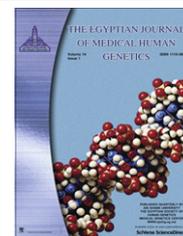




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ORIGINAL ARTICLE

Clinical and molecular findings in eight Egyptian patients with suspected mitochondrial disorders and optic atrophy

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8344A > G;
8993T > G/C;
11778G > A;
3460G > A;
14484T > C

Abstract Mitochondrial respiratory chain disorders (RCD) are a group of genetically and clinically heterogeneous diseases, caused due to defects of the respiratory chain. This study aimed to investigate the presence of common mtDNA point mutations in tRNA^{Leu} (UUR), tRNA^{Lys}, MT-ATPase 6, MT-ND4, MT-ND1, MT-ND6 genes in eight Egyptian patients suspected to have mtDNA disease and optic atrophy.

PCR-RFLP analysis was done for the detection of 3243A > G, 3271T > C, 8344A > G, and 8993T > G/C mtDNA point mutations. DNA direct sequencing was pursued for the detection of 11778G > A, 3460G > A and 14484T > C mtDNA point mutations. No point mutation of 3243A > G, 3271T > C, 8344A > G, and 8993T > G/C was detected in our group of patients. Four mtDNA polymorphisms in MT-ND1 and MT-ND4 genes (11467A > G, 11719G > A, 3348A > G and 3357G > A) were detected in three patients.

Mitochondrial disorders are caused by a variety of genetic and racial factors, which differ among populations. The negative results of this study indicate that the chosen mutations might not be specific in Egyptians. Another explanation might be due to the low heteroplasmic levels of the mtDNA mutation. A registry for the different mtDNA mutations in Egyptian patients is highly recommended.

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1. Introduction

Mitochondrial respiratory chain diseases (RCDs) are a heterogeneous group of inherited disorders characterized by impaired energy metabolism due to oxidative phosphorylation (OXPHOS) dysfunction [1,2]. Neurons and myocytes are highly dependent on ATP produced by mitochondria, so these tissues are primarily affected [3,4]. A reported minimum birth prevalence of mitochondrial RCDs was 1/5000 [1]. Studies in

adults and/or children suggest a prevalence approaching 1/7600 in Australia [5] and 1/21,000 in Sweden [6]. Mitochondrial RCDs can be caused by either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA) mutations. Mutations in the mitochondrial genome could be either large-scale rearrangements or point mutations [7,8].

A number of 'classical' syndromes have been described that are often, but not always, associated with a particular genotype. They include mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy and ragged red fibers (MERRF), maternal inherited Leigh's syndrome (MILS), neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP), Leber's hereditary optic neuropathy (LHON), and progressive external ophthalmoplegia (PEO). However, a substantial group of patients, particularly children, do not fulfill the clinical criteria for a particular syndrome and may have symptoms or signs that overlap one or more clinical syndromes [8,9].

There is a number of recurrent mtDNA point mutations, including the 3243A > G transition in the mitochondrial transfer RNA Leucine1 gene (tRNA^{Leu} (UUR); OMIM#590050), which is the most common mtDNA point mutation. It accounts for more than 85% of patients with MELAS and for a large number of PEO patients that do not have large-scale deletions. The less common 3271T > C mutation in the same gene was found in about 10% of MELAS patients [10,11]. The point mutation 8344A > G in the tRNA^{Lys} gene (OMIM#590060) has been found in >85% MERRF patients [12]. The 8993T > G/C mutations in the mitochondrial ATP synthase subunit 6 gene (MT-ATPase 6; OMIM#516060) are found in about 10–30% of individuals with MILS [13–15], while the same mutations account for >50% in NARP patients with elevated blood lactate concentration [13,16]. Several point mutations in protein coding genes have been described in LHON patients, specifically the transition 11778G > A in the mitochondrial NADH reductase subunit 4 gene (MT-ND4; OMIM#516003) that account for approximately 50% of all cases. The three point mutations 11778G > A, 3460G > A in the MT-ND1 gene (OMIM#516000) and 14484T > C in the MT-ND6 gene (OMIM#516006) account for 90–95% of LHON cases [17,18].

Obtaining a definitive and specific molecular diagnosis for a patient with clinically suspected mtDNA diseases allows more specific medical management, accurate genetic counseling, prenatal diagnosis, and overcomes the ambiguities often seen with biochemical analysis, histological, and enzymatic evaluations [19]. In this study we aimed to investigate the presence of the most common mtDNA point mutations (3243A > G, 3271T > C, 8344A > G, 8993T > G/C, 11778G > A, 3460G > A and 14484T > C) in Egyptian patients who are suspected to have mtDNA diseases and optic atrophy.

