Identification of causative mutations in patients with Leigh syndrome and MERRF by mitochondrial DNA-targeted next-generation sequencing

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Purpose: Mitochondrial diseases are clinically and genetically heterogeneous disorders, which make their exact diagnosis and classification difficult. The purpose of this study was to identify pathogenic mitochondrial DNA (mtDNA) mutations in 2 Korean families with myoclonic epilepsy with ragged-red fibers (MERRF) and Leigh syndrome, respectively.

Materials and Methods: Whole mtDNAs were sequenced by the method of mtDNA-targeted next-generation sequencing (NGS).

Results: Two causative mtDNA mutations were identified from the NGS data. An m.8344A>G mutation in the tRNA-Lys gene (*MT-TK*) was detected in a MERRF patient (family ID: MT132), and an m.9176T>C (p.Leu217Pro) mutation in the mitochondrial ATP6 gene (*MT-ATP6*) was detected in a Leigh syndrome patient (family ID: MT130). Both mutations, which have been reported several times before in affected individuals, were not found in the control samples.

Conclusion: This study suggests that mtDNA-targeted NGS will be helpful for the molecular diagnosis of genetically heterogeneous mitochondrial diseases with complex phenotypes.

Key words: Leigh disease, MERRF syndrome, Mitochondrial DNA, *MT-ATP6*, *Mitochondrially encoded tRNA lysine gene*, High-throughput nucleotide.

Introduction

Mitochondrial diseases caused by mitochondrial dysfunction are clinically and genetically a very heterogeneous group of disorders. These diseases primarily result from mutations

in the mitochondrial DNA (mtDNA) that lead to oxidative phosphorylation impairment, ATP depletion, cellular dysfunction, and ultimately cell death [1]. Most mitochondrial diseases involve impairment of multiple organs, particularly in tissues with a high energy demand such as nerve and muscle.

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The multi-organ involvement and the overlapping of symptoms among mitochondrial disorders make the exact diagnosis and classification difficult [2–4]. Thus, molecular testing for mtDNA mutations is now an important diagnostic tool for patients with mitochondrial disorders [5].

One of the many mitochondrial diseases, myoclonic epilepsy with ragged-red fibers (MERRF, MIM# 545000), is caused mainly by maternally inherited mutations in the mitochondrially encoded tRNA lysine gene (*MT-TK*, MIM# 590060), such as m.8344A>G, m.8356T>C, and m.8363G>A [6–9]. These point mutations are usually observed to be in the heteroplasmic state [10]. They result in decreased transfer of lysine by mitochondrial tRNA, which consequently disrupts the synthesis of proteins essential for oxidative phosphorylation. In addition to mutations in *MT-TK*, defects in many other genes have also been reported to present the MERRF phenotype. These include *MT-TL1* (mitochondrially encoded tRNA leucine 1, MIM# 590050) [4,11], *MT-TH* (mitochondrially encoded tRNA histidine, MIM# 590040) [12], *MT-TS1* (mitochondrially encoded tRNA serine 1, MIM# 590080) [3], *MT-TS2* (MIM# 590085), *MT-TF* (mitochondrially encoded tRNA phenylalanine, MIM# 590070) [13], and *MT-ND5* (NADH-ubiquinone oxidoreductase chain 5, MIM# 516005) [14]. Transfer of mtDNA carrying a tRNA gene mutation to cell lines results in a severe defect in mitochondrial translation in the recipient cells, implying that the tRNA mutation itself is sufficient to cause the disease.

Leigh syndrome (MIM# 256000) is an early-onset progressive neurodegenerative disorder with a characteristic neuropathology consisting of focal, bilateral lesions in one or more areas of the central nervous system [15,16]. Clinical symptoms vary depending on which areas of the central nervous system are involved. Besides its clinical heterogeneity, Leigh syndrome exhibits genetic heterogeneity, with different modes of inheritance: mitochondrial, autosomal recessive, and X-linked recessive [17,18]. Mutations in several mtDNA genes are associated with Leigh syndrome, such as *MT-FMT* (mitochondrially encoded methionyl-tRNA formyltransferase, MIM# 611766), *MT-ATP6* (mitochondrially encoded ATP synthase F_0 subunit 6, MIM# 516060), *MT-ND5*, and *MT-ND6* (MIM# 516006). In particular, m.8993T>G/C and m.10158T>C have been frequently reported in Leigh syndrome [19–23].

