Diagnostic efficacy of muscle biopsy in pediatric patients with genetically confirmed mitochondrial diseases

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ABSTRACT

Diagnostic efficacy of muscle biopsy in pediatric patients with genetically confirmed mitochondrial diseases

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Background: Mitochondrial diseases are caused by mutations in nuclear or mitochondrial genomes which encode oxidative phosphorylation system. Diagnostic gold standard had been muscle tissue biopsy, but next-generation sequencing is being preferred because of evolving genetic studies. We aimed to analyze the efficacy of muscle biopsy compared to mitochondrial DNA (mtDNA) sequencing in diagnosis of mitochondrial diseases.

Methods: We retrospectively reviewed medical charts of 274 patients with mitochondrial diseases who went through diagnostic evaluation in Gangnam Severance hospital from August 2005 to April 2019. All patients had serum lactate test, brain magnetic resonance imaging, muscle biopsy, biochemical tests, and mtDNA sequencing. We performed statistical analysis of pathologic findings between patients with mtDNA mutations and those without, then we calculated sensitivity, specificity, positive predictive value, and negative predictive value of each pathologic test compared to mtDNA sequencing.

Results: Among 274 patients with mitochondrial diseases, median age at symptom onset was 0.7 years old, and median age at muscle biopsy was 3.2 years old. Most common symptoms were developmental faltering (44%) and seizures (41%). Among them, 27 patients had mtDNA mutations confirmed. At last visit, all patients had systemic involvement of the disease including central nervous system (99%), gastrointestinal (24%) and ophthalmic (15%) involvement.

Between 27 patients with mtDNA mutations and those without, pathologic studies and diagnosis by electron microscopy were statistically insignificant. However, diagnosis by light microscopy, and by findings of ragged red fibers alone, had statistical significance. (p=0.009 and 0.049 each) Sensitivity of pathologic tests ranged from 41 to 56 percent in patients with mtDNA mutations. Specificity ranged from 38 to 80 percent, with light microscopy (78%) and pathologic findings with ragged red fibers (80%) being most specific.

Conclusion: Light microscopy findings, especially findings of ragged red fibers, could be indicative of primary mitochondrial etiology in mitochondrial diseases. Further studies would help determine the significance of diagnostic tests in mitochondrial diseases. As genetic diagnosis is becoming mainstream of the diagnosis, matching pathologic results with genetic results would help increasing yield of genetic diagnosis according to specific pathologic findings in primary mitochondrial diseases.

Keywords: mitochondrial disease, muscle biopsy, mitochondrial DNA, ragged red fibers

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I. INTRODUCTION

Human mitochondrial diseases are caused by defects in respiratory chain complex in the inner membrane of mitochondria, inducing decreased energy production in human cells[1,2]. Primary mitochondrial disease is the result of germline mutations in mitochondrial or nuclear deoxyribonucleic acid (DNA) mutation encoding elements of mitochondrial oxidative phosphorylation system, notably the respiratory chain. Secondary mitochondrial dysfunction is caused either by mutations that encode proteins which are not in mitochondrial oxidative phosphorylation system but which indirectly inhibit the system, or by acquired

oxidative stress[2].

There is no gold standard or consensus in diagnostic evaluation of mitochondrial disease[3]. Bernier *et al.* suggested diagnosis by clinical, histologic, enzymologic, functional, and genetic criteria[4]. Among multiple diagnostic methods, muscle biopsy has been considered as the gold standard before widespread use of genetic tests, despite insufficient sensitivity or specificity. With the advent of genomic technology, next-generation sequencing (NGS) has been preferred for diagnosis of mitochondrial disease[5]. However, there are limitations of the diagnostic evaluation in mitochondrial diseases. There are few references on efficacy of each diagnostic method[6], and that most diagnostic methods cannot differentiate primary and secondary origin of mitochondrial disorders except genomic tests.

In this study, we aimed to delineate the efficacy of muscle biopsy in patients diagnosed with mitochondrial diseases. We tried to compare the efficacy between other diagnostic methods with genetic analysis of mitochondrial DNA.

II. MATERIALS AND METHODS

1. Patient group

We reviewed medical records of 274 patients diagnosed with mitochondrial diseases in Gangnam Severance Hospital from August 2005 to April 2019. Clinical data of initial symptoms, organ involvement, and clinical outcomes were collected. All patients had serum lactate test, brain magnetic resonance imaging, muscle biopsy, and mtDNA sequencing. Most patients (272 patients) had biochemical studies done. Results were reviewed and classified by

available criteria. The study was approved by institutional review board in Gangnam Severance Hospital, Seoul, Korea.

2. Criteria of syndromic diagnosis

All patients were diagnosed as mitochondrial diseases by criteria of Bernier et al[4]. The patients were further classified by mitochondrial syndromes. Diagnosis of mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) was concordant with diagnostic criteria for definitive MELAS by MELAS study committee in Japan[7]. Diagnosis of Leigh syndrome was concordant with criteria suggested by Rahman *et al*[8]. MERRF was clinically diagnosed by canonic features suggested by DiMauro *et al*[9]. Others who did not fit in criteria for specific mitochondrial syndromes were grouped as nonspecific.

3. Pathologic diagnosis by muscle biopsy

Pathologic diagnosis was done from the patients' quadriceps muscle. Light microscopic findings of ragged red fibers, (RRF) focal cytochrome *c* oxidase (COX) deficiency, and increased succinate dehydrogenase stain were noted[10, 11]. Electron microscopic findings of pleoconia and megaconia suggested mitochondrial disease[12].

4. Biochemical diagnosis by muscle biopsy samples

Biochemical screening was done using muscle and fibroblast tissues obtained by muscle biopsy. The tissues were analyzed by standard spectrophotometric measurement suggested by Rustin *et al*[13]. We analyzed the activities in mitochondrial complex I (nicotinamide adenine dinucleotide-

coenzyme Q (CoQ) reductase), II (succinate-CoQ reductase and succinate-cytochrome *c* reductase), III (succinate-cytochrome *c* reductase and cytochrome *c* reductase), IV (cytochrome *c* oxidase), and V (oligomycin-sensitive ATPase), and citrate synthase.

