Current Topics in Genetics
Vol. 5, 2012

Original Communication

Leber's hereditary optic neuropathy - Molecular study of 540 Portuguese patients

S. Esteves[1], C. Nogueira[1], T. Evangelista[2], M. Encarnação[1], M. Teixeira[1], R. Neiva[3], C. Pereira[3], and L. Vilarinho[1,*]

[1]Unidade de Investigação & Desenvolvimento - Centro de Genética Medica, Instituto Nacional de Saude, Porto, [2]Serviço de Neuroloftalmologia - Hospital Santa Maria, Lisboa, [3]Unidade de Bioquímica Genética - Centro de Genética Médica, Instituto Nacional de Saude, Porto, Portugal

ABSTRACT

Leber's hereditary optic neuropathy (LHON) is a maternal hereditary disease that causes blindness due to optic atrophy, with typical onset during the second or third decade of life and affects predominantly males. The primary etiological factor relates to mutations in the mitochondrial genome. The purposes of this study were the screening of the most common mutations associated with LHON by PCR-RFLP (G11778A, G3460A, G15257A, and T14484A) in 540 Portuguese patients, the sequencing of MT-ND1 and MT-ND6 genes, considered as hot spot for LHON mutations by direct sequencing of PCR products and the establishment of genotype-phenotype correlation in the characterized patients. Of the 540 patients studied, 30 harbor three of the most common mutations investigated and in other seven patients we found seven pathogenic mutations, two of them present in MT-ND1 gene (G3688C and A4123G) and not described in the literature. In the approach taken to patients studied with LHON phenotype it was possible to perform a genotype-phenotype correlation in 6.9% of cases and only 5.5% had the most common mutations, while 1.4% presented other mitochondrial DNA (mtDNA) mutations between which, two not yet described in the literature. In addition to the mutations found, were also identified several polymorphisms, which reflect the high variability of mtDNA. This study also underlines the importance of further clinical and biochemical data of the patients to better clarify the pathology and thus provide a more accurate and precise diagnosis in addition to a more targeted molecular study.

KEYWORDS: optic atrophy, mitochondrial DNA, LHON, leber disease

*Corresponding author: Dr. Laura Vilarinho, Medical Genetics Center, Genetics Department, National Institute of Health, Praca Pedro Nunes, 88, 4099-028, Porto - Portugal.
laura.vilarinho@insa.min-saude.pt

INTRODUCTION

Leber's hereditary optic neuropathy (LHON) is a maternally inherited mitochondrial disorder characterized by bilateral loss of central vision, with typical onset during second or third decade of life and most frequently found in young adult males [1]. Usually, the disease comprises only ophtalmological findings. However, in some cases, the presence of additional neurological abnormalities, such as movement disorders, seizures, mental retardation or peripheral neuropathy, leads to the diagnoses of LHON-*plus* [2]. The severity differs between patients, even between members of the same family: sometimes light perception can be retained and partial vision recovery, even after several years, occurs in some

cases [3]. Other rare associations, such as LHON/multiple sclerosis - like clinical phenotype and a MELAS (Mitochondrial Encephalomyopathy and lactic acidoses with stroke-like episodes)/ LHON overlap syndrome - have also been described [4]. The first studies of the association of multiple sclerosis with LHON mutations, namely G11778A, have appeared in 1992 [5]. Both patients and asymptomatic carriers often have peripapillary telangiectasias. In some cases it is possible to detect a decrease of enzymatic activity of complex I of respiratory chain, in the muscle. These patients do not show significant changes in histological studies (absence of typical RRFs - Ragged Red Fibers). However, there are some patients that may develop them.

