ISTANBUL UNIVERSITY PRESS

RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1164334
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

EVALUATION OF MITOCHONDRIAL DNA MUTATIONS IN SIX FAMILIES BY RESEQUENCING ARRAY

MİTOKONDRİYAL DNA MUTASYONLARININ TEKRAR DİZİLEME ARRAY YÖNTEMİ İLE ALTI AİLEDE DEĞERLENDİRİLMESİ

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Cite this article as: Kolbasi Demircioglu G, Guntekin Ergun S, Gucuyener K, Percin FE, Ergun MA. Evaluation of mitochondrial DNA mutations in six families by resequencing array. J Ist Faculty Med 2023;86(1):78-87. doi: 10.26650/IUITFD.1164334

ABSTRACT

Objective: Human mitochondrial DNA is a circular, double stranded molecule which is inherited through maternal lineage. Point mutations in tRNA, rRNA or protein coding genes and structural rearrangements such as partial deletions or duplications can cause mitochondrial disorders. The prevalence of mitochondrial diseases is estimated to be 1/5000 worldwide. For the analysis of mtDNA mutations, Sanger sequencing, Southern blot, long and quantitative PCR, Resequencing Array and next-generation sequencing methods can be used. In this study, we analysed whole mitochondrial genomes of six children (along with their mothers) who were admitted to Gazi University Hospital with symptoms suggestive of mitochondrial disease.

Materials and Methods: After the extraction of genomic DNA from six children and their mothers, mtDNA resequencing with the analysis of obtained data was performed. In order to determine whether one of the mutations found in Patient 4 was homoplasmic or heteroplasmic, PCR and RFLP techniques were also used.

Results: Among six patients included in this study group, none of the variants detected could be attributed to any mitochondrial diseases, except the pathogenic mutation detected in Patient 4. The m.3460 G>A mutation detected in Patient 4 was located in the *MT-ND1* gene that was known to be responsible for LHON. This mutation detected in Patient 4 was also detected both in his mother and sister with homoplasmic state. The lack of clinical findings in his mother and sister was thought to be due

ÖZET

Amaç: İnsan mitokondriyal DNA'sı, maternal kalıtılan dairesel, çift sarmallı bir moleküldür. tRNA, rRNA veya protein kodlayan genlerdeki nokta mutasyonları ve kısmi delesyonlar veya duplikasyonlar gibi yapısal yeniden düzenlemeler mitokondriyal bozukluklara neden olabilir. Mitokondriyal hastalıkların dünya genelinde prevalansının 1/5000 olduğu tahmin edilmektedir. MtDNA mutasyonlarının analizi ile ilgili olarak Sanger dizileme, Southern blot, kantitatif PCR, tekrar dizileme ve yeni nesil dizileme yöntemleri kullanılabilir.

Gereç ve Yöntem: Bu çalışmada, mitokondriyal hastalığı düşündüren semptomları olan ve Gazi Üniversitesi Hastanesi'ne başvuran 6 çocuk ve annesinden genomik DNA'nın elde edilmesinin ardından mtDNA yeniden dizileme yöntemi ile elde edilen verilerin analizi yapıldı. Dördüncü hastada bulunan mutasyonlardan birinin homoplazmik veya heteroplazmik olduğunu belirlemek için PCR-RFLP tekniği kullanıldı.

Bulgular: Çalışma grubumuza dahil edilen altı hastadan 4. hastada saptanan patojenik mutasyon dışında, saptanan değişikliklerin hiçbiri hastalarımızdaki mitokondriyal hastalık ile ilişkilendirilmemiştir. Dördüncü hastada saptanan m.3460 G>A mutasyonu: *MT-ND1* geninde lokalize olup LHON'dan sorumludur. Dördüncü hastada saptanan bu mutasyon, hastanın anne ve kız kardeşinde de homoplazmik olarak saptandı. Anne ve kız kardeşinde klinik bulgu olmamasının, kadınlarda hastalığın penetransının azalmasına ve nükleer genomdaki genlerin modifiye edilmesine bağlı olduğu düşünüldü.

