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원 저

리증후군에서의 혈장 아미노산 및 소변 유기산 분석

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Plasma Amino Acid and Urine Organic Acid Analyses in Leigh Syndrome

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Purpose: Detection of abnormal metabolites in plasma amino acid (PAA) and urine organic acid (UOA) analyses has been used to diagnose clinical mitochondrial diseases, such as Leigh syndrome. In this study, the diagnostic values and effectiveness of PAA and UOA analyses were reviewed.
Methods: This was a retrospective study of patients with Leigh syndrome who were diagnosed between 2003 and 2018 in a single tertiary care center. Through a whole mitochondrial sequencing and nuclear DNA associated mitochondrial gene panel analysis, 19 patients were found to be positive for mitochondrial DNA (mtDNA) mutation-associated Leigh syndrome, and 57 patients were negative. Their PAA and UOA analyses results were then compared.
Results: In the comparison of the PAA and UOA analyses results between the two groups, no abnormal metabolites showed obvious differences between the mtDNA mutation-positive Leigh syndrome and mtDNA mutation-negative Leigh syndrome groups.
Conclusion: PAA and UOA analyses are inappropriate test methods for diagnosing Leigh syndrome or screening of mtDNA mutation-associated Leigh syndrome. However, UOA analysis might still be a suitable screening test for Leigh syndrome.

Key words: Plasma amino acid, Urine organic acid, Leigh syndrome, Mitochondrial disease

Introduction

Mitochondrial diseases are a large heterogeneous group of rare disorders caused by the primary dysfunction of the mitochondrial respiratory chain[1-3]. Among these mitochondrial diseases, Leigh syndrome (or subacute necrotizing encephalomyelopathy) is the most commonly seen mitochondrial diseases in children in a clinical setting, with a prevalence of approximately 40,000 newborns[3-6]. Severe neurologic symptoms, including psychomotor retardation, progressive neurologic decline, and extensive neurologic features, are common in Leigh syndrome[6].

Detection of abnormal metabolites in plasma amino acid (PAA) and urine organic acid (UOA) analyses has been used to diagnose clinical mitochondrial diseases, including Leigh syndrome, because such metabolic studies are known to be helpful in the assessment of mitochondrial energy

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production efficiency[7–9]. Several metabolites have been reported to be associated with certain genetic defects seen in mitochondrial diseases[10,11]. However, recently developed methods such as mitochondrial DNA (mtDNA) sequencing and massively parallel sequencing have surpassed PAA and UOA analyses as important methods for the diagnosis of Leigh syndrome[3,6,12].

In this study, we reviewed the diagnostic values of PAA and UOA analyses, which are representative metabolic tests that have been routinely performed in patients with mtDNA mutation–positive and –negative Leigh syndrome. Their effectiveness in the diagnosis of mtDNA mutation–associated Leigh syndrome was also examined retrospectively.

Materials and Methods

1. Selection of patients

This was a retrospective study of patients with Leigh syndrome diagnosed between 2003 and 2018 in a single tertiary care center, Gangnam Severance Hospital. The total number of initial clinically diagnosed patients included in this study was 79. The clinical diagnosis of Leigh syndrome was based on the stringent diagnostic criteria for Leigh syndrome defined by Rahman et al.[5,6]. The criteria include a progressive neurological disease with motor and intellectual developmental delays, signs and symptoms of brain stem and/or basal ganglia disease, raised lactate concentration in blood and/or cerebrospinal fluid, and bilateral symmetric hyperintense signal abnormalities in the brain stem and/or basal ganglia on T2–weighted images in brain magnetic resonance imaging (MRI)[4–6]. Whole mitochondrial sequencing and nuclear DNA associated mitochondrial gene panel tests were performed for all patients using next generation sequencing technology. Genetic test was conducted using the patients' serum blood. Three patients were diagnosed with nuclear DNA–associated Leigh syndrome and were excluded. In other words, those with Leigh syndrome associated with nuclear gene mutations reported in the literature so far were excluded from this study. Finally, 19 patients tested positive for mtDNA mutation–associated Leigh syndrome and 57 patients tested negative. The results of the PAA and UOA analyses were compared using a clinical Leigh syndrome case with a negative gene test as a control. This study was approved by the Institutional Review Board of the Gangnam Severance Hospital, Yonsei University College of Medicine (3–2017–0168). Informed consent for this retrospective study was waived by the board (Fig. 1).