5. Genetic diagnosis

Genetic diagnosis was done by whole exome sequencing of mitochondrial DNA. All variants were noted and searched for pathogenicity according to American College of Medical Genetics[14] and MitoMAP[15], a database recognized by ACMG as reference database of mitochondrial variants[14]. We performed sequencing of nuclear DNA in some patients, the results of which are not included in this study.

6. Statistical analysis

We performed statistical analysis using SPSS version 23.0. (SPSS Inc., Chicago, IL, USA) We analyzed clinical, pathologic, and biochemical findings between patients with mtDNA mutations and those without. Then, we calculated sensitivity, specificity, positive predictive value, and negative predictive value of each syndromic diagnosis and pathologic test compared to mtDNA sequencing.

III. RESULTS

1. Clinical characteristics of the patients

Table 1 overviews the clinical characteristics of patients with mitochondrial

diseases who were enrolled in this study. Of 274 patients, 166 were male. (61%) Median age at symptom onset was 0.7 years old, (interquartile range (IQR) 0.3-1.6) and median age at muscle biopsy was 3.2 years old. (IQR 1.5-5.5) Median duration from symptom onset to muscle biopsy was 1.6 years. (IQR 0.6-3.1) Most common symptoms at first visit were developmental delay or regression (121 patients, 44%) and seizures (112 patients, 41%), followed by mental change, (11 patients, 4.0%) ataxia or dysarthria, hypotonia or scoliosis, (10 patients each, 3.6%) and defects in ocular muscle or related nerves. (7 patients, 2.6%)

Table 1. Clinical characteristics of the patients (n=274)

General Characteristics	Total
Number of male:female (ratio)	166:108 (1.5)
Median age at symptom onset (yr) (Interquartile range, IQR)	0.73 (0.26-1.64)
Median age at muscle biopsy (yr) (IQR)	3.16 (1.50-5.51)
Duration from symptom onset to muscle biopsy (yr) (IQR)	1.59 (0.64-3.11)
Symptoms at first visit, n (%, multiple choice)	
Developmental delay or regression	121 (44)
Seizures	112 (41)
Mental change	11 (4.0)
Ataxia, gait disturbance, dysarthria	10 (3.6)
Hypotonia or scoliosis	10 (3.6)
Oculomotor defect (ptosis/strabismus/diplopia)	7 (2.6)
Visual defect (blindness, visual disturbance)	5 (1.8)
Focal neurologic deficit or headache	5 (1.8)
Movement disorder	4 (1.5)
Abnormal head circumference	3 (1.1)
Hearing impairment	1 (0.4)

2. Initial diagnostic evaluation of mitochondrial diseases

Patients went through assays for diagnostic evaluation as shown in Table 2. Half of the patients had serum lactic acidosis. (137 patients, 50%) Most patients had multiple abnormal brain MRI findings. Diffuse atrophy was the most common finding (162 patients, 59%), followed by focal or diffuse T2 hyperintensity in white matter (112 patients, 41%), basal ganglia (91 patients, 33%), cerebellum (77 patients, 28%), and cortex. (73 patients, 27%) Syndromic diagnosis yielded 87 patients with Leigh syndrome, (32%) 17 patients with MELAS, (6.2%) and 1 patient with MERRF. (0.4%)

Table 2. Initial diagnostic evaluation of mitochondrial diseases (n=274)

Evaluation	Number of patients (%)
Serum lactic acidosis	
No acidosis	137 (50)
Mild	78 (29)
Moderate	43 (16)
Severe	16 (5.8)
MRI involvement (multiple involvement)	
Basal ganglia	91 (33)
Thalamus	33 (12)
Midbrain	31 (11)
Pons	19 (6.9)
Medulla	23 (8.4)
Cerebellum	77 (28)
Cortex	73 (27)
White matter	112 (41)
Diffuse atrophy	162 (59)
Infarction	18 (6.6)
Syndromic Diagnosis	

Nonspecific	169 (62)
Leigh syndrome	87 (32)
MELAS	17 (6.2)
MERRF	1 (0.4)

MELAS: mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes

3. Confirmative diagnostic evaluation of mitochondrial disease patients

Table 3 shows the results of diagnostic evaluation done in 274 patients. Muscle biopsy showed pathologic findings in 141 patients. (51%) Light microscopic findings showed ragged red fibers and/or increased succinate dehydrogenase stain in 66 patients. (24%) Electron microscopy showed pleoconia or megaconia in 136 patients. (50%) Majority of patients had abnormal results in biochemical assay. (247 patients, 90%) Most common finding was defect in mitochondrial respiratory chain (MRC) complex I only. (213 patients, 78%) Genetic variants were found in 48 patients. Among them, variants were confirmed pathogenic in 27 patients (9.9%) and likely pathogenic in 1 patient. (0.4%) Eleven patients had variants with unknown significance. (4.0%)

Table 3. Confirmative diagnostic evaluation of mitochondrial disease patients

Diagnostic evaluation	**Number of patients (%)**
Muscle biopsy (n=274)	
Light microscopy	
Normal	188 (69)
RRF and/or SDH positive	66 (24)
Nonspecific	20 (7.3)
Electron microscopy	
Normal	138 (50)
Pleoconia only	12 (6.9)

Megaconia only	14 (8.0)
Pleoconia and Megaconia	110 (40)
MRC complex enzyme assay (n=272)	
Normal	25 (9.2)
Defect in MRC complex I only	213 (78)
Defect in MRC complex II	1 (0.4)
Defect in MRC complex IV only	32 (12)
Defect in both MRC complex I and IV	1 (0.4)
mtDNA variant status (n=274)	
Pathogenic	27 (9.9)
Likely pathogenic	1 (0.4)
Possibly benign	3 (1.1)
Likely benign	6 (2.2)
Unknown significance	11 (4.0)
Negative findings	226 (82)

RRF: Ragged red fibers, SDH: Succinate dehydrogenase, MRC: mitochondrial respiratory chain, mtDNA: mitochondrial deoxyribonucleic acid

4. Clinical outcomes of the patients

Table 4 portrays systemic involvement of the mitochondrial disease in the patients and their functional state at last visit. All patients had multi-organ involvement. Central nervous system involvement was noted in 266 patients. (97%) Gastrointestinal (65 patients, 24%) involvement was also common. Less frequent were ophthalmic (40 patients, 15%), pulmonary (37 patients, 14%), and cardiac (31 patients, 11%) involvement. Functional state of the patients was poor. At last visit, 133 patients (49%) were bedridden, and 83 patients (30%) were wheelchair-bound.