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Submitted/Başvuru: 19.08.2022 • **Revision Requested/Revizyon Talebi:** 14.09.2022 •
Last Revision Received/Son Revizyon: 10.10.2022 • **Accepted/Kabul:** 13.10.2022 • **Published Online/Online Yayın:** 26.01.2023

to decreased penetrance of the disease in females and modifying genes in nuclear genome.

Conclusion: Screening of mtDNA using resequencing method could provide fast, effective and more reliable results in the diagnosis of mitochondrial diseases. Also, currently, the NGS technology analysis of nuclear DNA along with mtDNA will provide more reliable results in diagnosis of mitochondrial diseases, thus allowing more accurate genotype-phenotype correlation.

Keywords: Mitochondrial DNA, mutation, Resequencing Array

Sonuç: mtDNA'nın yeniden dizileme yöntemiyle taranması mitokondriyal hastalıkların tanısında hızlı, etkili ve daha güvenilir sonuçlar sağlayabilir. Ayrıca, şu anda NGS teknolojisi ile nükleer DNA'nın mtDNA ile birlikte analiz edilmesi mitokondriyal hastalıkların tanısında daha güvenilir sonuçlar verecek ve böylece daha doğru genotip-fenotip korelasyonuna izin verecektir.

Anahtar Kelimeler: Mitokondriyal DNA, mutasyon, array

INTRODUCTION

Mitochondria are double-membraned organelles in eukaryotic cells that mainly function in oxidative phosphorylation and production of ATP by transportation of electrons through the electron transport chain. They also have a role in cellular signalling, apoptosis, beta oxidation and lipid and cholesterol synthesis (1). Each cell contains approximately 500-2,000 mitochondria; tissues with high energy demand, such as extrinsic eye muscles, heart muscle and neurons, contain a greater number of mitochondria compared to other tissues (2).

Mitochondrial proteins are encoded by mitochondrial and nuclear genomes. Human mitochondrial DNA (mtDNA) is a circular, double stranded molecule consisting of 16569 bp, which is inherited through maternal lineage. The mitochondrial genome encodes 22 transfer RNAs (tRNA), 2 ribosomal RNAs (rRNA), 13 proteins and subunits of respiratory chain complexes I, III, IV and V, while the remaining OXPHOS proteins, along with other proteins necessary for mitochondrial metabolism and maintenance, are encoded by the nuclear genome and transferred to the mitochondria through special import systems (3). MtDNA is highly polymorphic, so that variations can be observed even among people of the same ethnic group. Particular combinations of mtDNA variations observed in an individual form a haplogroup, which are thought to cause differences in terms of oxidative phosphorylation capacity and formation of reactive oxygen radicals (1, 4). The mtDNA has about a 10-17-fold high mutation rate compared to the nuclear genome, partly due to the absence of protective histones and the existence of endogenous reactive oxygen species. Some deleterious mtDNA mutations are homoplasmic, while others are found in the heteroplasmic state. The ratio of wild type to mutant mtDNA determines the phenotype. Clinical symptoms and tissue dysfunction occur after the mutant mtDNA rate reaches the threshold level, which varies among tissue types according to dependency on the OXPHOS metabolism (3). Point mutations in tRNA, rRNA or protein coding genes and structural rearrangements such as partial deletions or duplications can cause mitochondrial disorders. Mutations in the nuclear genome can also cause mitochondrial disorders by interfering with mtDNA replication, repairment or mitochondrial function (5). Although the exact prevalence of mitochondrial diseases cannot be ascertained, it is estimated to be about 1/5000 worldwide (6).

Regarding the analysis of mtDNA mutations, Sanger sequencing, Southern blot and long and quantitative PCR had been used. Resequencing Array has been reported to be faster and less expensive, allowing better resolution with respect to DNA sequencing (7). However, as these technologies are expensive and are limited in speed, throughput and sensitivity, next-generation sequencing (NGS) can also be used (8). Recently, long-read sequencing has been performed for the whole sequence of mtDNA molecules (9).