Ever since the human mtDNA had been completely sequenced [24], several hundreds of different mtDNA mutations have been reported to be associated with various mitochondrial diseases in the Human Mitochondrial Genome Database (MITOMAP; <http://mitomap.org/MITOMAP>). However, mtDNA analysis

studies have not been actively performed in Korean patients with mitochondrial diseases [25–27]. Whole-mtDNA sequencing is usually performed by polymerase chain reaction (PCR) amplification of mtDNA with 46 fragments, with subsequent Sanger sequencing using a mitoSEQr resequencing system (Life Technology, Grand Island, NY, USA). Next-generation sequencing (NGS) technology, which has revolutionized the field of molecular biology, has recently been applied in the diagnosis of mitochondrial diseases [28,29]. The NGS technique is now recognized to reduce sequencing cost and analysis time, with high coverage and fidelity, thereby enabling the decoding of a number of human mitochondrial diseases. Calabrese et al. [30] developed an MToolBox pipeline to reconstruct and analyze human mtDNA from high-throughput sequencing data.

In the present study, we analyzed 2 Korean patients with MERRF and Leigh syndromes, respectively, by applying an mtDNA-targeted NGS method into whole-mtDNA sequencing. From the analysis, 2 mtDNA mutations were identified as the underlying causes of each disorder: an m.8344A>G mutation in *MT-TK* from the MERRF patient, and an m.9176T>C (Leu217Pro) mutation in *MT-ATP6* from the Leigh syndrome patient.

Materials and Methods

1. Subjects

This study included mitochondrial diseases from 2 families

Fig. 1. Pedigrees of 2 patients with mitochondrial disorders and brain magnetic resonance imaging (MRI) of patient 1. (A) Pedigrees of the MT130 and MT132 families. The open symbols represent unaffected individuals and filled symbols represent affected individuals. The probands are indicated by the arrows. Asterisks indicate individuals whose DNAs were used in this study. (B) Brain MRI of patient 1 with the Leigh syndrome. T2 weighted images revealed the hyperintense lesion mainly involving the midbrain level (arrow). These lesions were symmetrical and longitudinally extended from the bilateral caudate nucleus to the dorsal pons area with sparing of the mammillary body.

of Korean origin: 1 with Leigh syndrome (family ID: MT130) and 1 with MERRF (family ID: MT132) (Fig. 1A). In addition to these 2 families, 300 healthy individuals were examined as the healthy controls. Written informed consent was obtained from all participants according to a protocol approved by the institutional review board for Sungkyunkwan University, Samsung Medical Center (2013-11-057-021).

2. Clinical assessment

Clinical information included assessments of age at onset, mental status, cranial nerve function, muscle impairments, sensory loss, and deep tendon reflexes. Age at onset was determined by asking patients what their age was when symptoms first appeared. Neurophysiological studies were carried out on 4 affected individuals (3 males and 1 female). Motor and sensory conduction velocities of median, ulnar, peroneal, tibial, and sural nerves were determined, and needle electromyography was performed in the bilateral upper and lower limb muscles. Laboratory tests included levels for creatine kinase, copper, ceruloplasmin, lactic acid, and thiamine.

Brain magnetic resonance images were evaluated using a 1.5-T system (Siemens Vision; Siemens, Erlangen, Germany). Whole brains were scanned using a slice thickness of 7 mm and a 2-mm interslice gap, to produce 16 axial images. The imaging protocol consisted of T2-weighted spin echo (TR/TE=4,700/120 ms), T1-weighted spin echo (TR/TE=550/12 ms), and fluid-attenuated inversion recovery (TR/TE=9,000/119 ms; inversion time 2,609 ms) images.

