Mutation	2.857%(2)	-17	-16.7	*Changed*	Anticodon stem	40%
m.5772G>A	$tRNA^{Cys}$	Polymorphism	1.429%(1)	-18.1	-15.9	*Changed*	T-stem	100%
m.5775T>C	$tRNA^{Cys}$	Polymorphism	1.429%(1)	-18.1	-17.8	Normal	T-loop	80%
m.7521G>A	$tRNA^{Asp}$	Polymorphism	1.429%(1)	-9.1	-9.7	Normal	Acceptor stem	50%
m.7567C>A	$tRNA^{Asp}$	Novel	1.429%(1)	-9.1	-10.9	*Changed*	T-loop	40%
m.10034T>C	$tRNA^{Gly}$	Polymorphism	4.286%(3)	-8.5	-8.1	*Changed*	Variable region	90%
m.10410T>C	$tRNA^{Arg}$	Polymorphism	1.429% (1)	-10.1	-11.7	Normal	Acceptor stem	20%
m.10463T>C	$tRNA^{Arg}$	Polymorphism Somatic Mutation	2.8557%(2)	-10.1	-10.1	Normal	Acceptor stem	100%
m.12166T>C	$tRNA^{His}$	Polymorphism	1.429%(1)	-10	-9.5	*Changed*	Anticodon loop	80%

Table 3: *In silico* analysis of the sequence variations found in mt-tRNA enconding genes (cont.).

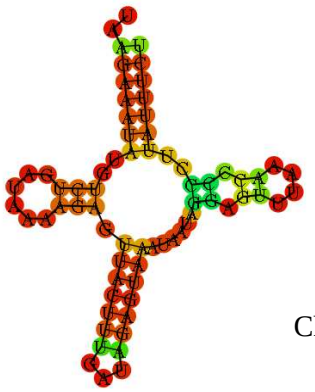
m.12179A>G	*tRNA^{His}*	Polymorphism	1.429%(1)	-10	-10	Normal	Variable region	100%
m.12184A>G	*tRNA^{His}*	Polymorphism	1.429%(1)	-10	-15	*Changed*	T-stem	70%
m.12192G>A	*tRNA^{His}*	Polymorphism Mutation (MICM)	1.429%(1)	-10	-9.9	Normal	T-loop	30%
m.12308A>G	*tRNA^{Leu2}*	Polymorphism Somatic Mutation	14.286%(10)	-15.1	-14.9	*Changed*	Variable region	100%
m.15904C>T	*tRNA^{Thr}*	Polymorphism	5.714%(4)	-13.6	-13.3	*Changed*	D-loop	60%
m.15905C>T	*tRNA^{Thr}*	Polymorphism	4.286%(3)	-13.6	-13.3	*Changed*	D-loop	40%
m.15908T>C	*tRNA^{Thr}*	Polymorphism Mutation (DEAF)	1.429%(1)	-13.6	-13.6	Normal	D-stem	100%
m.15924A>G	*tRNA^{Thr}*	Polymorphism Mutation (LIMM)	4.286%(3)	-13.6	-13.6	Normal	Acceptor stem	100%
m.15927G>A	*tRNA^{Thr}*	Polymorphism Mutation (Multiple Sclerosis/DEAF)	2.857%(2)	-13.6	-13.4	Normal	Acceptor stem	50%
m.15928G>A	*tRNA^{Thr}*	Polymorphism Mutation (Multiple Sclerosis/idiopathic repeat miscarriage/AD)	2.857%(2)	-13.6	-13.6	Normal	Acceptor stem	70%
m.15930G>A	*tRNA^{Thr}*	Polymorphism	1.429%(1)	-13.6	-11.2	*Changed*	Variable region	20%
m.15946C>T	*tRNA^{Thr}*	Polymorphism	1.429%(1)	-13.6	-11.4	*Changed*	Acceptor stem	80%

Abbreviations: ADPD = Alzheimer's Disease and Parkinson Disease; LHON= Leber's Hereditary Optic Neuropathy ; MICM = Maternally Inherited Cardiomyopathy; DEAF= Deafness; LIMM=Mitochondrial Myopathy Lethal Infantile.



