# Clinical validity of biochemical and molecular analysis in diagnosing Leigh syndrome: a study of 106 Japanese patients

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ORIGINAL ARTICLE

# Clinical validity of biochemical and molecular analysis in diagnosing Leigh syndrome: a study of 106 Japanese patients

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**Abstract** Leigh syndrome (LS) is a progressive neurodegenerative disorder of infancy and early childhood. It is clinically diagnosed by typical manifestations and characteristic computed tomography (CT) or magnetic resonance imaging (MRI) studies. Unravelling mitochondrial respiratory chain (MRC) dysfunction behind LS is essential for deeper understanding of the disease, which may lead to the development of new therapies and cure. The aim of this study was to evaluate the clinical validity of various diagnostic tools in confirming MRC disorder in LS and Leigh-like syndrome (LL). The results of enzyme assays, molecular analysis, and cellular oxygen consumption rate (OCR) measurements were examined. Of 106 patients, 41 were biochemically and genetically verified, and 34 had reduced MRC activity but no causative mutations. Seven patients with normal MRC complex activities had mutations in the *MT-ATP6* gene. Five further patients with normal activity in MRC were identified with causative mutations. Conversely, 12 out of 60 enzyme assays performed for genetically verified patients returned normal results. No biochemical or genetic background was confirmed for 19 patients. OCR was reduced in ten out of 19 patients with negative enzyme assay results. Inconsistent enzyme assay results between fibroblast and skeletal muscle biopsy samples were observed in 33% of 37 simultaneously analyzed cases. These data suggest that highest diagnostic rate is reached using a combined enzymatic and genetic approach, analyzing more than one type of biological materials where suitable. Microscale oxygraphy detected MRC impairment in 50% cases with no defect in MRC complex activities.

**Keywords** Mitochondrial respiratory chain disorder · Leigh syndrome · Enzyme assay · Genetic analysis · Oxygen consumption rate

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## Introduction

Leigh syndrome (LS) (OMIM 256000), also known as subacute necrotizing encephalopathy, is a progressive neurodegenerative disorder associated with primary or secondary dysfunction of mitochondrial oxidative phosphorylation. Clinical manifestations include psychomotor regression or retardation and signs of brainstem dysfunction, such as respiratory disturbance, nystagmus, ophthalmoplegia, or dysphagia (Thorburn and Rahman 1993). Symptoms often start in infancy, and many patients do not survive into childhood (Sofou et al. 2014). LS was originally defined neuropathologically by bilateral necrotic lesions in the basal ganglia and/or brainstem that were found at autopsy (Leigh 1951). Such lesions can now be observed in vivo with brain magnetic resonance imaging (MRI) or computed tomography (CT) (Gropman 2013). LS is clinically diagnosed based on typical manifestations and neuroimaging, accompanied by an elevated lactate or lactate-to-pyruvate (L/P) ratio in the blood or cerebrospinal fluid (CSF). The clinical diagnosis is followed by enzyme assays and genetic analysis to confirm the biochemical and molecular background (Baertling et al. 2014).

With advances in biochemical techniques and genomic medicine, enzyme assays and genetic analyses are now standard procedures for confirming mitochondrial respiratory chain (MRC) disorders. Numerous reports on the biochemical and molecular profiles of LS have been published, but there are limited studies on clinically diagnosed LS with negative biochemical or molecular findings (Sofou et al. 2014), and the clinical validity of these diagnostic methods remains unknown. In this report, we present the results of 106 Japanese patients with LS and Leigh-like syndrome (LL) to evaluate the clinical validity of various diagnostic methods. We also assessed the detection rate of each type of biological material for the enzyme assays to determine which was optimal for diagnosing LS/LL patients. We also assessed the usefulness of microscale oxygraphy.

## Patients and methods

### Patients

A total of 106 patients were included in this study. Patients were referred to either Chiba Children's Hospital or Saitama Medical University for enzyme assay and genetic analysis of MRC disorders from February 2007 to February 2015 by pediatricians and neurologists across Japan. Written informed consent was obtained from the parents of each patient. Both institutions received approval for comprehensive MRC analysis and genetic analysis from their appropriate ethics review boards. Data on the present illness, laboratory results, and neuroimaging findings were extracted from case summaries that accompanied the samples.

We used the stringent criteria defined by Rahman as the inclusion criteria for LS (Rahman et al. 1996). Those with atypical or normal neuroimaging results, or those with typical neuroimaging but with normal lactate levels in serum and CSF were classified as LL patients (Rahman et al. 1996). Patients were excluded from the study when they were diagnosed with pyruvate dehydrogenase complex deficiency or eventually diagnosed as having other metabolic diseases.

### Measurements

Activities of MRC complexes I, II, III, and IV were assayed in mitochondria isolated from skin fibroblasts or in the crude supernatant following centrifugation at 600 g from tissues, as previously described (Kirby et al. 1999; Murayama et al. 2009). Enzyme activities of each complex were presented as the percentage of normal control mean relative to appropriate reference enzyme activities, such as citrate synthase or MRC complex II. Enzyme activity was defined as being decreased at <40% in a cell line or <30% in a tissue, as reported (Bernier et al. 2002).