Table 4. Clinical outcomes of the patients (n=274)

Systemic involvement at last visit	Number of patients (%)
Central nervous system	272 (99)
Gastrointestinal	65 (24)
Ophthalmic	40 (15)
Pulmonary	37 (14)
Cardiac	31 (11)
Musculoskeletal	24 (8.8)
Renal/Urologic	23 (8.4)
Otologic	22 (8.0)
Endocrine	14 (5.1)
Peripheral nervous system	1 (0.4)
Functional state at last visit	
Normal or self-ambulatory	38 (14)
Wheelchair-bound	83 (30)
Bedridden	133 (49)
Expired	20 (7.3)

5. Mitochondrial DNA variants of the patients

Table 5 shows profile of the mtDNA variants found in 48 patients. Pathogenic variants were identified in 27 patients. (56%) The most common allele was m.3243A>G found in 9 patients, (19%) associated with MELAS, Leigh syndrome, and variable phenotypes of mitochondrial myopathy. Mutation m.10191T>C was noted in 5 patients (10%), which is associated with Leigh syndrome or epilepsy, strokes, optic atrophy and cognitive decline (ESOC) phenotype. In 4 patients, (8%) m.8993T>C was noted, which is related with neurogenic muscle weakness, ataxia, and retinitis pigmentosa and (maternally-associated) Leigh syndrome. Allele m.13513G>A was seen in 2 patients (4%), associated with Leigh syndrome and MELAS. There was 1 patient with likely

pathogenic mutation of m.15995G>A, which is correlated with nonspecific mitochondrial cytopathy. (2%) Variants of unknown significance were noted in 11 patients, (23%) none of which was associated with specific disease phenotype. The remaining 9 patients had possibly or likely benign variants. (19%)

Table 5. Mitochondrial DNA variants of the patients (n=48)

Position	Locus	Associated Disease(s)	Allele	RNA	Nucleotide change	Amino acid change	Mito TIP	Status	n (%)
3243	MT-TL1	MELAS / LS / DMDF / MIDD / SNHL / CPEO / MM / FSGS / ASD / Cardiac+multi-organ dysfunction	A3243G	tRNA Leu (UUR)	A-G		P	Cfm	9 (19)
8344	MT-TK	MERRF	A8344G	tRNA Lys	A-G		P	Cfm	1 (2.1)
8993	MT-ATP6	NARP / LS / MILS / other	T8993C		T-C	L-P	P	Cfm	2 (4.2)
8993	MT-ATP6	NARP / LS / MILS / other	T8993G		T-G	L-R	P	Cfm	2 (4.2)
9176	MT-ATP6	Familial bilateral striatal necrosis/ LS	T9176C		T-C	L-P	P	Cfm	1 (2.1)
9185	MT-ATP6	LS / Ataxia syndromes / NARP-like disease	T9185C		T-C	L-P	P	Cfm	1 (2.1)
10158	MT-ND3	LS / MELAS	T10158C		T-C	S-P	P	Cfm	1 (2.1)
10191	MT-ND3	LS / Leigh-like Disease / ESOC	T10191C		T-C	S-P	P	Cfm	5 (10)
11777	MT-ND4	LS	C11777A		C-A	R-S	P	Cfm	1 (2.1)
13513	MT-ND5	LS / MELAS / LHON-MELAS overlap syndrome / negative association with carotid atherosclerosis	G13513A		G-A	D-N	P	Cfm	2 (4.2)

14459	MT-ND6	LDYT / LS / dystonia / carotid atherosclerosis risk	G14459A		G-A	A-V	P	Cfm	1 (2.1)
14487	MT-ND6	dystonia / LS / ataxia / ptosis / epilepsy	T14487C		T-C	M-V	P	Cfm	1 (2.1)
15995	MT-TP	Mitochondrial cytopathy	G15995A	tRNA Pro	G-A		LP	Rep	1 (2.1)
12311	MT-TL2	CPEO	T12311C	tRNA Leu (CUN)	T-C		PB	Rep	1 (2.1)
14693	MT-TE	MELAS / LHON / DEAF / hypertension helper	A14693G	tRNAGlu			PB	Rep	2 (4.2)
3290	MT-TL1	Possible hypertension factor	T3290C	tRNA Leu (UUR)			LB	Rep	1 (2.1)
4343	MT-TQ	Possible hypertension factor	A4343G	tRNA Gln			LB	Rep	1 (2.1)
5821	MT-TC	DEAF helper mutation	G5821A	tRNA Cys			LB	Rep	1 (2.1)
15927	MT-TT	LHON / Multiple Sclerosis / DEAF1555 increased penetrance / CHD	G15927A	tRNA Thr	G-A		LB	Rep	2 (4.2)
15951	MT-TT	LHON / LHON modulator	A15951G	tRNA Thr			LB	Conf	1 (2.1)
3971	MT-ND1?	Un	T3971C		T-C	Un	Un	Un	1 (2.1)
3982	MT-ND1?	Un	G3982A		G-A	Un	Un	Un	1 (2.1)
5600	MT-TA?	Un	AC5600A	tRNA Ala?	AC-A	Un	Un	Un	1 (2.1)
8368	Un	Un	G8368A	Un-known	G-A	Un	Un	Un	1 (2.1)
10744	Un	Un	A10744G		A-G	Un	Un	Un	1 (2.1)
12145	MT-TH?	Un	T12145C	tRNA His?	T-C	Un	Un	Un	1 (2.1)
13339	MT-ND5?	Un	T13339C		T-C	Un	Un	Un	1 (2.1)
15163	MT-CYB?	Un	A15163G	Un-known	A-G	Un	Un	Un	2 (4.2)

