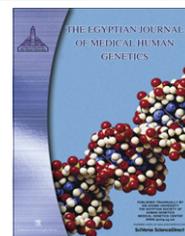




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CASE REPORT

Mitochondrial DNA depletion syndrome presenting with ataxia and external ophthalmoplegia: Case report

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Abstract The mitochondrial DNA depletion syndromes are autosomal recessive disorders characterized by decreased mitochondrial DNA copy number in affected tissues. Mutations in 2 genes involved in deoxyribonucleotide metabolism, the deoxyguanosine kinase gene and the thymidine kinase 2 gene, had been related to this syndrome. This study aims to describe the clinical, histochemical, biochemical and molecular diagnosis of one Egyptian pediatric patient with the myopathic form of mitochondrial depletion syndrome. The patient presented to Cairo University Pediatric Hospital with the clinical suspicion of mitochondrial encephalomyopathy. Histochemical and biochemical studies of the respiratory chain complexes were performed on the muscle biopsy specimen from the patient. Molecular diagnosis was done by quantitative radioactive Southern blot and sequencing analysis of the whole coding regions of the TK2 gene. Histochemical staining revealed cytochrome oxidase negative fibers and increased staining for succinate dehydrogenase. The activity of complex I was not detected and complex IV activity was about 46% of age matched controls. Southern blot analysis showed reduction of the mitochondrial/nuclear DNA ratio, the degree of depletion was around 30% of aged-matched controls. Sequencing analysis of the TK2 gene revealed no sequence variation. Targeted molecular diagnosis based on the biochemical analysis of the respiratory chain enzymes makes the molecular evaluation of mitochondrial disorders much easier. Involvement of other nuclear genes rather than TK2 gene in the pathogenesis of the myopathic form of mitochondrial depletion syndrome should be considered.

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1. Introduction

Mitochondria are keys to many cellular processes. One of the most important mechanisms is oxidative phosphorylation (OXPHOS) resulting in the production of cellular energy in the form of ATP. The OXPHOS system consists of five complexes (I–V) and two mobile electron carriers (coenzyme Q and cytochrome c) embedded in the inner mitochondrial membrane [1].

The mitochondrial genome encodes 13 essential polypeptides of the OXPHOS system and the necessary RNAs machinery. The remaining structural proteins and those involved in import, assembly and mitochondrial DNA (mtDNA) replication are encoded by the nuclear DNA and are targeted to the mitochondria [2].

Disorders of mitochondrial origin are a heterogeneous group of diseases commonly manifesting in tissues with a high-energy demand, for example, muscle and nerve, hence the name “mitochondrial encephalomyopathies” [3].

Mutations in respiratory chain protein subunits encoded by either mitochondrial (mt) DNA or nuclear (n) DNA are responsible for such diseases [4]. Over 100 point mutations of mtDNA that are known to cause mitochondrial dysfunction had been identified [5].

Recent developments in the molecular diagnostics allowed for the exploration of many of pathogenic mutations, thus providing more clues about the molecular basis of these disorders. The aim of the present study is to describe the clinical, histochemical, biochemical and molecular diagnosis of one Egyptian pediatric patient with the myopathic form of mitochondrial DNA depletion syndrome.

2. Case report

A 2.5 years old girl, the 3rd child of unrelated parents, born by a caesarean section at term after normal pregnancy. She had

normal developmental history. By the age of 1.5 years, mother noticed unsteadiness of gait and repeated falling followed at 2 years by loosing the ability of independent walking, with the appearance of head nodding, nystagmus and squint followed by seizures. Her clinical examination revealed ataxia and generalized trunkal and limb hypotonia, bilateral ptosis, external ophthalmoplegia and fine nystagmoid movements. Her laboratory investigations showed persistent lactic acidemia, 2.8 mmol/L (normal, up to 2.2 mmol/L). Liver and kidney functions, creatine kinase (CK) and plasma ammonia were normal. Brain magnetic resonance imaging (MRI) showed abnormal lesions in the basal ganglia (appearing hypodense in T1 weighted images (WIs) and hyperintense on T2 WIs, cerebellar demyelination and abnormal signal intensity in the brain stem. This picture was suggestive of SURF1 gene mutation Fig. 1. Echocardiographic (ECHO) and chest radiographic findings were normal. An electroencephalogram (EEG) showed epileptiform activity. The parents have one healthy daughter (1 year old). Their first child (III-1) had presented in a similar manner at 8 months of age and died by the age of 2.5 years, the cause of death was uncertain. They had a boy (III-2) who died by 32 weeks of gestation (S.B.) Fig. 2.