The cellular oxygen consumption rate (OCR) of fibroblast-derived cell lines was measured using microscale oxygraphy (Seahorse XF96 system; Seahorse Bioscience, Billerica, MA, USA) in cases with negative enzyme assay results. Material was prepared as reported (Kopajtich et al. 2014). After measurement of the basal OCR, oligomycin, carbonyl cyanide phenylhydrazone, and rotenone were added sequentially, and OCR was recorded after each addition. Maximum respiration rate (MRR) corresponds to the OCR after the addition of carbonyl cyanide phenylhydrazone minus rotenone-insensitive OCR (Invernizzi et al. 2012). Samples were measured in a 96-well plate, using 16 wells for each sample. Each sample's data were normalized as 20,000 cells per well. We analyzed five control samples, each one being measured at least five times. Cells in passages five through nine were used for controls and patient samples. In each run, we measured one or two controls with patient samples. OCR was expressed as percentage relative to the average of control(s).

Patients with MRC defects by enzyme assay were analyzed for mitochondrial DNA (mtDNA) mutations by whole mtDNA sequencing. Where no causative mtDNA mutations were found, we proceeded to whole-exome sequencing with next-generation sequencing for nuclear DNA (nDNA) mutations. Detailed information on this procedure was previously reported (Kohda et al. 2016). Those with negative enzyme assay results were screened for mutations using targeted gene panel of 251 nuclear genes known to cause mitochondrial diseases as well as the whole mitochondrial genome. In a few cases where referring clinicians had screened for and identified common mtDNA mutations before referring

patients to our institutions, findings were negative in our enzyme assay. There was also one case in whom an outside laboratory identified an nDNA mutation, although it was biochemically negative in our assay. The results of these cases were incorporated into the study to estimate the detection rate of each diagnostic method.

### Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). The Kruskal–Wallis *H* test was used to evaluate differences in continuous variables between groups, chi-squared and Fisher's tests were used to evaluate differences between categorical variables, and Wilcoxon test was used to evaluate differences between control and patient samples. All statistical tests were two sided, and $p$ values <0.05 were considered statistically significant.

## Results

### Overview

All 106 analyzed patients were from different families, and no consanguinity was reported. Seventy-five patients showed MRC defects that satisfied Bernier's criteria (Table 1). Forty-one of those patients received a molecular diagnosis: nDNA mutations in 19 and mtDNA mutations in 22. In 34 patients, the underlying genetic mutation was not identified. Of the 31 patients with no apparent reduction in MRC activities, seven had mutations in *MT-ATP6*, one in *MT-ND6*, three in *ECHS1*, and one in *SLC19A3*. The remaining 19 patients had no biochemical defect in MRC and no confirmed genetic diagnosis, including two patients whose gene analysis was not performed due to lack of material. Microscale oxygraphy was performed in 19 available fibroblast cell lines, with no reduction in enzyme activities and a significant reduction in OCR observed in ten.

**Table 1** Mitochondrial respiratory chain (MRC) complex activities and associated genetic mutations

| Mutation | Complex I–IV activity (enzyme assay) | | Total |
|---|---|---|---|
| | Decreased | Not decreased | |
| nDNA | 19 | 4 | 23 |
| mtDNA | 22 | 8 | 30 |
| None confirmed | 34 | 19 | 53 |
| Total | 75 | 31 | 106 |

*nDNA* nuclear DNA, *mtDNA* mitochondrial DNA

### Clinical presentation

Patient clinical features and metabolic status are summarized in Table 2 according to their biochemical and genetic backgrounds:

1. Positive assay and mutation identified (41 patients)
2. Mutation only (12 patients)
3. Positive assay only (34 patients)
4. Negative assay and no confirmed genetic diagnosis (19 patients).

There was no apparent clinical difference between groups. Patient status, age of living patients, LS/LL ratio, and median age at onset were similar. Besides regression and developmental delay, seizure and respiratory distress were the two major clinical symptoms observed in each group. There were no differences in serum or CSF lactate levels between groups. The mean serum and CSF L/P ratios for the whole cohort were 23.0 ± 13.2 and 24.1 ± 18.9, respectively, which were higher than the L/P ratios in normal individuals (Saudubray and Charpentier 2001), with no significant difference between groups.

### Enzyme assay

A total of 154 samples (92 fibroblast, 56 skeletal muscle, four liver, one cardiac muscle, and one lymph node) were submitted for enzyme assay, and a total of 151 assays (91 fibroblasts, 55 skeletal muscle, four liver, and one cardiac muscle sample) were completed. Of these, 89 assays (59%) exhibited decreased activity: fibroblasts, 54/91 (59%); skeletal muscle, 31/55 (56%); liver, 4/4 (100%); and cardiac muscle, 0/1 (0%), confirming MRC disorder in 75 (71%) of the 106 patients analyzed. No significant difference was found between the detection rate of fibroblasts and skeletal muscle biopsy samples. Isolated complex I defect was most frequently observed (37 patients), followed by isolated complex IV (17). Combined complex defects were observed in 20 patients, and the most frequently observed combination was defects of complexes I and IV (13).