2. Subjects and investigations

2.1. Subjects

The study included eight Egyptian patients (four males and four females) with clinical suspicion of mitochondrial disease and optic atrophy. The purpose and the extent of the study were explained to the parents, and consent forms were obtained and approved by the National Research Center

(NRC) Ethics Research Committee. Patients were clinically examined to detect any malformation and anomalies. Pedigree was constructed. Mitochondrial diseases were diagnosed depending on a high index of suspicion based on neurologic, muscular, ophthalmologic, hearing loss, neurophysiological impairment and increase in lactate and pyruvate plasma levels. Since this is mainly based on a diagnosis of exclusion, all other causes were considered and ruled out. The following investigations were done for all patients:

*Neurophysiological investigations:

Electroencephalogram (EEG)
Electromyogram (EMG)
Nerve conduction studies (NCS)
Complete eye evaluation including electroretinogram (ERG) and visual-evoked potential (VEP)
Hearing test
Magnetic resonance imaging (MRI)
Magnetic resonance spectroscopy (MRS)

*Pathological examination:

Muscle biopsy was stained by the modified Gomori trichrome stain [20]

*Biochemical assessments:

Plasma lactate (9.0–16.0 mg/dL) [21]
Plasma pyruvate (0.3–0.7 mg/dL) [22]

3. Methods

3.1. DNA extraction

Five ml venous blood was used for DNA extraction by the salting-out procedure of [23].

3.2. PCR amplification

Seven uniplex PCR amplifications were performed using the following primers for the detection of the seven common reported mutations.

(1) Mutation 3243A > G [24]

F: 5'- CCT CCC TGT ACG AAA GGA C -3'

R: 5'- GCG ATT AGA ATG GGT ACA ATG-3'

(2) Mutation 3271T > C [25]

F: 5'- AGG ACA AGA GAA ATA AGG C -3'

R: 3'- AAT TCC AGT CTC CAA GTT AAG GAG AAG AAT -5'

(3) Mutation 8344A > G [26]

F: 5'- CCC CCA TTA TTC CTA GAA CCA GGC G -3'

R: 5'- GGG GCA TTT CAC TGT AAA GAG GTG TGG G -3'

(4) Mutation: 8993T > G/C [27]

F: 5'- CCG ACT AAT CAC CAC CCA AC -3'

R: 3'- TTC GGA GAT GGA CGT GCT GT -5'

(5) Mutation: 11778G > A [28]

F: 5'- CCC ATC GCT GGG TCA ATA GT -3'

R: 3'- G GT GCA AGA GGA CTA GTT TA -5'

(6) Mutation: 3460G > A [29]

F: 5'- CAG TCA GAG GTT CAA TTC CTC -3'

R: 3'- ATG ATA CTT GGG GGG AGG GGT -5'

(7) Mutation: 14484T > C [30]

F: 5'- CCC CAC TAA AAC ACT CAC CAA G -3'
 R: 3'- ATT TGG GTA TAT TGG AGG GGG TTT -5'

3.3. Restriction enzyme digestion

Amplified PCR products containing suspected mutations, **3243A > G**, **3271T > C**, **8344A > G** and **8993T > G/C** were digested using four restriction endonucleases HaeIII, AflII, BlnII and HpaII (Fermentas, European union), respectively. Digested PCR products were analyzed by polyacrylamide gel electrophoresis.

3.4. DNA sequencing

Amplified PCR products containing suspected mutations 11778G > A, 3460G > A and 14484T > C mtDNA were purified using the QIAquickT (Qiagen©, Germany) PCR Purification Kit for further analysis by DNA sequencing. Cycle sequencing reactions were performed in 20 µl total volumes containing 8 µl ABI PRISM® BigDye® Terminator v3. 1 Ready Reaction Mix, Applied Biosystems ABI 310 DNA Sequencer using 310 Data Collection Software version 3.1.0. Sequencing products were analyzed using DNA Sequencing Analysis Software version 5.4. Sequencing results were evaluated both manually and automatically with the Sequence Navigator.

4. Results

The study included eight patients with mitochondrial disorder and optic atrophy; four were females and four were males. Pa-

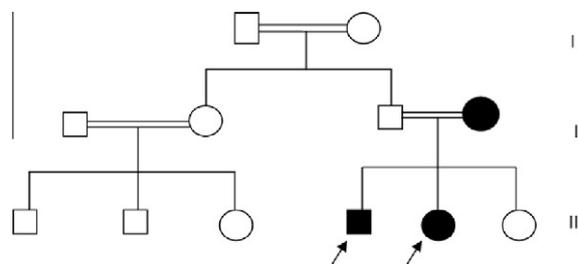


Figure 1 Pedigree of patient 2 showing maternal inheritance.