In this study, we analysed whole mitochondrial genomes of 6 children (and their mothers) who were admitted to Gazi University Hospital between 2010-2012 and had symptoms suggestive of mitochondrial disease, using a microarray-based resequencing method.

MATERIALS AND METHOD

Six children who were clinically diagnosed with mitochondrial diseases at the Gazi University Department of Pediatrics, Division of Child Neurology and their mothers were included in this study. Mitochondrial disease diagnostic criteria that were used in this study were Nijmegen Clinical Criteria for mitochondrial diseases (10, 11). Affymetrix Mitochip v2.0, a microarray-based resequencing method, was used for detection of variants. This study was accepted by the Local Ethics Committee of Gazi University Faculty of Medicine (Date: 29.09.2010, No:135). This study was supported by Gazi University Scientific Research Projects Coordination Unit (01/2011-54).

DNA isolation

After written consent was obtained from the patients and their mothers, 5 ml of venous blood was withdrawn from their antecubital veins. Genomic DNA was extracted using the high concentration salt precipitation method (12). The concentration of the DNA samples was measured using a spectrophotometer.

Human MtDNA resequencing

Genomic DNA of 50 ng/µl was used for one Long-Range PCR. After PCR reaction, the products were checked on a 1% agarose gel. The PCR products were purified and quantified using a spectrophotometer. Then, the fragmentation reaction was performed and checked on a 4% agarose gel. After labelling, the samples were loaded into the array and the hybridisation procedure was performed for 16 h in a hybridisation oven. Finally, the array was washed using the GeneChip® Fluidics Station 450 and scanned using the GeneChip® Scanner 3000. After scanning of the array, data analysis was performed with the GSEQ 4.1 program (Affymetrix®). Raw data (files with cell extension) was converted to an analysable file format (chp extension). Obtained sequences were then compared to revised Cambridge Reference Sequence (rCRS), which is the reference mtDNA sequence, and mtDNA variants were defined (13). All the variants detected in the patients were homoplasmic.

In this study, we did not analyse the nuclear genomes of the patients and their mothers.

Analysis of obtained data

The variants were named based on Mitomap online database (https://www.mitomap.org/). In order to interpret mtDNA variations, the American College of Medical Genetics (ACMG) and Association of Molecular Pathology (AMP) guidelines were used. The variants were classified into categories of Pathogenic, Likely Pathogenic, Uncertain Significance, Likely Benign and Benign (14-15). The incompatibility between some of the results of the patients and their mothers were due to the no-calls of the resequencing system.

PCR-RFLP analysis

In order to determine whether the m.3460G>A missense mutation found in Patient 4 was homoplasmic or heteroplasmic, the PCR and RFLP technique was used. After the isolation of DNA from Patient 4 with his mother and sister, forward and reverse primers with the BsaHI restriction enzyme were used. The primers were designed as follows: 5′-ATGGCCAACCTCCTACTCCT-3′ and 5′-GCGGTGATGTAGAGGGTGAT-3′.

RESULTS

In this study, we investigated mtDNA variations using the Mitochip resequencing method in patients that were clinically diagnosed with mitochondrial diseases. Since mitochondrial diseases are maternally inherited, mothers of the patients were also included in the study. Obtained raw data was converted into a suitable format using specific software, and all variants were classified based on Mitomap online database (www.mitomap.org) with ACMG/AMP guidelines.

Patient 1

A nine-month-old female patient was referred to the genetics department with hypotonia, muscle weakness and poor sucking. Her mother had a history of oligohydramniosis. A patent foramen ovale was detected by echocardiography. Cranial magnetic resonance (MRI) revealed that hemispheric grooves were prominent in anterior frontal and temporal lobes of the brain and myelinization was compatible with her age. Magnetic resonance (MRS) revealed minimal lipid and lactate peaks; persistence of this sign was considered compatible with neurometabolic diseases by child neurologists. The patient scored two points according to the mitochondrial disease scoring system. The missense variants detected in patient 1 and mother 1 are listed in table 1. No pathogenic mutations were detected either for the patient or her mother (table 1).