3. Methods

3.1. Ethical issue

Verbal consent was obtained from the parents for all the procedures performed. Parental written informed consent was obtained for the undergoing muscle biopsy.

3.2. Family history

The patient who is a regular visitor of the neurometabolic clinic of Cairo University Pediatric Hospital (CUPH) presented clinically with encephalomyopathy. The underlying

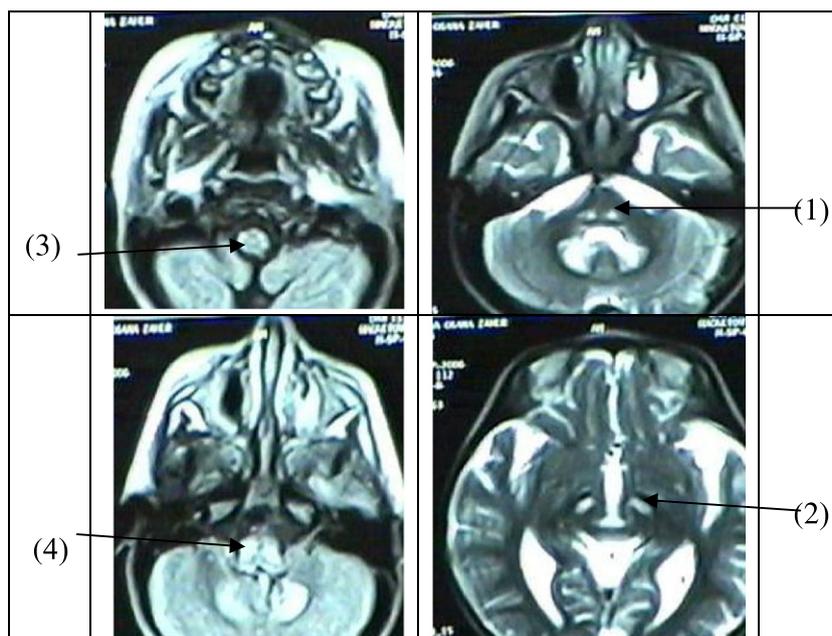


Figure 1 MRI brain of the proband showing abnormal T2 and FLAIR hyperintense signals are noted at: dentate nuclei (1), periaqueductal gray matter (2), medulla oblongata (3) and lower pons (4) (arrows).

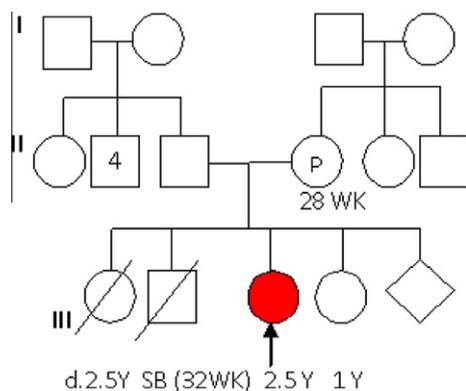


Figure 2 Family pedigree of the studied patient.

Table 1 Sequence of the primers used for amplification and sequencing of ATPase 6 gene.

PCR primers	Sequence (5'–3')
MTF8200	TTTCATGCCCATCGTCCTAGAATTA
MTR9550	TGCCCTCCTAATTGGGGGGTA
MTF 8648	GACTAATCACCACCAACAATGAC

cause of encephalomyopathy was suspected clinically to be of mitochondrial origin. Members of the family pedigree were interviewed to identify both personal or family medical histories and other clinical abnormalities.