tients' ages of onset ranged from 2 months to 3 years (mean age was 1 year and 3 months). Patient 1 (P1) had a maternal relative with psychiatric disease, P2 had a affected mother and a similarly affected sib, (Fig. 1). P4 had a family history of a similarly affected sib, P3 had two sibs manifested with seizure and died before the age of 1 year, and P6 had a family history of three neonatal deaths by congenital heart disease. All patients in the study presented with hypotonia and optic atrophy. Seizures were present in six cases and mental retardation in two cases. Dystonia was found in one case, microcephaly in one case, moderate sensorineural hearing loss in one case, nystagmus in one case and papilledema and ptosis in another. Pathological examination of muscle biopsy showed ragged red fibers (RRF) in one case. Neurophysiological investigations: EMG revealed myopathy in all cases; MRI brain showed cortical atrophy, white matter demyelination and/or basal ganglia degeneration. NCS showed demyelinating neuropathy in peripheral nerves in four cases. The EEG showed epileptogenic dysfunction in three cases. MRS showed a high lactate peak in two cases, (Figs. 2-4) Table 1.

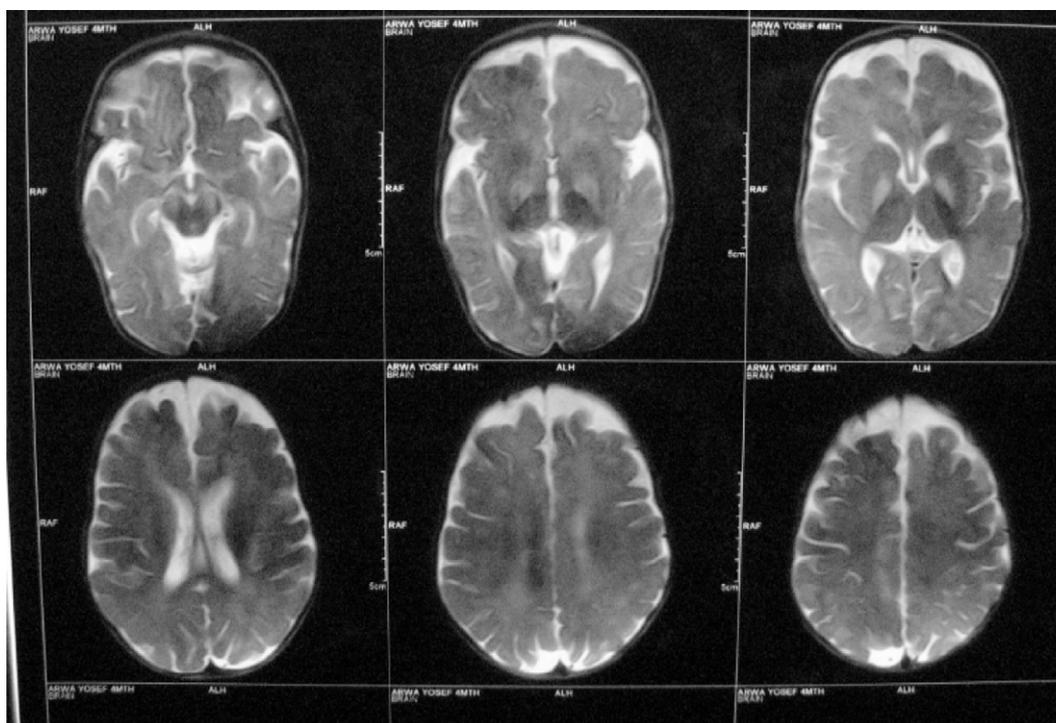


Figure 2 Axial T2 of patient 2 showing bilateral high signal of lentiform nucleus (mainly globus pallidus), deep white matter changes and mild cortical atrophy changes.

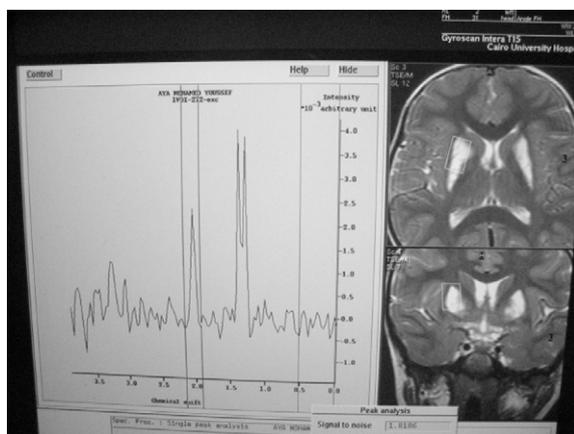


Figure 3 MR spectroscopy for patient 6 showing high signal of lentiform nucleus and high lactate peak indicating mitochondrial disorders.

PCR-RFLP analysis for detection of four of the common mitochondrial mutations was negative in all patients (Figs. 5–8). In all cases, only a band corresponding in size to that of the fragment containing the normal nucleotide was observed: a 196 bp band for the 2343A digested fragment (Fig. 5), a 172 bp band for the 3271T fragment (Fig. 6), two bands (299 and 78 bp) for the 8344A digested fragment (Fig. 7), and a 554 bp band for the 8993T fragment (Fig. 8).

