



미토콘드리아 DNA 돌연변이에 따른 Leigh 증후군의 임상 양상 분석

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Leigh Syndrome: Subgroup Analysis according to Mitochondrial DNA Mutation

Purpose: Leigh syndrome (LS) is a rare, progressive neurodegenerative disorder with characteristic abnormalities in the central nervous system. Such patients present with heterogeneous clinical symptoms and genetic abnormalities; thus, prognosis is difficult to anticipate. The present study investigates whether distinct patient characteristics are associated with mitochondrial DNA (mtDNA) mutation in LS patients.

Methods: We retrospectively analyzed data from patients diagnosed with LS at our hospital who were assessed using genomic sequencing of mtDNA. A subgroup analysis was performed to divide patients according to the mtDNA sequencing results.

Results: Among the 85 patients enrolled, 18 had mtDNA mutations. Most patients had lactic acidosis and a lactate/pyruvate ratio above 20, indicating respiratory chain abnormalities. In the subgroup analysis, the mutation group had a significantly higher female-to-male ratio, alanine level, ocular involvement, and midbrain and medulla abnormalities on magnetic resonance imaging (MRI).

Conclusion: The subgroup analysis indicates that mtDNA sequencing is recommended for female patients, or those who exhibit ocular involvement, high alanine levels, or MRI findings with lesions in the midbrain and medulla.

Key Words: Mitochondria, Mitochondrial DNA, Leigh syndrome, Alanine, Brainstem

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Introduction

Leigh syndrome (LS) is a rare, genetically heterogeneous, and progressive neurodegenerative disorder¹⁻³. LS involves the progression of focal and necrotizing lesions in the central nervous system, including the basal ganglia, cerebellum, and brainstem⁴. Clinical presentations include psychomotor delay, weakness, hypotonia, ataxia, dystonia, brainstem dysfunction, respiratory difficulty, swallowing dysfunction, and ophthalmological abnormalities such as nystagmus and optic atrophy⁵. The age at onset of LS is typically between 3 and 12 months. Most patients die within a few years of being diagnosed⁶.

Since the identification of the first pathogenic mutation in a patient with LS in 1991, more than 75 disease-associated genes have been identified. Several studies have reported mitochondrial DNA (mtDNA) mutations in approximately 10–20% of LS cases⁶⁻⁸. These mutations are usually associated with defects involving

Submitted: 14 September, 2017
Revised: 29 September, 2017
Accepted: 30 September, 2017

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mitochondrial oxidative phosphorylation⁹). Mutations in 13 of 37 mtDNA-encoded genes that encode subunits of mitochondrial respiratory chain complexes have been associated with LS⁸).

LS is a rare and heterogeneous disease; therefore, it is difficult to obtain large amounts of data from patients. The objective of the present study is to compare differences in clinical features, laboratory findings, and imaging characteristics of LS in individuals with and without mtDNA mutations.

Materials and Methods

1. Participants

This study was a retrospective analysis of patients diagnosed with LS and followed up at Gangnam Severance Hospital from March 2006 to February 2016. Patients who fulfilled the following criteria were included: (1) characteristic features of LS on neuroimaging, i.e., symmetrical hyperintense lesions in the basal ganglia and/or brainstem on T2-weighted magnetic resonance imaging (MRI); (2) abnormal energy metabolism indicated by a severe defect in oxidative phosphorylation or pyruvate dehydrogenase complex activity⁹), and (3) genetic analysis of whole mtDNA performed at a diagnostic workup. All procedures conducted were approved by the Institutional Review Board of Gangnam Severance Hospital in Seoul, Korea.(3-2017-0618).

2. mtDNA analysis

DNA was extracted from peripheral blood leukocytes or primary cultured fibroblast cells, using the QIAcube System and QIAamp DNA Blood Mini Extraction Kit (Qiagen, CA) and was stored in 10 mM of Tris buffer solution at -20°C. mtDNA was amplified using long-range polymerase chain reaction (PCR). PCR conditions were as follows: 98°C for 30 seconds, 30 cycles at 98°C for 10 seconds, 72°C for 8 minutes 15 seconds, and a final extension at 72°C for 10 minutes. PCR products were run on a 1% agarose gel, and the expected 16.5-kb fragments were excised. DNA was purified using Agencourt AMPure XP (Beckman Coulter, IN). Quantification was performed using 4200 TapeStation (Agilent, UK). We fragmented the PCR product into 150–200-bp segments with NEBNext dsDNA Fragmentase[®] (New England Biolabs, MA), according to the manufacturer's protocol. The enzyme-fragmented PCR product was used as the input for the Accel-NGS[®] 2S PCR-Free DNA Library Kit following the manufacturer's protocol. Final libraries were evaluated on the 4200 TapeStation and quantitated using Qubit (Thermo Fisher Scientific, MA). Libraries were sequenced by synthesis on MiSeq for paired 150-bp read lengths using Illumina MiSeq V3 Kits