2. Clinical characteristics of mtDNA mutation–associated Leigh syndrome patients

The clinical characteristics, including age at first clinical presentation, age at diagnosis of Leigh

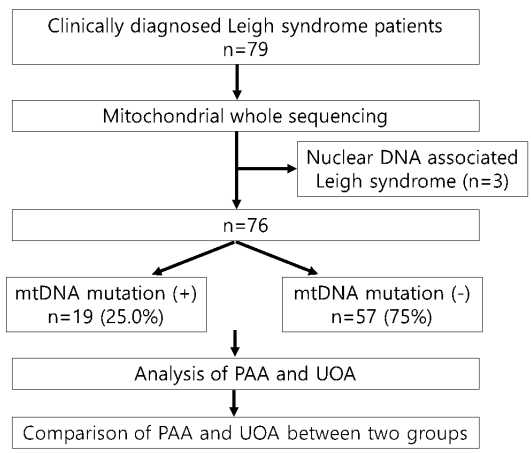

Fig. 1. This figure shows the flow leading to final patient selection. mtDNA, mitochondrial DNA; PAA, plasma amino acid; UOA, urine organic acid.

syndrome, time interval of the follow-up period, familial history of mitochondrial diseases, symptoms, and status of organ involvement, of patients with mtDNA mutation-associated Leigh syndrome were examined (Table 1).

3. Mitochondrial characteristics of patients with mtDNA mutation-associated Leigh syndrome

The mitochondrial dysfunction profiles, including subtypes of mtDNA mutations, serum lactate/pyruvate ratio, and severity of serum lactic acidosis, were graded as follows for all patients: mild increase, ≥2-fold change from the reference point and moderate increase, ≥3-fold change from the reference point[13,14]. Serum lactate and pyruvate levels were measured in arterial blood samples. Muscle biopsies of patients were obtained, and muscle biopsies were subjected to routine morphological and histochemical staining, including periodic acid-Schiff, modified Gomori trichrome, ATPase 9.4, nicotinamide adenine dinucleotide tetrazolium reductase, and succinate dehydrogenase staining. All samples were examined for electron microscopic changes, such as pleoconia and megaconia. Abnormalities on MRI and magnetic resonance spectroscopy were examined[13,14].

The clinical severity of the patients at the last follow-up was graded as follows: mild, self-ambulatory with or without independence during daily activities; moderate, wheelchair-bound full-time or partially dependent during daily activities, with brief communication abilities; and severe, bedridden, totally dependent on help to perform daily activities, or death. In addition, each patient's stroke history, ratio of psychomotor retardation, oxygen dependency, and gastrointestinal support status were investigated[13,14].

Table 1. Clinical characteristics of patients with mtDNA mutation-associated Leigh syndrome (n=19)

Sex (male:female) (n)	11:8
Age at the first clinical presentation (month, median, range)	15 (1-118)
Age at diagnosis of Leigh syndrome (month, median, range)	25 (5-244)
Time interval from the first clinical presentation to the diagnosis of Leigh syndrome (months)	13 (1-173)
Time interval from the first visit to the last visit (months)	124 (8-290)
Familial history of mitochondrial diseases (n, %)	2 (10.5)
Symptoms at disease onset (n, %)	
Delayed development	8 (42.1)
Seizure	4 (21.1)
Ataxia	4 (21.1)
Motor weakness	2 (10.5)
Visual disturbance	1 (5.3)
Organ involvement (n, %)	
Central nervous system	19 (100)
Muscle	8 (42.1)
Lung	8 (42.1)
Eye	7 (36.8)
Gastrointestinal system	5 (26.3)
Renal system	3 (15.8)
Heart	1 (5.3)
Ear	1 (5.3)