DNA sequencing revealed the absence of mitochondrial mutations **11778G > A**, **3460G > A** and **14484T > C** in all patients. Four mitochondrial variants in MT-ND1 and MT-ND4 genes were found in patients 3, 4, and 8. Gene loci, codon changes, amino acid changes and status of the four variants 11467A > G, 11719G > A, 3348A > G and 3357G > A are summarized in Table 2.

5. Discussion

Diagnosing mitochondrial RCDs remains a major challenge to the clinician due to their varied age at onset, clinical

presentations and their genetic heterogeneity [8,19]. Thus, mitochondrial diseases may present with “any symptom in any organ at any age” [31]. The age of onset in patients of patients included in this study ranged between 2 months and 3 years, a range that was reported with several mitochondrial diseases including MELAS (<2 to >20 years in ~70% of cases [32]), MERRF (childhood age [12]), MILS (typically 3–12 months, later onset >1 year or in adulthood occurs in up to 25% of patients [33]) and NARP (early childhood [13]). The peak age of onset in LHON varies between the second and the third decades of life [17]. However, visual deterioration can occur at any time during the first to the seventh decade of life and LHON should be part of the differential diagnosis of all cases of bilateral, simultaneous or sequential optic neuropathy, irrespective of age and especially in male patients [34,35].

Taking a family history is difficult when we consider the relatively non-specific features of mitochondrial diseases. Patients with unaffected maternal relatives may be due to variation in the expression of the disease or in the presence of low levels of heteroplasmic mutations [8,36]. Also, the clinical variability among siblings that was found in patients 3 and 6 is one of the most remarkable features of mitochondrial disease caused by mtDNA defects. This is thought to reflect the mitochondrial “genetic bottleneck”. However, this difference in the transmitted mutation load does not explain why one sibling might present with neurological disease while another might develop heart failure. Clearly additional factors must come into play [37].

Seven out of the eight patients included in this study showed multisystem involvement (brain, eye, and muscles) reflecting, in part, the dependence on energy derived from oxidative phosphorylation in a wide variety of tissues; a feature that is a hallmark of mitochondrial diseases and may be the initial indication to the correct diagnosis [9,19]. Our patients did not fulfill the clinical criteria of any specific classical syndrome. However, they manifested with the common symptoms of mitochondrial diseases that include seizures, stroke-like episodes, neuropathy, ptosis, dystonia and unexplained hypotonia in newborn, infant, or young child [9,31] in conjunction with other symptoms such as hearing loss and visual

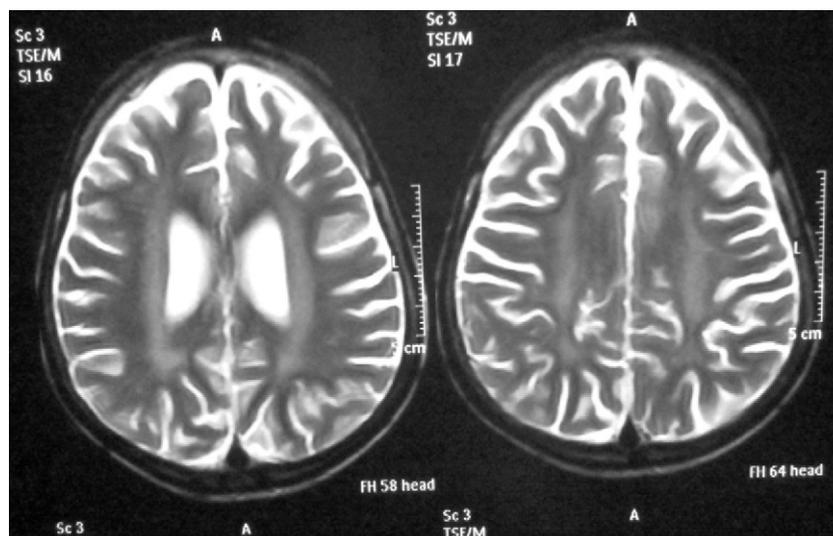


Figure 4 Axial T2 for patient 6 showing deep white matter changes and mild cortical atrophy.