3. DNA purification and determination of mtDNA common deletion

DNA was extracted from peripheral blood, using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). Long-template PCR was performed to determine the common deletion of mtDNA (8,469-13,447), with forward (m.8182-8208) and reverse (m.13981-13957) primer pairs, by using the Expand 20 kb PLUS PCR system (Roche Diagnostics GmbH, Mannheim, Germany). The PCR condition consisted of initial denaturation at 92°C for 2 min, followed by 30 cycles of 92°C for 10 s, 58°C for 30 s, 68°C for 10 min, and a final extension at 68°C for 7 min.

4. Mitochondrial DNA-targeted next-generation sequencing

The mtDNA-targeted NGS was applied to 2 affected individuals from both families (III-1 in MT130 and II-2 in MT132). The probe design for target capture was performed according to SureDesign website (<https://earray.chem.agilent.com/suredesign>; Agilent,

Santa Clara, CA, USA), and sequencing was performed with the HiSeq 2000 Genome Analyzer (Illumina, San Diego, CA, USA). The UCSC hg19 assembly (GRCh37, mtDNA GenBank Accession No. NC_001807.4) was used as the reference sequence for mapping and alignment. All variant calls from whole mtDNA were checked to see whether they were registered in the dbSNP142 (<http://www.ncbi.nlm.nih.gov>) and MITOMAP database. All insertion/deletions (indels) and candidate causative variants were confirmed by Sanger's sequencing method, using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Life Technology, Grand Island, NY, USA) and the ABI3130XL Genetic Analyzer (Life Technology). Because the mtDNA sequences from the NGS were primarily aligned by the hg19 assembly (NC_001807.4), variants were compared with the revised Cambridge reference sequence (rCRS, NC_012920.1), which is commonly used as the reference sequence for mtDNA studies [31].

5. Determination of heteroplasmic rate

The heteroplasmic rate was measured by dual methods. First, the rate was determined as the ratio of altered reads per total read depth at the mutation site from the NGS data. Second, PCR-amplified DNA fragments including the mutation site were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), which was then used to transform *Escherichia coli* (DH5 α). Plasmids were isolated from about 100 colonies/mutation, and the mutation rate was then determined by counting the clones that had a mutation.

6. Haplogrouping and conservation analysis

The mtDNA haplogroup was determined by using the HaploGrep software based on PhyloTree Build 16 (<http://haplogrep.uibk.ac.at>) and the mtDNAMANAGER (<http://mtmanager.yonsei.ac.kr>) tool. Multiple alignments of the amino acid sequences from different species were performed using the MEGA5 program (ver. 5.05; <http://www.megasoftware.net/>) by executing the ClustalW algorithm.

Results

1. Clinical manifestations

1) Patient 1

A 21-year-old female (Fig. 1A; MT130, III-1) presented at the emergency room with stupor mentality. She was born at full term from healthy parents, and early milestones were normal. From the age of 14 years, she had difficulty in making friends

and her mood fluctuated severely. She had acted like a child since 18 years of age. She was admitted to a college 2 years ago but did not adapt well. In addition, she began to eat only what she wanted, such as milk, broccoli, and sweet potatoes. Twelve days before the hospital visit, she had a high fever, vomiting, and diarrhea, and on the visiting day, she could not awake spontaneously. On neurological examination at 21 years, her mental status was stuporous and her orientation to time, place, and person was severely impaired. The blood creatine kinase level was 660 IU/L and other values, including complete blood count, liver and kidney function test, copper, ceruloplasmin, lactic acid, and thiamine, were normal. Brain magnetic resonance imaging (MRI) revealed high signal intensity on the T2-weighted image in the bilateral paramedian thalamus, midbrain, pons, and paraventricular area (Fig. 1B).

Table 1. Mitochondrial DNA-targeted next-generation sequencing

Item	Information
Target sequence	NC_001807.4 UCSC hg19 assembly (GRCh37)
Coverage	100%
Average read depth	6,586 reads
Number of SNPs (including indels) ^a	MT130, 42; MT132, 31

^aSingle nucleotide polymorphism (SNP) numbers were obtained from comparison with revised Cambridge reference sequence (NC_012920.1). Indel, insertion and deletion polymorphism.