4. Comparison of UOA and PAA analyses results

Blood and urine samples at the time of diagnosis were collected from all patients with clinically diagnosed Leigh syndrome, and PAA and UOA analyses were performed. All samples were collected under fasting conditions to minimize artifacts in the results[8]. The difference in the ratio of abnormal PAA and UOA metabolites at initial diagnosis was statistically compared between the mtDNA mutation-positive and mtDNA mutation-negative groups.

5. Statistical analysis

All analyses were conducted using the Statistical Package for the Social Sciences (SPSS version 22.0; IBM Corp., Armonk, NY, USA). Descriptive statistics, including medians, ranges, and percentages, were used. Chi-square tests and Fischer's exact tests were used to evaluate differences between groups. Statistical significance was set at $p<0.05$.

Results

1. Clinical characteristics of patients with mtDNA mutation-associated Leigh syndrome

The male-to-female ratio of patients with mtDNA mutation-associated Leigh syndrome was 11:8, and the median age at the time of the first clinical presentation was 15 years. The median age at diagnosis of Leigh syndrome was 25 months, and the range was from 5 to 244 months. The median time interval from the first clinical presentation to the diagnosis of Leigh syndrome was 13 months, and the median time interval from the first visit to the last visit was 124 months. A familial history of mitochondrial diseases was observed in two patients. The most common symptoms at disease onset were delayed development and seizures (63.2%). Problems with the central nervous system, which occurred in all patients were the most frequently observed, followed by problems associated with muscle, lung, eye, gastrointestinal, renal, heart, and ear in the order of frequency (Table 1).

2. Mitochondrial characteristics of patients with mtDNA mutation-associated Leigh syndrome

In the genetic test, a total of 10 mutation subtypes were found, and these included m.10191T>C, m.8993T>G, m.8993T>C, and m.3697G>A; m.10191T>C was the most common mutation and was observed in 4 out of 19 patients (21.1%). Regarding serum lactic acidosis, 12 patients were classified as having a mild to moderate increase (63.2%). Among 19 patients, 17 provided muscle biopsy samples, and light microscopic changes and electron microscopic changes were found in 5 and 10 patients, respectively. MRI showed bilateral symmetric hyperintense signal abnormalities of the basal ganglia in 94.7% of patients, involvement of the thalamus in 42.1%, brainstem signal abnormality in 68.4%, cerebellar signal abnormality in 68.4%, and cerebral atrophy in 52.6%. When examining the functional clinical severity of the patients, 17 patients were classified as having moderate clinical severity and above. Developmental regression and deterioration were observed in 10 patients (52.6%). Six patients had oxygen dependency, one patient needed temporary tube feeding, and five patients required percutaneous endoscopic gastrostomy (Table 2).

3. Comparison of PAA analyses findings at initial diagnosis: mtDNA mutation-positive vs. mtDNA mutation-negative groups

In PAA analysis, abnormal levels of ethanolamine, alanine, ammonia, asparagine, and alpha-aminobutyric amino acids were detected in both groups. However, no statistically significant difference was found between the mtDNA mutation-positive and mtDNA mutation-negative groups. Glutamic acid levels, which were abnormally high in both groups, were significantly higher in patients with mtDNA-negative Leigh syndrome than in patients with mtDNA-mutation positive Leigh syndrome. In addition, although not statistically significant, abnormal levels of ornithine, leucine, serine, cystine, citrulline, aspartic acid, and taurine tended to be more frequent in the mtDNA mutation-negative group than in the mtDNA mutation-positive group (Table 3).