(Illumina, CA). The sequenced reads were mapped to the human mitochondria reference (NC_012920) with the Burrows-Wheeler Aligner (BWA), and variants were identified with the Genome Analysis ToolKit (GATK). Sequence variants were filtered in accordance with various quality parameters.

3. Subgroup definitions and data analysis

Patients were divided into either a mtDNA mutation-positive or mtDNA mutation-negative group. The two groups were statistically compared and analyzed on the basis of biochemical screening tests, neuroradiological findings, and pathologic findings. Patient data included sex, age of first symptoms, organ involvement, biochemical study (i.e., serum lactate, serum pyruvate, lactate-pyruvate ratio, creatine kinase (CK), plasma amino acids, and urine organic acid), brain MRI findings, respiratory chain enzyme activities, muscle histochemistry, and mtDNA sequencing findings. Patients with known nuclear DNA (nDNA) mutations were excluded¹⁶⁻⁸).

4. Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (SPSS Inc., Chicago, IL). Descriptive statistics included the mean, standard deviation (SD), median, and range. Continuous variables are presented as means and were compared between groups using two-sample *t*-tests. Categorical variables are presented as counts and percentages and were compared between groups using the chi-square test and Fisher's exact test. *P* values less than 0.05 were considered statistically significant.

Results

Among the 85 patients who underwent mitochondrial gene analysis, 67 (78.8%) had normal mtDNA and 18 (21.2%) had

Table 1. The Position of mitochondrial DNA (mtDNA) Mutations in Patients with Leigh Syndrome (LS) (n=18)

mtDNA position	Prevalence
T10191C	5 (5.9%)
T8993C	4 (4.7%)
G13513A	2 (2.4%)
T8993G	2 (2.4%)
T10158C	1 (1.2%)
G14459A	1 (1.2%)
C11777A	1 (1.2%)
T9185C	1 (1.2%)
T9176C	1 (1.2%)
Total	18/85 (21.2%)

mtDNA, mitochondrial DNA.

Table 2. Comparison of Pediatric Clinical Variation in Leigh Syndrome (LS) between mitochondrial DNA (mtDNA) Mutation Positive and Negative Groups (n=85)

Clinical feature	Specification	Total (n=85)	mtDNA (+) (n=18)	mtDNA (-) (n=67)	P-value
Sex (female)		44 (40.7%)	15 (75.0%)	30 (44.8%)	0.022*
Age at onset of first symptom (years)		1.56±2.08	2.34±2.69	1.30±1.79	0.028*
Organ involvement	CNS	85 (100%)	18 (100.0%)	67 (100.0%)	
	Muscular	43 (50.6%)	9 (50.0%)	34 (50.7%)	0.955
	Respiratory	24 (28.2%)	6 (33.3%)	18 (26.9%)	0.555
	GI tract	33 (38.8%)	5 (27.8%)	28(41.8%)	0.279
	Ear	4 (4.7%)	0 (0%)	4 (6.0%)	0.379
	Eye	8 (9.4%)	5 (27.8%)	3 (4.5%)	0.009*
	Heart	6 (7.1%)	1 (5.6%)	5 (7.5%)	1.000
	Hematology	1 (1.2%)	0 (0.0%)	1 (1.5%)	1.000
	Nephrology	5 (5.9%)	2 (11.1%)	3 (4.5%)	0.285

*P-values <0.05 were considered statistically significant.