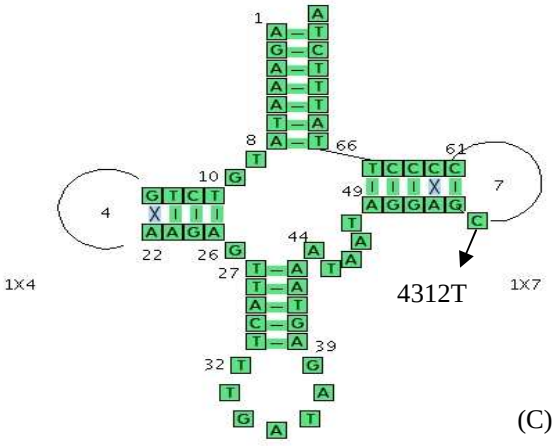
Normal

ΔG=-7,80 kcal/mol (A)

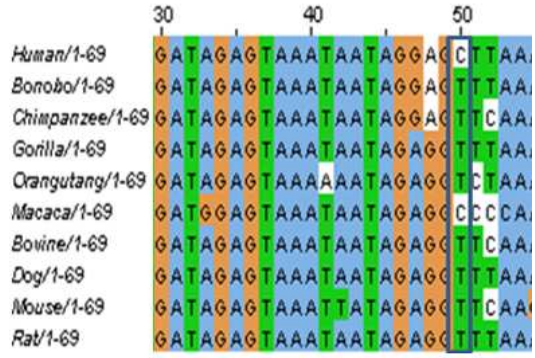


Changed

ΔG=-8,50 kcal/mol (B)

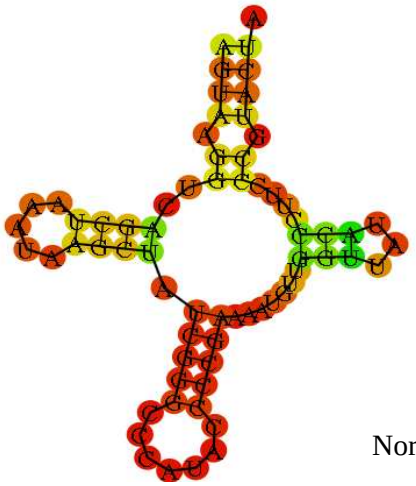


(C)



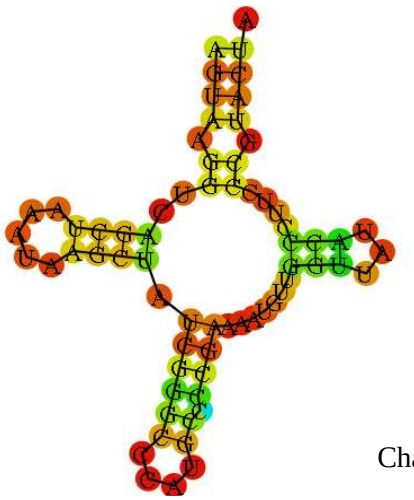
(D)

Figure 2: Results from *in silico* analysis for nucleotide (m.4312C>T mt-*tRNA*^{Ile}) A- normal and B- "mutated" structure (RNAfold); C- Location of the sequence variation in the clover-shaped structure; D- Evolutionary conservation for the nucleotide position (signed with blue rectangle).



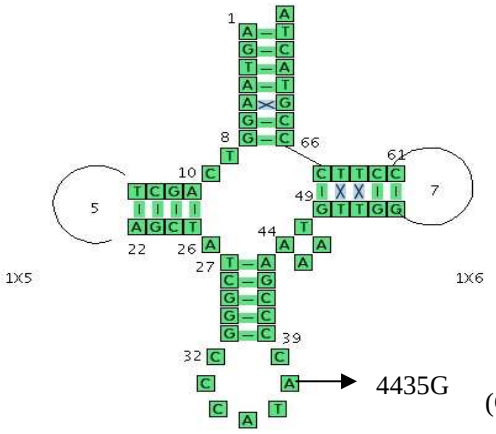
Normal

ΔG=-12,80 kcal/mol (A)