15359	MT-CYB?	Un	T15359C	Un-known	T-C	Un	Un	Un	1 (2.1)
16062	Un	Un	A16062G	Un-known	A-G	Un	Un	Un	1 (2.1)

MELAS: mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes, LS: Leigh syndrome, DMDF: diabetes mellitus + deafness, MIDD: maternally inherited diabetes and deafness, SNHL: sensorineural hearing loss, CPEO: chronic progressive external ophthalmoplegia, MM: mitochondrial myopathy, FSGS: focal segmental glomerulosclerosis, ASD: Autism spectrum disorder, DEAF: Maternally inherited deafness or aminoglycoside-induced deafness, MERRF: myoclonic epilepsy with ragged red fibers, NARP: neurogenic muscle weakness, ataxia, and retinitis pigmentosa, MILS: maternally-inherited Leigh's syndrome, ESOC: epilepsy, strokes, optic atrophy and cognitive decline, LHON: Leber hereditary optic neuropathy, LDYT: Leber's hereditary optic neuropathy and dystonia, CHD: coronary heart disease, P: Pathogenic, LP: Likely pathogenic, PB: Possibly benign, LB: Likely benign, Un: Unknown, Cfm: Confirmed, Rep: Reported, Conf: Conflicting reports

6. Comparison of diagnostic evaluation between mitochondrial disease patients with and without variants (including mutations) in mitochondrial DNA

Table 6 shows result of syndromic, pathologic and biochemical diagnostic evaluation in patients with variants in mtDNA. Higher ratio of 48 mtDNA variant-positive patients had statistically significant mitochondrial disease syndromes and characteristic light microscopic findings, compared to 226 patients without identified mtDNA variants. In detail, 31 of 48 mtDNA variant-positive patients (65%) were diagnosed as mitochondrial disease syndromes compared to 74 of 226 mtDNA variant-negative patients. (33%, p=.000) Especially, 12 patients with identified mtDNA variants (25%) were diagnosed as MELAS, in contrast with 5 patients without mtDNA variants. (2%, p=.000) On the other hand, 17 of 48 patients with mtDNA variants (35%) were phenotypically nonspecific, compared to 152 of 226 patients without DNA variants. (67%, p=.000)

Pathologically, 19 of 48 variant-positive patients (40%) had light microscopic findings of ragged red fibers and/or increased succinate

dehydrogenase stain, compared to 47 of 226 variant-negative patients. (21%, *p*=.006) Notably, 17 patients with mtDNA variants (35%) had ragged red fibers, compared to 47 patients without mtDNA variants. (19%) (*p*=.010) Half of the patients in both groups had characteristic electron microscopic findings of pleoconia and/or megaconia (*p*=.709)

Biochemically, most patients with mtDNA variants (46 of 48 patients, 96%) and most without mtDNA variants (201 of 224 patients, 90%) yielded enzymatic defect in mitochondrial respiratory chain (MRC) complex. (*p*=.187) Most common defect of MRC complex I only was found in 40 mtDNA variant-positive patients (83%) and 173 variant-negative patients. (77%, *p*=.305)

Table 6. Comparison of diagnostic evaluation between mitochondrial disease patients with and without variants (including mutations) in mitochondrial DNA

	mtDNA variant (+) (n=48)	**mtDNA variant (-) (n=226)**	**Total (n=274)**	***p*-values**
Syndromic Diagnosis (%)	31 (65)	74 (33)	105 (38)	.000
Leigh Syndrome	18 (38)	69 (31)	87 (32)	.346
MELAS	12 (25)	5 (2.2)	17 (6.2)	.000
MERRF	1 (2.1)	0 (0.0)	1 (0.4)	.175
Nonspecific	17 (35)	152 (67)	169 (62)	.000
Pathologic diagnosis (LM and/or EM) (%)	28 (58)	113(50)	141 (52)	.294
LM (RRF and/or SDH)	19 (40)	47 (21)	66 (24)	.006
RRF	17 (35)	42 (19)	59 (22)	.010
EM (pleoconia and/or megaconia)	25 (52)	111 (49)	136 (50)	.709
Pleoconia	24 (50)	98 (44)	122 (45)	.401
Megaconia	23 (48)	101 (45)	124 (45)	.683
Biochemical diagnosis (n=272) (%)	46 (96)	201 (90)	247 (91)	.187
MRC complex I only	40 (83)	173 (77)	213 (78)	.305
MRC complex II	0 (0.0)	1 (0.4)	1 (0.4)	1.000

MRC complex I+IV	1 (2.1)	0 (0.0)	1 (0.4)	.175
MRC complex IV only	5 (10)	27 (12)	32 (12)	.764

mtDNA: mitochondrial deoxyribonucleic acid, MELAS: mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes, MERRF: myoclonic epilepsy with ragged red fibers, LM: light microscopy, EM: electron microscopy, RRF: Ragged red fibers, SDH: Succinate dehydrogenase, MRC: mitochondrial respiratory chain complex

7. Comparison of diagnostic evaluation in mitochondrial disease patients with and without mutations in mitochondrial DNA

Table 7 shows result of diagnostic evaluation in 28 patients with mtDNA mutations, i.e. pathogenic or likely pathogenic mtDNA variants. The 28 patients also had statistically significant higher ratio of mitochondrial disease syndrome and light microscopic findings, compared to 246 patients without mtDNA mutations. Most patients with mtDNA mutations (26 of 28, 93%) had syndromic diagnoses, compared to one-third of mutation-negative patients. (79 of 246, 32%) (p=.000) Specifically, the ratio of MELAS in patients with mtDNA mutations (9 of 28, 32%) was 10 times higher than in patients without mutations (8 of 246, 3%) were diagnosed with MELAS. (p=.000) Also, the ratio of Leigh syndrome in mutation-positive patients (16 of 28, 57%) was twice as high as in mutation-negative patients. (71 of 246, 29%) (p=.002)

