Mitochondrial DNA involvement in frontotemporal lobar degeneration –
analysis of sequence variants in *MTND* genes

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Abstract

Mitochondria dysfunction and oxidative damage have been suggested to have an important role in ageing-related neurodegenerative diseases. One of the possible mechanisms is related to mitochondrial DNA (mtDNA) alterations that may impair mitochondrial respiratory chain function. Contrary to other neurological disorders, frontotemporal lobar degeneration (FTLD) pathophysiology is still poorly understood, and the etiology of most cases remains unknown. Recently, two mtDNA alterations were reported in a patient with FTLD and another study proposed an association between FTLD and a specific mtDNA haplogroup.

To determine if mtDNA is involved in FTLD, we sequenced 4 mtDNA genes encoding subunits of NADH dehydrogenase from 17 patients. The alterations detected were submitted to in silico analysis for evaluating possible pathogenicity.

In 82% patients we found 29 different alterations, including polymorphisms (62.1%), mutations already associated to other diseases (27.1%) and unpublished variants (13.8%). Many of the alterations detected (69%) were not associated to a change in the amino acid translated and so are not expected to cause mitochondrial dysfunction. The non-synonymous variants are predicted to be benign, according to the in silico analysis. However, even if these alterations are not primarily pathogenic, an interaction with other mutations may occur, leading to the disease, worsening its expression or influencing age of onset.

Our study is still preliminary, but the high number of mtDNA alterations identified suggests a possible role of this genome in FTLD. However, it is not yet possible to determine whether these variants are part of the etiology or an epiphenomenon.

Keywords

mitochondrial DNA; frontotemporal lobar degeneration; neurodegenerative diseases; dementia; mutations; NADH dehydrogenase
Abbreviations

FTLD - frontotemporal lobar degeneration; MRC – mitochondrial respiratory chain; MTND – mitochondrial NADH dehydrogenase; mtDNA - mitochondrial DNA; nDNA – nuclear DNA; OXPHOS – oxidative phosphorylation; ROS – reactive oxygen species

Introduction

Mitochondria are fundamental organelles in the cell, not only responsible for ATP production by OXPHOS pathway, occurring in the MRC system, but also intervening in many other cell mechanisms, namely apoptosis and signal transduction. Each cell contains about $10^3$-$10^4$ molecules of mtDNA (Greaves et al., 2012), a circular double-strand molecule of 16,568 base pairs (Andrews et al., 1999), encoding 37 genes: 13 peptides of MRC (7 from complex I, 1 from complex III, 3 from complex IV, 2 from complex V), and also 22 tRNAs and 2 rRNAs, needed for mitochondrial protein synthesis.

mtDNA has specific characteristics, distinct from the ones of nDNA and that justify the particularities of mtDNA associated diseases. The more determinant are (1) maternal inheritance, (2) heteroplasmmy, (3) random mitotic segregation during cell division, (4) replication “independently” of the cell cycle, occurring even in post-mitotic cells – relaxed replication (Greaves et al., 2012) - and (5) variable number of mitochondria in each cell, depending on the tissue (Greaves et al., 2012; Schapira, 2006; DiMauro and Davidzon, 2005; Schon and Manfredi, 2003). Moreover, mtDNA has a high mutation rate, which can be explained since this molecule is almost totally encoding, it is localized near the main source of ROS production, it does not have protective histones and its repair mechanisms are limited. For those reasons, spontaneous mutations are frequent, mainly being neutral polymorphisms (DiMauro and Davidzon, 2005).
Even though mtDNA plays an important role in mitochondrial function, mitochondria do not work autonomously. In fact, most MRC subunits are encoded by nuclear genes. Furthermore, nDNA also has the genes necessary to the synthesis of other proteins needed for replication, transcription and stabilization of mtDNA and for the import, transport and assembly of MRC components (Greaves et al., 2012).