In 42 patients, more than one type of tissue material was assayed; results were inconsistent in 17. Excluding those with mutations in the *MT-ATP6* gene, 37 patients had both skeletal muscle biopsy samples and fibroblasts assayed; results were inconsistent in 13 (Supplementary Table 1). Inconsistency was observed in four patients with nDNA mutations, in one with mtDNA mutation, and in eight with no genetic background confirmed. For genetically verified patients excluding those with mutations in the *MT-ATP6* gene, 60 samples were analyzed by enzyme assay; 12 returned normal or nonsignificant results, the majority of which were from patients with nDNA mutations (Supplementary Table 2).

**Table 2** Clinical presentations of patients with Leigh syndrome

| | Defect and mut | Mut only | Defect only | No defect, no validated mut | Total |
|---|---|---|---|---|---|
| Number of patients | 41 | 12 | 34 | 19 | 106 |
| Leigh-like | 6 | 4 | 10 | 4 | 24 |
| Living[a] | 71% (20/28) | 78% (7/9) | 62% (16/26) | 83% (10/12) | 71% (53/75) |
| Age of living patients[a] [median (range)] | 9 (3–17) years | 8 (3–15) years | 9.5 (3–38) years | 8.5 (6–20) years | 8 (3–38) years |
| Age at onset [median (range)] | 10.5 months (0 months–8 years) | 9 months (0 months–5 - years) | 5.5 months (0 months–6 - years) | 10 months (0 months–2 - years) | 9 months (0 months–8 - years) |
| Neonatal onset | 2 (5%) | 2 (17%) | 7 (21%) | 1 (5%) | 12 (11%) |
| Seizure | 20% | 33% | 41% | 42% | 32% |
| Involuntary movement | 10% | 25% | 18% | 16% | 15% |
| Hypotonia | 24% | 42% | 9% | 32% | 23% |
| Nystagmus/ ophthalmoplegia | 17% | 33% | 26% | 11% | 21% |
| Dysphagia | 10% | 17% | 29% | 32% | 21% |
| Respiratory distress | 24% | 17% | 41% | 37% | 31% |
| Serum L/P (mean ± SD) (number of data available) | 26.4 ± 16.4 (36) | 22.9 ± 14.9 (10) | 21.4 ± 9.9 (27) | 18.2 ± 5.7 (16) | 23.0 ± 13.2 (89) |
| CSF L/P (mean ± SD) (number of data available) | 27.2 ± 28.0 (30) | 20.7 ± 4.5 (9) | 25.0 ± 9.6 (20) | 18.7 ± 7.1 (14) | 24.1 ± 18.9 (73) |

*Mut* mutations in mitochondrial and nuclear DNA, *L/P* lactate-to-pyruvate ratio, *SD* standard deviation, *CSF* cerebrospinal fluid

[a] As of November 2016

### Oxygen consumption rate

The OCR was measured in 19 of the 31 LS/LL patients who presented normal enzyme assay results. Seven cases with mtDNA mutations were omitted. Analysis was precluded in three cases from whom fibroblast cell lines were not available. In an additional two patients, cell lines did not react properly to the experiment, and results were not obtained. Based on MRR distribution in our five controls, a reduction to <71.6% was considered a significant decline ($p < 0.05$). In 19 patients, it ranged from 36% to 136%, with a median of 69% of normal control(s). Ten patients showed a significant decline, suggesting mitochondrial respiratory dysfunction (Table 3).

### mtDNA analysis

Analysis of mtDNA mutation was performed for 103 patients and were identified in 30 patients across seven different genes (Table 4), resulting in a yield of 29%. *MT-ATP6* was the gene most frequent (ten patients). We also identified 19 patients with 11 different mutations in mtDNA genes related to complex I.

Previously unreported variants were considered as potential novel causative mutations of LS/LL when they coincided with positive enzyme assay results. Mutation m.14439G>A was shown to be pathogenic using cybrid analysis (Uehara et al. 2014). One of two cases with a mutation in m.14487T>C showed a reduction in enzyme activity of complex I. Mutations m.3946G>A and m.14687A>G had been reported to cause other mitochondrial diseases (Kirby et al. 2004; Spruijt et al. 2007; Bruno et al. 2003) and were considered as causative in our patients who showed defects in respective MRC complexes. Enzyme analysis of patients with confirmed pathogenic mutations m.3697G>A, m.10158T>C, m.10191T>C, m.13513G>A, and m.14459G>A all showed defects in complex I (Kohda et al. 2016).

### nDNA analysis

Seventy-six patients proceeded to nDNA analysis, and 17 patients were identified with mutations in nine genes related to MRC complexes (*SURF1, NDUFA1*, *NDUFAF6*, *NDUFS4*, *NDUFS6, NDUFV2, BOLA3*, *SCO2*, and *GTPBP3*, see Table 4). Mutations in *NDUFAF6* and *SURF1* were most frequent (five patients each), with all patients showing reduced activity in complex I *(NDUFAF6*) or IV (*SURF1*). Mutations in genes related to complex I constituted more than half of the nDNA mutations. The genetic defects were all in agreement with the biochemical defects.