Around 60% of patients in both groups had pathologic findings of mitochondrial disease. (p=.663) The ratio of characteristic light microscopic findings among 28 patients with mtDNA mutations (12 patients, 43%) was twice as those without. (54 of 246, 22%) (p=.014) Also, 13 mutation-positive patients (46%) and 149 mutation-negative patients (61%) had electron microscopic findings of pleoconia and/or megaconia. (p=.149) All 28 patients with mtDNA mutations and 90 percent of 244 mutation-negative patients had biochemical defects. (p=.088) The ratio of defect in MRC complex I only was similar between

mutation-positive patients (22 patients, 79%) and mutation-negative patients (191 patients, 78%) (*p*=.972)

Table 7. Comparison of diagnostic evaluation in mitochondrial disease patients with and without mutations in mitochondrial DNA

	mtDNA mutation (+) (n=28)	mtDNA mutation (-) (n=246)	Total (n=274)	*p*-values
Syndromic Diagnosis (%)	26 (93)	79 (32)	105	.000
Leigh Syndrome	16 (57)	71 (29)	87 (32)	.002
MELAS	9 (32)	8 (3.3)	17 (6.2)	.000
MERRF	1 (3.6)	0 (0.0)	1 (0.4)	.102
Nonspecific	2 (7.1)	167 (68)	169 (62)	.000
Pathologic diagnosis (LM and/or EM) (%)	16 (57)	151 (61)	167 (61)	.663
LM (RRF and/or SDH)	12 (43)	54 (22)	66 (24)	.014
RRF	10 (36)	49 (20)	59 (22)	.054
EM (pleoconia and/or mega-conia)	13 (46)	149 (61)	162 (59)	.149
Pleoconia	13 (46)	109 (44)	122 (45)	.831
Megaconia	12 (43)	112 (46)	124 (45)	.788
Biochemical diagnosis (n=272) (%)	28 (100)	219 (90)	247 (91)	.088
MRC complex I only	22 (79)	191 (78)	213 (78)	.972
MRC complex II	0 (0.0)	1 (0.4)	1 (0.4)	1.000
MRC complex I+IV	1 (3.6)	0 (0.0)	1 (0.4)	.103
MRC complex IV only	5 (18)	27 (11)	32 (12)	.291

mtDNA: mitochondrial deoxyribonucleic acid, DNA, MELAS: mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes, MERRF: myoclonic epilepsy with ragged red fibers, LM: light microscopy, EM: electron microscopy, RRF: Ragged red fibers, SDH: Succinate dehydrogenase, MRC: mitochondrial respiratory chain complex

8. Diagnostic values of muscle biopsy compared to diagnosis by mitochondrial DNA variants and mutations

Table 8 compares pathologic and syndromic diagnoses with diagnosis by mtDNA variants. Comparing pathologic evaluation with diagnosis by mtDNA variants, pathologic findings showed low sensitivity, from 35 to 58 percent, indicating that patients with mtDNA variants have 35 to 58 percent possibility of having characteristic pathology. The specificity of the studies was highest in light microscopy (79%) and findings of RRF, (81%) showing that mtDNA variant-negative patients are less likely to have light microscopic findings, especially RRF. Positive predictive values (PPVs) of pathologic findings ranged from 18 to 29%, showing that having positive pathologic findings would only yield up to 29% possibility of being diagnosed by mtDNA variant. Negative predictive values (NPVs) of all pathologic studies were over 80%, with light microscopy and RRF having the highest values of 86%, indicating that pathology-negative patient would likely be variant-negative.

Syndromic diagnosis showed overall mediocre sensitivity, low PPVs, (except MERRF) relatively high specificity, and higher NPVs compared to diagnosis by mtDNA variants. Especially, MELAS syndrome showed high specificity of 98% and high NPV of 86%. Patients without mtDNA variants would be ruled against MELAS, and patients without features of MELAS would likely be variant-negative.

Table 8. Diagnostic values of muscle biopsy compared to diagnosis by mitochondrial DNA variants

mtDNA variant	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Pathology	58	50	20	85

Light microscopy	40	79	29	86
RRF	35	81	29	86
Electron microscopy	52	51	18	83
Pleoconia	50	57	20	84
Megaconia	48	55	19	83
Syndromic Diagnosis	65	67	30	90
Leigh Syndrome	38	69	21	84
MELAS	25	98	71	86
MERRF	2.1	100	100	83
Nonspecific	35	33	10	70

mtDNA: mitochondrial deoxyribonucleic acid, PPV: positive predictive value, NPV: negative predictive value, RRF: ragged red fibers, MELAS: mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes, MERRF: myoclonic epilepsy with ragged red fibers

9. Diagnostic values of muscle biopsy compared to diagnosis by mitochondrial DNA mutations

Pathologic evaluation showed low sensitivity (range 36 to 57%) and PPVs (range 8 to 18%) when compared with diagnosis by mtDNA mutations. Patients with mtDNA mutations would have up to 57% likelihood of having characteristic pathology. Moreover, patient with positive pathologic findings would have only 18% possibility of having mtDNA mutation. Specificity ranged from 38 to 80% with light microscopic findings and RRF showing highest values. (78% and 80% each) NPVs was high from 87 to 92%, with light microscopy and RRF both having NPV of 92%. Therefore, patients with light microscopic finding or RRF would likely have mtDNA mutation, and vice versa, i.e. patients without mtDNA mutation are most likely without RRF or other characteristic light microscopic findings.

Comparing with mtDNA mutations, syndromic diagnosis had high sensitivity of 93%, indicating that most patients with mtDNA mutation would be likely to be classified in a mitochondrial disease syndrome. High NPV of 99%

indicates that patients diagnosed as syndrome-negative would most likely be free of mtDNA mutation. MELAS had high specificity of 97% and NPV of 93%, indicating that mtDNA mutation-negative patients would be ruled against MELAS, and vice versa.

Table 9. Diagnostic values of muscle biopsy compared to diagnosis by mitochondrial DNA mutations

mtDNA mutation	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Pathology	57	38	9.6	89
Light microscopy	43	78	18	92
RRF	36	80	17	92
Electron microscopy	46	39	8.0	87
Pleoconia	46	56	11	90
Megaconia	43	54	9.7	89
Syndromic Diagnosis	93	68	25	99
Leigh Syndrome	57	71	18	94
MELAS	32	97	53	93
MERRF	3.6	100	100	90
Nonspecific	7.1	32	1.2	75

mtDNA: mitochondrial deoxyribonucleic acid, PPV: positive predictive value, NPV: negative predictive value, RRF: ragged red fibers, MELAS: mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes, MERRF: myoclonic epilepsy with ragged red fibers

IV. DISCUSSION

1. Analysis of clinical data

The median age at first symptoms of 274 patients was 0.73 years old.