Table 1 Clinical and laboratory data of the eight patients with mitochondrial disorder.

| Patient number/ gender | Age of onset | Clinical symptoms | Eye manifestation | Blood lactate (9.0–16.0 mg/ dL)/pyruvate (0.3–0.7 mg/dL) | Investigations |
|---------------------------|--------------|--|----------------------------------|---|---|
| 1/M | 1 yr | Hypotonia hyporeflexia dystonia | Optic atrophy | 32/1.4 | EMG: myopathy. MRI: white matter demyelination, cerebral and cerebellar atrophy and basal ganglia degeneration |
| 2/ F | 2 yr | Severe hypotonia seizure stroke | Optic atrophy | 33/2 | EMG: myopathy. NCS: demyelinating neuropathy. MRI: white matter changes and mild cortical atrophic changes |
| 3/F | 2 mo | Hypotonia brisk reflexes seizures microcephaly | Optic atrophy | 22/3.2 | EEG: bilateral temporoparietal epileptogenic dysfunction. EMG: myopathy. MRI: simplified gyral pattern anteriorly with atrophic changes, hypogenesis of corpus callosum, white matter demyelination |
| 4/M | 4 mo | Hypotonia brisk reflexes ankle clonus seizures sensorineural hearing loss (SNHL) | Optic atrophy | 34/2.2 | EMG: myopathy. NCS: demyelinating neuropathy in peripheral nerves. Muscle biopsy: ragged red fibers. MRI: cortical atrophic changes and basal ganglia degeneration |
| 5/M | 6 mo | Hypotonia seizures | Optic atrophy | 33.5/3.4 | EMG: myopathy. MRI: basal ganglia degeneration and white matter demyelination |
| 6/ F | 1 yr | Hypotonia hyporeflexia seizures | Optic atrophy papilledema ptosis | 35/2 | EEG: bilateral focal epileptogenic. EMG: myopathy. NCS: demyelinating neuropathy in peripheral nerves. MRI: white matter changes and mild cortical atrophy. MRS: high lactate peak |
| 7/F | 2 yr | Mental retardation hypotonia | Optic atrophy | 25/1 | EMG: myopathy. MRI: white matter demyelination and basal ganglia degeneration. MRS: high lactate peak |
| 8/M | 3 yr | Mental retardation hypotonia seizures | Nystagmus optic atrophy | 32/2 | EMG: myopathy, EEG: epileptogenic dysfunction, NCS: demyelinating neuropathy in peripheral nerves. MRI: Cortical atrophic changes |

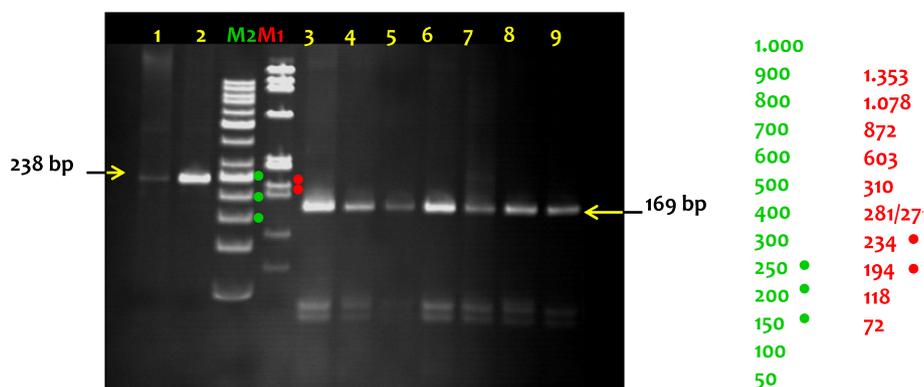


Figure 5 8% Polyacrylamide gel electrophoretic analysis of HaeIII digestion of the PCR product for detection of mutation 3243A > G. Fragments containing normal 3243A nucleotide have two enzyme recognition sites that cleave it into three bands (169, 37 and 32 bp). The 3243G mutated nucleotide introduces another enzyme recognition site into the amplified fragments that are digested producing 97, 72, 37 and 32 bp bands. Lane (1): undigested PCR product (238 bp). Lanes (2–8): digested PCR products from seven different patients showing digestion pattern of normal fragments only. M1: 50 bp DNA ladder; its fragment lengths are demonstrated left to the figure (in green). M2: ØX174 HaeIII molecular weight marker; its fragment lengths are demonstrated left to the figure (in red). The dot marks represent the reference bands that correspond to the analyzed fragments.