Patient 2

A four-month-old female patient was referred to our department with the prediagnosis of Leber Hereditary Optic Neuropathy (LHON), due to vision loss detected in routine examination. Routine metabolic tests were normal. Visual evoked potential was bilaterally not responsive. The patient scored two points according to the mitochondrial disease scoring system. The variants detected in patient 2 and mother 2 are listed in table 2. No pathogenic mutations were established in the patient or her mother (table 2).

Patient 3

This five years and three months old male patient's development was compatible with his age until the age of 4.5 years; at that age, he was referred to our department due to ataxia and difficulty in holding his head up and sitting up, all manifesting in a six months' period. Cranial MRI revealed increased density in cerebellum and basal ganglia, involvement in bilateral globus pallidus and cerebral crus, compatible with mitochondrial encephalomyelopathic diseases. MRS revealed hyperintense symmetrical signal alterations in bilateral globus pallidus in T2 and FLAIR sequences, elevated lactate peak and moderate cerebral atrophic changes compatible with mitochondrial metabolic disease. EEG revealed slow waves of 2 Hz in bilateral temporoparietal regions; there was no asymmetry between two hemispheres. Muscle biopsy specimen was stained irregularly with oxidase enzyme dyes (NADH, SDH, COX). Hearing was partially impaired in the left ear, and the right ear could not be evaluated due to epileptic jerks at the time of examination. Medical treatment was started with the prediagnosis of mitochondrial cytopathy. The patient scored six points according to the mitochondrial disease scoring system. The variants detected in patient 3 and mother 3 are listed in table 3. There were no pathogenic mutations in the patient or his mother (table 3).

Table 1: Variants detected in patient 1 and her mother

Variants	Patient 1	Mother 1	Locus	ClinGen pathogenicity
m.73A>G	+	+	D-LOOP	Benign
m.153A>G	+	+	D-LOOP	Benign
m.195T>C	+	-	D-LOOP	Benign
m.225G>A	+	-	D-LOOP	Benign
m.226T>C	+	-	D-LOOP	Likely benign
m.263A>G	+	-	D-LOOP	Benign
m.750A>G	+	-	MT-RNR1	Benign
m.1438A>G	+	-	MT-RNR1	Benign
m.1719G>A	+	-	MT-RNR2	Benign
m.3705G>A	+	-	MT-ND1	Benign
m.4769A>G	+	+	MT-ND2	Benign
m.6371C>T	+	+	MT-CO1	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.8393C>T	+	+	MT-ATP8	Benign
m.8860A>G	+	+	MT-ATP6	Benign
m.11377G>A	+	-	MT-ND4	Benign
m.11719G>A	+	-	MT-ND4	Benign
m.12406G>A	+	-	MT-ND5	Benign
m.12705C>T	+	-	MT-ND5	Benign
m.13708G>A	+	-	MT-ND5	Benign
m.13966A>G	+	-	MT-ND5	Benign
m.14470T>C	+	-	MT-ND6	Benign
m.14766C>T	+	-	MT-CYB	Benign
m.15326A>G	+	-	MT-CYB	Benign
m.15927G>A	+	+	MT-TT	Likely benign
m.16189T>C	+	-	D-LOOP	Benign
m.16223C>T	+	+	D-LOOP	Benign
m.16278C>T	+	+	D-LOOP	Benign
m.16519T>C	+	+	D-LOOP	Benign

Patient 4

The patient was referred to our department at the age of 15 due to bilateral visual impairment. Visual impairment had started in the right eye, and the left eye was affected a month later. Ophthalmological examination revealed obscuration of bilateral optic disc margins. Medical history revealed that the patient's mother's grandmother also had bilateral visual loss at the age of 15. The patient scored 2 points according to the mitochondrial disease scoring system. The variants detected in patient 4 and mother 4 are listed in table 4.