Many diseases with mitochondrial involvement have been reported, affecting mainly the nervous system, heart and skeletal muscle, since these are the tissues with higher energy needs (Greaves et al., 2012; Schapira, 2006; Zeviani and Donato, 2004; Schon and Manfredi, 2003). OXPHOS abnormalities that are characteristic of these disorders can be caused by alterations in nuclear or mitochondrial genes, namely substitutions, deletions, duplications, insertions or depletion of mtDNA (DiMauro and Schon, 2008; Schapira, 2006; Zeviani and Donato, 2004; Schon and Manfredi, 2003).

Beyond mitochondrial OXPHOS syndromes, there are neurological diseases in which physiopathological mechanisms are considered to involve an interaction between genes and environmental factors (Mattson et al., 2008), with reports of structural and functional mitochondrial abnormalities (DiMauro and Schon, 2008; Mattson et al., 2008; Lin and Beal, 2006; Schapira, 2006; Beal, 2005; Wallace, 2005; Schon and Manfredi, 2003). This is the case of Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and amyotrophic lateral sclerosis. mtDNA polymorphisms and specific haplogroups have also been associated to neurodegenerative diseases, dementia and longevity (Mancuso et al., 2008; Grazina et al., 2006; Wallace, 2005). Mitochondrial dysfunction is related to ROS production and mtDNA somatic mutations that accumulate throughout the years, leading to energy insufficiency, signaling defects, apoptosis and replicative senescence, culminating in loss of cell function (Taylor and Turnbull, 2005). Moreover, accumulation of somatic mutations can exacerbate inherited defects of MRC.
FTLD is among the neurodegenerative disorders, with pre-senile onset, more commonly associated with dementia (Seelaar et al., 2011). The etiology of this pathology seems to include genetic, behavioral and environmental factors, accounting for the clinical and histological heterogeneity of this disease (Seelaar et al., 2011; Sleegers et al., 2010; Mackenzie et al, 2010, 2009). Clinically, the designation FTLD includes behavioral variant frontotemporal dementia, semantic dementia and progressive nonfluent aphasia (Neary et al., 2005). About 15% of the patients are also diagnosed with amyotrophic lateral sclerosis or Parkinson’s disease (Sleegers et al., 2010). Clinical overlap with Alzheimer´s disease (van der Zee et al., 2008), corticobasal syndrome and supranuclear palsy (Rabinovici and Miller, 2010) is not uncommon. Various nuclear genes mutations are associated with the different variants of FTLD, namely microtubule associated protein tau (MAPT) and progranulin (GRN) genes and, less frequently, valosin containing protein (VCP), charged multivesicular body protein 2B (CHMP2B), TAR-DNA binding protein (TARDP) and fused in sarcoma (FUS) genes (Seelaar et al., 2011; Sleegers et al., 2010) but these fail to explain most cases.

Grazina et al. (2004) have already reported two homoplasmic mtDNA variants in one patient with diagnosis of FTLD, in nucleotides 3316 and 3337 of the MTND1 gene, correlating with complex I deficiency. Recently an association has been described between FTLD and mtDNA haplogroup cluster IWX (Krüger et al., 2010), which has a higher non-synonimous/synonymous rate in the MTND genes than other European haplogroup clusters. However, as for most correlations between specific mtDNA haplogroups and neurological disorders, results are not consensual and a previous study did not come with the same conclusion (Rose et al., 2008).

In this perspective, the aim of the present work is to analyze mtDNA genes encoding MRC complex I (or NADH dehydrogenase) subunits in patients diagnosed with FTLD, in order to
determine whether *MTND* genes variants are involved in this disease, contributing for pathology.

**Material and methods**

We have studied 17 patients (eleven female and six male) diagnosed with probable FTLD according to standard criteria (McKhann et al., 2001), 16 with frontotemporal dementia – behavioral variant and 1 with semantic dementia, followed at the Department of Neurology of “Centro Hospitalar e Universitário de Coimbra”. The mean age at onset was 60.7 years (range 41 – 79).

Written informed consent was obtained from all the participants and the study was approved by the local Ethical Committee.