Table 3. Comparison of Biochemical Laboratory Findings in Patients with Leigh Syndrome (LS) with or without mitochondrial DNA (mtDNA) Mutations (n=85)

Biochemical laboratory findings		Total (n=85)	mtDNA (+) (n=18)	mtDNA (-) (n=67)	P-value	Reference value
Lactate	At the time of diagnosis	2.91±1.71	2.85±1.40	2.93±1.79	0.877	0.5-2.2mmol/L
	Last visit	2.43±1.27	2.61±1.01	2.39±1.33	0.523	
Pyruvate	At the time of diagnosis	0.17±0.10	0.13±0.07	0.18±0.11	0.072	0.034-0.102 mmol/L
	Last visit	0.16±0.07	0.15±0.06	0.16±0.07	0.941	
Lactate/ pyruvate ratio	At the time of diagnosis	21.89±28.37	36.53±13.52	17.96±10.18	0.189	
	Last visit	18.17±18.87	18.02±6.62	18.21±21.02	0.970	
CK	At the time of diagnosis	148.94±133.16	122.67±107.87	156.00±139.04	0.349	44-245 IU/L
	Last visit	162.25±161.78	106.35±61.49	176.72±176.95	0.112	
Plasma amino acid (n=72)	Alanine	432.93±189.03	517.19±220.99	408.86±173.65	0.042*	143-439 nmol/mL
	Glycine	329.4±920.72	705.94±1,948.72	221.82±75.08	0.336	81-436 nmol/mL
	Proline	181.39±90.56	178.69±84.77	182.16±92.87	0.894	52-298 nmol/mL
	Threonine	125.72±86.72	151.75±168.77	118.29±41.08	0.443	189.8 nmol/mL
Urine organic acid (n=59)	Abnormal findings	28(47.5%)	6/11 (54.5%)	22/48 (45.8%)	0.602	

mtDNA, mitochondrial DNA; CK, creatine kinase.

*P-values <0.05 were considered statistically significant.

mtDNA mutations. The most frequent mutation, T1019C, a mutation in the *ND3* gene of the mitochondrial respiratory chain (MRC) complex I, was present in 5 patients (5.9%)^{10,11}. The prevalence of the position of a mtDNA mutation is shown in Table 1.

Of the 85 patients with LS, 41 were male and 44 were female (Table 2). The average age of the first clinical presentation was 1.56 (SD=2.08) years old. All 85 patients (100%) had central nervous system involvement, and other organs that were affected are shown in Table 2. Subgroup analysis was performed to compare the mtDNA mutation positive and negative groups. The proportion of females was significantly higher in the positive group than in the negative group ($P=0.022$). The age at onset of first symptoms was significantly greater in the positive group than in the negative group ($P=0.028$). The incidence of ocular involvement was also significantly higher in the mtDNA mutation positive group ($P=0.009$). In contrast, there was no

Table 4. Comparison of Leigh Syndrome (LS) Magnetic Resonance Imaging (MRI) Findings between mitochondrial DNA (mtDNA) Mutation Positive and Negative Groups (n=85)

Involved lesion	Total (n=85)	mtDNA (+) (n=18)	mtDNA (-) (n=67)	P-value
Basal ganglia	80 (94.1%)	17 (94.4%)	63 (94.0%)	1.000
Thalamus	28 (32.9%)	8 (44.4%)	20 (29.9%)	0.242
Brainstem	31 (28.7%)	10 (55.6%)	21 (31.3%)	0.058
Midbrain	30 (35.3%)	11 (61.1%)	19 (28.4%)	0.010
Pons	14 (16.5%)	4 (22.2%)	10 (14.9%)	0.483
Medulla	19 (22.4%)	8 (44.4%)	11 (16.4%)	0.022*
Cerebellum	23 (27.1%)	6 (33.3%)	17 (25.4%)	0.555
Atrophy	44 (51.8%)	7 (38.9%)	37 (55.2%)	0.218
Cortex	23 (27.1%)	6 (33.3%)	17 (25.4%)	0.555
White matter	35 (41.2%)	3 (16.7%)	32 (47.8%)	0.017*

mtDNA, mitochondrial DNA.

*P-values <0.05 were considered statistically significant.

significant difference between the two groups in the involvement of other organs.

The laboratory data of all patients are summarized in Table 3.

Biochemical laboratory measurements showed that the mean lactate and pyruvate levels were elevated, but in most patients, creatine kinase activity was not elevated relative to the reference value. In addition, no differences between the two groups were found for lactate, pyruvate, the lactate-pyruvate ratio, and CK levels and abnormal findings in urine organic acid. Plasma amino acid analysis revealed that the mean alanine level was elevated compared to the reference value and that the alanine levels of the two groups were significantly different ($P=0.042$).