Table 2. Mitochondrial characteristics of patients with mtDNA mutation-associated Leigh syndrome (n=19)

mtDNA mutation subtypes (n, %)	
m.10191T>C	4 (21.1)
m.8993T>G	3 (15.8)
m.8993T>C	2 (10.5)
m.3697G>A	2 (10.5)
m.9176T>C	2 (10.5)
m.13513G>A	2 (10.5)
m.9185T>C	1 (5.3)
m.10744A>G	1 (5.3)
m.11777C>A	1 (5.3)
m.10744A>G	1 (5.3)
Serum lactic acidosis (n, %)	
Normal	7 (36.8)
Mild	6 (31.6)
Moderate	6 (31.6)
Muscle biopsy obtained (n=17, %)	
Light microscopic changes (+)	5 (29.4)
Electron microscopic changes (+)	10 (58.8)
Magnetic resonance imaging (n, %)	
Basal ganglia	18 (94.7)
Thalamus	8 (42.1)
Brainstem	13 (68.4)
Midbrain	13 (68.4)
Pons	8 (42.1)
Medulla	10 (52.6)
Cerebellum	13 (68.4)
Cerebral atrophy	10 (52.6)
Cortex signal abnormality	10 (52.6)
White matter signal abnormality	6 (31.6)
Clinical severity (n, %)	
Mild	2 (10.5)
Moderate	6 (31.6)
Severe	11 (57.9)
Regression/Deterioration (n, %)	10 (52.6)
O_2 dependency (n, %)	6 (31.6)
Tube feeding (n, %)	
Temporary tube feeding	1 (5.3)
PEG/gastrostomy	5 (26.3)

PEG, percutaneous endoscopic gastrostomy.

4. Comparison of UOA analyses results at initial diagnosis: mtDNA mutation-positive vs. mtDNA mutation-negative groups

In UOA analysis, abnormal levels of dicarboxylic acid, tricarboxylic acid (TCA) intermediates, and lactate were observed with relatively high frequency in both groups. However, no significant differences were observed between the two groups (Table 4).

Discussion

PAA analysis has been used to diagnose diseases caused by mitochondrial dysfunction. According to previous studies, abnormal elevation of lactate or alanine levels strongly suggests a mitochondrial disease[7,8]. Abnormal elevation of lactate level was observed in all patients with Leigh syndrome, and abnormal elevation of alanine concentration was observed in 21 patients (27.6 %) in this study. However, abnormal metabolites that distinguish between mtDNA mutation-positive Leigh syndrome and mtDNA mutation-negative Leigh syndrome were not observed in previous

Table 3. Comparison of plasma amino acid analysis results at initial diagnosis: mtDNA mutation-positive vs. mtDNA mutation- negative groups

Metabolite	Total patients (n=76)	mtDNA mutation-positive Leigh syndrome (n=19)	mtDNA mutation-negative Leigh syndrome (n=57)	p-value
Ethanolamine	41	8	33	0.232
Alanine	21	7	14	0.300
Ammonia	31	7	24	0.686
Asparagine	26	6	20	0.780
alpha-aminobutyric acid	16	5	11	0.516
Glutamine	13	3	10	0.860
Valine	12	3	9	1.000
Isoleucine	14	3	11	0.733
Glycine	8	2	6	1.000
Phenylalanine	8	2	6	1.000
Glutamic acid	17	1	16	0.038
Proline	6	1	5	0.623
Ornithine	11	1	10	0.188
Leucine	12	1	11	0.146
Threonine	5	1	4	0.789
Lysine	4	1	3	1.000
Phosphoethanolamine	1	0	1	1.000
Serine	4	0	4	0.567
Phosphoserine	0	0	0	-
Cystine	5	0	5	0.323
Hydroxylysine	0	0	0	-
Arginine	1	0	1	1.000
Citrulline	7	0	7	0.182
Hydroxyproline	0	0	0	-
Taurine	6	0	6	0.327
Tyrosine	1	0	1	1.000
Aspartic acid	4	0	4	0.567

mtDNA, mitochondrial DNA; PAA, plasma amino acid.