Four cases were identified with a mutation in *ECHS1*, a gene involved in valine degradation. An outside laboratory identified one more patient with a mutation in the same gene (Yamada et al. 2015). Accumulation of toxic intermediates

**Table 3** Oxygen consumption rate (OCR) measured with a Seahorse analyzer

| Patient | Enzyme analysis | MRR (%) |
|---|---|---|
| Pt139 | ns (Fb) | 136 |
| Pt156 | ns (Fb) | **69** |
| Pt161 | ns (Fb) | **36** |
| Pt207 | ns (M, Fb) | 90 |
| Pt216 | ns (M, Fb) | 94 |
| Pt394 | ns (Fb) | 94 |
| Pt430 | ns (M, Fb) | **62** |
| Pt536 | ns (M, Fb) | 96 |
| Pt545 | ns (M, Fb) | **53** |
| Pt668 | ns (M, Fb) | **62** |
| Pt696 | ns (Fb) | 127 |
| Pt701 | ns (Fb) | **61** |
| Pt703 | ns (M, Fb) | 81 |
| Pt794 | ns (Fb) | **48** |
| Pt822 | ns (M, Fb) | 108 |
| Pt840 | ns (Fb) | **43** |
| Pt1038 | ns (Fb) | **51** |
| Pt1065 | ns (M, Fb) | 78 |
| Pt1120 | ns (Fb) | **51** |

MRR reduction to <71.6% of normal control value was considered to indicate mitochondrial impairment and is shown in bold

*OCR* oxygen consumption rate, *MRR* maximum respiration rate, *ns* not significant, *M* skeletal muscle, *Fb* cultured fibroblast, *CIV* complex IV, *P* partial decline

caused by impairment in this pathway is suspected to cause MRC complex defect (Peters et al. 2014). Three of our five patients showed no decline in enzyme activities, one patient showed a defect in complex IV and another in complex I. Lastly, one patient was identified with a mutation in *SLC19A3*, a gene encoding a thiamine transporter, which is essential for cerebral thiamine metabolism.

Mutations in all these genes except *BOLA3* had been reported to cause LS (Tiranti et al. 1998; Budde et al. 2000; Fernandez-Moreira et al. 2007; McKenzie et al. 2011; Kopajtich et al. 2014; Peters et al. 2014; Gerards et al. 2013). *BOLA3* had been identified in patients with other mitochondrial diseases (Cameron et al. 2011; Haack et al. 2013), and our case was previously reported as the first evidence of this mutation in an LS patient (Kohda et al. 2016).

## Discussion

We demonstrated the importance of combining multiple methods of diagnosing LS/LL patients. Genetic analysis identified a causative mutation in 51% (53/104) of analyzed cases. Enzyme assay recognized MRC complex defects in 71% (75/106) of patients. With those approaches combined, MRC defects were confirmed in 82% (87/106) of cases. The highest diagnostic rate was reached by a combined enzymatic and genetic approach. Seven patients with normal enzyme activities had mutations in the *MT-ATP6* gene, which encodes for complex V, which is measured in few laboratories. Screening for *MT-ATP6* mutations should be performed in such settings at an early stage of diagnosis, as they comprise a significant proportion of LS/LL etiology, and screening is readily available.

Detection rates in our study of various biopsy samples were <60% individually, which confirms previous results. Most importantly, the rate in muscle biopsies was no higher than in fibroblast cell lines, a finding not reported previously. For the diagnosis of mitochondrial diseases, skeletal muscle is often considered the tissue of choice (Thorburn and Rahman 1993), and fibroblasts have been considered less sensitive than skeletal muscle biopsy samples, detecting MRC defects in only half of cases with positive skeletal muscle assay results (Thorburn et al. 2004; Heuvel et al. 2004). A similar sensitivity was observed in our study, although skeletal muscle biopsy samples returned negative results in six out of 19 cases with reduced MRC activity in fibroblasts, resulting in similar overall detection rates. Tissue specificity of mitochondrial diseases was attributed to heteroplasmy of mtDNA, but inconsistencies between materials were frequently observed in nDNA-mutated cases. These findings suggest that, when possible, more than one type of patient biological sample should be analyzed, regardless of genetic background, to improve the detection rate of mitochondrial disorder.

In pediatric practice, it can be difficult to obtain multiple biological samples, and physicians must choose selectively. Although tissues used for analysis should be taken from the most affected organ (Munnich and Rustin 2001), this is difficult to apply in principle to LS/LL, a neurodegenerative disorder of the central nervous system. So the choice would be between skeletal muscle biopsy samples and cultured fibroblast cell lines in most cases. Skeletal muscle biopsy is invasive and requires general anesthesia, which poses a risk to pediatric patients (Baertling et al. 2014). Fibroblasts, on the other hand can be obtained in office settings with local anesthesia. If only one type of material can be obtained, fibroblasts should be prioritized, as cell lines from cultured fibroblasts can be used in future studies such as those involving cybrid analysis and rescue experiments to verify the pathogenicity of novel variations (Haas et al. 2008). Should no defect be observed in fibroblasts, or if the clinical status calls for a rapid result, skeletal muscle biopsy should also be considered.