The neuroimaging findings are summarized in Table 4. Of the 85 patients, 80 (94.1%) had basal ganglia involvement, and 31 (28.7%) had brainstem involvement, of which the midbrain was the most commonly affected site (30/31; 96.7%). In addition, 23 of the 85 patients (27.1%) showed cortex involvement, and 35 patients (41.2%) showed white matter involvement. MRI findings showed no significant differences in the involvement of the basal ganglia, thalamus, cortex, and cerebellum between the two groups. The ratio of abnormalities in the brainstem was significantly higher in the mtDNA mutation-positive group, especially in the midbrain ($P=0.010$) and medulla ($P=0.014$). The ratio of white matter involvement was lower in mtDNA mutation-positive group ($P=0.017$).

A muscle biopsy biochemical enzyme assay identified 46 patients (60.5%) with MRC 1 complex deficiency, 26 patients (34.2%) with MRC 4 deficiency, and 4 normal patients (5.3%). Moreover, 17 patients (21.5%) showed mitochondrial disease-specific findings, such as ragged red fiber and abnormal histochemical staining. Three patients (3.8%) had nonspecific findings and 59 patients (74.7%) were normal. An electron microscope examination revealed 33 patients (41.8%) with pleioconia and 30 (38.0%) with megaconia. No differences were found between the two groups (Table 5).

Discussion

Previous studies have identified clinical differences according to the MRC complex type in LS¹². More recently, however, studies have attempted to classify clinical features that are associated with genetic variation in patients with LS¹¹⁻¹³. Phenotype analysis with the same mutation is limited because of the rarity of the disease. Therefore, our study performed a genome-wide analysis of mtDNA rather than analyze a single mutation. We analyzed age, sex, biochemical markers, and the pathology of the patients who underwent mtDNA analysis. We diagnosed and treated patients with LS for the past 10 years using the same analytical procedures at a single institution.

LS typically presents clinical features in the first 2 years of life¹⁴. The mean age of clinical presentation in the patients in our study was 1.56 years, similar to the results of previous studies. Although previous studies of LS did not report sex differences^{6,15}, we found that the proportion of females in the mtDNA mutation group was significantly higher than that in the non-mutation group ($P=0.022$). The age of onset was significantly higher in the mtDNA mutation positive group. We performed a genome-wide analysis of mtDNA for all the patients included in our study. Therefore, the patients without a mtDNA mutation likely have a nDNA mutation. LS with a nDNA mutation often presents with more severe outcomes than LS with a mtDNA mutation⁴. Therefore, due to nDNA mutations, the age of onset of the mtDNA mutation-free group may be younger.

The clinical manifestation reported in our study is similar to that reported in previous studies, involving symptoms such as delayed development, seizure, motor weakness, loss of consciousness, muscle atrophy, and ataxia⁸. The incidence of ocular involvement in the mtDNA mutation-positive group was significantly higher; therefore, additional mitochondrial genetic testing should be considered.

Screening tests for LS include an analysis of lactate and pyruvate

Table 5. Comparison of Diagnostic Evaluation in mitochondrial DNA (mtDNA) Mutation Positive and Negative Groups of Patients with Leigh Syndrome (LS) (n=85)

Evaluation	Findings	Total (n=85)	mtDNA (+) (n=18)	mtDNA (-) (n=67)	P-value
Biochemical enzyme assay (n=76)	Normal	4 (5.3%)	0 (0.0%)	4 (6.7%)	0.915
	MRC complex I defect	46 (60.5%)	10 (62.5%)	36 (60.0%)	
	MRC complex IV defect	26 (34.2%)	6 (37.5%)	20 (33.3%)	
Light microscope findings (n=79)	Normal	59 (74.7%)	9 (56.3%)	50 (79.4%)	0.055
	Specific	17 (21.5%)	5 (31.3%)	12 (19.0%)	
	Nonspecific	3 (3.8%)	2 (12.5%)	1 (1.6%)	
Electron microscope findings (n=79)	Normal	44 (55.7%)	10 (62.5%)	34 (54.0%)	0.540
	Pleioconia	33 (41.8%)	6 (37.5%)	27 (42.9%)	
	Megaconia	30 (38.0%)	5 (31.3%)	25 (39.7%)	

mtDNA, mitochondrial DNA; MRC, mitochondrial respiratory chain.

*P values <0.05 were considered statistically significant.

levels, the lactate/pyruvate ratio, serum amino acid abnormalities, and urine organic acid^{16,17}. Oxidative phosphorylation deficiency may lead to lactic acidosis. Pyruvate accumulates and is eventually metabolized into lactate by lactate dehydrogenase or transaminated into alanine by alanine aminotransferase, leading to an increase in these two substances in the blood, urine, and cerebrospinal fluid¹⁸. Therefore, assessment of alanine levels is useful in screening and diagnosing mitochondrial diseases^{16,19,20}. In our study, the alanine level was significantly higher in the mtDNA mutation group, indicating that alanine levels may be helpful in differentiating patients with mtDNA mutations.