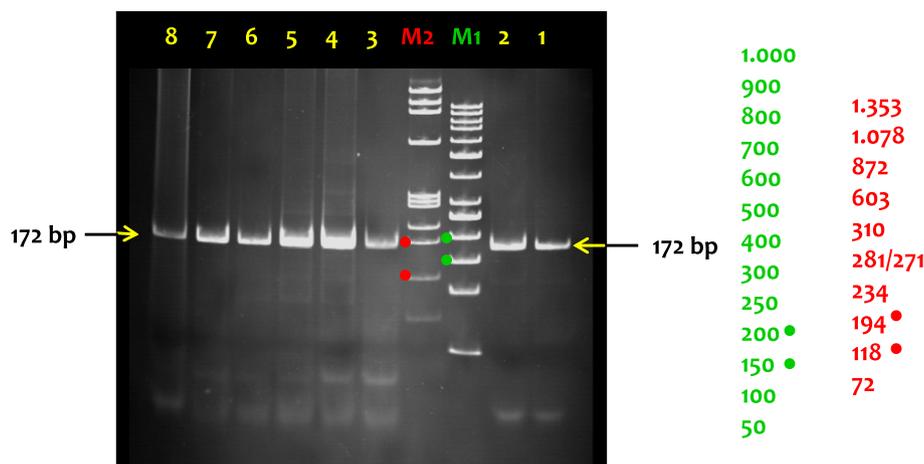


Figure 6 8% Polyacrylamide gel electrophoretic analysis of AflII digestion of the PCR product for detection of mutation 3271T > C. Fragments containing normal 3271T nucleotide have no enzyme recognition site. The 3271C mutated nucleotide introduces one enzyme recognition site into the amplified fragments that are digested producing 142 and 30 bp bands. Lanes (1–2): undigested PCR products. Lanes (3–8): digested PCR products from 6 different patients showing digestion pattern of normal fragments only. M1: 50 bp DNA ladder; its fragment lengths are demonstrated left to the figure (in green). M2: ØX174 HaeIII molecular weight marker; its fragment lengths are demonstrated left to the figure (in red). The dot marks represent the reference bands that correspond to the analyzed fragments.

impairment. This coincides with the findings of McFarland and Turnbull [9] who reported similar mitochondrial cases with atypical phenotype.

All of our patients manifested with optic atrophy and hypotonia; two red flag findings in mitochondrial diseases [31]. Although, optic atrophy is the primary and generally the only clinical manifestation in most LHON pedigrees [38], it is manifested also as a secondary feature, overshadowed by other more prominent neurological and ocular manifestations, in several mitochondrial diseases including MELAS, MERRF, CPEO, KSS, MILS, and mitochondrial neuro-gastrointestinal encephalomyopathy (MNGIE) [17,39]. Several studies of patients with LS showed hypotonia as a feature of their disease [40–43]. Hypotonia, dystonia, muscle weakness, hypo or

hyperreflexia, seizures, mental retardation, movement disorders, peripheral neuropathy, eye movement disorders and optic atrophy are all abnormalities due to focal and necrotizing lesions characterizing LS [13,15,44]. Optic atrophy, hypotonia and seizures alone or in association with brisk reflexes were found in two of our patients whose ages of onset were 2 and 6 months. The patient with brisk reflexes also showed microcephaly that is a non-specific feature that increases the likelihood of a mitochondrial disorder [31] and was found in association with brainstem dysfunction and growth retardation in LS patients [45].

Seizures were also found in four other patients included in this study. In one of them, seizures manifested at an age of 2 years, the patient had experienced a stroke attack and had

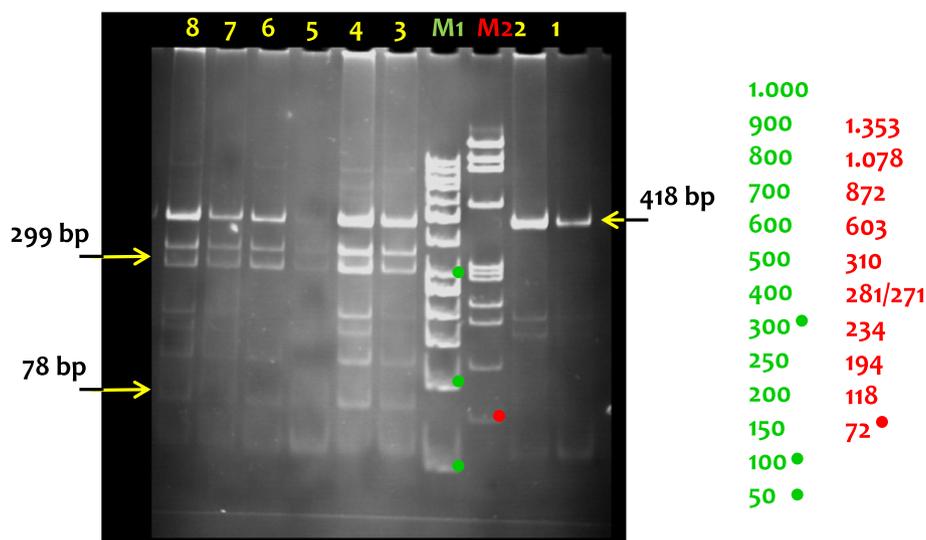


Figure 7 10% Polyacrylamide gel electrophoretic analysis of BanII digestion of the PCR product for detection of mutation 8344A > G. Fragments containing normal 8344A nucleotide have two enzyme recognition sites that cleave it into three bands (299, 78 and 41 bp). The 8334G mutated nucleotide introduces another enzyme recognition site into the amplified fragments that are digested producing 299, 52, 41 and 26 bp bands. Lanes (1–2): undigested PCR product (418 bp). Lanes (3–8): digested PCR products from five different patients showing digestion pattern of normal fragments only. M1: 50 bp DNA ladder; its fragment lengths are demonstrated left to the figure (in green). M2: ØX174 HaeIII molecular weight marker; its fragment lengths are demonstrated left to the figure (in red). The dot marks represent the reference bands that correspond to the analyzed fragments.