Relatively high numbers of enzyme assays return negative results in genetically verified cases of LS/LL (Sofou et al. 2014). In our study, the rate of negative assay results in genetically verified cases was 20%, excluding *MT-ATP6* mutated cases. This observation implies that a normal MRC result in

**Table 4** Mutations in mitochondrial DNA (mtDNA) and nuclear DNA (nDNA)

| Patient | Gene | Mutation | LS/LL | Enzyme assay | Heteroplasmy rate (%) | Tissue |
|---|---|---|---|---|---|---|
| Pt27 | *SURF1* (NM_003172.2) | c.743C>A:p.A248D<br>c.743C>A:p.A248D | LS | CIV | | |
| Pt756 | *SURF1* (NM_003172.2) | c.367_368del:p.R123Gfs<br>c.54+1G>T | LS | CIV | | |
| Pt981 | *SURF1* (NM_003172.2) | c.743C>A:p.A248D<br>c.54+1G>T | LL | CIV | | |
| Pt1066 | *SURF1* (NM_003172.2) | c.367_368del:p.R123Gfs<br>c.867G>A:p.W289X | LS | CIV | | |
| Pt1143 | *SURF1* (NM_003172.2) | c.743C>A:p.A248D<br>c.826_827ins18:p.V276_T277ins6 | LS | CIV | | |
| Pt312[a] | *NDUFA1* (NM_004541) | c.55C>T:p.P19S | LS | CI | | |
| Pt286 | *BOLA3* (NM_212552) | c.287A>G:p.H96R<br>c.287A>G:p.H96R | LS | CC (I, II) | | |
| Pt376 | *ECHS1* (NM_004092) | c.98T>C:p.F33S<br>c.176A>G:p.N59S | LS | CIV | | |
| Pt536 | *ECHS1* (ENST00000368547) | c.5C>T:p.A2V<br>c.1A>G:p.M1V | LS | ns | | |
| Pt1038 | *ECHS1* (NM_004092) | c.5C>T:p.A2V<br>c.176A>G:p.N59S | LS | ns | | |
| Pt1135 | *ECHS1* (NM_004092) | c.5C>T:p.A2V<br>c.176A>G:p.N59S | LS | CI | | |
| Pt101 | *NDUFAF6* (NM_152416) | c.371T>C:p.I124T<br>c.805C>G:p.H269D | LS | CI | | |
| Pt330 | *NDUFAF6* (NM_152416) | c.820A>G:p.R274G<br>c.820A>G:p.R274G | LS | CI | | |
| Pt512 | *NDUFAF6* (NM_152416) | c.226T>C:p.S76P<br>c.805C>G:p.H269D | LS | CI | | |
| Pt598 | *NDUFAF6* (NM_152416) | c.206A>T:p.D69V<br>c.371T>C:p.I124T | LL | CI | | |
| Pt866 | *NDUFAF6* (NM_152416) | c.371T>C:p.1124T<br>c.805C>G:p.H269D | LS | CI | | |
| Pt711 | *NDUFS4* (NM_002495) | c.340T>C:p.W114R<br>c.340T>C:p.W114R | LS | CI | | |
| Pt1087 | *NDUFS6* (NM_004553) | c.309+5G>A<br>c.343T>C:p.C115R | LS | CC (I, IV) | | |
| Pt1177 | *NDUFV2* (NM_021074) | c.427C>T:p.R143X<br>c.580G>A:p.E194K | LS | CI | | |
| Pt628 | *SCO2* (NM_001169109) | c.577G>A:p.G193S<br>c.773T>C:p.M258T | LS | CC (I, IV) | | |
| Pt751 | *GTPBP3* (NM_032620) | c.8G>T:p.R3L<br>c.923_947del:p.E309Rfs | LS | CC (I, IV) | | |
| Pt156 | *SLC19A3* (NM_025243) | c.372C>G:p.Y124X<br>c.265A>C:p.S89R | LS | ns | | |
| Pt416 | *MT-ND1* | m.3697G>A:p.G131S | LS | CI | 100 | F |
| Pt619 | *MT-ND1* | m.3946G>A:p.E214K | LS | CC (I, IV) | 66 | M |
| Pt179 | *MT-ATP6* | m.8993T>G:pL156R | LL | ns | nearly 100 | B |
| Pt274 | *MT-ATP6* | m.8993T>C:p.L156P | LS | CC (I, III) | 100 | F |
| Pt453 | *MT-ATP6* | m.8993T>G:p.L156R | LS | CC (I, IV) | 100 | F |
| Pt341 | *MT-ATP6* | m.8993T>C:p.L156R | LS | ns | 100 | M |
| Pt720 | *MT-ATP6* | m.8993T>G:p.L156R | LS | ns | nearly 100 | B |
| Pt772 | *MT-ATP6* | m.8993T>G:p.L156R | LS | ns | nearly 100 | M |
| Pt968 | *MT-ATP6* | m.8993T>G:p.L156R | LS | ns | nearly 100 | B |
| Pt400 | *MT-ATP6* | m.9176T>C:p.L217P | LS | ns | 100 | B |

