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원 저

멜라스 증후군 진단에서의 혈장 아미노산과 소변 유기산 분석

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Plasma Amino Acid and Urine Organic Acid in Diagnosis of MELAS

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Purpose: In the past, detection of metabolic abnormalities in plasma amino acid (PAA) and urine organic acid (UOA) has been widely used to diagnose clinical mitochondrial diseases, such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). In this study, the diagnostic values of PAA and UOA were reviewed, and their effectiveness in the diagnosis of MELAS was examined retrospectively.

Methods: Blood and urine samples at the time of diagnosis were collected from all clinically diagnosed MELAS patients (n=31), and PAA and UOA tests were performed. All samples were collected in a fasting state to minimize artifacts in the results. The difference in the ratio of abnormal metabolites of PAA and UOA at initial diagnosis was statistically compared between the MELAS with genetic confirmation (n=19, m.3243A>G mutation) and MELAS without genetic confirmation (n=12) groups. The MELAS without genetic confirmation group was used as control.

Results: Comparison of PAA and UOA between the two groups revealed that no abnormal metabolites showed characteristic differences between gene-confirmed MELAS patients with and those without genetic confirmation.

Conclusions: Abnormal values of metabolites in PAA or UOA might be useful as a screening test but are not sufficient to diagnose MELAS patients.

Key words: Plasma amino acid, Urine organic acid, MELAS, Mitochondrial disease

Introduction

Mitochondrial diseases are those in which structural or functional disabilities of the mitochondria occur due to dysfunction of the mitochondrial respiratory chain, resulting in various clinical phenotypes[1,2]. Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) are characterized by symptoms such as stroke-like episodes, encephalopathy with seizures and/or dementia, muscle weakness, and early psychomotor development. The m.3243A>G pathogenic variant is found in approximately 80% of these patients' genetic information[3-5].

Mitochondrial dysfunction causes various metabolic abnormalities. Therefore, detection of abnormal metabolites in serum lactate/pyruvate, plasma amino acid (PAA), and urine organic acid (UOA) has been widely used to diagnose clinical mitochondrial diseases, such as MELAS. This is because such metabolic studies are helpful for the assessment of mitochondrial energy pro-

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duction efficiency. Recently, however, with the development of mitochondrial DNA (mtDNA) and nuclear DNA genome sequencing, targeted genomic tests have evolved into confirmative tests for mitochondrial disease[6-9].

In this study, the diagnostic values of PAA and UOA, which are representative metabolic tests that are routinely performed among patients with mtDNA mutation-positive and-negative MELAS, were reviewed, and their effectiveness in the diagnosis of MELAS was examined retrospectively.

Materials and Methods

1. Selection of patients

This was a retrospective study of MELAS patients diagnosed between 2003 and 2017 in a single tertiary care center at Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. The total number of clinically diagnosed MELAS patients included in this study was 31. The inclusion criteria for MELAS patients in this study were based on the diagnostic criteria of the Japanese MELAS study committee, which is currently the most commonly used in clinical practice[10]. With reference to these criteria, we defined the patients with two or more findings in category A and high lactate level in category B as 'MELAS without genetic confirmation'. Meanwhile, patients diagnosed with definitive gene mutation were defined as 'MELAS with genetic confirmation'. Their clinical and mitochondrial dysfunction-related characteristics and the results of PAA and UOA were studied. Whole mitochondrial gene sequence analysis was performed for all patients. NGS was used to genetically establish the diagnosis of mtDNA-associated LS and to quantify the heteroplasmic mutant load of mtDNA. The sequence results were compared with those of the human mitochondrial reference (GenBank ID: NC_012920.1). As a result, Nineteen patients were positive for the m.3243 A>G mutation, while 12 patients were negative for the gene test (Fig. 1). 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.