LHON was the first disease to be molecularly characterized in mitochondrial DNA (mtDNA) in 1988 [6], with the identification of pathogenic G11778A mutation present in the *MT-ND4* gene (NADH dehydrogenase subunit 4) of complex I of the respiratory chain. Subsequently, other mtDNA mutations, both primary and secondary, were identified, mostly located in encoding structural genes for complex I subunits (*MT-ND1*, *MT-ND2*, *MT-ND3*, *MT-ND4*, *MT-ND4L*, *MT-ND5* e *MT-ND6*). Primary mutations are strongly associated with LHON phenotype and are sufficient to cause the disease. The three most common mutations are G11778A (30-90%), G3460A (15%) and T14484C (10%), frequencies depending on the ethnic group [7].

The other group comprises secondary mutations that may interact and act synergistically to induce this phenotype.

Several studies have shown that the mitochondrial *MT-ND6* is a hot spot gene for LHON mutations, since ten mutations that cause optic neuropathy have already been described in this gene: this is the case of the A14495G which is associated with one of the most severe phenotypes [8]. Later, the MT-ND1 mitochondrial gene was also identified as a hot spot for LHON mutations [9].

Most pathogenic LHON mutations affect complex I of respiratory chain but there are also some secondary mutations described in genes coding for complex III, IV and V. Mutations are usually homoplasmic, however, cases of heteroplasmic mutations have been observed in some specific patients or families [10]. The prevalence of LHON in males ranges from 80% to 90% in Europe, up 60% in Japanese families. Among the female subjects at risk, the rate of occurrence varies between 8% and 32% [11]. The variable expression of LHON may be due to the association of pathogenic mutations with specific mtDNA haplogroups [10]. For example, haplogroup J (specifically European) is found more frequently in individuals with primary LHON mutations G11778A and T14484C than in control populations of other ethnic groups, thus suggesting that this haplogroup may increase the penetrance of the disease [10, 12, 13]. The primary G3460A mutation does not appear to be related to any particular haplogroup.

Although LHON is one of the best studied mitochondrial disorders, the exact pathological mechanism is still not fully known. Since the mitochondrion is the power factory of the cell, the most obvious explanation is that the mtDNA defect leads to a significant decrease in energy production and failure in optic nerve function.

At present there is no causal treatment of mitochondrial diseases, and in case of LHON there is no symptomatic one. Clinicians generally advise patients not to smoke or drink alcohol. Some of the patients receive anti-inflammatory drugs, with no positive effect, often before the final diagnosis is made [14]. The probability of visual recovery can also vary with mutations associated [11, 15]. A combination of all clinical, biochemical, physiological and molecular data clearly show that, while mtDNA mutations are essential for disease development, they are far from being the only cause. The interaction of different genetic and environmental factors is responsible for the expression of these highly variable and still mysterious disorders, placing it among multifactorial rather than strictly mitochondrial diseases [14].

The purposes of this study carried out in Portuguese patients were: i) screening of most common mutations associated with LHON, ii) sequencing of the MT-ND1 and MT-ND6 genes considered as hot spots for mutations of this

pathology and iii) establishment of genotype-phenotype correlation in the characterized patients.

PATIENTS AND METHODS

Patients

We investigated 540 Portuguese patients of both sexes, with a suggestive phenotype of LHON or LHON-like, from several hospitals around the country, aged between 3 months and 67 years.

As a control, we studied healthy and unrelated individuals.

Methods

DNA was extracted from leukocytes, whole blood and/or muscle biopsy and there were initially surveyed four most common mutations: G11778A, G3460A, T14484C and G15257A by *Polymerase Chain Reaction - Restriction Fragment Length Polymorphism* (PCR-RFLP). The amplified fragments were digested with the respective restriction enzymes (*MaeIII*; *AcyI*; *AccI* e *DpnII*). The products of enzymatic digestion were separated by horizontal electrophoresis on polyacrylamide gel (T = 12%, C = 3%) and then revealed with silver nitrate for visualization of bands corresponding to DNA fragments amplified. Positive results were later confirmed by automatic sequencing in an ABI 3130XL Genetic Analyser PRISMTM using Big Dye Terminator Cycler Sequencing Ready reaction Kit protocol (Applied Biosystems). In cases where no primary mutations were detected, we performed the sequencing of the MT-ND1 and MT-ND6 genes. The respective genes were amplified by PCR using a commercial mixture with DNA polymerase - ImmoMix Red (Bioline), primers at 50 pmol/mL, approximately 60 ng of DNA and bidistilled water. Primers were designed to overlap the fragments and the sequence covering the genes found in their flanking regions (*MT-TI* and *MT-TL1*). PCR products were purified by ExoSap (usb ®) enzyme and sequenced by the method described above. We used MvuI and Tsp509I restriction enzymes for the search of new MT-ND1 mutations, G3688C and A4123G respectively, by PCR-RFLP in 100 control subjects.