**Table 4** (continued)

| Patient | Gene | Mutation | LS/LL | Enzyme assay | Heteroplasmy rate (%) | Tissue |
|---|---|---|---|---|---|---|
| Pt698 | *MT-ATP6* | m.9176T>C:p.L217P | LS | CIV | 100 | B |
| Pt127 | *MT-ATP6* | m.9185T>C:p.L220P | LL | ns | 80 | B |
| Pt728 | *MT-ND3* | m.10158T>C:p.S34P | LS | CI | 80 | B |
| Pt994 | *MT-ND3* | m.10158T>C:p.S34P | LS | CI | 100 | B |
| Pt43 | *MT-ND3* | m.10191T>C:pS45P | LS | CI | 100 | F |
| Pt44 | *MT-ND3* | m.10191T>C:pS45P | LS | CI | 69 | F |
| Pt58 | *MT-ND3* | m.10191T>C:pS45P | LS | CI | na | |
| Pt83 | *MT-ND3* | m.10191T>C:pS45P | LS | CI | 100 | F |
| Pt108 | *MT-ND3* | m.10191T>C:p.S45P | LS | CI | 95 | B |
| Pt965 | *MT-ND3* | m.10197G>C:pA47P (VUS)[b] | LL | CC(I,III,IV) | na | |
| Pt190 | *MT-ND4* | m.11246G>A:pA163T (VUS) | LS | CC (I, IV) | 73 | F |
| Pt153 | *MT-ND5* | m.13094T>C:pV253A | LS | CC (I, IV) | na | B,M |
| Pt467 | *MT-ND5* | m.13513G>A:p.D393N | LL | CI | 59 | B |
| Pt744 | *MT-ND5* | m.13513G>A:p.D393N | LL | CC (I, IV) | 50 | B |
| Pt377 | *MT-ND6* | m.14439G>A:pP79S | LS | CI | 100 | F |
| Pt28 | *MT-ND6* | m.14459G>A:pA72V | LS | CI | 54 | F |
| Pt593 | *MT-ND6* | m.14459G>A:p.A72V | LS | CI | 96 | F |
| Pt224 | *MT-ND6* | m.14487T>C:p.M63V | LS | CI | 99 | B |
| Pt1063 | *MT-ND6* | m.14487T>C:p.M63V | LS | ns | Nearly 100 | B |
| Pt396 | $tRNA^{Glu}$ | m.14687A>G | LS | CI | 85 | M |

Pt255, identified with a mutation in *ECHS1* gene, is not listed here, and therefore the number of patients does not add up to the total number of patients with nDNA mutations on Table 1. The patient was omitted from this table because the gene analysis was processed in an outside laboratory

Segregation analyses have been completed for all autosomal recessive mutation cases

*mtDNA* mitochondrial DNA, *nDNA* nuclear DNA, *LS* Leigh syndrome, *LL* Leigh-like syndrome, *CI* isolated complex I deficiency, *CIV* isolated complex IV deficiency, *CC* combined complex deficiency, *VUS* variant of unknown significance, *ns* not significant, *na* not available, *F* fibroblasts, *M* skeletal muscle, *B* blood

[a] Pt312 is a male patient

[b] m.10197G>C is designated as VUS because the mutation confirmed in MITOMAP is m.10197G>A

muscle and/or fibroblast cell line does not exclude the possibility of a mitochondrial disorder. A reasonable proportion of MRC defects may remain undetected if negative enzyme assay results prevent us from proceeding to genetic analysis. Interestingly, negative assay results were more frequently observed in cases with nDNA than mtDNA mutations. In addition, genetic causes such as *ECHS1* mutations, which are not directly related to components of the MRC complexes, have been associated with LS/LL. In such cases, each separate MRC complex may not show reduced activity and thus remain undetected by enzyme assay. If marker substances detected by basic metabolic analysis leads directly to diagnosis, as is the case with urinary organic acids in *ECHS1* mutation, the next step is to proceed directly to analyzing the candidate gene.

In addition to genetic screening and spectrophotometric assays that measure the activity of individual respiratory complexes, we used microscale oxygraphy to help analyze mitochondrial activity. Microscale oxygraphy has a high efficiency for detecting mitochondrial respiratory defects in genetically proven mitochondrial disease patients, an observation by Invernizzi but not adopted by many diagnostic laboratories (Invernizzi et al. 2012). Half the cases in our cohort with no apparent defect in activities of MRC complexes showed a significant decline in OCR. Moreover, two nDNA mutations were identified in this group. Although evidence needs to be accumulated, this finding suggests the promising value of microscale oxygraphy as a screening tool to detect MRC defect, especially in cases in whom each complex remains intact. If cellular OCR shows a significant reduction, genetic screening should be considered, even if MRC defects were not detected by enzyme assays of fibroblasts or peripheral organs.