Bilateral, symmetrical hyperintensities in T2-weighted images are a characteristic finding in LS¹⁵. These lesions are commonly found in the basal ganglia, especially in the putamen, or in variable areas within the brainstem, such as the substantia nigra, nucleus ruber, or medulla oblongata^{12,21}. In our study, brain involvement was present in all patients, and most of the patients had lesions in the basal ganglia or brainstem. In particular, the ratio of midbrain and medulla lesions in the brainstem was significantly higher in the group with mtDNA mutations than those in the group without mtDNA mutations ($P=0.010$ and 0.022 , respectively). In the mtDNA mutation-positive group, the proportion of white matter involvement, a nonspecific finding in LS, was significantly lower than that in the non-mutation group ($P=0.017$). Previous studies that analyzed MRI features and the MRC complex reported no specific correlations between MRI findings and MRC complex type²². In contrast, a previous investigation has reported a difference in MRI findings between groups with and without nDNA mutations, such as the *SURF1* mutation²³. Our study found a significant difference in MRI findings between mtDNA mutation-positive and -negative groups. Thus, MRI findings correlate better with genetic findings than with pathologic findings. Imaging differences can signal differences in the natural course of the disease, suggesting that clinical differences in treating individuals with LS may depend on the presence of mtDNA mutations.

Although LS is a single syndrome, over 75 genes are known to cause this disease⁹. These genes are important for oxidative phosphorylation⁷. The gold standard of LS diagnosis is gradually progressing from neuropathologic to genetic analysis because previous analyses have not uncovered clinically relevant correlations²⁴⁻²⁶. Previous biochemical and clinical diagnostic methods for LS may require reanalysis and reclassification based on genotype. Diagnosis using genotype would improve predictions of the clinical course from the time of diagnosis. However, genome-wide mtDNA and nDNA analysis are difficult and costly. Thus, identifying the characteristics of the patients with LS that have mtDNA mutations could present relevant criteria for determining whether mtDNA or

nDNA analysis should be performed. The results of our study suggest that mtDNA sequencing is recommended to evaluate patients with LS who are female or who exhibit ocular involvement, high alanine levels, or MRI findings of lesions in the midbrain and medulla.

A limitation of our study is that we could not compare the clinical features of the mtDNA mutation group and the nDNA mutation group. Further studies are required to analyze the characteristics of patients with mutations in nDNA.

요약

목적: Leigh 증후군은 진행되는 드문 신경퇴행성 질환으로 중추신경계의 이상을 특징으로 한다. Leigh 증후군 환자들은 다양한 임상 증상 및 유전적 이상을 나타내기 때문에 예후를 예측하기 어렵다. 따라서, 본 연구에서는 Leigh 증후군 환자들에서의 미토콘드리아 DNA 돌연변이와 환자들의 임상 양상과의 연관성에 대해 분석하였다.

방법: Leigh 증후군으로 진단받은 환자 중에서 미토콘드리아 DNA 유전자 돌연변이 검사를 시행한 85명의 환자들의 자료를 후향적으로 분석하였다. 미토콘드리아 DNA 돌연변이 결과에 따라 환자군을 나누고, 집단 간의 임상 양상 차이를 통계 분석을 시행하였다.

결과: 연구에 참여한 85 명의 환자 중 18 명이 미토콘드리아 DNA 돌연변이를 가지고 있었다. 대부분의 환자는 젖산수치 및 젖산/피루베이트 비율이 20 이상이었으며 이는 호흡 연쇄 반응의 이상에 부합하는 결과였다. 미토콘드리아 DNA 돌연변이가 있는 환자군은 미토콘드리아 DNA 돌연변이가 없는 환자군과 비교하여, 여성 비율, 알라닌 수치, 안구 침범, 자기 공명 영상(MRI)상 중뇌 및 숨뇌 이상이 통계학적으로 유의하게 높았다.

결론: 임상적으로 Leigh 증후군이 의심될 때, 여성환자, 안구침범을 보이는 환자, 높은 알라닌 수치를 보이는 환자, MRI상 중뇌나 숨뇌에 이상소견이 보이는 환자에서는 미토콘드리아 DNA 돌연변이 검사를 우선적으로 고려할 수 있다.

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