2. Clinical characteristics of MELAS patients with genetic confirmation

The clinical characteristics of 19 MELAS patients with genetic confirmation of the m.3243A>G mutation

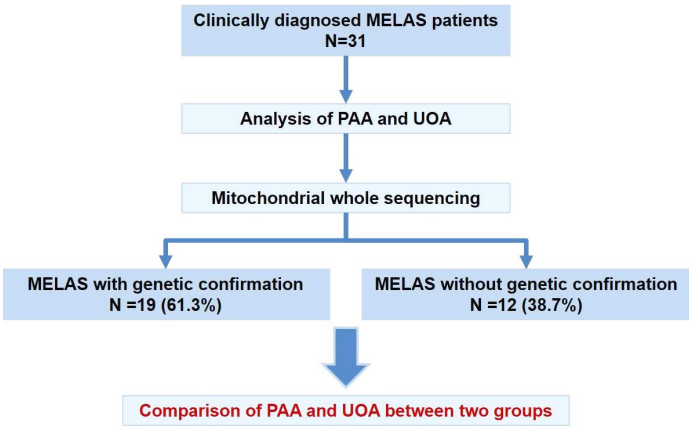

Fig. 1. Study design and patient selection.

were examined, including age at first clinical presentation, age at diagnosis of MELAS, time interval of follow-up period, familial history of MELAS, presentation of symptoms, and organ involvement.

3. Mitochondrial characteristics of MELAS patients with genetic confirmation

The mitochondrial dysfunction profiles of all patients, including serum lactate/pyruvate ratio and severity of serum lactic acidosis, were graded as follows: mildly increased, ≥2-fold of normal reference; moderately increased, ≥3-fold of normal reference; severe, ≥4-fold of normal reference values[11,12]. Serum lactate and pyruvate levels were measured in arterial blood samples. Muscle biopsies of some patients were obtained and were processed through routine morphological and histochemical staining, including periodic acid-Schiff, modified Gomori trichrome, ATPase 9.4, nicotinamide adenine dinucleotide tetrazolium reductase, and succinate dehydrogenase stains. All samples were examined for changes, such as pleoconia and megaconia, using electron microscopy. Abnormalities in magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) were also examined[13,14].

The clinical severity of the patients was graded as follows: mild, self-ambulatory with or without independence for daily activities; moderate, full-time wheelchair-bound or partially dependent for daily activities, with ability for brief communication; severe, bedridden, totally dependent for daily activities, or expired. In addition, the ratio of psychomotor retardation, oxygen dependency, and enteral tube feeding status were also investigated[13,14].

4. Comparison of UOA and PAA

Blood and urine samples at the time of diagnosis were collected from all clinically diagnosed patients with MELAS, and PAA and UOA tests were performed. All samples were collected under fasting conditions to minimize artifacts in the results[8]. The difference in the ratio of abnormal metabolites of PAA and UOA at initial diagnosis was statistically compared between the MELAS with genetic confirmation and MELAS without genetic confirmation groups. The MELAS without genetic confirmation group was used as control.

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 were used, including median, range, and percentage. 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 the MELAS with genetic confirmation group

The male-to-female ratio of patients in the MELAS with genetic confirmation group was 10:9, and the median age of the first clinical presentation was 13 years (Table 1). The median age at diagnosis of MELAS was 14 years, and the range was investigated from 3 to 29 years. The median time interval from the first clinical presentation to the diagnosis of MELAS was 5 months, and the median time interval from the first visit to the last visit was 107 months. Familial history of MELAS was observed in five patients. The most common presentations at disease onset were seizures and headaches (42.1%). Problems with the central nervous system were observed in all patients, along with ophthalmic, endocrinologic, cardiologic, hearing, psychological, myopathic, gastrointestinal, and renal

problems. Severity was classified according to the degree of organ involvement, and 42.1% of the patients had multiple organ involvement, as well as central nervous system and muscle problems.

2. Mitochondrial characteristics of the MELAS with genetic confirmation group

Table 2 presents the mitochondrial characteristics of patients that have the m.3243A>G mutation. In the MELAS patients with genetic confirmation, 11 patients had serum lactic acidosis at a moderate to severe degree (57.9%). Among the 19 patients, muscle biopsy samples of eight patients were observed, and light and electron microscopy changes were found in three patients. MRI findings showed infarction in 94.7%, white matter signal abnormality in 84.2%, and cortex signal abnormality and cerebral atrophy in 78.9% patients. All 19 patients had abnormal MRI findings. In addition, 13 patients showed a lactate peak in MRS. On examining the functional clinical severity of the patients, nine patients were found above moderate levels. Delayed development was observed in 14 patients (73.7%); among them, 11 had developmental regression, one had oxygen dependency, and three required enteral tube feeding.