3.3. Muscle biopsy

Muscle biopsy specimens from Quadriceps Femoris were performed under general anesthesia at the CUPH. The specimens were immediately frozen in liquid nitrogen after collection, stored at -80°C .

3.4. Histochemical and biochemical investigations

The activities of the respiratory chain (RC) complexes and the Citrate Synthase (CS) as a marker for the mitochondrial

content, were determined in the muscle homogenate using standard spectrophotometric methods [6,7]. Enzyme activities are expressed with respect to both the total protein and CS activity [8]. Muscle biopsy specimens were subjected to histochemical staining for COX and SDH using Bio-Optica Staining Kits; Milan, Italy.

3.5. Molecular genetic studies

Total nuclear DNA and mtDNA were extracted from peripheral blood leukocytes of the patient along with age and sex matched healthy controls. The control pediatric group was attending the hospital for other different reasons. All control subjects reported no symptomatic metabolic, genetic or ocular disorders regarding family history, past medical problems and current health. Extraction was done using the DNA isolation kit from Qiagen (TM), Germany, following the manufacturer's instructions. DNA was extracted from the muscle biopsy specimens of the proband along with other age-matched patients and controls using the phenol–chlorophorm–isoamyl alcohol method, according to the published methods [9–12].

As neuroimaging of the proband was initially suggestive of the mitochondrial disorder, Leigh syndrome, ATPase 6 gene was examined by direct sequencing being commonly mutated in such disorder [13]. The gene was PCR-amplified from blood DNA using the forward and reverse primers MTF8200 and MTR9550 respectively. The purified products were sequenced by the Applied Biosystems (ABI) 3130xl genetic analyzer using the capillary electrophoresis. The MTF8200, MTF 8648 and MTR9550 primers were used for sequencing (Table 1). The sequences were analyzed using Staden software. Sequencing results were compared with the revised Cambridge reference sequence (rCRS) [14]. All sequence variants were compared with the MITOMAP database (<http://www.mitomap.org>), the Human Mitochondrial Genome Database (<http://www.genpat.uu.se/mtDB>) and the GiiB-JST mtSNP (single nucleotide polymorphism) database (http://mitsnp.tmg.or.jp/mitsnp/index_e.shtml), all are provided in the public domain.

Based on the clinical suspicion of mitochondrial DNA depletion syndromes (MDS) and according to the histochemical and

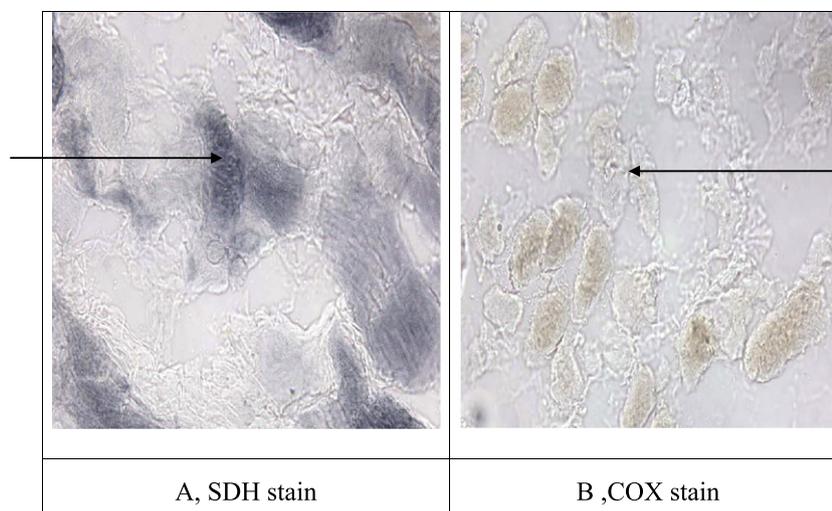


Figure 3 Histochemical analysis of the muscle biopsy of the proband. (A) SDH stain shows increased staining, (B) COX staining shows reduced enzymatic activity in several myofibers (for both, the magnification $\times 40$) (arrows). Increased SDH stain and reduced COX stain are highly suggestive of mitochondrial disease.