2) Patient 2

A 27-year-old female (Fig. 1A; MT132, II-2) had complaints of ataxic gait and myoclonus. She was born at full term from healthy parents, and early milestones were normal. At 13 years of age, she began to stagger and easily fall, and the symptoms worsened progressively. Myoclonus was elicited by photic stimulus or voluntary movement of the limbs. On neurological examination at 27 years, ataxia, disability of tandem gait, bilateral hand tremor, and intermittent myoclonus were observed. Her muscle strengths and sensory functions were normal and deep tendon reflexes were normoreflexive. Electrophysiological studies, including nerve conduction study and electromyography, did not reveal any abnormal findings.

2. Identification of causative mutations by mtDNA-targeted next-generation sequencing

To identify genetic causes, whole-mtDNA-targeted NGS was performed using mtDNA from the 2 patients described above (Table 1). The whole target was completely covered (mtDNA coverage: 100.0%) with an average read depth of 6,586X. MT130 (III-1) and MT132 (II-2) showed 42 and 31 variants (including indels) in total, respectively, when their mtDNA sequences were compared with the rCRS mtDNA [31]. All the sequencing variants are shown in Table 2. The haplogroups of the 2 mtDNA sequences were estimated to be D5a1 (MT130) and N9a5 (MT132), respectively.

Table 2. Variants identified from mtDNA-targeted next-generation sequencing in two patients

Locus/gene	Mutation		MT130 (III-1)	MT132 (II-2)	Report	
	Nucleotide	Amino acid			dbSNP142	MITOMAP
D-loop	m.68G>A	-	A	G	-	Pol
	m.73A>G	-	G	G	rs3087742	Pol
	m.150C>T	-	T	T	rs62581312	Pol
	m.263A>G	-	G	G	rs2853515	Pol
	m.303.1insC	-	-	InsC	rs369786048	Pol
	m.310T>C	-	T	C	-	Pol
	m.315.1insC	-	InsC	InsC	-	Pol
	m.489T>C	-	C	T	rs28625645	Pol
MT-RNR1	m.750A>G	-	G	G	rs2853518	Pol
	m.752C>T	-	T	C	rs2853518	Pol
	m.1107T>C	-	C	T	rs111033323	Pol
MT-RNR2	m.1438A>G	-	G	G	rs2001030	Pol
	m.2706A>G	-	G	G	rs2854128	Pol
MT-ND1	m.3496G>T	p.Ala64Ser	T	G	-	Pol/LHON
	m.3729A>G	Syn	A	G	-	Pol
	m.4136A>G	p.Tyr277Cys	G	A	rs199476121	Pol/LHON