Total DNA was extracted from venous peripheral blood using standard methods and quantified by UV spectrophotometry (λ=260 nm). Automated sequencing analysis was performed according to manufacturer's instructions (3130 ABI Prism sequencing system), using BigDye® Terminator Ready Reaction Mix v3.1 (Applied Biosystems). We have studied mtDNA regions to screen *MT-ND1, MT-ND2, MT-ND4L* and *MT-ND4* genes coding for complex I subunits for confirmed pathogenic mutations, polymorphisms and novel sequence variations. The database MITOMAP (www.mitomap.org) was used to classify the variants found. All sequences were analyzed using Sequencing Analysis® v5.4 and SeqScape® v.2.5 software (Applied Biosystems), by comparison with reference sequence obtained from database GenBank. *In silico* analysis was performed for all non synonymous sequence variations found, using PolyPhen-2® to predict the possible impact of amino acid substitutions on the structure and function of the protein translated.
Results

A total of 29 different alterations were identified and 59% of the patients had multiple alterations, in multiple genes. 82% of the patients had at least one mtDNA sequence variation in the genes studied.

We have found 9 alterations in ND1 gene, 7 in ND2 gene, 2 in ND4L gene and 11 in ND4 gene (Table 1). In ND1 gene, there were 5 polymorphisms, 3 variants reported in disease (one of which is a haplogroup marker) and 1 alteration not reported in the literature. In ND2 gene, there were 6 polymorphisms (2 are haplogroup markers) and 1 variant reported in disease. In ND4L gene, there were 2 polymorphisms (one is a haplogroup marker). In ND4 gene, there were 5 polymorphisms (one is a haplogroup marker), 3 variants reported in disease (one is a haplogroup marker) and 3 novel alterations. In total, 62.1% of the different alterations corresponded to polymorphisms, 27.1% to variants reported in disease and 13.8% to novel variants.

Each alteration was detected in one patient only, except the ones in positions 4580, 5460, 10550, 11299, 11467 and 11914, found in 2 patients each, and in position 11719, identified in 7 patients.

In silico analysis predicts that all the non-synonymous variants identified are possibly benign.
Table 1 – Characteristics of the mtDNA variants detected (according to MITOMAP)

<table>
<thead>
<tr>
<th>Nucleotide Position</th>
<th>Locus</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Reported mtDNA Base Substitution Diseases</th>
<th>mtDNA Somatic Mutations</th>
<th>In silico analysis prediction (score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3316</td>
<td>MT-ND1</td>
<td>G-A</td>
<td>A-T</td>
<td>DM II/ LHON/ PEO (status unclear)</td>
<td></td>
<td>Benign (0.001)</td>
</tr>
<tr>
<td>3337</td>
<td>MT-ND1</td>
<td>G-A</td>
<td>V-M</td>
<td>Cardiomyopathy (Possibly synergistic)</td>
<td></td>
<td>Benign (0.020)</td>
</tr>
<tr>
<td>3483</td>
<td>MT-ND1</td>
<td>G-A</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3772*</td>
<td>MT-ND1</td>
<td>A-G</td>
<td>M-V</td>
<td></td>
<td></td>
<td>Benign (0.421)</td>
</tr>
<tr>
<td>3847</td>
<td>MT-ND1</td>
<td>T-C</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3915</td>
<td>MT-ND1</td>
<td>G-A</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4025</td>
<td>MT-ND1</td>
<td>C-T</td>
<td>T-M</td>
<td></td>
<td></td>
<td>Benign (0.017)</td>
</tr>
<tr>
<td>4104</td>
<td>MT-ND1</td>
<td>A-G (het)</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4216</td>
<td>MT-ND1</td>
<td>T-C</td>
<td>Y-H (hg JT)</td>
<td>LHON/Insulin Resistance</td>
<td>Acute leukemia platelets, leukocytes &amp; bone marrow</td>
<td>Benign (0.006)</td>
</tr>
<tr>
<td>4529</td>
<td>MT-ND2</td>
<td>A-T</td>
<td>syn (hg I)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4580</td>
<td>MT-ND2</td>
<td>G-A</td>
<td>syn (hg V)</td>
<td></td>
<td></td>
<td>Pancreatic cancer cell line</td>
</tr>
<tr>
<td>4727</td>
<td>MT-ND2</td>
<td>A-G</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4745</td>
<td>MT-ND2</td>
<td>A-G</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4976</td>
<td>MT-ND2</td>
<td>A-G</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5046</td>
<td>MT-ND2</td>
<td>G-A</td>
<td>V-I</td>
<td></td>
<td></td>
<td>Benign (0.024)</td>
</tr>
<tr>
<td>5460</td>
<td>MT-ND2</td>
<td>G-A</td>
<td>A-T</td>
<td></td>
<td></td>
<td>Benign (0.000)</td>
</tr>
<tr>
<td>10550</td>
<td>MT-ND4L</td>
<td>A-G</td>
<td>syn (hg K)</td>
<td></td>
<td></td>
<td>Endometrium control tissue</td>
</tr>
<tr>
<td>10589</td>
<td>MT-ND4L</td>
<td>G-A</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Mitochondrial DNA involvement in frontotemporal lobar degeneration – analysis of sequence variants in MTND genes