Table 2 Results of the biochemical analysis of mitochondrial respiratory chain's enzymes.

Enzyme activity nmol/min/mg prot	CS	COX	COX/CS	SDH	SDH/CS	II	II/CS	III	III/CS	I	I/CS	II + III	II + III/CS
The proband	210	156	0.74	16	0.08	20	0.1	62	0.3	N.D.	N.D.	15	0.014
Mean ± SD of controls	197 ± 47	559 ± 158	3.1 ± 0.8	20 ± 7	0.10 ± 0.03	35 ± 100	18 ± 0.05	54 ± 14.0	33 ± 0.13	29 ± 10	0.18 ± 0.004	14 ± 5	0.09 ± 0.03
Reference interval	141-311	330-900	1.7-4.6	11-45	0.07-0.16	22-63	0.11-0.26	34-76	0.16-0.52	12-50	0.10-0.31	6-21	0.06-0.14

Abbreviations: COX: cytochrome-o oxidase; I: complex I; II: complex II; III: complex III; NA: not available; N.D.: not detected; succinate dehydrogenase; CS: Citrate Synthase. Data are given as single values for the patient and as mean ± SD for controls.

biochemical analyses results, P32-radioactive Southern blot and quantitation of the mt/n DNA ratio were conducted as previously described [15,16]. For confirmation of the myopathic form of MDS, TK2 gene was sequenced using the 3130xl ABI automated sequencer [17].

4. Results

Results of histochemical staining of the muscle biopsy specimen were compatible with mitochondrial myopathy. There was increased staining for SDH Fig. 3A and COX negative fibers Fig. 3B. The SDH stain evaluates complex II of the RC (totally encoded by the nuclear DNA) while [18], the COX stain evaluates complex IV [19] encoded for by both n/mt DNA. The presence of increased SDH activity and decreased COX activity is highly suggestive of mtDNA involvement [20,21].

Biochemical analysis of the muscle biopsy specimen was suggestive of mtDNA involvement. Marked reduction of the activities of the RC complexes had been detected. Complex I

Table 3 Nucleotide changes found in ATPase 6 genes.

Nucleotide locus	Nucleotide position	Sequence change (a)	Amino acid change (b)	Previously reported references
ATPase 6	8292	g-a	Non-coding	[22]
ATPase 6	8527	a-g	M-V	[23]
ATPase 6	8723	g-a	R-Q	[24]

(a) Changes at the following nucleotides positions are frequently occurring variants in the general population. (b) The first letter is the amino acid residue predicted by the revised Cambridge sequence. The last letter is the predicted amino acid in the protein of the patient. Amino acid changes are expressed as one letter code: <http://molbio.info.nih.gov/gcode.html>.

Table 4 Quantitation of mt/n DNA ratio of the proband and other myopathic patients (2-6) along with control cases using phosphorus imager. The results are expressed as the copy number of mitochondrial DNA per haploid nuclear gene. The control value of the muscle mtDNA copy number was obtained through the pooling of muscle specimens of 9 age-matched controls (1-9). The degree of depletion in the proband of the present study was around 50% of age-matched controls.

mtDNA/nDNA ratio			
7.916940226	CONTROL 1	CONTROLS	
6.497194225	CONTROL 2		
6.697378218	CONTROL 3	Mean:	Std. Dev.:
6.716325279	CONTROL 4	5865	1245
6.158424718	CONTROL 5	Minimum:	Maximum:
4.197091359	CONTROL 6	4197	7917
5.256952307	CONTROL 7		
4.291275294	CONTROL 8		
5.051666402	CONTROL 9		
2.958601912	The proband (1)	PATIENTS	
3.59282748	PATIENT2		
3.47408944	PATIENT3	Mean:	Std. Dev.:
3.347482147	PATIENT4	3228	0.285
2.900524709	PATIENT5	Minimum:	Maximum:
3.096489032	PATIENT6	2901	3593