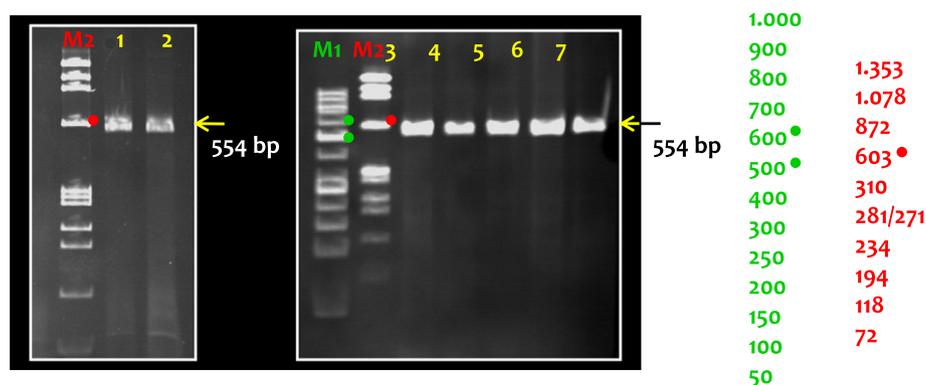


Figure 8 8% Polyacrylamide gel electrophoretic analysis of HpaII digestion of the PCR product for detection of mutation 8993T > C. Fragments containing normal 8993T nucleotide have no enzyme recognition site. The 8993C mutated nucleotide introduces one enzyme recognition site into the amplified fragments that are digested producing 347 and 207 bp bands. Lanes (1–2): undigested PCR product (554 bp). Lanes (3–7): digested PCR products from 5 different patients showing digestion pattern of normal fragments only. M1: 50 bp DNA ladder; its fragment lengths are demonstrated left to the figure (in green). M2: ØX174 HaeIII molecular weight marker; its fragment lengths are demonstrated left to the figure (in red). The dot marks represent the reference bands that correspond to the analyzed fragments.

Table 2 mtDNA variants found in the eight Egyptian patients as compared to the human mtDNA revised Cambridge reference sequence.

| Patient number | Nucleotide number and substitution | Codon Change | Locus | Amino acid change | Status |
|----------------|------------------------------------|--------------|--------|-------------------|-------------|
| 4 | 11467A > G | TTA > TTG | MT-ND4 | L236L | Homoplasmic |
| 4 and 8 | 11719G > A | GGG > GGA | MT-ND4 | G320G | Homoplasmic |
| 4 | 3348A > G | CTA > CTG | MT-ND1 | L14L | Homoplasmic |
| 3 | 3357G > A | ATG > ATA | MT-ND1 | M17M | Homoplasmic |

a family history of an affected brother who showed similar symptoms. This family was suspected to suffer from MELAS syndrome. Thus, encephalopathy characterized by seizures, dementia or both and stroke-like episode before the age of 40 years are two of three almost invariant criteria for diagnosing MELAS [32]. Seizures were found in 96% of MELAS patients while 99% had stroke-like episodes in the study of Hirano and Pavlakis [46].

The three other patients with seizures had features of hearing loss and RRF on muscle biopsy, ptosis, papilledema and hyporeflexia, nystagmus and mental retardation. Hearing loss is a common disabling feature of mitochondrial disease with a variable age of onset. It frequently occurs in families harboring the 3243A > G mutation [47], other mtRNA gene mutations [48–51] and in LHON, Kearns Sayre, CPEO [52], and with non-classical mitochondrial diseases [53]. RRF on muscle biopsy demonstrating abnormal subsarcolemmal accumulations of mitochondria is suggestive of mitochondrial disease. Thus, the presence of >2% RRFs on skeletal muscle biopsy is taken as one of the criteria for the diagnosis of mitochondrial disorders [54]. Mental retardation and seizures were detected in 52.6% of Egyptian patients with non classical mitochondrial diseases [53], in a MELAS patient with hypotonia, convulsion and right hemiparesis [55]. While mental retardation alone was found in 59% of LS patients [56] and in MERRF patients [57]. Nystagmus is one of the clinical features of OXPHOS defects presenting in the neonatal period [58], commonly associated with LS [9,40] and was found in LHON patients [59]. Ptosis was reported in probands with MELAS/MERRF overlap disease [60], LS [56,61] and non-classical mitochondrial disease [53,62].