RESULTS

In this study we identified nine mutations, which allowed molecular characterization of 37 patients with suspicions of LHON (Table 1 and 2).

Three of the most frequent mutations were identified in 30 patients (23 male and 7 female) which means 5.5% of the total of studied patients (Table 1). The G3460A mutation was not identified in any of the patients studied. The direct sequencing of the *MT-ND1* and *MT-ND6* genes in the remaining 510 patients revealed the presence of seven pathogenic mutations in seven patients, two of them not yet described in the literature (Table 2).

The new mutations, G3688C and A4123G, located in the *MT-ND1* gene were found in homoplasmic state, as is typical of mutations associated with LHON phenotype (Figure 1).

Additionally to the previously referred mutations we have also identified 80 polymorphisms, 13 of them which have not yet been described in the literature [26] (Table 3).

Table 1. Most frequent mutations associated with LHON found in this study.

Mutation	Gene	Amino acid change	Incidence in this study	Number of patients/sex	Reference
G11778A	*MT-ND4*	R340H	4.4 % (24/540)	17/M 7/F	[6]
T14484C	*MT-ND6*	M64V	0.2 % (1/540)	1/M	[16]
G15257A[a]	*MT-CYB*	D171N	0.9 % (5/540)	4/M 1/F	[17]

[a]Associated with this mutation, two more mutations G13708A (A458T) and G15812A (V356M) in *MT-ND5* and *MT-CYB* genes were identified, respectively.

M - Male; F - Female.

Table 2. Other pathogenic mtDNA mutations found in this study.

Mutation	Gene	Amino acid change	Incidence in this study	Clinical symptoms	Sex	Age (years)	Reference
C3275A	*MT-TL1*	-	0.2 % (1/540)	Optic atrophy	M	53	[18]
G3688C	*MT-ND1*	A128P	0.2 % (1/540)	Retrobulbar optic neuritis in acute onset	F	66	n/d
A3796G	*MT-ND1*	T164A	0.2 % (1/540)	Retinal despigmentation; amaurosis and albinism	F	3/12	[19]
A4123G	*MT-ND1*	I273V	0.4 % (2/540)	LHON	M F	32 35	n/d
A4136G	*MT-ND1*	Y277C	0.2 % (1/540)	LHON	F	26	[20]
G4284A	*MT-TI*	-	0.2 % (1/540)	progressive bilateral decrease of visual acuity; Bilateral papillary pallor	M	61	[21]
T14325C	*MT-ND6*	N117D					[22]

n/d – not described; M – Male; F – Female.