Table 2. Continued

Locus/gene	Mutation		MT130 (III-1)	MT132 (II-2)	Report	
	Nucleotide	Amino acid			dbSNP142	MITOMAP
<i>MT-ND2</i>	m.4688T>C	Syn	T	C	-	Pol
	m.4769A>G	Syn	G	G	rs3021086	Pol
	m.4883C>T	Syn	T	C	rs200763872	Pol
	m.5178C>A	p.Leu237Met	A	C	rs28357984	Pol
	m.5231G>A	Syn	G	A	rs371345850	Pol
	m.5301A>G	p.Ile278Val	G	A	rs199794187	Pol
	m.5417G>A	Syn	G	A	rs386828968	Pol
<i>MT-CO1</i>	m.7028C>T	Syn	T	T	rs2015062	Pol
<i>MT-TK</i>	m.8344A>G	-	A	G	rs118192098	MERRF
<i>MT-ATP6</i>	m.8701A>G	p.Thr59Ala	G	A	rs2000975	Pol
	m.8860A>G	p.Thr112Ala	G	G	rs2001031	Pol
	m.9176T>C	p.Leu217Pro	C	T	rs199476135	LS
	m.9180A>G	Syn	G	A	rs2298011	Pol
<i>MT-CO3</i>	m.9540T>C	Syn	C	T	rs2248727	Pol
<i>MT-ND3</i>	m.10397A>G	Syn	G	A	-	Pol
	m.10398A>G	p.Thr114Ala	G	A	rs2853826	Pol
	m.10400C>T	Syn	T	C	rs28358278	Pol
<i>MT-ND4</i>	m.10873T>C	Syn	C	T	rs2857284	Pol
	m.11719G>A	Syn	A	A	rs2853495	Pol
	m.11944T>C	Syn	C	T	rs3087901	Pol
	m.12026A>G	p.Ile423Val	G	A	rs202136725	Pol/DM
	m.12040A>G	Syn	G	A	-	Pol
<i>MT-ND5</i>	m.12705C>T	Syn	T	C	rs267606893	Pol
	m.12358A>G	p.Thr8Ala	A	G	rs201027657	Pol
	m.12372G>A	Syn	G	A	rs2853499	Pol
	m.13708G>A	p.Ala458Thr	A	A	rs28359178	Pol /LHON
<i>MT-CYB</i>	m.14766C>T	p.Thr7Ile	T	T	rs28359178	Pol
	m.14783T>C	Syn	C	T	rs527236042	Pol
	m.15043G>A	Syn	A	G	rs527236043	Pol
	m.15301G>A	Syn	A	G	rs527236045	Pol
	m.15326A>G	p.Thr194Ala	G	G	rs2853508	Pol
	m.15883G>A	Syn	G	A	-	Pol
	m.16046T>C	-	T	C	-	Pol
D-Loop	m.16172T>C	-	T	C	rs2853508	Pol
	m.16182A>C	-	C	C	-	Pol
	m.16209T>C	-	T	C	rs386829278	Pol
	m.16223C>T	-	T	T	rs2853513	Pol
	m.16257C>A	-	C	A	rs376682258	Pol
	m.16261C>T	-	C	T	rs138126107	Pol
	m.16309A>G	-	A	G	rs373517769	Pol
	m.16390G>A	-	A	A	rs41378955	Pol
	m.16519T>C	-	C	C	rs3937033	Pol

MITOMAP: Human Mitochondrial Genome Database (<http://mitomap.org/MITOMAP>).

Syn, synonymous mutation; Pol, polymorphic; LHON, Leber's hereditary optic neuropathy; MERRF, myoclonic epilepsy and ragged-red fibers; DM, diabetes mellitus; LS, Leigh syndrome.

Fig. 2. Identification of causative mutations in mtDNA from 2 mitochondrial patients. (A) Sequencing chromatograms of two mutations, m.8344A>G in the myoclonic epilepsy with ragged-red fibers (MERRF) patient (MT132) and m.9176T>C in the Leigh syndrome patient. The unaffected individuals (proband's mother: I-2 in MT132) shows heteroplasmy for the m.8344A>G. (B) Diagrammatic representation of tRNA-Lys. The position of the A-to-G base substitution (m.8344A>G) in the pseudouridine (TψC) loop is indicated by a red box. The nucleotides within boxes are conserved in mammals (human, cow, mouse, rat, and hamster), whereas the nucleotides within circles are conserved throughout a wider range of species including chicken, *Xenopus*, cod, honeybee, and fruit fly. (C) Conservation analysis of the two mutation sites.

From the screen, 2 causative mtDNA mutations were identified in the examined families. An m.8344A>G mutation in *MT-TK* was detected in the MERRF patient (family ID: MT132), and an m.9176T>C (p.Leu217Pro) mutation in *MT-ATP6* was detected in the Leigh syndrome patient (family ID: MT130). Both mutations were found in the affected probands as the homoplasmy, whereas m.8344A>G was found in the patient's mother (I-2) as a heteroplasmic state with an approximate mutation rate of 80% (Fig. 2A). Both mutations, which have been reported several times in MERRF and Leigh syndrome patients [32-34], were not found in the 300 control samples. The m.8344A>G mutation was located in the less conserved region, but it was within the pseudouridine arm which plays an important role on the protein synthesis (Fig. 2B) [35]. The m.9176T>C (p.Leu217Pro) mutation was located in the highly conserved region between different species (Fig. 2C).