The pathogenic m.3460G>A missense mutation was detected in patient 4 and his mother (table 4). After the detection of pathogenic mutation of m.3460G>A in patient 4 and his mother, the patient's sister was also analysed, and the same mutation was detected in his sister, as well. In order to determine the homoplasmic state of the mutation, PCR- RFLP were used. The primers mentioned in the Materials and method section were used to amplify a 215 bp PCR product. In order to genotype the amplified PCR products, BsaHI restriction enzyme was used at 37°C. The wild type allele (G) revealed two bands of 146 bp and 69 bp, whereas the mutant (A) allele revealed as 215 bp. This genotyping result confirmed that the patient, his sister and his mother had the G3460A mutation in homoplasmic state, with respect to two control subjects (figure 1).

Table 2: Variants detected in patient 2 and her mother

Variants	Patient 2	Mother 2	Locus	ClinGen pathogenicity
m.73A>G	+	+	D-LOOP	Benign
m.150C>T	+	+	D-LOOP	Benign
m.195T>C	+	+	D-LOOP	Benign
m.204T>C	+	+	D-LOOP	Benign
m.263A>G	+	+	D-LOOP	Benign
m.279T>C	+	+	D-LOOP	NR*
m.750A>G	+	+	MT-RNR1	Benign
m.961T>C	+	+	MT-RNR1	Likely benign
m.1119T>C	+	+	MT-RNR1	Likely benign
m.1438A>G	+	+	MT-RNR1	Benign
m.2706A>G	+	+	MT-RNR2	Benign
m.3497C>T	+	+	MT-RNR2	Benign
m.4769A>G	+	+	MT-ND2	Benign
m.5441A>G	+	+	MT-ND2	NR*
m.6221T>C	+	+	MT-CO1	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.8860A>G	+	+	MT-ATP6	Benign
m.10398A>G	+	+	MT-ND3	Benign
m.11252A>G	+	+	MT-ND4	Benign
m.11719G>A	+	+	MT-ND4	Benign
m.13629A>G	+	+	MT-ND5	NR*
m.14766C>T	+	+	MT-CYB	Benign
m.15326A>G	+	+	MT-CYB	Benign
m.15346G>A	+	+	MT-CYB	Likely benign
m.15941T>C	+	+	MT-TT	Benign
m.16217T>C	+	+	D-LOOP	Benign

*No records/ Not included in Mitomap's confirmed pathogenic mutations

Table 3: Variants detected in patient 3 and his mother

Variants	Patient 3	Mother 3	Locus	ClinGen pathogenicity
m.152T>C	+	+	D-LOOP	Benign
m.263A>G	+	+	D-LOOP	Benign
m.750A>G	+	+	MT-RNR1	Benign
m.1438A>G	+	+	MT-RNR1	Benign
m.2380C>T	+	+	MT-RNR2	NR*
m.2706A>G	+	+	MT-RNR2	Benign
m.4769A>G	+	+	MT-ND2	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.7094T>C	+	+	MT-CO1	NR*
m.7805G>A	+	+	MT-CO2	Likely benign
m.8860A>G	+	+	MT-ATP6	Benign
m.9797T>C	+	+	MT-CO3	NR*
m.15326A>G	+	+	MT-CYB	Benign
m.15670T>C	+	+	MT-CYB	Benign
m.16129G>A	+	+	D-LOOP	Benign