<table>
<thead>
<tr>
<th>Nucleotide Position</th>
<th>Locus</th>
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<th>Reported mtDNA Base Substitution Diseases</th>
<th>mtDNA Somatic Mutations</th>
<th>In silico analysis prediction (score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10771*</td>
<td>MT-ND4</td>
<td>A-G</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10899*</td>
<td>MT-ND4</td>
<td>A-G</td>
<td>N-S</td>
<td></td>
<td></td>
<td>Benign (0.017)</td>
</tr>
<tr>
<td>11251</td>
<td>MT-ND4</td>
<td>A-G</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11299</td>
<td>MT-ND4</td>
<td>T-C</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11467</td>
<td>MT-ND4</td>
<td>A-G</td>
<td>syn</td>
<td>Altered brain pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11470</td>
<td>MT-ND4</td>
<td>A-G</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11488*</td>
<td>MT-ND4</td>
<td>A-G</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11674</td>
<td>MT-ND4</td>
<td>C-T</td>
<td>syn</td>
<td></td>
<td></td>
<td>Pancreatic cancer cell line, prostate tumor</td>
</tr>
<tr>
<td>11719</td>
<td>MT-ND4</td>
<td>G-A</td>
<td>syn</td>
<td>(hg H=G)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11914</td>
<td>MT-ND4</td>
<td>G-A</td>
<td>syn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11947</td>
<td>MT-ND4</td>
<td>A-G</td>
<td>syn</td>
<td>(hg W)</td>
<td></td>
<td>Prostate tumor</td>
</tr>
</tbody>
</table>

* Novel alterations; het – alteration detected in heteroplasmy; Syn – synonymous; hg – haplogroup marker; DM II – diabetes mellitus type II; LHON - Leber’s hereditary optic neuropathy; PEO – progressive external ophthalmoplegia; AD – Alzheimer’s disease; PD – Parkinson’s disease

Discussion

Many of the mtDNA alterations found (69%) were synonymous and hence are not expected to be the major cause of disease or to have repercussions on mitochondria function, even though that may not always be the case, with some synonymous mutations reported in several diseases (Rollins et al., 2009; Jeronimo et al., 2001).
Concerning the 4 novel variants not previously reported in literature, two were non synonymous and in silico analysis predicts that they are possibly benign. The non synonymous polymorphisms detected were also submited to in silico analysis, given the fact that, even if so far no disease has been associated to those nucleotide variants, it does not mean that they are not potentially pathogenic. However, the analysis of the variants that we have detected predicts that they are possibly benign (table 1). However, the polymorphisms detected may modulate the effects of other DNA variants and be indirectly involved in disease by that mechanism. The accumulation of polymorphisms can also reveal a tendency for the occurrence of mtDNA alterations, since this may mean that repair systems are less effective in these subjects.