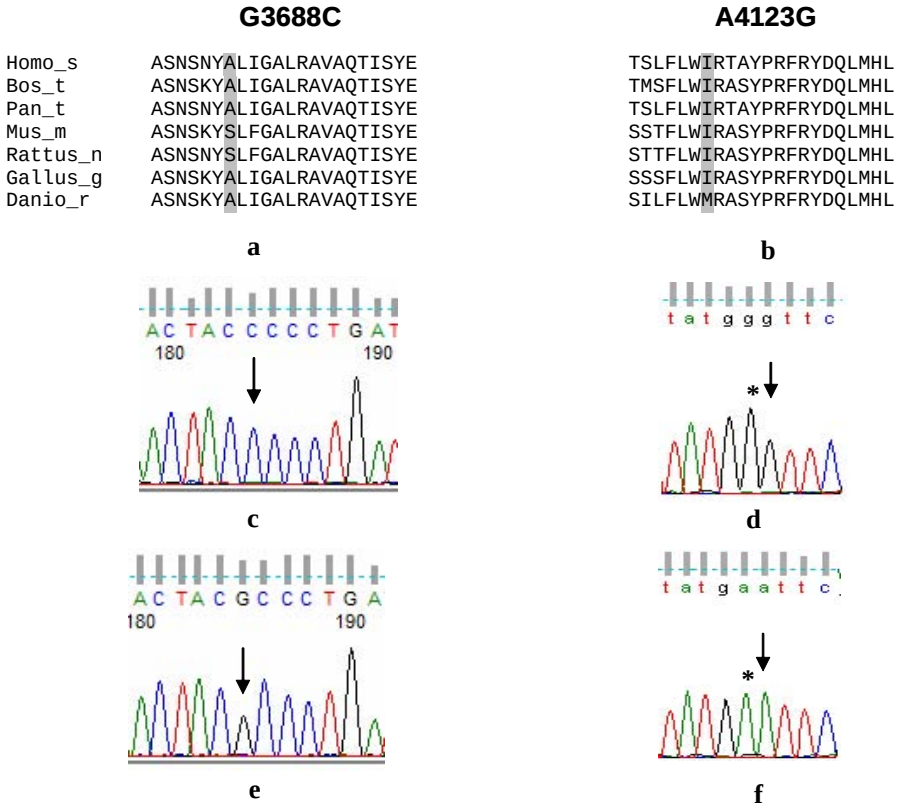

Figure 1. **a**,**b** - Amino acid alignment of human *MT-ND1*, for the novel mutations, G3688C e A4123G, respectively, with homologous of *Bos taurus*, *Pan troglodytes*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus* and *Danio rerio*. Conserved residues are highlighted. Protein sequences were the ones referred in *Ensembl Genome Browser* [23]. Conservation of the mutated residues was assessed by alignment of orthologous and human protein sequences with ClustalW program [24]. **c,d** - Partial sequences of the *MT-ND1* gene in two normal controls for each novel mutation. **e,f** - Partial sequences of the *MT-ND1* gene with the mutations G3688C and A4123G, respectively. * A4122G polymorphism, already described in the literature [25].

Table 3. Novel polymorphisms identified in this study.

Gene	mtDNA position	Nucleotide change
MT – ND1	3388	C - T
MT – ND1	3494	C - T
MT – ND1	3535	T - C
MT – ND1	3642	C - G
MT – ND1	3744	G - A
MT – ND1	3825	A - G
MT – ND1	3912	G - A
MT – ND1	4035	A - G
MT – ND1	4095	C - T
MT – ND1	4218	T - C
MT – ND6	14155	C - T
MT – ND6	14539	A - G

The most common polymorphisms already published and found in this work were the T4216C and T3394C, located in the *MT-ND1* gene.

DISCUSSION

The study of 540 Portuguese patients with suspicions of LHON or LHON-like revealed a positivity of 4.6% for primary mutations G11778A and T14484C, and 0.9% for secondary mutation G15257A, initially screened as they are most frequently associated with this phenotype. The G11778A mutation was found in homoplasmic state in 4.4% of cases, which is consistent with the literature data, since it is the most frequent and more severe primary mutation associated with LHON [6, 15]. In 0.2% of cases, corresponding to a male patient was identified a T14484C mutation in homoplasmic state. Five patients being three related presented the mutation G15257A together with G13708A and G15812A mutations, as described by Johns and collaborators (1991) [17]. These mutations are considered secondary, however, by acting in synergy, may be considered the cause of optic atrophy presented in these patients.