*No records/ Not included in Mitomap's confirmed pathogenic mutations

Table 4: Variants detected in patient 4 and his mother

Variants	Patient 4	Mother 4	Locus	ClinGen pathogenicity
m.64C>T	+	-	D-LOOP	Benign
m.152T>C	+	+	D-LOOP	Benign
m.263A>G	+	+	D-LOOP	Benign
m.750A>G	+	+	MT-RNR1	Benign
m.827A>G	+	+	MT-RNR1	Benign
m.1438A>G	+	+	MT-RNR1	Benign
m.2442T>C	+	+	MT-RNR2	Likely benign
m.2706A>G	+	+	MT-RNR2	Benign
m.3460G>A	**+**	**+**	**MT-ND1**	**Pathogen**
m.3847T>C	+	+	MT-ND1	Likely benign
m.4769A>G	+	+	MT-ND2	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.8674A>G	+	+	MT-ATP6	NR*
m.8860A>G	+	+	MT-ATP6	Benign
m.13188C>T	+	+	MT-ND5	NR*
m.13731A>G	+	+	MT-ND5	NR*
m.14766C>T	+	+	MT-CYB	Benign
m.15326A>G	+	+	MT-CYB	Benign
m.15930G>A	+	+	MT-TT	Benign
m.16126T>C	+	+	D-LOOP	Benign
m.16519T>C	+	+	D-LOOP	Benign

*No records/ Not included in Mitomap's confirmed pathogenic mutations

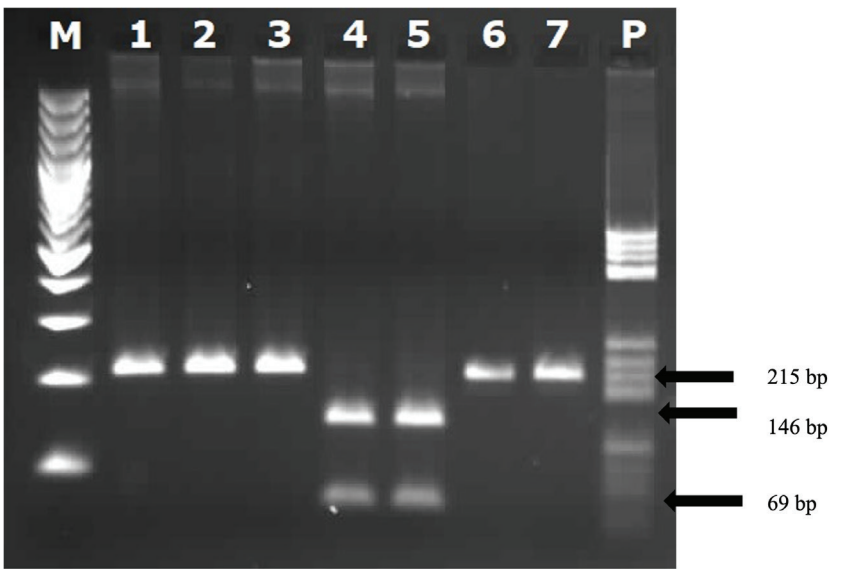

Figure 1: PCR-RFLP analysis of Patient 4, healthy sister and mother, after BsaHI restriction enzyme digestion. M: 100 bp marker; Lane 1: Patient, Lane 2: Sister, Lane 3: Mother, Lanes 4 and 5: control subjects, Lanes 6 and 7: uncut, Lane P: PBR3222 marker

Patient 5

A three-year-old female patient, who had no symptoms until the age of eight months, was referred to our department due to myoclonic jerks in extremities and seizures. The seizures were not responsive to antiepileptic treatment. Her developmental milestones were delayed; she could sit up at 10 months, walk at 17 months and say a few words at 2 years of age; she was still unable to form a sentence at the time of administration. Her liver edge was palpable 5 cm below the right costal margin. Metabolic screening tests revealed elevated urine lactic acid, 3-hydroxy butiric acid and acetoacetic acid. The patient scored 5 points in mitochondrial disease scoring system. The variants detected in patient 5 and mother 5 are listed in table 5. No pathogenic mutations were established in the patient or her mother (table 5).