Table 1. Clinical characteristics of m.3243A>G mutation-positive MELAS patients at diagnosis (n=19)

Gender (male:female) (n)	10:9
Age at first clinical presentation (years, median, range)	13 (0-24)
Age of diagnosis of MELAS (years, median, range)	14 (3-29)
Time interval from first clinical presentation to the diagnosis of MELAS (months)	5 (0-132)
Time interval from 1st visit to last visit (months)	107 (28-180)
Familial history of MELAS (n, %)	5 (26.3)
Presented symptom at disease onset (n, %)	
Seizure	8 (42.1)
Headache	8 (42.1)
Motor weakness	2 (10.5)
Visual disturbance	2 (10.5)
Ataxia	1 (5.3)
Delayed development	1 (5.3)
Organ involvement (n, %)	
Central nervous system	19 (100)
Ophthalmic	15 (78.9)
Endocrinologic	13 (68.4)
Cardiologic	12 (63.2)
Auditory	10 (52.6)
Psychological	9 (47.4)
Myopathy	9 (47.4)
Gastrointestinal system	9 (47.4)
Renal system	5 (26.3)
Organ involvement severity (n, %)	
CNS only	8 (42.1)
CNS+muscle	3 (15.8)
CNS+muscle+multiple	8 (42.1)

Abbreviation: CNS, central nervous system

Table 2. Mitochondrial characteristics of m.3243A>G mutation-positive MELAS patients (n=19)

Serum lactic acidosis (n, %)	
Mild	8 (42.1)
Moderate	9 (47.4)
Severe	2 (10.5)
Magnetic resonance imaging (n, %)	
Infarction	18 (94.7)
Cortex signal abnormality	15 (78.9)
Diffuse cerebral atrophy	
Mild	6 (31.6)
Severe	7 (36.8)
Cerebellar atrophy	15 (78.9)
Basal ganglia signal abnormality	8 (42.1)
Thalamus signal abnormality	2 (10.5)
White matter signal abnormality	16 (84.2)
Magnetic resonance spectroscopy obtained (n, %)	
Presence of lactate peak	13 (68.4)
Muscle biopsy obtained (n=8)	
Light microscopic changes (+)	3 (37.5)
Electron microscopic changes (+)	3 (37.5)
Clinical severity (n, %)	
Mild	10 (52.6)
Moderate	3 (15.8)
Severe	3 (15.8)
Expire	3 (15.8)
Delayed development (n, %)	14 (73.7)
Regression / Deterioration (n, %)	11 (57.9)
Oxygen dependency (n, %)	1 (5.3)
Enteral tube feeding (n, %)	3 (15.8)

3. Comparison of PAA between groups with and without genetic confirmation at initial diagnosis

PAA tests were performed as initial diagnostic techniques at the time of the first diagnosis, and the results were compared between the groups with and without genetic confirmation. The number of patients in each group with values above normal range in the PAA test was compared. Among the metabolites examined, the major metabolites in which abnormalities were observed are summarized in Table 3. No significant difference was found between the two groups for most of the metabolites in PAA. However, ethanolamine and glutamine levels were significantly higher in the mutation-negative group than in the group with mutations.

4. Comparison of UOA between groups with and without genetic confirmation at initial diagnosis

Similar to that for PAA, UOA testing were performed as initial diagnostic tools at the time of the first diagnosis, and the results were compared between the groups with and without genetic confirmation (Table 4). The number of patients in each group with values above normal range in the PAA test was compared. The major metabolites wherein abnormalities were observed are summarized in Table 4. No significant difference was found between the two groups for most of the UOA metabolites. Among the UOAs, 3-hydroxy butyrate, acetoacetate, and lactate showed a higher frequency in the m.3243 A>G mutation-positive group, although this was not statistically significant.

Table 3. Comparison of PAA metabolites in groups with and without confirmation at initial diagnosis