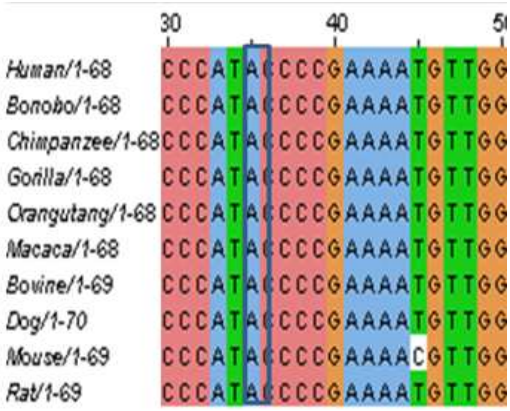


Changed

ΔG=-12,90 kcal/mol (B)

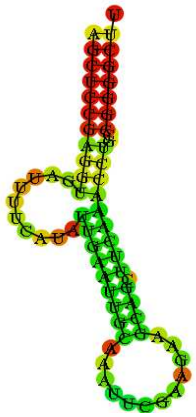


(C)

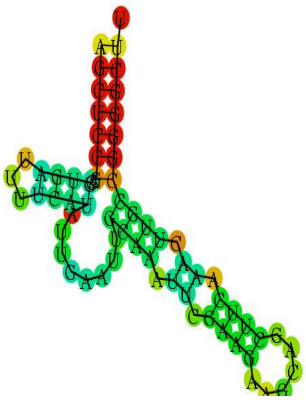


(D)

Figure 3: Results from *in silico* analysis for nucleotide (m.4435A>G of *mt-tRNA*^{Met}) A- normal and B- "mutated" structure (RNAfold); C- Location of the sequence variation in the clover-shaped structure; D- Evolutionary conservation for the nucleotide position (signed with blue rectangle).



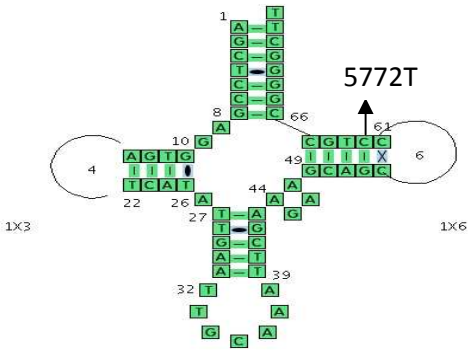
Normal



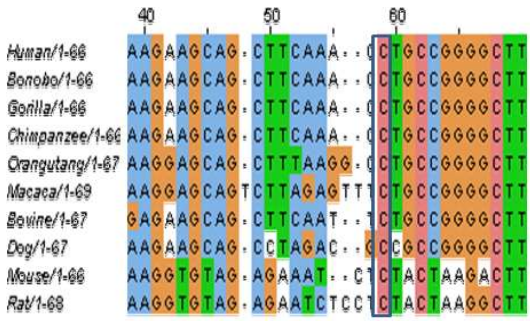
Changed

ΔG=-18,10 kcal/mol (A)

ΔG=-15.90 kcal/mol (B)

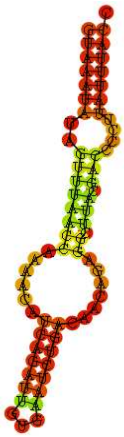


(C)

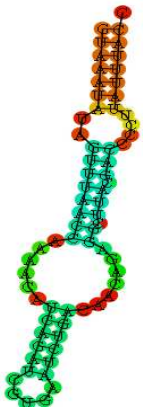


(D)

Figure 4: Results from *in silico* analysis for nucleotide (m.5772G>A of *mt-tRNA*^{Cys}) A- normal and B- "mutated" structure (RNAfold); C- Location of the sequence variation in the clover-shaped structure; D- Evolutionary conservation for the nucleotide position (signed with blue rectangle).