The results found in this study regarding the most common mutations identified are proportionally in accordance to the literature, although presenting with a lower frequency. This lower frequency may be due to a rather selective screening of patients sent to this study, probably because several patients have been included with a clinical picture of LHON-like.

In addition to the most common mutations, seven rare mutations were also identified, in 1.4% of the cases studied, two of which (G3688C and A4123G) have not yet been described. Of these rare mutations, four are present in *MT-ND1* gene and the other three in *MT-ND6* gene and in genes corresponding to the flanking regions, *MT-TL1* and *MT-TI*.

The homoplasmic C3275A mutation identified in one patient is located in the *MT-TL1* gene coding for tRNA leucine 1 and was described by Garcia-Lozano and collaborators (2001) [18], associated with the LHON phenotype.

The pathogenic mutations A3796G (MT-ND1), A4136G (MT-ND1), G4284A (MT-TI) and T14325C (MT-ND6) were also found in homoplasmic state in this cohort of patients. The A3796G mutation was identified in a patient with retinal depigmentation, amaurosis and albinism. However, there are still some questions whether this mutation is responsible for such clinical phenotype, since it is described either as a pathogenic mutation in heteroplasmy state associated with dystonia with adulthood onset [19] or as a polymorphism, when present in homoplasmic state [26].

The A4136G mutation identified in a patient with clinical symptoms of LHON is characterized by the substitution of a tyrosine for a cysteine at residue 277 (Y277C). It was described either as a pathogenic mutation associated with LHON linked to neurological disorders and deficits in complex I [20], or as a secondary mutation [27]. To clarify the presumable pathogenicity of this mutation, further studies are being conducted, including its screening on 100 control subjects by PCR-RFLP. Another patient harbours the T14325C and G4284A missense mutations. The latter one located in *MT-TL1* gene was identified due to the fact that we studied the flanking regions

of *MT-ND1* gene. The first one is described as a candidate for LHON pathogenic mutation, whereas the second is associated with a heterogeneous clinical phenotype [21]. The simultaneous presence of two probable pathogenic mtDNA mutations in the same patient suggests that G4284A may be a secondary mutation when associated with LHON phenotype. The patient, in whom they were identified, presents a clinical phenotype of papillary pallor and progressive bilateral decrease of visual acuity. In this case, we speculate that the mutation responsible for that phenotype is the T14325C, since, according to the literature, it will be more related to the clinical phenotype of our patient.

In this investigation were also detected the mutations G3688C and A4123G, not yet described in the literature, in homoplasmic state, both in *MT-ND1* gene. The G3688C mutation was identified in a patient with retrobulbar optic neuritis in acute onset and is characterized by the substitution of an alanine for a proline at residue 128 (A128P). Alanine is an amino acid with aliphatic hydrophobic character and being replaced by an amino acid with hydrophilic character such as proline, in a conserved region, may lead to a change in the structure and function of this subunit of complex I. For this same residue, a pathogenic mutation is described in the literature, which involves the exchange of an alanine for a threonine (A128T), being associated with mitochondrial encephalomyopathy [28]. The A4132G mutation was identified in two patients with clinical phenotype of LHON. This novel mutation is characterized by the substitution of an isoleucine for a valine at residue 273 (I273V), both aliphatic amino acids in a conserved region of mtDNA molecule. These two new mutations were searched by PCR-RFLP in a control sample of 100 healthy subjects and no positive case was found. This result suggests the pathogenicity of these two mutations, but the further functional, biochemical and/or family linkage studies will confirm the true pathogenicity of these new mutations.