This is comparable to 0.75 years old of 106 patients with Leigh syndrome[16] and 0.83 years old in 118 patients with mitochondrial disease[4]. Common symptoms at first visit were developmental delay or regression (44%) and seizures. (41%) This is in line with Ogawa's study where developmental regression or delay, seizures, and respiratory distress were three main symptoms of the Leigh syndrome patients[16]. Also, the three symptoms are counted as diagnostic CNS symptoms in disease criteria[17].

Serum lactate elevation was noted in 50.0% of the patients. This is higher than 22.0% of Bernier *et al*.'s cohort[4] and lower than 94.1% of 35 patients with Leigh syndrome[8] or 93% of 110 patients with MELAS[18]. MRI involvement was diffuse, with most common findings of atrophy (59%), white matter involvement (41%), and basal ganglia involvement (33%). This is similar to a report on 25 patients with mitochondrial disease, with common findings of supratentorial atrophy, white matter changes, and basal ganglia changes[19].

In our cohort, one-third of the patients were diagnosed as Leigh syndrome (32%), followed by MELAS (6.2%) and one patient with MERRF. (0.4%) In Bernier's cohort, 6.7% had Leigh syndrome and 1.6% had MELAS. Prevalence of mtDNA-associated Leigh syndrome is estimated as 0.7 to 1:100,000[20]. Prevalence of MELAS is estimated as 0.2:100,000[7]. Prevalence of mitochondrial disorders is considered to be at least 13:100,000 live births[21], and primary mtDNA mutation estimated as 20:100,000 of live births[22]. Therefore, estimated Leigh syndrome would be 3.5 to 7.7%, and estimated MELAS would be 1.0 to 1.5%, which is more concurrent with Bernier's cohort. The difference of prevalence in our cohort is because as a retrospective study, we performed mtDNA sequencing in patients with suspected mitochondrial DNA defects, which are more likely in patients with clinical syndromes, such as Leigh syndrome and MELAS, than in nonspecific patients.

Most patients had central nervous system involvement, followed by gastrointestinal, ophthalmic and pulmonary involvement which are mostly caused by muscle weakness. This is concurrent with major symptoms of mitochondrial disease. Bernier et al suggested major clinical criteria of at least 3 systemic involvement including neurologic, muscular, nutritional presentations pathognomonic of respiratory chain disorder[4]. Also, modified Wolf's criteria denoted CNS presentation including seizures or developmental delay, multisystem disease including GI tract and vision, and muscular presentation including ophthalmoplegia, exercise intolerance or muscle weakness[17].

At the last visit, half of the patients were bedridden, and 7% were expired. The mortality rate is lower than previous study of Bernier's cohort, where 40% had expired[4]. This may be due to improvement of overall patient care in mitochondrial medicine in last two decades. Patients who were diagnosed with mitochondrial diseases have taken coenzyme Q and multivitamin, mainly vitamin B and C complex. They went through routine follow-up with systemic screening, management, and nutrition care every 3 to 6 months, which could have improved their quality of life and prevented some of unexpected respiratory or cardiac failure.

2. Analysis of pathologic and biochemical data

In our study, light microscopic findings showed ragged red fibers or abnormal stain in succinate dehydrogenase (SDH) in 24% of total cohort, 40% of the mtDNA variant-positive patients and 43% of patients with mtDNA mutation. As the percentage is higher in patients with mtDNA mutation, light microscopic findings may be indicative of primary origin. However, previous reports show controversial findings. RRF are most common in MERRF or MELAS, from 58 to 85% of the patients, but rare in complex I deficiency. In another study, RRF

was scarce in patients with mitochondrial diseases under 3 years old[6, 23, 24]. In our cohort, 78% of the patients had defect in MRC complex I and 7% of the patients had MELAS or MERRF syndrome, so light microscopic findings are more common than estimated in previous studies.

Electron microscopy showed pleoconia and/or megaconia in 46% of the mutation-positive group, 52% of the variant-positive group, and 59% of total patients, most of them showing both findings. The percentage is lower as primary mitochondrial disease patients are specified. Parikh *et al.* reported that pediatric patients have more electron microscopic findings than light microscopic findings of mitochondrial disease[5]. Abnormally large or odd-shaped mitochondria may signify mitochondrial disease, but also indicate diffuse myopathy regardless of its etiology[24]. As Vogel maintained, Electron microscopic abnormalities combined with RRF are indicative of mitochondrial disease[24]. However, combined with our and previous studies, electron microscopy alone may not be effective in determining primary mitochondrial disease from secondary causes.

Enzymatic defects were present in all patients with mtDNA mutation, 96% of patients with mtDNA variants, and 91% in total patients. This is higher than 71% of 92 patients with mtDNA mutation-negative mitochondrial diseases[25]. Although the ratio is increased in our group with genetically confirmed mitochondrial disease, the ratios of all groups are over 90%, which cause difficulty in deciding whether a positive enzymatic defect indicates primary origin. Parikh et al has noted on how enzymatic activities are decreased by secondary causes such as physical inactivity[5]. As half of our patients are bedridden and other 30% are wheelchair-bound, other causes may have affected enzymatic defect. Therefore, enzymatic defects are inconclusive in distinguishing primary or secondary origin of mitochondrial disease.

3. Analysis of genetic data

Among the 274 patients who went through mtDNA sequencing, 28 patients (10.2%) had mutations in mtDNA, and additional 20 patients (7.3%) had mitochondrial variants which were of unknown significance, or possibly or likely benign. The yield of mutation is higher than 4 patients (3.3%) with mitochondrial gene mutations of 118 mitochondrial disease patients (Bernier et al 2002) and 1 patient (2.4%) with mtDNA mutation among 46 infants with mitochondrial disease[26].