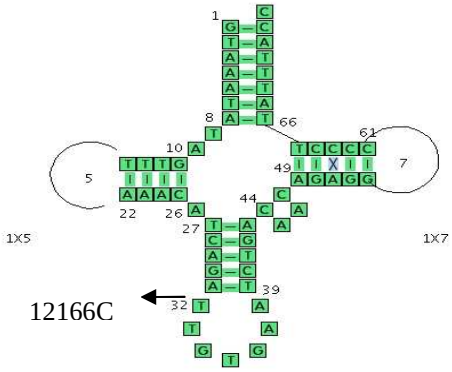


Normal

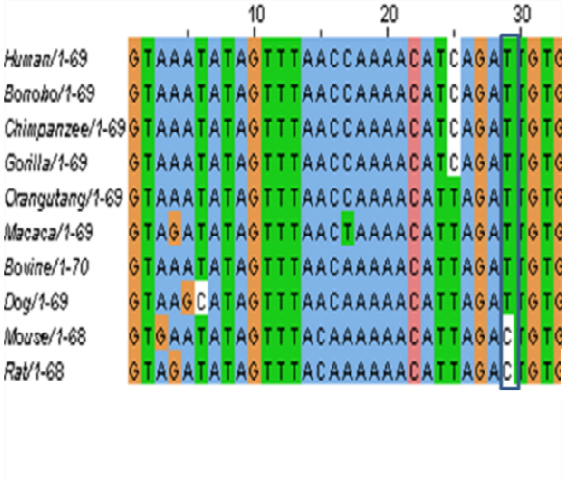


Changed

ΔG=-10,00 kcal/mol (A) ΔG=-9.5 kcal/mol (B)



(C)



(D)

Figure 5: Results from *in silico* analysis for nucleotide (m.12166T>C of *mt-tRNA*^{His}) A- normal and B- "mutated" structure (RNAfold); C- Location of the sequence variation in the clover-shaped structure; D- Evolutionary conservation for the nucleotide position (signed with blue rectangle).

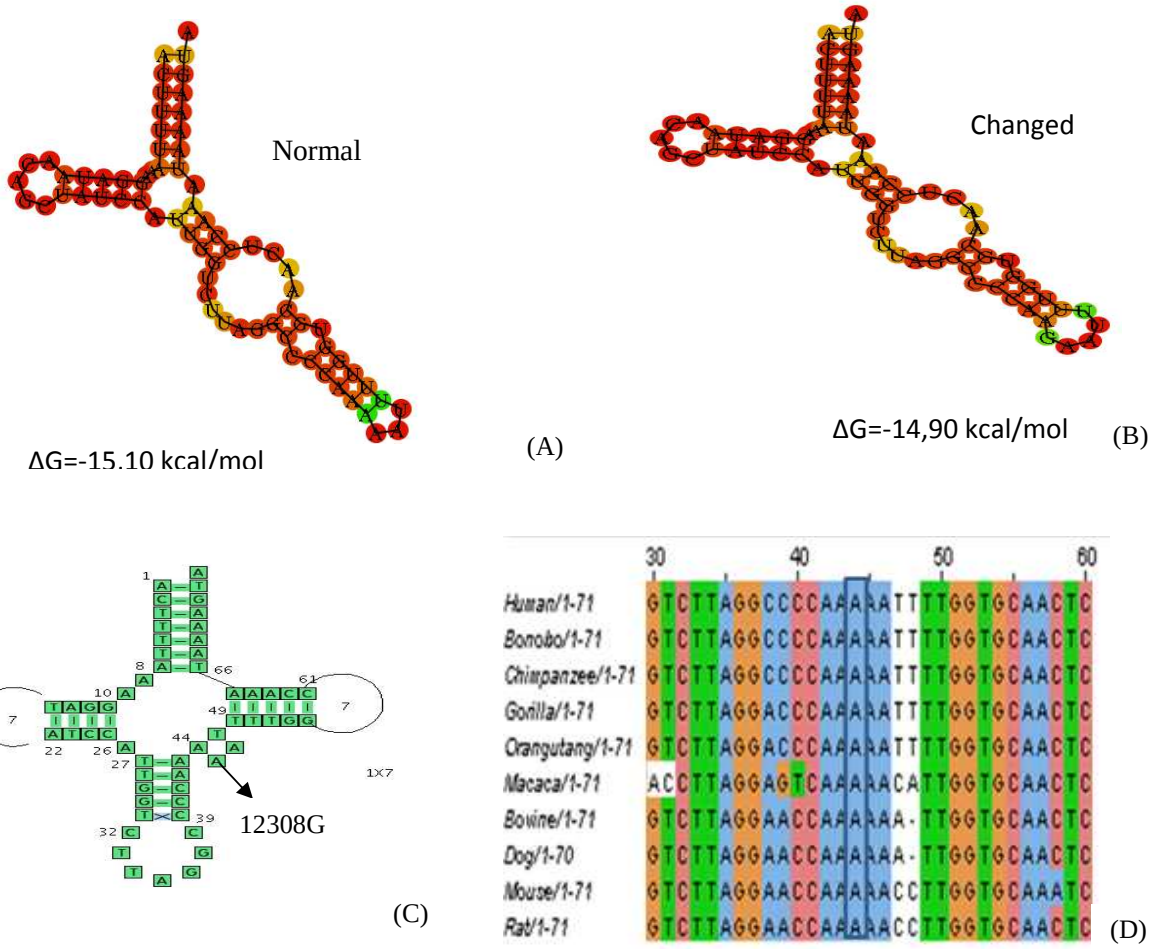
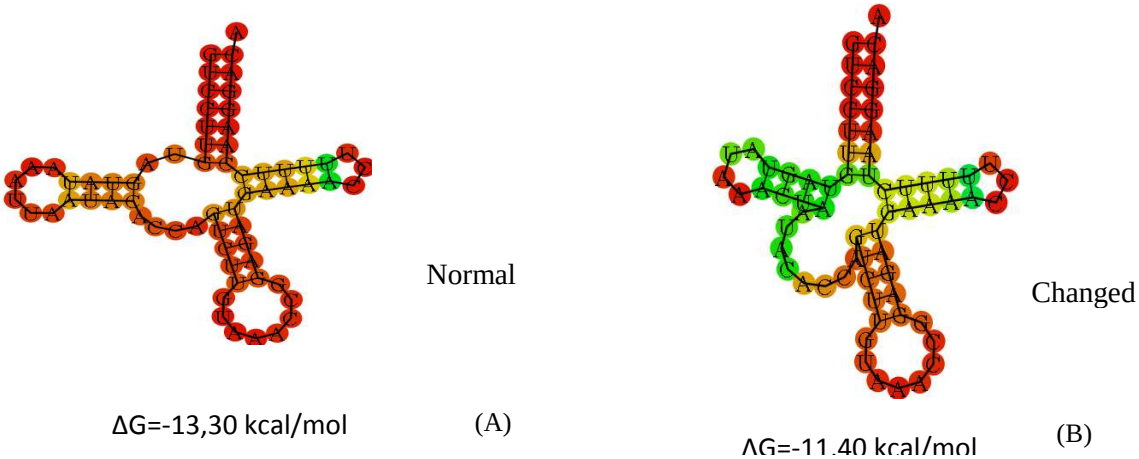