In addition to the previously referred mutations, we have also identified several polymorphisms, some of which have not yet been described, reflecting the high variability of mtDNA. These mitochondrial variations may somehow affect oxidative phosphorylation, resulting in decreased mitochondrial respiration and generation of reactive oxygen species. However, these results should be confirmed by further studies in other populations, as reported by other authors [29]. Some polymorphisms such as T3394C and T4216C (*MT-ND1*), appear more frequently in the studied patients, being also described, in other studies, as secondary mutations associated with LHON. There is still some controversy about the pathogenic effect of secondary mutations of mtDNA in LHON. These nucleotide substitutions are found in higher frequency in LHON patients than in control subjects and some researchers argue that they can act synergistically with the primary mutations thus, increasing the risk of disease expression. These secondary mutations and other polymorphisms found could possibly be related to different mitochondrial haplogroups, especially J or T, which is consistent with our sample, since these are Portuguese patients.

The diagnosis of LHON is not always easy because there are other diseases with similar symptoms, such as Autosomal Dominant Optic Atrophy (ADOA) [30], triggered by changes in OPA1 gene, whose clinical phenotype is characterized by degeneration of retinal ganglion cells associated with optic atrophy. This gene is located on chromosome 3 and encodes a protein related to the dynamin GTPase, inserted into the inner mitochondrial membrane. Consequently, this work will continue with the study of this gene in patients who did not reveal mutations for mitochondrial genes.

The approach taken to patients studied with LHON phenotype turned possible to perform a genotype-phenotype correlation in 6.9% of cases and only 5.5% had the most common mutations, while 1.4% presented other mtDNA mutations between which, two not yet described in the literature. This study also underlines the importance of further clinical and biochemical data of the patients to better clarify the pathology and thus provide a more accurate and precise diagnosis in addition to a more targeted molecular study.

ACKNOWLEDGMENTS

The authors acknowledge the collaboration of clinicians in sending the patient samples studied: Drª Ana Maria Silva, Dr. Luís Monteiro, Drª Carla Fraga, Drª Edite M. Silva, Drª Isabel Leite and Drª Fátima Campos of Hospital Santo António, Dr. Ferro of Hospital Santa Maria, Drª Ana Moreira of Hospital D. Estefânia, Drª Cândida Barroso of Hospital Egas Moniz, Drª Isabel M. Aires of Hospital of Aveiro, Dr Tedim Cruz of Hospital São Sebastião, Drª Olinda Faria of Hospital São João, Drª Carolina Araújo and Dr. Helder Simões of Hospital of Capuchos, Dr. Jorge Becho of Hospital of Santarém and Drª Carla Ferreira of Hospital of São Marcos.