With advances in molecular technologies, genetic screening is becoming increasingly utilized over enzyme analysis and invasive biopsies (Lake et al. 2016; Taylor et al 2014). Enzyme assays are considered a confirmatory method for diagnosis of LS/LL in cases with ambiguous genetic results or where genetic analysis fails to detect causative mutations (Morava and Brown 2015). However, in our study, gene analysis could not identify underlying mutations in 45% of cases with reduced MRC complex activities. The genetic spectrum

of LS/LL is still expanding, and biochemical data obtained via enzyme assays enable the efficient selection of candidate genes (Thorburn et al. 2004) and provide essential information in the pathogenicity of identified gene variants. Thus, enzyme analysis remains an important part of the diagnostic process of mitochondrial disorders.

Based on our increasing understanding of the biological and molecular background of the disease, new therapeutic methods are being proposed (Martinelli et al. 2012; Morava and Brown 2015). Precise biochemical and genetic diagnosis is imperative in considering the possible gene-specific therapeutic options. It is also essential to provide appropriate genetic counseling. All available biochemical and molecular methods should be combined to not only diagnose the disease but also to provide optimal care to the LS/LL patients.

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**Compliance with ethical standards**

**Conflict of interest** None.

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## References

Baertling F, Rodenburg RJ, Schaper J et al (2014) A guide to diagnosis and treatment of Leigh syndrome. J Neurol Neurosurg Psychiatry 85(3):257–265

Bernier FP, Boneh A, Dennett X, Chow CW, Cleary MA, Thorburn DR (2002) Diagnostic criteria for respiratory chain disorders in adults and children. Neurology 59(9):1406–1411

Bruno C, Sacco O, Santorelli FM et al (2003) Mitochondrial myopathy and respiratory failure associated with a new mutation in the mitochondrial transfer ribonucleic acid glutamic acid gene. J Child Neurol 18(4):300–303

Budde SM, van den Heuvel LP, Janssen AJ et al (2000) Combined enzymatic complex I and III deficiency associated with mutations in the nuclear encoded NDUFS4 gene. Biochem Biophys Res Commun 275(1):63–68

Cameron JM, Janer A, Levandovskiy V et al (2011) Mutations in iron-sulfur cluster scaffold genes NFU1 and BOLA3 cause a fatal deficiency of multiple respiratory chain and 2-oxoacid dehydrogenase enzymes. Am J Hum Genet 89(4):486–495

Fernandez-Moreira D, Ugalde C, Smeets R et al (2007) X-linked NDUFA1 gene mutations associated with mitochondrial encephalomyopathy. Ann Neurol 61(1):73–83

Gerards M, Kamps R, van Oevelen J et al (2013) Exome sequencing reveals a novel Moroccan founder mutation in SLC19A3 as a new cause of early-childhood fatal Leigh syndrome. Brain 136(3):882–890

Gropman AL (2013) Neuroimaging in mitochondrial disorders. Neurotherapeutics 10(2):273–285

Haack TB, Rolinski B, Haberberger B et al (2013) Homozygous missense mutation in BOLA3 causes multiple mitochondrial dysfunctions syndrome in two siblings. J Inherit Metab Dis 36(1):55–62

Haas RH, Parikh S, Falk MJ et al (2008) The in-depth evaluation of suspected mitochondrial disease. Mol Genet Metab 94(1):16–37

Heuvel LP, Smeitink JA, Rodenburg RJT (2004) Biochemical examination of fibroblasts in the diagnosis and research of oxidative phosphorylation (OXPHOS) defects. Mitochondrion 4(5-6):395–401

Invernizzi F, D'Amato I, Jensen PB, Ravaqlia S, Zeviani M, Tiranti V (2012) Microscale oxygraphy reveals OXPHOS impairment in MRC mutant cells. Mitochondrion 12(2):328–335

Kirby DM, Crawford M, Cleary MA, Dahl HH, Dennett X, Thorburn DR (1999) Respiratory chain complex I deficiency: an underdiagnosed energy generation disorder. Neurology 52(6):1255–1264

Kirby DM, McFarland R, Ohtake A et al (2004) Mutations of the mitochondrial ND1 gene as a cause of MELAS. J Med Genet 41(10):784–789

Kohda M, Tokuzawa Y, Kishita Y et al (2016) A comprehensive genomic analysis reveals the genetic landscape of mitochondrial respiratory chain complex deficiencies. PLoS Genet 12(1):e1005679. doi:10.1371/journal.pgen.1005679

Kopajtich R, Nicholls TJ, Rorbach J et al (2014) Mutations in GTPBP3 cause a mitochondrial translation defect associated with hypertrophic cardiomyopathy, lactic acidosis, and encephalopathy. Am J Hum Genet 95(6):708–720

Lake NJ, Compton AG, Rahman S, Thorburn DR (2016) Leigh syndrome: one disorder, more than 75 monogenic causes. Ann Neurol 79(2):190–203