The mtDNA 4,977-bp deletion is one of the most frequently observed mtDNA mutations in human tissues, but neither patient showed the common deletion when long-template PCR was performed (Fig. 3). Although the whole-mtDNA sequencing

Fig. 3. Determination of the common mtDNA deletion (4,977 bp) by long-template polymerase chain reaction. The 5.8 kb and 822 bp bands indicate the wild type and the common deletion type of mtDNA, respectively. Marker, 1 kb ladder; CTL (Mut), mtDNA mutant with 4,977 bp common deletion; CTL (WT), mtDNA wild type with no deletion.

revealed many other variants, we considered them non-causative polymorphic variants because they were also observed in the healthy controls.

Discussion

The present study identified 2 mtDNA mutations (m.8344A>G in *MT-TK* and m.9176T>C [p.Leu217Pro] in *MT-ATP6* from the MERRF patient and Leigh syndrome patient, respectively), by applying an mtDNA-targeted NGS tool. We believe that these mutations are the underlying cause of each disease phenotype, because both mutations have been reported to be the underlying causes of MERRF and Leigh syndromes [32-34] and no healthy control showed the same mutation. In addition, both mutations were located in the well-conserved protein region or functionally important pseudouridine arm of t-RNA.

The whole-mtDNA sequencing revealed several mtDNA variants that have been reported as polymorphisms as well as association (or secondary) factors in MITOMAP: these include m.3496G>T (more frequent in Japanese patients with Leber's hereditary optic neuropathy [LHON]) [36], m.4136A>G (a secondary genetic cause for LHON) [37], m.12026A>G (more frequent in Japanese diabetic patients) [38], and m.13708G>A (a secondary genetic factor in Chinese LHON patients) [39]. In this study, because all these variants were also found in the healthy controls, we considered them as non-causative polymorphic variants. However, we could not completely exclude them as risk factors of the corresponding phenotypes.

Clinically, the patients with the m.9176T>C *MT-ATP6* or m.8344A>G mutations revealed typical clinical symptoms of Leigh syndrome and MERRF, respectively. Patient 1 afflicted with the Leigh syndrome had experienced neurological deterioration and psychomotor regression since 14 years of age. On brain MRI, the symmetrical paramedian thalamus, brainstem, and periventricular area, which are hallmarks of the disease, were shown. Furthermore, patient 2 with the m.8344A>G mutation showed progressive ataxia and myoclonus, which were compatible phenotypes with the MERRF.

Pathogenic mtDNA mutations are usually observed as a heteroplasmic state [40,41]. However, in this study, both causative mutations were observed in the affected probands as almost homoplasmic states. Moreover, the MERRF patient's unaffected mother (I-2 in MT132) showed a considerably high mutation load with an approximate rate of 80%. It is known that the m.8344A>G mutation accounts for 80% to 90% of MERRF cases [40,41]. In spite of the high mutation load, the

reason that the MERRF patient's mother was unaffected may be due to the DNA source used in this study. We used total DNA purified from the peripheral blood instead of affected tissues. The heteroplasmic state usually shows a tissue-specific rate. Recently, asymptomatic m.8344A>G carriers with high mutant loads have been reported [42].

The multi-organ involvement and overlapping symptoms make exact diagnosis of mitochondrial diseases difficult. Therefore, mutation screening is an important tool for the diagnosis of rare mitochondrial disorders. In this study, we applied mtDNA-targeted NGS to identify genetic causes of the 2 studied diseases. NGS technology has been recently introduced in mtDNA testing for mitochondrial diseases [28-30]. The application of NGS may reduce the testing cost and time, with high fidelity. In particular, heteroplasmic rates could be directly determined by counting altered reads from the total read depth at the corresponding mutation site. Therefore, we believe that whole-mtDNA sequencing by NGS will be a powerful tool for the molecular diagnosis of genetically and clinically heterogeneous mitochondrial diseases.

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