studies and this study. In addition, improperly handled specimens can frequently cause abnormal elevations in glutamic acid, ornithine, phosphoserine, aspartic acid, and taurine levels as well as decreases in glutamine, cystine, asparagine, arginine, and homocysteine concentrations[8]. Therefore, the abnormal glutamic acid, ornithine, leucine, serine, cystine, citrulline, aspartic acid, and taurine levels seen in the patients with mtDNA mutation-negative Leigh syndrome should be interpreted with caution. However, ethanolamine was found in 41 patients (53.9%), and relatively high rates of abnormal ethanolamine were found in both groups. Given that studies on plasma ethanolamine levels and mitochondrial dysfunction have not been previously conducted, further studies are necessary.

UOA is a byproduct of protein, carbohydrate, and fat catabolism; UOA analysis is a representative metabolic test along with PAA analysis. However, UOA analysis is known to have a better diagnostic value than that of PAA analysis. Nevertheless, UOA analysis has the limitation of instability, where its accuracy is greatly reduced when the test is performed with a highly diluted urine sample or if the urine comes from a person who has taken multiple drugs[8]. However, elevation of dicarboxylic acid, TCA intermediates, ethylmalonic

Table 4. Comparison of urine organic acid analysis results at initial diagnosis: mtDNA mutation-positive vs. mtDNA mutation- negative groups

Metabolite	Total patients (n=76)	mtDNA mutation-positive Leigh syndrome (n=19)	mtDNA mutation-negative Leigh syndrome (n=57)	p-value
Dicarboxylic acid	16	4	12	1.000
TCA intermediates	9	3	6	0.539
Lactate	10	3	7	0.695
Ketone	7	1	6	0.492
3-methylglutaconic acid	4	1	3	1.000
4-hydroxyphenyl lactate	3	1	2	0.734
Paracetamol	3	1	2	0.734
Ethylmalonic acid	2	1	1	0.408
3-hydroxyphenyl lactate	4	1	3	1.000
3-hydroxy-3-methylglutaric acid	3	0	3	0.569
3-hydroxybutyric acid	3	0	3	0.569
Acetoacetate	3	0	3	0.569
Pyruvate	1	0	1	1.000
3-hydroxyisovaleric acid	2	0	2	1.000
Hippurate	2	0	2	1.000
Citric acid	2	0	2	1.000
Isovalerylglycine	2	0	2	1.000
Succinate	0	0	0	-
beta-Alanine	0	0	0	-
Ketoglutaric acid	0	0	0	-
Glutaric acid	1	0	1	1.000
3-methylglutaric acid	2	0	2	1.000
Fumarate	2	0	2	1.000
Tyrosine	0	0	0	-
3-hydroxy-phenylhydracrylate	1	0	1	1.000
2-(n-propyl)glutaric acid	1	0	1	1.000
3-methylcrotonylgycine	0	0	0	-
17-hydroxyisovaleric acid	0	0	0	-
Methylcrotonylglycine	0	0	0	-
Isobutyrylglycine	1	0	1	1.000
Homovanillic acid	1	0	1	1.000
Hydroxyglutaric acid	0	0	0	-
Malic acid	1	0	1	1.000
7-hydroxyoctanoate	1	0	1	1.000
Sebacic acid	1	0	1	1.000
c6-c10 dicarboxylic acid	1	0	1	1.000
Ascorbic acid	0	0	0	-

mtDNA, mitochondrial DNA; UOA, urine organic acid; TCA, tricarboxylic acid.