Metabolite	m.3243 A>G mutation (+) (n=19)	m.3243 A>G mutation (-) (n=12)	*P*-value	Metabolite	m.3243 A>G mutation (+) (n=19)	m.3243 A>G mutation (-) (n=12)	*P*-value
Alanine	6	3	0.694	Aspartic acid	0	1	0.387
Phosphoserine	5	5	0.373	Glutamic acid	0	2	0.142
Ammonia	4	4	0.447	Methionine	0	1	0.387
Asparagine	4	5	0.282	Citrulline	0	1	0.387
1-methylhistidine	3	1	0.546	Glycine	0	0	-
Cystine	2	1	0.841	Lysine	0	0	-
Ethanolamine	2	5	0.043	Phosphoethanolamine	0	0	-
Histidine	1	1	0.735	Threonine	0	0	-
alpha-aminobutyric acid	2	3	0.286	Sarcosine	0	0	-
Ornithine	2	0	0.510	alpha-aminoadipic acid	0	0	-
Taurine	2	2	0.619	Cystathionine	0	0	-
Isoleucine	2	0	0.510	beta-alanine	0	0	-
Hydroxyproline	2	0	0.510	beta-aminoisobutyric acid	0	0	-
Glutamine	1	4	0.038	Homocystine	0	0	-
Proline	1	0	1.000	gamma-aminobutyric acid	0	0	-
Arginine	1	0	1.000	Ethanolamine	0	0	-
Tryptophan	1	0	1.000	Hydroxylysine	0	0	-
Valine	1	0	1.000	3-methylhistidine	0	0	-
Serine	0	2	0.142	Anserine	0	0	-
Leucine	0	1	0.387	Carnosine	0	0	-
Phenylalanine	0	1	0.387	Alloisoleucine	0	0	-
Tyrosine	0	1	0.387				

Abbreviation: PAA, plasma amino acid

Discussion

PAA analysis indicates disorders of mitochondrial metabolism in the presence of increased alanine levels in both plasma and CSF, but its sensitivity is low. Increased levels of proline, glycine, and sarcosine have also been associated with mitochondrial dysfunction[8,15]. However, to date, PAA metabolites associated with MELAS have not yet been reported. The PAA level can be changed by physiological stress or regression, and a normal PAA level cannot be the basis for reliably excluding mitochondrial disease. Moreover, in the process of collecting PAA or in the case of hemolysis, an error may occur. Improperly stored specimens cause abnormal elevations in glutamate, aspartate, ornithine, phosphoserine, and taurine with a decrease in glutamine, cystine, asparagine, arginine, and homocystine[8]. In this study, most of the PAA metabolites did not show any characteristic differences depending on the presence or absence of mutations. It is difficult to rule out the possibility that the significant increase in ethanolamine and glutamine observed in the mutation-negative group was an artifact generated during handling of the specimen.

Organic acids are byproducts of protein, carbohydrate, and fat catabolism. UOA has been widely used in the diagnosis of sudden-onset encephalopathy patients, such as MELAS, due to high ease and accuracy in its urine extraction compared to that from plasma. Generalized aminoaciduria, along with renal tubular acidosis and glycosuria, may accompany renal Fanconi syndrome. These findings can be observed in mtDNA deletion mitochondrial syndromes. However, thus far, the metabolites of UOA associated with MELAS remain unknown. There are many artifacts in the results, which can be greatly influenced by medication and dietary conditions[8]. Increased excretion of TCA cycle intermediates, ethylmalonic acid, dicarboxylic aciduria, and

Table 4. Comparison of UOA metabolites in groups with and without confirmation at initial diagnosis

Metabolite	m.3243 A>G mutation (+) (n=19)	m.3243 A>G mutation (-) (n=12)	*P*-value	Metabolite	m.3243 A>G mutation (+) (n=19)	m.3243 A>G mutation (-) (n=12)	*P*-value
3-hydroxy butyrate	9	4	0.595	3-methylglutaconic acid	0	0	-
Acetoacetate	9	4	0.595	2-hydroxy-3-methylbutyric acid	0	0	-
Lactate	9	4	0.595	Tiglyglycine	0	0	-
2-hydroxybutyrate	2	1	0.841	2-ethylhydracrylic acid	0	0	-
Fumarate	1	1	0.735	2-hydroxyglutaric acid	0	0	-
2-propylglutaric acid	1	0	1.000	Hexanoylglycine	0	0	-
Phenylpyruvic acid	1	0	1.000	Suberylglycine	0	0	-
Malate	0	0	-	2-methyl-3-hydroxyacetoacetic acid	0	0	-
Methylmalonic acid	0	0	-				
2-oxoadipic acid	0	0	-	3-methylglutaric acid	0	0	-
2-aminoadipic acid	0	0	-	N-acetylaspartic acid	0	0	-
Methylcitric acids	0	0	-	Mevalonic acid	0	0	-
Pyruvate	0	0	-	2-hydroxyisovaleric acid	0	0	-
3-hydroxypropionic acid	0	0	-	2-hydroxy-3-methylvaleric acid	0	0	-
				2-ketoisocaproic acid	0	0	-
Propionylglycine	0	0	-	Phenyllactic acid	0	0	-
Isovalerylglycine	0	0	-	5-oxoproline (pyroglutamic acid)	0	0	-
3-hydroxyglutaric acid	0	0	-	3-hydroxyisovaleric acid	0	0	-
Dicarboxylic acid	0	0	-	3-methylcrotonylglycine	0	0	-
Acylglycine	0	0	-	Methylsuccinic acid	0	0	-