Figure 6: Results from *in silico* analysis for nucleotide (m.12308A>G of *mt*-tRNA^{Leu2}) A-normal and B- "mutated" structure (RNAfold); C- Location of the sequence variation in the clover-shaped structure; D- Evolutionary conservation for the nucleotide position (signed with blue rectangle).



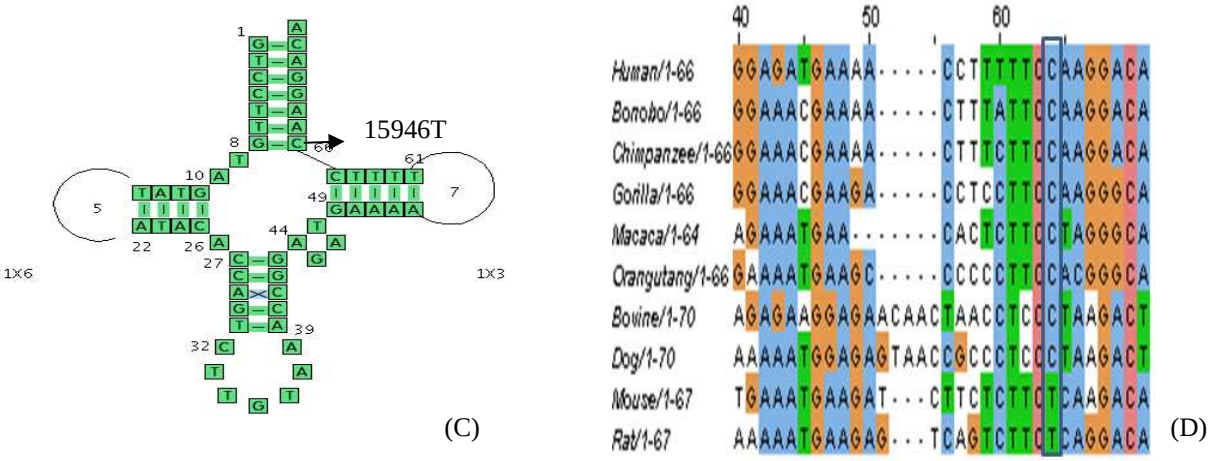


Figure 7: Results from *in silico* analysis for nucleotide (m.15946C>T of *mt*-tRNA^{Thr}) A-normal and B- "mutated" structure (RNAfold); C- Location of the sequence variation in the clover-shaped structure; D- Evolutionary conservation for the nucleotide position (signed with blue rectangle).