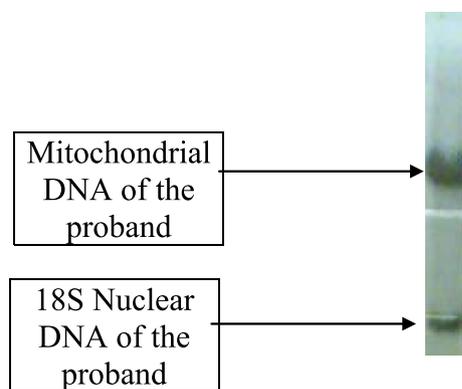


Figure 4 Autoradiograph of Southern blot of total DNA extracted from muscle of the proband. The total (nuclear and mitochondrial) DNA was hybridized simultaneously with two ^{32}P -labeled probes—human mtDNA and a cloned fragment of the human 18S ribosomal RNA gene (nuclear DNA). Arrows.

activity was not detected. Complex IV activity was about 46% of age-matched healthy controls. The activities were also found to be reduced when referred to CS. Activity of SDH was normal. Results of biochemical analysis are shown in Table 2.

Sequencing analysis of the ATPase 6 gene revealed 3 recognized benign polymorphisms [22–24] Table 3. Southern blot analysis showed reduction of the mt/n DNA ratio of the studied case and confirmed mitochondrial DNA depletion, the degree of depletion was around 50% of age-matched controls Table 4, Fig. 4. Sequencing analysis of the TK2 gene revealed no sequence variations.

5. Discussion

Mitochondrial DNA depletion syndromes (MDS) are autosomal recessive disorders characterized by a reduction in mitochondrial DNA copy number in the clinically affected tissues [4,15].

A balanced supply of deoxyribonucleoside triphosphates (dNTPs) is required for DNA polymerase γ to faithfully replicate the mitochondrial genome, the mitochondrial deoxynucleotide triphosphates (dNTPs) pool is maintained both through the import of cytosolic dNTPs and by salvaging deoxyribonucleosides within the organelle itself [4,25].

A number of nuclear-encoded enzymes are involved in these processes, including thymidine kinase 1 (TK1) within the cytosol, thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) within mitochondria. The last two are key enzymes of the mitochondrial salvage pathway [4,26].

In the past few years, there had been a substantial increase in the understanding of the molecular basis of mtDNA depletion syndromes [8]. The *TK2* gene is located on chromosome 16 and encodes a 234-amino acid polypeptide, which is synthesized in the cytoplasm, then imported into the mitochondrial matrix [20]. It catalyzes the transfer of a phosphate group from adenine triphosphate to thymidine or deoxycytidine [28,29], whereas dGK efficiently phosphorylates deoxyguanosine and deoxyadenosine to the corresponding deoxynucleotide monophosphates [30]. The combined action of the 2 enzymes allows the synthesis of all 4 dNTPs needed for mtDNA replication [30,31].

Mutations in the *TK2* gene in patients with myopathic MDS [28] and mutations in the *dGK* gene in patients with hepatocerebral MDS [32,33] were identified confirming the concept that imbalances in mitochondrial dNTP pool can affect mtDNA integrity. This imbalance leads to inefficient replication and, therefore, depletion of mtDNA [18,34]. More recently, mutations in *RRM2B*, encoding the small subunit of p53-inducible ribonucleotide reductase, have also been identified in patients with myopathic mtDNA depletion disorder [35].

The MDS differs from other mitochondrial disorders because it is a quantitative defect rather than a qualitative defect. The low level of mtDNA in some tissues causes insufficient synthesis of respiratory chain components [15]. Depletion of mtDNA can occur as a secondary phenomenon, for example as a result of antiretroviral nucleoside analogue therapy [36]. In MDS, however, mtDNA depletion is considered a primary process, and the degree of depletion is generally proportional to the severity of the phenotype [27].

Patients with the myopathic form of MDS usually present at or soon after birth with progressive weakness, hypotonia, hyporeflexia, and die of respiratory failure before 1 year of age (congenital form) or before 10 years of age (juvenile form) [27].