Abbreviation: UOA, urine organic acid

3-methyl glutaconic acid commonly occurs in mitochondrial disease but is rarely diagnostic of a specific mitochondrial disorder[9,16-18].

Therefore, because PAA and UOA have diagnostic limitations, genetic studies are considered the gold standard for diagnosis of mitochondrial disease, and their use is accelerating with the development of massively parallel sequencing. In addition to the classical diagnostic criteria for MELAS, genetic defects such as missense mtDNA mutations, like m.3243A>G, are essential for diagnosis[2,3].

This study showed that the PAA and UOA of MELAS patients with genetically confirmed m.3243A>G mutation through whole mitochondrial sequencing showed a slightly significant difference compared to those of clinically diagnosed MELAS patients negative for the mutation. It is rare to compare the results of PAA and UOA with the results of MELAS gene, confirming that the results for genetic defects accompanying clinical features should be used rather than PAA and UOA in future diagnostic methods for MELAS. One limitation of the present work is that a more accurate study could be achieved by comparing the results of our patients' PAA and UOA with those of the normal control group. However, the focus of this study is to explore the differences in metabolic abnormalities depending on the genetic confirmation of MELAS. Studies exploring the relationship between PAA and UOA tests that have been carried out for the diagnosis of mitochondrial diseases such as MELAS patients in the past, and genetic tests can help understand MELAS.

We concluded that abnormal values of metabolites of PAA or UOA might be useful as a screening test but are not sufficient to diagnose MELAS patients. Through this study, we reaffirm that genetic confirmation is very important for the diagnosis of MELAS. In addition, we think that this study provides an opportunity to consider the effectiveness of PAA and UOA, which have been used to diagnose mitochondrial diseases such as MELAS. Further research is warranted to establish whether PAA or UOA determination is useful as a screening test for mitochondrial disease.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. Each parent received a detailed explanation of the study and signed an informed consent form before their child participated in the study. This study was approved by the Institutional Review Board of Gangnam Severance Hospital, Yonsei University College of Medicine (3-2017-0168).

한 글 요 약

목적: 과거에는 혈장 아미노산 및 소변 유기산의 대사 이상 검출이 멜라스 증후군과 같은 임상적인 미토콘드리아 질환을 진단하는 데 널리 사용되었다. 본 연구에서는 혈장 아미노산 및 소변 유기산의 진단적 가치를 고찰하고, 멜라스 증후군 진단에 있어 이들의 유효성을 후향적으로 살펴보았다.

방법: 임상적으로 진단된 모든 멜라스 증후군 환자(n=31)로부터, 진단 당시의 혈액 및 소변 검체를 채취하여 혈장 아미노산 및 소변 유기산 검사를 시행하였다. 모든 샘플은 결과의 인위적 오류를 최소화하기 위해 금식 상태에서 수집되었습니다. 유전자로 진단된 멜라스 증후군 환자(n=19, m.3243A>G 돌연변이)와 유전자로 진단되지 않은 멜라스 증후군 환자(n=12) 그룹 간에 초기 진단 시 혈장 아미노산 및 소변 유기산의 비정상 대사물질 비율의 차이를 통계적으로 비교하였다. 유전자로 진단되지 않은 멜라스 증후군 환자군을 대조군으로 사용하였다.

결과: 두 그룹 간의 혈장 아미노산과 소변 유기산을 비교한 결과, 유전자로 진단된 멜라스 증후군 환자와 유전자로 진단되지 않은 멜라스 증후군 환자 간에 특징적인 차이를 보이는 비정상적인 대사 산물이 없는 것으로 나타났다.

결론: 혈장 아미노산 또는 소변 유기산의 비정상적인 대사물질 값은 멜라스 증후군의 진단에 있어서 선별 검사로 유용할 수 있지만 진단하기에는 충분하지 않다.

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