Discussion

Human mitochondrial tRNAs acquired interest with the discovery of correlations between point mutations in its genes and neurodegenerative diseases (Zifa et al., 2007). The clinical evaluation related to mtDNA is often complicated and *mt-tRNAs* mutations present unusual difficulties in clinical diagnosis because of the inability to determine if the changes are pathogenic or not (Glatz et al., 2011). The minimum free energy is a criterion to predict the stability of mt-tRNA structure, since a pathogenic mutation in mt-tRNA can alter the secondary structure. Lower minimum free energy is related to a more stable structure, but it is not an absolute fact, because of biological complexity (Zhang et al., 2011). Evolutionary conservation is also an important feature of pathogenicity (Yarham et al., 2011). High percentage of evolutionary conservation suggests that sequence variation may be deleterious. In 28 variations, 17 (m.1640A>G, m.1659T>C, m4312C>T, m.4318C>T, m.4435A>G ,m.4456C>T, m.5633C>T, m.5772G<A, m.7567C>A, m.10034T>C, m.12166T>C, m.12184A>G, m.12308A>G, m.15904C>T, m.15905C>T, m.15930G>A and m.15946C>T) are predicted to have

changes of structure and minimum free energy, but only 8 variations have an elevated evolutionary conservation (Table 3). In 5 variations, despite of having high percentage of conservation, its structures do not change (m.5775T>C, m.10463T>C, m.12179A>G, m.15908T>C and m.15924A>G). There are 7 alterations (m.1640A>G, m.5772G>A, m.5775T>C, m.10034T>C, m.12166 T>C, m.12179A>G and m.15946C>T) that are reported in MITOMAP as polymorphisms but the percentage of conservation is high. Variations previously described as pathogenic mutations, affect mostly high conserved nucleotides, whereas most polymorphic alterations affect rather nonconserved nucleotides (Table 3). However, there are exceptions: some pathogenic mutations affect nonconserved positions and some polymorphic variations affect conserved positions. This suggests that the rate of conservation of a nucleotide by itself cannot be considered as a threshold for which the mutation will be effectively pathogenic (Zifa et al., 2007). Other criteria must be considered.

From the 28 variations detected, m.4312C>T, m.4318C>T and m.4456C>T are present in heteroplasmy, and were found in the same patient, but the percentage of heteroplasmy was not determined. On the other hand, heteroplasmy of variants in *mt-tRNA* genes has been generally regarded as direct evidence for pathogenicity (McFarland et al., 2004).

Location of variations in the tRNA structure is also very important. For a mutation affecting directly the function of *mt-tRNA*, this should occur in a critical base to the recognition of the codon and aminoacylation. However, of all the pathogenic mutations that occur in *mt-tRNA* genes and that have been previously associated with mitochondrial diseases, only a few occur in these critical positions (Suzuki et al., 2011). From 28 variations, there are 14 alterations that are located in the stems, 10 variations are localized in the loops and 4 are in the variable region. Many *mt-tRNA* pathological mutations are mainly located, on the stem portions (Pereira et al., 2008). An elevated number of variations were found in $tRNA^{Thr}$ coding genes, in comparison with other tRNAs. This is in agreement to the study by Pereira and colleagues (2008), which indicates that the variability in this gene is much higher, especially in the stem regions. Other *mt-tRNAs* genes have also more variations in the stems, such as $tRNA^{Val}$ and $tRNA^{Arg}$. Other genes have higher numbers of variations in the loops ($tRNA^{Ile}$ and $tRNA^{Met}$). The $tRNA^{Met}$ presents variations in the loops, namely one substitution, m.4435A>G, is located in the anticodon loop, which is relevant, since this tRNA performs an important role as the initiator of all mtDNA proteins.