Since the first description of *TK2* mutations leading to the myopathic form of mtDNA depletion syndrome in 2001, approximately 20 different pathogenic mutations have now been reported, distributed throughout the *TK2* coding sequence [37].

The proband of the present study was clinically suspected to have mitochondrial encephalomyopathy. Based on MRI findings, the initial diagnosis was suggestive of Leigh syndrome (LS) with *SURF1* gene mutation. Bilateral putaminal T2 prolongation has long been considered a consistent feature of LS on MRI studies [38]. LS with COX deficiency caused by *SURF1* gene mutations represents a relatively homogeneous clinical entity. Similar to the proband of this study, affected patients present at around 1 year of age with progressive encephalopathy, generalized hypotonia, trunk ataxia, oculomotor abnormalities and central respiratory problems [39,40]. However, the biochemical evidence of deficiency of complex I and complex IV in such case was against the suggestion of *SURF1* gene mutation which is exclusively associated with isolated complex IV deficiency.

As high index of suspicion of MDS is warranted in any child with proximal weakness and hypotonia of unclear etiology, the diagnosis needed to be confirmed by Southern blot analysis for mtDNA quantitation and subsequent *TK2* mutation screening [37].

Based on the results of histochemical, biochemical and molecular analyses, the proband had fulfilled the diagnostic criteria for the myopathic form of MDS [41,42]. These criteria include clear evidence of mtDNA depletion by Southern blot, histochemical evidence of COX deficiency, plus at least two of the following features: ragged-red fibers (RRF), lactic acidosis, multiple defects of respiratory chain enzymes [25]. Based on these findings, it was mandatory to screen this patient for mutations in *TK2* gene, in order to better define the frequency of mutations in this gene.

Similar studies had been conducted by Tiranti et al., Johanson et al., Mandel et al. and Finsterer [4,25,37,43]. Similar to the probands of the present study, those patients developed

myopathy and depletion of muscular mtDNA in infancy. In contrast to results of the present study, several mutations in *TK2* gene had been detected confirming the hypothesis that mutations in *TK2* represent a new etiology for mitochondrial DNA depletion, underscoring the importance of the mitochondrial dNTP pool in the pathogenesis of mitochondrial depletion. Therefore, MDS should be included in the differential diagnosis of all unexplained myopathies of infancy and childhood [27,44].

Similar to the conclusion of the present study, it had been found that the myopathic form of MDS is genetically heterogeneous. *TK2* mutations account for only a small percentage of patients with the myopathic form of MDS [25]. This suggests that defects in other genes must be involved in the etiology of myopathic MDS. Therefore, it is recommended to screen negative patients for mutations in other candidate genes rather than *TK2* and *dGK* genes involved in dNTP metabolism.

The CK level of the proband of the present study was normal. Similar finding had been found in a study that involved 4 patients with MDS [37]. Similarly, they concluded that marked increase in serum CK values seen in most patients is an unusual finding in mitochondrial myopathies and a useful diagnostic clue.

6. Conclusion

Holistic approach through clinical, neuroimaging, histochemical, biochemical and molecular investigations is important to reach the final diagnosis. Targeted molecular diagnosis based on the biochemical analysis of RC enzymes makes the molecular evaluation of pediatric mitochondrial disorders more easier. Negative results of molecular diagnosis for mtDNA genes do not exclude mitochondrial diseases and involvement of nuclear genes should be always suspected.

To the best of our knowledge, this study is the first study that reported on the Egyptian patient with MDS syndrome through molecular analysis which further reinforces the fundamental role of molecular analysis. Recruitment of the latest techniques in the molecular genetic analysis of these disorders can reveal novel mutations. In the future, more information about the role of molecular genetic analysis will be provided by large scale family studies and investigation of an increased number of possible candidate genes identified by the human genome project.

Last and not least, Egypt as a country with high consanguinity and where genetic disorders are a very common cause of morbidity and mortality, molecular genetic analysis including the latest molecular techniques should be highly encouraged.

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