Table 5: Variants detected in patient 5 and her mother

Variants	Patient 5	Mother 5	Locus	ClinGen pathogenicity
m.73A>G	+	+	D-LOOP	Benign
m.263A>G	+	+	D-LOOP	Benign
m.A512G	+	+	D-LOOP	NR*
m.709G>A	+	+	MT-RNR1	Benign
m.750A>G	+	+	MT-RNR1	Benign
m.1438A>G	+	+	MT-RNR1	Benign
m.1888G>A	+	+	MT-RNR2	Benign
m.2706A>G	+	+	MT-RNR2	Benign
m.4026A>G	+	+	MT-ND1	NR*
m.4216T>C	+	+	MT-ND1	Benign
m.4769A>G	+	+	MT-ND2	Benign
m.4917A>G	+	+	MT-ND2	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.8697G>A	+	+	MT-ATP6	Benign
m.8860A>G	+	+	MT-ATP6	Benign
m.10463T>C	+	+	MT-TR	Benign
m.11251A>G	+	+	MT-ND4	Benign
m.11623C>T	+	+	MT-ND4	NR*
m.11719G>A	+	-	MT-ND4	Benign
m.12633C>A	+	-	MT-ND5	Benign
m.12634A>G	+	-	MT-ND5	Likely benign
m.13368G>A	+	+	MT-ND5	Benign
m.14034T>C	+	+	MT-ND5	NR*
m.14766C>T	+	+	MT-CYB	Benign
m.14905G>A	+	+	MT-CYB	Benign
m.15326A>G	+	+	MT-CYB	Benign
m.15452C>A	+	+	MT-CYB	Benign
m.15607A>G	+	+	MT-CYB	Benign
m.15928G>A	+	+	MT-TT	Benign
m.16126T>C	+	+	D-LOOP	Benign
m.16163A>G	+	+	D-LOOP	Benign
m.16186C>T	+	+	D-LOOP	Benign
m.16274G>A	+	+	D-LOOP	Benign
m.16294C>T	+	-	D-LOOP	Benign
m.16519T>C	+	+	D-LOOP	Benign

*No records/ Not included in Mitomap's confirmed pathogenic mutations

Patient 6

This thirteen-year-old female patient was on antiepileptic treatment due to atonic seizures and the abnormal EEG. Medical history revealed she could walk at two years, after the operation she had due to bilateral hip dislocation, and she had ataxic gait. She started talking at the age of two but her speech was not fluent from the beginning; she had articulation defects and muttering. Deep tendon reflexes were hyperactive in the lower extremities and pathologic reflexes were present. CSF lactate level was elevated. Her condition was assessed as mild mental retardation according to psychometric evaluation. Cranial MRI revealed insignificant pathological signal increase in bilateral globus pallidus, predominant on the left side. MRS was normal. EMG revealed mixed type demyelinating neuropathy of sensorial and motor nerve fibres; needle EMG was consistent with myopathy. The patient scored five points according to the mitochondrial disease scoring system. The variants detected in patient 6 and mother 6 are listed in table 6. There were no pathogenic mutations in the patient or her mother (table 6).

Although mtDNA can be sequenced using the Sanger sequencing method, it can be inadequate to detect all the areas of mutation in mtDNA; since the percentage of heteroplasmic mutations, especially when analysed in mtDNA obtained from blood, can be under the threshold level of detection (18). Mitochondrial chip is a high throughput method, which helps to sequence all mitochondrial genomes and detect probable pathogenic variants with reasonable accuracy (19). The advantages of the Mitochip method over conventional sequencing are short analysis time and cost-effectivity. Furthermore, especially in heteroplasmic tissues, where normal and mutant mtDNA copies are both present, the microarray method can detect mutant alleles of even below 2% percentage, where detection with conventional sequencing requires a minimum allele percentage of approximately 30% (20). It is also used for haplotyping in population genetic studies (21). One of the disadvantages of microarray-based sequencing is that deletions or insertions cannot be detected with this method (19).