Regarding the 7 mutations that have been reported in other diseases (three synonymous), none of the patients has been diagnosed with those disorders. According to the in silico analysis, the non synonymous mutations are not expected to be the cause of significant alterations in the proteins translated. Therefore, they are not predicted to be a major etiological factor in any disease, at least if not associated with other mutations. Accordingly, the status of alteration in position 3316, detected in two mitochondrial syndromes and in diabetes mellitus, is unclear. The mutation in nucleotide 3337 is reported to have a possibly synergistic role in cardiomyopathy (Zifa et al., 2008). The T-C transition in position 4216 has been found in leukemia (Linnartz et al., 2004). It has also been considered a secondary mutation in Leber's hereditary optic neuropathy (Johns and Berman, 1991) but Lodí et al. (2000) found no further impairment in mitochondrial oxidative metabolism in patients with this mutation. It is currently classified as a polymorphism and it is also a marker of haplogroup JT. One of the mutations (in position 5460), which was present in two patients, has been reported in two neurodegenerative disorders (Kosel et al., 1996; Schnopp et al., 1996; Lin et al., 1992; Petruzzella et al., 1992) in which dementia is one of the symptoms. Moreover, Parkinson’s
disease can be associated to FTLD and Alzheimer´s disease has been proposed to be part of a spectrum of disorders which also includes FTLD (van der Zee et al., 2008). These data are in favor of an eventual association of this mutation with FTLD. On the other hand, it can also mean that these two patients are more likely to have the FTDL subtype associated with Parkinson´s disease, even though they have not experienced any parkinsonian symptoms so far. The in silico analysis predicts the variant in position 5460 to be benign, suggesting that it is more likely just a polymorphism frequent in the neurological diseases in which it has been reported rather than part of the primary etiology of those disorders. Another possibility is that this alteration may increase the penetrance of other pathogenic mutations. The synonymous mutation in position 11467 has been associated with altered brain pH (Rollins et al., 2009). This is one of the three positions that define the super- haplogroup U, K, UK. The other two are located in genes not included in the current study. Haplogroups U and UK have less coupled mitochondria, leading to a lower level of ROS production, which could explain the increased postmortem brain pH (Rollins et al., 2009). These haplogroups have been reported to have a protective effect against aging and neurodegeneration. Two mutations (in positions 11674 and 11947) have been reported in prostate tumor cells (Jeronimo et al., 2001). As they are synonymous, their role in oncogenesis is not clear. The first one, as well as a polymorphism in position 4580, have been detected in a pancreatic cancer cell line (Jones et al., 2001), but they have not been identified in patients with pancreatic cancer so far.

Besides the alteration in positions 5460 and 11467, three other variants were detected in more than one patient, and these are markers of haplogroups common in Europe. Concerning the variant in position 11719, although it is not the defining polymorphism for any haplogroup, it is more frequent in haplogroups G and H, which is the most frequent haplogroup in Western Europe, explaining why it was found in 41% of the patients. Interestingly, a study on the association between haplogroups and longevity in a Finnish population (Niemi et al., 2003)
reported a lower frequency of haplogroup H in the elderly, comparing to middle-aged subjects and infants, which supports a possible haplogroup related predisposition to neurodegenerative diseases and early aging. This is consistent with the hypothesis that this haplogroup is associated to tightly coupled mitochondria, with higher levels ATP production rates but also of ROS (Wallace, 2005). Therefore, haplogroup H might be associated to an increased susceptibility to mtDNA mutations, explaining the reports of a high frequency in subjects with neurodegenerative and psychiatric disorders. Interestingly, Niemi et al. (2003) also found a higher frequency of cluster WIX in nonagenarians comparing to younger people, implying a possible protective role of this mtDNA lineage against aging. This is contrary to the correlation found between the same cluster and FTLD (Krüger et al., 2010), also in Finland. Further studies are needed to evaluate whether these associations are valid and reproducible in other European populations.

An important feature of most pathogenic mutations (Montoya et al., 2009; DiMauro and Davidzon, 2005; Zeviani and Donato, 2004) is their presence in heteroplasmy, which, in our study, was only detected for one of the variants, that was synonymous. Even though synonymous mRNA variants are considered to be functionally neutral, their selection in some diseases, including neurodegeneration and cancer, may challenge this assumption. In fact, it is possible that nucleotide variants that do not change an amino acid might change some yet unidentified functions in the mRNA. As an example, internal mRNA sequences seem to be required to initiate mRNA translation and these could be rendered non-functional by synonymous variants (Brandon et al., 2006).