The breaking of Watson–Crick base pairing is an important characteristic to identify pathogenic mutations that occur in the stem structures. The disruption of C-G (cytosine-guanine) base pair linking is significantly more common in pathogenic mutations. The A–T (adenine–thymine) bonding has a lower thermodynamic energy than C–G bonding and it is possible that breaking A–T bonds has less effect on the structure of the *mt-RNA* than an equal break of a C–G pair bases (Figure 4 and 7) (McFarland et al., 2004). Variation m.5772G>A is in T-stem of $tRNA^{Cys}$ (within LS of mtDNA). Variations m.15927G>A, m.15928G>A and m.15946C>T are located in stems of $tRNA^{Thr}$. Since these variations break the links C-G, these changes are probably more pathogenic.

After *in silico* analysis, there are 6 sequence variations that present high probability of being pathogenic: m.4312C>T, due the heteroplasmy presented, to the change of minimum free energy and its structure, and high percentage of conservation; m.4435A>G alteration induces structure and binding minimum free energy changes, it is localized in a critical position and it is totally conserved in all species analysed; m.5772G>A modifies the structure and binding minimum free energy, being located a local of disruption of Watson–Crick base pairing (C-G) of T-stem and it is 100% conserved in all species; the alteration m.12166T>C presents changes in structure and binding minimum free energy, it is located in anticodon loop and is highly conserved; the most frequent variation identified in our cohort is m.12308A>G, in the variable region of *mt-*$tRNA^{Leu2}$ gene. This variation leads to structure alteration and it is totally conserved; the variation m.15946C>T also causes change in the structure and minimum free energy; it is localized in the acceptor stem and has high conservation rate.

Conclusions

The analysis of the *mt-tRNAs* variations indicates that there is not sufficient evidence to classify the variations as pathogenic causative of FTLD. However, through this study it is possible to gather important data.

Most of the detected variations altered the structure and minimum free energy of tRNAs. There is a higher number of substitutions in the stems than in the loops, which is in agreement with the literature, concerning the involvement of mt-tRNA folding genes in diseases. The evolutionary conservation is not always in agreement with the

results obtained for the structure, for prediction of pathogenicity. Therefore, it is difficult to detect pathogenic mutations due to heterogeneity of results. Nevertheless, according to all the pathogenicity criteria studied, in 28 variations detected, the more likely to be pathogenic are m.4312C>T in $tRNA^{Ile}$, m.4435A>G in $tRNA^{Met}$, m.5772G>A in $tRNA^{Cys}$, m.12166T>C in $tRNA^{His}$, m.12308A>C in $tRNA^{Leu2}$ and m.15946C>T in $tRNA^{Thr}$.

It is yet unclear, at the molecular level, how the mutant *mt-tRNAs* can cause mitochondrial dysfunction. There is a current notion that not only *mt-tRNA* mutations, but a combination of different mutations present in mitochondrial genes is responsible for a variety of clinical diseases (McFarland et al., 2004).

Additionally, given the role of *mt-tRNAs* in MRC function, mutations in these genes may affect the ability to produce mitochondrial proteins. These protein synthesis anomalies result in OXPHOS deficiency, since the enzymatic activity of all MRC complexes could be affected. Furthermore, according to the "Mitochondrial cascade hypothesis" (Swerdlow and Khan, 2004), polymorphic variations in MCR subunits encoding genes establish MCR efficiency and basal mitochondrial ROS production, that correlates with mtDNA damage. Acordingly, somatic mtDNA mutation decreases MCR efficiency leading to reduced OXPHOS and/or increased ROS production. For this reason, mtDNA mutations possibly modify age of onset, contributing to neurodegeneration process, probably due to an impairment of MCR and/or translation mechanisms.

Moreover, deeper biochemical investigations are needed to better understand the relationships between mtDNA and FTLD, considering the involvement of nuclear genes. Genotype/phenotype correlation can involve nuclear and mitochondrial interactions, but the exact mechanism is still unknown.

In conclusion, more research is needed to determine whether the *mt-tRNA* variations play a direct pathogenic role in FTLD. A functional study would certainly help to prove the possible pathogenicity of these alterations.

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3. Annex 1: Author information pack (Neurobiology of Disease)

NEUROBIOLOGY OF DISEASE

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