One reason for the low yield of mtDNA in suspected mitochondrial disease patients is possibility of nuclear DNA (nDNA) mutation. Among 251 genetic defects known as of 2015 leading to mitochondrial diseases, mitochondrial DNA encodes 37 genes, compared to 210 nuclear genes[27]. As mtDNA genome is small compared to nDNA, it may affect no more than 30% of pediatric mitochondrial diseases[26]. A Dutch center found 16% to 19% of the mtDNA-negative mitochondrial disease patients (44 and 109 patients, respectively) had nuclear gene mutations associated with the disease confirmed by exome sequencing[25, 28]. Legati *et al*. revealed 20% yield of mutations in nDNA among patients with mtDNA-negative mitochondrial disease patients, by NGS and further whole exome sequencing among NGS-negative patients highly suspected with mitochondrial disease[22]. As we did not perform nDNA panel sequencing in patients, further study on mtDNA-negative cohort may yield more patients with mutations related to oxidative phosphorylation system.

Also, the patients may have other diseases with similar clinical presentation, such as muscle weakness, regression, or seizures, causing secondary mitochondrial dysfunction. Wortmann et al found that 19% of mtDNA-negative patients suspected with mitochondrial diseases had mutations in genes not associated with mitochondrial diseases, including *SCN1A* of Dravet syndrome,

ARID1B and *SMARCA4* of Coffin-Siris syndrome, and *ACTA1* of nemaline myopathy[25]. The percentage is same as that of patients with nDNA causing mitochondrial diseases in the study. With advanced genetic testing, secondary mitochondrial dysfunction may be much more common than primary mitochondrial disease, because multiple etiology may present as mitochondrial dysfunction, including neuromuscular, chromosomal, neurodegenerative, autoimmune, or metabolic disorders, with many associated gene mutations[2].

In addition, mtDNA mutation-negative patients could have mitochondrial disease by mutations found after the study. With genetic evolution in the recent decades, it took 13 years, from 1995 to 2008 to identify nearly 100 genes associated with mitochondrial diseases, and it took only 7 years to identify another 150 genes associated with mitochondrial diseases[27, 29]. Therefore, NGS panels should be routinely updated, and review of the patients' sample by new panels may reveal novel mutations.

Further, mutation-negative patients may have defect in the pathway between genetic transcription and protein encoding. For example, Kemp *et al.* found cases with defects in mitochondrial translation. They studied 52 patients with mtDNA mutation-negative respiratory chain deficiency, and found nDNA mutation in only 1 patient. Functional study of 22 patients without either mtDNA or nDNA mutation presented 7 patients (32%) with diffuse or selectively decreased translation in mitochondria[30]. Further studies may reveal new genes or other mechanism leading to defects in the pathway from gene to protein.

Most common mutation was m.3243A>G in 9 patients, followed by m.10191T>C in 5 patients, and m.8993T>C and T>G in total 4 patients. Study of prevalence of mutations is centered in a British team of adult mitochondrial disease patients. Gorman *et al.* performed pedigree analysis of adult patients with mitochondrial diseases, and detected adults and children at risk of mitochondrial

diseases, who had first-degree relatives of mitochondrial diseases or who had mtDNA mutations found without symptoms during pedigree analysis. Among them, 40.8% had m.3243A>G. Also, m.8993T>G was noted in 2 of 282 (0.7%) at-risk adults and children. However, m.10191T>C was not noted in any patient[31]. This may be because m.10191T>C is associated with Leigh syndrome, which has childhood onset with low survival rate at adulthood. Also, a report on m.10191T>C shows 16 cases as of 2012, of which at least half were *de novo*[32]. Combining the characteristics of the disease and the sporadic nature of the mutation, there is low possibility that m.10191T>C would have been noted in pedigree analysis of adult proband.

4. Comparison of diagnostic methods

Comparing with diagnosis by mtDNA mutations, syndromic diagnosis, especially of Leigh syndrome or MELAS, and characteristic light microscopic findings were statistically significant. This is correlated with relative high specificity of the diagnostic methods, (Table 9) showing that mtDNA-negative patients are less likely to have syndromic diagnosis or light microscopic findings. This is concurrent with previous study which compared genetically confirmed mitochondrial disease patients with mutation-negative patients[17]. Researchers used modified Wolf criteria which defined scores 5 to 7 as probable mitochondrial disorder and scores over 8 to be definite mitochondrial disorder. They found 44 primary mitochondrial disease patients had diagnostic scores[33] of mean 7.6 (range 6 to 12), and mean 7.0 not including histologic score. On the other hand, 17 mutation-negative patients had mean score of 5, (range 4 to 6) and all patients had histology score of 0. Matching the previous studies on clinical syndrome of mitochondrial disease, symptoms of majority of MELAS patients, seizure, migraine, stroke-like episode, short stature, elevated lactate, and stroke-like

picture in MRI[7] count as 6 points. Including muscle weakness, exercise intolerance, or loss of skills, common in over one third of juvenile MELAS patients, could add up to 3 points. In Leigh syndrome, diagnostic criteria of developmental delay, loss of skills, brainstem involvement and/or extrapyramidal signs, neuropathy, elevated lactate, and characteristic MRI findings of Leigh syndrome[8], sum up as 6 to 7 points. Also, light microscopic findings of ragged red fibers or reduced COX staining counts as 4 points each, and SDH positive blood vessels count as 2 points, which are negative in mutation-negative patients.

However, electron microscopic findings and biochemical diagnosis had no statistical significance, with similar ratio of patients with positive findings in mutation-positive and negative groups. The insignificance of electron microscopy is contrary to Wolf's criteria which counted abnormal mitochondria in electron microscopy as 2 points, and Morava's study where mutation-negative patients had negative histologic findings, suggesting high specificity of histologic studies. This could be explained by studies indicating that pathologic findings or biochemical studies could also represent secondary mitochondrial dysfunction[4, 34, 35]. Reasons why light microscopic findings are more distinctive of primary disease need further investigation.