Regarding the non synonymous alterations detected, the prediction of benignancy does not exclude their involvement in FTLD. Even without causing major changes in protein structure, the mtDNA variants may interact with other mutations, leading to the disease, worsening its expression or influencing age of onset. The reports of some “benign” mutations in several
disorders are consistent with this hypothesis. Nevertheless, we cannot exclude that mtDNA alterations might be an epiphenomenon of the diseases.

The *in silico* analysis of non synonymous variants takes into account the sequence, philogenetic and structural information characterizing the substitutions. However, there are other criteria to evaluate the pathogenicity of mtDNA variants (DiMauro and Davidzon, 2005; Zeviani and Donato, 2004). Therefore, studies with cybrids and determination of MRC activity should be a complementary approach to our study. However, since the variants found are not expected to be directly pathogenic, it would probably be more useful to compare the frequency of these alterations in a healthy population of the same age, in order to evaluate whether there is a significant difference in frequencies between FTLD patients and controls. This might be difficult to investigate due to the small number of patients. This problem could be overcome by increasing the number of subjects.

The high mutation rate of mtDNA also makes it more complex to determine whether mtDNA alterations have significant effects in the cell. It is also necessary to take into account the extension of tissues affected, haplotype, environmental factors and nDNA background (DiMauro and Davidzon, 2005; Zeviani and Donato, 2004). Moreover, the presence of alterations in blood does not predict whether the same alterations are present in the nervous system. One possibility is to study other tissues, for example by performing a muscle or skin biopsy, since the presence of an alteration in multiple tissues makes it more likely to be also present in brain. This is only valid for inherited mtDNA alterations. Even so, we have to consider the possibility of heteroplasmy and the possible variability in levels of mutant mtDNA in different organs. Therefore, the best way to evaluate mtDNA involvement in FTLD, especially of sporadic cases, is through post-mortem study of the brain, which is often not possible.
Conclusion

Our analysis revealed a considerable number of mtDNA alterations in the FTLD population studied, present in 82% of the patients. 59% had multiple alterations, in several genes. This may be an indicator of mtDNA involvement in FTLD, not yet known whether as an etiologic agent or as consequence of other alterations. The polymorphisms and mutations can also be solely associated with aging, according to the mitochondrial theory of aging.

Similarly to what has been proposed in Alzheimer’s disease (Grazina et al., 2006; Onyang et al., 2006), mtDNA mutations may induce different phenotypic alterations related to MRC dysfunction in FTLD patients, depending on the metabolic characteristics of the subject, the individual antioxidant panel and the exposure to toxic agents. Mitochondrial dysfunction may cause or exacerbate some of the histopathological characteristics of neurodegenerative diseases, according to the “Mitochondrial Cascade Hypothesis”, which has been proposed in Alzheimer’s disease (Swerdlow et al., 2010; Swerdlow and Khan, 2009) and also in Parkinson’s disease (Domingues et al., 2008). This hypothesis is also supported by the fact that neurodegeneration caused directly by mitochondrial dysfunction is common in primary mtDNA disorders, with the specific neuropathological findings depending on the underlying genetic defect (Greaves et al., 2012).

Further studies are needed to clarify the possible contribution of mtDNA to FTLD, including sequencing of the remaining mtDNA genes and correlating genetic data with results of MRC enzyme activities.
References


Appendix - Guide for Authors from Neurobiology of disease

Use of wordprocessing software

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Subdivision - unnumbered sections

Divide your article into clearly defined sections. Each subsection is given a brief heading. Each heading should appear on its own separate line. Subsections should be used as much as possible when cross-referencing text: refer to the subsection by heading as opposed to simply 'the text'.
Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.
Essential title page information

- **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

- **Author names and affiliations.** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors’ affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

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**Abstract**

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