All of our patients had high blood lactate and pyruvate levels. Two patients also showed high lactate peak on MRS. Despite their lack of specificity, an elevated plasma lactate or pyruvate level can be an important marker of mitochondrial disease [31].

Demyelination around occipital horns found in patient 6 was reported in MELAS patients [32]. Brain atrophy and basal ganglia degeneration found in patients 1, 2 and 4 are often seen in MERRF patients [63]. Basal ganglia with prominent cerebellar involvement found in patient 1 have been reported to be consistent with Leigh syndrome [64]. Another study by El-Bassyouni et al. [53] found cerebral white matter affection and cerebellar atrophy in 12/19 patients (63.2%) and basal ganglia calcification in 2/19 patients (10.5%) with mitochondrial disorders.

One of our patients showed abnormal subsarcolemmal accumulations of mitochondria on skeletal muscle biopsy stained with Gomori trichrome, a unique feature of mitochondrial disease described as the 'RRF'. However, RRFs are rarely seen in early childhood, when subsarcolemmal accumulation of mitochondria may be mild or absent. Additionally, abnormal accumulation of mitochondria may be absent in patients with proven mitochondrial disease such as LS and NARP [9,13,19].

Molecular analysis in our patients did not reveal any of the common mutations associated with mitochondrial respiratory chain disorders. This might be due to the low level heteroplasmy of the mtDNA mutation in peripheral blood samples, a situation reported by Haas et al. [2] to be responsible for false negative results. The 3243A > G MELAS mutation can be found in about 50% of the cases when blood samples are used

for PCR-RFLP. This is thought to be the result of the rapidly replicating ability of the blood cells, which will gradually eliminate the leukocytes with a higher mutation load [65]. 10–15% of LHON patients have heteroplasmic mutations in different tissues [38,66,67]. On the other hand, white blood cells or any other tissue type can be used to test for the 8993T > G/C mutations [13]; as they do not show any significant variation in mutation load among tissues [68].

Detecting the causative mutation is another challenge in molecular diagnosis. Determining which one of dozens, if not hundreds of genes spanning two genomes responsible for mitochondrial dysfunction in a given patient is the challenge [2]. MELAS was reported to be associated with many other mutations concentrated on the mtRNA_{Leu} (UUR) gene, even though there are more than thirty pathogenic mtDNA mutations in other mtRNA or protein-coding genes seen with the disease [10]. Mutations causing MERRF appear to be restricted to tRNA gene [69]. However, single cases of other non-tRNA mitochondrial point mutations, multiple mtDNA deletions [70] or nuclear DNA mutation in the polymerase gamma gene (POLG) have been described [71]. In LHON, many mtDNA point mutations have been described, but each account for only one or a few pedigrees worldwide. Most often, these other mutations involve genes encoding the ND1 and ND6 subunits of complex I as they are considered "mutational hotspots" region for the disease [38] accounting for 50–90% of LHON pedigrees in different ethnic groups [72]. Approximately 10%–20% of individuals with MILS have mutations in other mitochondrial genes than MT-ATP6 [14,15,73].

DNA sequence analysis in order to detect mutations 11778 and 3460 in our patients showed the presence of 4 mitochondrial variants named 11467A > G, 11719G > A, 3348A > G and 3357G > A. Both 11467A > G and 11719G > A are silent polymorphisms located on the mitochondrial genome within the ND4 gene. They had been previously described in control individuals [74]. Two studies of Kumar et al. [72] and Puomila et al. [75] reported that none of the two variants fulfilled the criteria for a primary pathogenic mtDNA mutation. The 11467A > G is a mitochondrial SNP that belongs to ethnic-specific haplogroups K which showed a significantly higher association to altered brain pH in patients suffering from psychiatric disorders [76]. Also, it is reported as haplogroup U5 and U6 specific polymorphism [77]. The 11719G > A polymorphism was reported in all T, J, U, X, and K haplogroups [78]. Howell et al. [79] found 11719G > A in the matrilineal pedigree of the 14482 LHON family. The 3348A > G and 3357G > A transitions are synonymous mitochondrial SNPs located in the mitochondrial ND1 gene [80]. The 3348A > G change characterizes the U6a haplogroup that is one of the African mitochondrial haplogroups [77,78].

In conclusion, establishing a specific diagnosis in a patient with the suspected mitochondrial disease is a complex endeavor that requires the integration of clinical assessments, family history, electrophysiologic investigations, biochemical testing, histopathological examination, and molecular testing. Close collaboration between primary clinicians, geneticists, pathologists, other clinical specialists, and diagnostic laboratories with expertise in mitochondrial biochemical and molecular testing is critical to maximize the likelihood of obtaining a correct diagnosis. Not finding the chosen common mutations in our

study could be due to low heteroplasmic levels of the mtDNA mutations in leukocytes or the presence of other mutations in either of the two genomes. Screening for more mutations is recommended to reach a specific diagnosis.

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