In our study, 105 patients were clinically diagnosed with mitochondrial syndromes, amongst whom 31 patients had mtDNA variants (29.5%) and 26 patients had mtDNA mutations found. (24.7%) Mutation ratio of each syndrome (i.e. PPVs as shown in Table 9) differed among mitochondrial syndromes. Among 87 patients with Leigh syndrome, 16 patients (18.4%) had known mutations, most commonly at MT-ATP6. (6 of 16 patients) Previous study by Ogawa et al. of 103 patients with Leigh syndrome yielded 30 patients with mtDNA mutations, (29.1%) most frequently MT-ATP6[16]. Our study has lower yield, but with similar trend of mutation sites. Among 17 MELAS patients, 9 patients (52.9%) had mutations. This is lower than reported 84% in Japanese cohort of 31 MELAS patients[36] The

one patient with MERRF had mutation m.8344A>G, as in a previous study where point mutation in nucleotide pair 8344 was found in all 5 patients with MERRF[36].

As genetic studies are discovering more patients with primary mitochondrial origin[2], further studies on matching current diagnostic methods with mutation-positive mitochondrial disease patients would help find indicators of primary mitochondrial disease in clinical, biochemical or pathologic methods. Especially, the sensitivity of syndromic diagnosis could be increased by refurbishing diagnostic criteria of clinical syndromes according to patients with mutations found. Further, previous diagnostic criteria of mitochondrial diseases are based on cohort mainly diagnosed with clinical and biochemical studies. The genetic yield of the Bernier's cohort was less than 10%[4]. While 90% of the patients could have secondary mitochondrial dysfunction, further review may detect more patients with primary origin, as dozens of novel genes were found to cause mitochondrial diseases during the last two decades.

Also, as genetic sequencing is becoming more comprehensive and less expensive with shorter turnaround time, further studies are needed to modify the diagnostic criteria according to patients with genetically confirmed mitochondrial diseases. For example, although Morava's study shows difference in scores of patients with primary mitochondrial diseases (6 to 12) and secondary mitochondrial disorders, (4 to 6) there was on overlap of the score range[17]. Patients with 6 points would need genetic results before confirmation of disease. Therefore, review and adjustment of modified Wolf criteria in patients with recent genetic diagnosis would enable more accurate differentiation of primary and secondary mitochondrial diseases.

V. CONCLUSION

Muscle biopsy has guided diagnosis of mitochondrial diseases for decades[5, 12]. Light microscopy findings, especially findings of ragged red fibers, could be indicative of primary mitochondrial etiology in mitochondrial diseases. Further studies would help determine the significance of each diagnostic tests in mitochondrial diseases.

As genetic studies are more rapidly evolving, diagnosis of mitochondrial diseases would be more centered on genetic diagnosis. Therefore, diagnostic criteria involving clinical, pathological, biochemical and radiologic studies should be reviewed according to presence and types of genetic mutations. Especially, matching pathologic results with genetic results would help increasing yield of genetic diagnosis according to specific pathologic findings in primary mitochondrial diseases.

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ABSTRACT (IN KOREAN)

미토콘드리아 질환이 유전적으로 진단된 소아 환자들에서 근육 조직 검사의 진단적 유용성

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최 한 솜

배경: 미토콘드리아 질환은 산화적 인산화 체계를 부호화하는 핵 내 또는 미토콘드리아 유전체의 돌연변이에 의해 발생한다. 진단적 표준 기법으로 근육 조직 검사가 이용되었으나, 유전 연구가 진화하면서 차세대 염기서열 분석법이 각광받고 있다. 이에 연구진은 미토콘드리아 질환의 진단에서 미토콘드리아 디옥시리보핵산 염기서열(mtDNA) 분석과 근육 조직 검사의 진단적 유용성을 비교 분석하고자 하였다.

방법: 2005년 8월부터 2019년 4월까지 강남세브란스병원에서 미토콘드리아질환에대한 진단적 평가를 한 274명의 환자들의 의무기록을 후향적으로 분석하였다. 모든 환자들은 혈청 젖산 검사, 뇌 자기공명영상, 근육 조직 검사, 생화학 검사 및 mtDNA 염기서열 분석을 진행하였다. 환자들 중 mtDNA 돌연변이가 확인된 환자들과 확인되지 않은 환자들 사이의 병리학적 소견에 대한 통계적 분석 후, mtDNA 염기서열 분석과 비교하였을 때 각 병리학적 검사의 민감도, 특이도, 양

성예측도 및 음성예측도를 계산하였다.

결과: 미토콘드리아 질환이 진단된 274명의 환자들 중 증상이 발현한 중간 연령은 0.7세였으며, 근육 조직 검사를 시행한 중간 연령은 3.2세였다. 가장 흔한 증상은 발달 지연 및 퇴행(44%) 그리고 경련(41%)이었다. 그들 중 27명에서 mtDNA 돌연변이가 확인되었다. 마지막 내원 시 모든 환자들에서 질병의 전신적 침범을 확인하였으며, 중추신경계 (99%), 소화기(24%), 안과 (15%) 등의 증상을 보였다.

돌연변이가 확인된 27명의 환자들과 확인되지 않은 환자들을 비교하였을 때, 병리학적 검사 및 전자 현미경적 진단은 통계학적으로 의미가 없었다. 그러나 광학 현미경 및 ragged red fibers 소견에 의한 진단은 통계적 의미가 있었다. (각각 p=0.009 및 0.049) 병리학적 검사의 민감도는 mtDNA 돌연변이가 확인된 환자들에서 최저 41%, 최고 56%였다. 병리 검사의 특이도는 38에서 80%였으며, 이 중 광학현미경 소견(78%) 및 병리학적 소견(80%)이 가장 특이도가 높았다.

결론: 광학 현미경 소견, 특히 ragged red fibers 소견은 미토콘드리아 질환에서 일차 미토콘드리아 질환을 시사할 수 있다. 추가 연구를 통해 미토콘드리아 질환에서 각 진단 검사의 중요성을 감별하는 데 도움을 얻을 수 있다. 또한 점점 진단의 주가 되어가는 유전 진단과 병리학적 소견을 대입하는 연구를 지속함으로써 일차 미토콘드리아 질환에서 특정 병리학적 소견에 따른 유전 진단율을 높이는 데 도움을 얻을 수 있을 것이다.

핵심되는 말: 미토콘드리아 질환, 근육 조직 검사, 미토콘드리아 DNA, ragged red fibers