REFERENCES

1. Harding, A. E. 1994, Rev. Neurol., 22, 186.
2. Grazina, M. M., Diogo, L. M., Garcia, P. C., Silva, E. D., Garcia, T. D., Robalo, C. B., and Oliveira, C. R. 2007, Eur. J. Paediatr. Neurol., 11, 115.
3. Man, P. Y., Turnbull, D. M., and Chinery, P. F. 2002, J. Med. Genet., 39, 162.
4. Nikoskelainen, E. K., Martilla, R. J., Huoponen, K., Juvonen, V., Lamminen, T., Sonninen, P., and Savontaus, M. L. 1995, J. Neurol. Neurosurg. Psychiatry, 59, 160.
5. Harding, A. E., Sweeney, M. G., Miller, D. H., Mumford, C. J., Kellar-Wood, H., Menard, D., McDonald, W. I., and Compston, D. A. 1992, Brain, 115, 979.
6. Wallace, D. C., Singh, G., Lott, M. T., Hodge, J. A., Schurr, T. G., Lezza, A. M., Elsas II, L. J., and Nikoskelainen, E. K. 1988, Science, 242, 1427.
7. Brown, M. D. and Wallace, D. C. 1994, J. Clin. Neurosci., 2, 138.
8. Chinnery, P. F., Brown, D. T., Andrews, R. M., Singh-Kler, R., Riordan-Eva, P., Lindley, J., Applegarth, D. A., Turnbull, D. M., and Howell, N. 2001, Brain, 124, 209.
9. Valentino, M. L., Barboni, P., Ghelli, A., Bucchi, L., Rengo, C., Achilli, A., Torroni, A., Lugaresi, A., Lodi, R., Barbiroli, B., Dotti, M., Federico, A., Baruzzi, A., and Carelli, V. 2004, Ann. Neurol., 56, 631.
10. Brown, M. D., Starikovskaya, E., Derbeneva, O., Hosseini, S., Allen, J. C., Mikhailovskaya, I. E., Sukernik, R. I., and Wallace, D. C. 2002, J. Hum. Genet., 110, 130.
11. Newman, N. J. 1993, Arch. Neurol., 50, 540.
12. Hofmann, S., Jaksch, M., Bezold, R., Mertens, S., Aholt, S., Paprotta, A., and Gerbitz, K. D. 1997, Hum. Mol. Genet., 6, 1835.
13. Torroni, A., Petrozzi, M., D'Urbano, L., Sellitto, D., Zeviani, M., Carrara F., Carducci, C., Leuzzi, V., Carelli, V., Barboni, P., De Negri, A., and Scozzari, R. 1997, Am. J. Hum. Genet., 60, 1107.
14. Tonska, K., Kodrón, A., and Bartnik, E. 2010, Biochim. Biophys. Acta, 1797, 1119.
15. Newman, N. J., Lott, M. T., and Wallace, D. C. 1991, Am. J. Ophthal., 111, 750.
16. Brown, M. D., Voljavec, A. S., Lott, M. T., Torroni, A., Yang, C. C., and Wallace, D. C. 1992, Genetics, 130, 163.
17. Johns, D. R. and Neufeld, M. J. 1991, Biochem. Biophys. Res. Commun., 181, 1358.
18. Garcia-Lozano, J. R., Aguilera, I., Bautista, J., and Nunez-Roldan, A. 2001, Hum. Mutat., 15, 120.
19. Simon, D. K., Friedman, J., Breakefield, X. O., Jankovic, J., Brin, M. F., Provias, J., Bressman, S. B., Charness, M. E., Tarsy, D., Johns, D. R., and Tarnopolsky, M. A. 2003, Neurogenetics, 4, 199.
20. Howell, N., Kubacka, I., Xu, M., and McCullough, D. A. 1991, Am. J. Hum. Genet., 48, 935.
21. Corona, P., Lamantea, E., Greco, M., Carrara, F., Agostino, A., Guidetti, D., Dotti, M. T., Mariotti, C., and Zeviani, M. 2002, Ann. Neurol., 51, 118.
22. Howell, N., Oostra, R. J., Bolhuis, P. A., Spruijt, L., Clarke, L. A., Mackey, D. A., Preston, G., and Herrnstadt, C. 2003, Am. J. Hum. Genet., 72, 1460.
23. www.ensembl.org
24. www.ebi.ac.uk/Tools/clustalw2
25. Mishmar, D., Ruiz-Pesini, E. E., Golik, P., Macaulay, V., Clark, A. G., Hosseini, S., Brandon, M., Easley, K., Chen, E., Brown, M. D., Sukernik, R. I., Olckers, A., and Wallace, D. C. 2003, P. Natl. Acad. Sci. USA, 100, 171.

26. www.mitomap.org
27. Obermaier-Kusser, B., Lorenz, B., Schubring, S., Paprotta, A., Zerres, K., Meitinger, T., Meire, F., Cochaux, P., Blankenagel, A., and Kommerell, G. 1991, Am. J. Hum. Genet., 55, 1063.
28. Valente, L., Piga, D., Lamantea, E., Carrara, F., Uziel, G., Cudia, P., Zani, A., Farina, L., Morandi, L., Mora, M., Zeviani, M., and Tiranti, V. 2009, Biochim. Biophys. Acta, 1787, 491.
29. Kumar, M., Tanwar, M., Saxena, R., Sharma, P., and Dada, R. 2010, Mol. Vis., 16, 782.
30. Kjer, P. 1959, Acta. Ophthalmol. Suppl., 164, 1.