acid, and 3-methylglutaconic acid levels in urine are often found in primary mitochondrial diseases, as reported in many studies[1,11,15-17]. Patients with Leigh syndrome in this study also had a relatively high frequency of these abnormal metabolites. Studies have shown that these metabolites are sometimes linked to mutations in specific genes. Mutations are often suspected in *MT-ATP6* when abnormal levels of TCA intermediates are observed; *ETHE1*, abnormal levels of ethylmalonic acid; SERAC1, abnormal 3-methylglutaconic acid levels; and *SUCLG1* or *SUCLA2*, abnormal methy-

lmalonic acid levels, and mtDNA single deletions are suspected for cases with abnormal 3-methylglutaconic and 3-methylglutaric acid levels[10]. In this study, in the mtDNA mutation-positive Leigh syndrome group, two patients with abnormal TCA intermediate levels had m.9185T>C mutation, suggesting a mutation in *MT-ATP6*. In addition, mutations in genes such as *ETHE1, SERAC1, SUCLG1*, and *SUCLA2* have been reported to be causative mutations in nuclear gene-associated Leigh syndrome[18].

However, as mentioned earlier, UOA analysis is an unstable test, and since findings characteristic of Leigh syndrome have not been clearly reported, it is impossible to reliably diagnose Leigh syndrome through UOA analysis alone. In addition, as seen in the results of this study, there seems to be no characteristic metabolites that can be identified specifically in patients with mtDNA mutation-positive or mtDNA mutation-negative Leigh syndrome. However, since some metabolites of UOA are associated with gene defects that are suggestive of Leigh syndrome, UOA analysis may be useful as a screening test for Leigh syndrome.

Leigh syndrome is a representative mitochondrial disease, which include a wide range of diseases that can affect both mtDNA and nuclear DNA; however, the genetic cause of Leigh syndrome has not been fully identified[6,18]. Nevertheless, with the further development of genetic analysis methods in the future, it is expected that the presence or absence of genetic mutations will become an essential diagnostic criterion for Leigh syndrome. Based on the existing literature and our research, PAA and UOA analyses are inappropriate test methods for diagnosing Leigh syndrome or screening for mtDNA mutation-associated Leigh syndrome. However, since some abnormal UOA metabolites are related to causative genes of Leigh syndrome, UOA analysis might still be suitable as a screening test for Leigh syndrome[10].

A limitation of this study is that the PAA and UOA analyses results of patients with Leigh syndrome were not compared to those of a normal control group. Further studies that compare these values to those of a normal control will be valuable to confirm our findings.

In conclusion, we confirmed that PAA and UOA analyses are not feasible for diagnosing Leigh syndrome. Further studies on the relationship between abnormal PAA and UOA metabolites and gene-confirmed Leigh syndrome will be helpful in understanding Leigh syndrome.

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요 약

목적: 혈장 아미노산(PAA) 및 소변 유기산(UOA) 분석에서 비정상적인 대사 산물의 검출은 리 증후군과 같은 임상 미토콘드리아 질환을 진단하는 데 사용되었다. 본 연구에서는 PAA 및 UOA 분석의 진단적 가치와 유효성을 검토하였다.

방법: 이 논문은 2003년에서 2018년 사이에 단일 3차 진료 센터에서 진단된 리 증후군 환자에 대상으로 후향적 연구로 진행되었다. 전체 미토콘드리아 시퀀싱 및 핵 DNA 관련 미토콘드리아 유전자 패널 분석을 통해 미토콘드리아 DNA (mtDNA) 돌연변이 관련 리 증후군에 대해 19명의 환자가 양성이었고 57명의 환자는 음성인 것으로 밝혀졌다. 그 이후에 PAA 및 UOA 분석 결과를 비교하였다.

결과: 두 그룹 간의 PAA 및 UOA 분석 결과를 비교한 결과, mtDNA 돌연변이 양성 Leigh 증후군과 mtDNA 돌연변이 음성 Leigh 증후군 그룹 간에 비정

상적인 대사 산물은 뚜렷한 차이를 보이지 않았다.

결론: PAA 및 UOA 분석은 리 증후군을 진단하거나 mtDNA 돌연변이 관련 리 증후군을 선별하기 위한 부적절한 검사 방법이다. 그러나 UOA 분석은 여전히 리 증후군에 대한 적합한 선별 검사일 수 있다.

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