Table 6: Variants detected in patient 6 and his mother

Variants	Patient 6	Mother 6	Locus	ClinGen pathogenicity
m.263A>G	+	+	D-LOOP	Benign
m.480T>C	+	+	D-LOOP	NR*
m.750A>G	+	+	MT-RNR1	Benign
m.1438A>G	+	+	MT-RNR1	Benign
m.2706A>G	+	+	MT-RNR2	Benign
m.4655>A	+	+	MT-ND2	NR*
m.4769A>G	+	+	MT-ND2	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.8860A>G	+	+	MT-ATP6	Benign
m.12192G>A	+	+	MT-TH	NR*
m.15115T>C	+	+	MT-CYB	Benign
m.15326A>G	+	+	MT-CYB	Benign
m.16311T>C	+	+	D-LOOP	Benign

*No records/ Not included in Mitomap's confirmed pathogenic mutations

DISCUSSION

The prevalence of mtDNA mutations among clinically affected patients is estimated to be approximately 1/5000 (6). Pathogenic mitochondrial DNA mutations result in mitochondrial DNA disorders, which are among the most common inherited human diseases (16). Both nuclear and mitochondrial genome mutations may cause mitochondrial diseases (17).

With the recent development, NGS is expected to become the method of choice for genetic analysis on mtDNA because it allows a rapid sequencing of the whole mtDNA with concurrent quantification of heteroplasmy levels for point mutations, down to low percentages (8).

Among six patients included in this study group, none of the variants detected in our study could be attributed to any mitochondrial diseases, except the pathogenic mutation detected in patient 4. The m.3460 G>A muta-

tion detected in patient 4 is located in the *MT-ND1* gene and it is one of the three primary mutations known to be responsible for LHON. Of all the LHON patients in the world, about 90% of the patients carry one of the mutations in nucleotides 11778, 3460 or 14484 (16). Incidence of these mutations are estimated to be 70, 15 and 10 percent for m.11778G>A, m.3460G>A and m.14484T>C, respectively. Heteroplasmy, secondary genomic or mitochondrial factors and environmental factors are suggested to affect the disease progression (22-23).

In patient 4, as the pathogenic m.3460G>A mutation was also detected in his sister and mother, the homoplasmic state of the mutation was confirmed by PCR- RFLP. Based on this finding, the lack of clinical findings in the mother and sister was thought to be due to decreased penetrance of the disease in females and modifying genes in nuclear genome. Also, the reason for this was thought to be the higher penetrance in males and possible existence of modifying loci in the nuclear genome (24). Since only 10% of female carriers exhibit LHON symptoms, whereas 50% of male carriers are affected, it was thought that other environmental or genetics factors that attribute to phenotypical expression must be present (25).

The small number of participants is one of the disadvantages of our study. In addition, the nuclear genome mutations that are associated with mitochondrial diseases could not be analysed in this study.

CONCLUSION

The MtDNA resequencing technique is a valuable diagnostic tool due to its reliability, short duration and the small quantity of required DNA. On the other hand, the heteroplasmy/homoplasmy state of detected mutations must be confirmed by a second method, such as PCR-RFLP, also used in this study. By increasing the number of studies like this one, a database for mitochondrial variants in the Turkish population could be founded. Also, currently, the NGS technology analysis of nuclear DNA along with mtDNA will provide more reliable results in diagnosis of mitochondrial diseases, thus allowing more accurate genotype-phenotype correlation.

Ethics Committee Approval: This study was approved by Gazi University Faculty of Medicine Institutional Review Board (Date: 29.09.2010, No: 135).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- G.K.D., K.G., F.E.P, M.A.E.; Data Acquisition- G.K.D., S.G.E.; Data Analysis/Interpretation- K.G., F.E.P., M.A.E.; Drafting Manuscript- G.K.D., K.G., F.E.P, M.A.E.; Critical Revision of Manuscript- G.K.D., S.G.E.; Final Approval and Accountability- G.K.D., K.G., F.E.P, M.A.E.; Material or Technical Support- G.K.D., K.G., F.E.P, M.A.E.; Supervision- K.G., F.E.P., M.A.E.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: This study was supported by Gazi University Scientific Research Projects Unit (Project number: 01/2011-54).

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