Leigh D (1951) Subacute necrotizing encephalomyelopathy in an infant. J Neurol Neurosurg Psychiatry 1(14):216–221

Martinelli D, Catteruccia M, Piemonte F et al (2012) EPI-743 reverses the progression of the pediatric mitochondrial disease–genetically defined Leigh syndrome. Mol Genet Metab 107(3):383–388

McKenzie M, Tucker EJ, Compton AG et al (2011) Mutations in the gene encoding C8orf38 block complex I assembly by inhibiting

production of the mitochondria-encoded subunit ND1. J Mol Biol 414(3):413–426

Morava E, Brown GK (2015) Next generation mitochondrial disease: change in diagnostics with eyes on therapy. J Inherit Metab Dis 38(3):387–388

Munnich A, Rustin P (2001) Clinical spectrum and diagnosis of mitochondrial disorders. Am J Med Genet 106(1):4–17

Murayama K, Nagasaka H, Tsuruoka T et al (2009) Intractable secretory diarrhea in a Japanese boy with mitochondrial respiratory chain complex I deficiency. Eur J Pediatr 168(3):297–302

Peters H, Buck N, Wanders R et al (2014) ECHS1 mutations in Leigh disease: a new inborn error of metabolism affecting valine metabolism. Brain 137(Pt 11):2903–2908

Rahman S, Blok RB, Dahl H-HM et al (1996) Leigh syndrome: clinical features and biochemical and DNA abnormalities. Ann Neurol 39(3):343–351

Saudubray J-M, Charpentier C (2001) Clinical phenotypes: diagnosis/algorithms. In: Scriver CR, Sly WS (eds) The metabolic and molecular bases of inherited diseases, 8th edn. McGraw-Hills, New York, pp 1327–1403

Sofou K, De Coo IF, Isohanni P et al (2014) A multicenter study on Leigh syndrome: disease course and predictors of survival. Orphanet J Rare Dis 9:52

Spruijt L, Smeets HJ, Hendrickx A et al (2007) A MELAS-associated ND1 mutation causing leber hereditary optic neuropathy and spastic dystonia. Arch Neurol 64(6):890–893

Taylor RW, Pyle A, Griffin H et al (2014) Use of whole-exome sequencing to determine the genetic basis of multiple mitochondrial respiratory chain complex deficiencies. JAMA 312(1):68–77

Thorburn DR, Rahman S (1993) Mitochondrial DNA-associated Leigh syndrome and NARP. In: Pagon RA, Adam MP, Ardinger HH et al (eds) GeneReviews. University of Washington, Seattle

Thorburn DR, Chow CW, Kirby DM (2004) Respiratory chain enzyme analysis in muscle and liver. Mitochondrion 4(5–6):363–375

Tiranti V, Hoertnagel K, Carrozzo R et al (1998) Mutations of *SURF-1* in Leigh disease associated with cytochrome c oxidase deficiency. Am J Hum Genet 63(6):1609–1621

Uehara N, Mori M, Tokuzawa Y et al (2014) New *MT-ND6* and *NDUFA1* mutations in mitochondrial respiratory chain disorders. Ann Clin Transl Neurol 1(5):361–369

Yamada K, Aiba K, Kitaura Y et al (2015) Clinical, biochemical and metabolic characterization of a mild form of human short-chain enoyl-CoA hydratase deficiency: significance of increased N-acetyl-S-(2-carboxypropyl)cysteine excretion. J Med Genet 52(10):691–698

主論文の和文の要約

論文タイトル:Clinical validity of biochemical and molecular analysis in diagnosing Leigh syndrome; a study of 106 Japanese patients

邦題:Leigh 脳症の診断における生化学的・分子生物学的分析の臨床的妥当性に関する研究

【はじめに】

ミトコンドリアはエネルギー産生を担う細胞内小器官で、赤血球を除くほとんどすべての細胞に存在する。特にエネルギー需要の大きい細胞には多数存在し、独自の DNA（ミトコンドリア DNA）を持つと同時に、核 DNA による支配も受けている。構造的には、脂質二重膜である外膜と内膜からなり、内膜の内側をマトリックス、内膜と外膜の間のスペースを膜間腔という。エネルギー産生は、内膜に埋め込まれている呼吸鎖が担っており、呼吸鎖は複合体 I から V まである。複合体 I～IV が電子伝達系を、複合体 V が ATP 合成を担っており、それぞれ多数のサブユニットから構成されている。複合体 II を除き、各複合体はミトコンドリア DNA と核 DNA の二重支配を受けているが、核遺伝子にコードされている蛋白のほうが多い（複合体 II は、核遺伝子にのみコードされている。）。

ミトコンドリア病は、ミトコンドリアによる ATP 産生が低下することにより各種臓器に障害がでる疾患または症候群の総称であり、特にエネルギー需要の大きい臓器に障害が出やすい。ミトコンドリアはさまざまな臓器においてエネルギー産生を担っていることから、あらゆる臓器であらゆる症状を呈し、あらゆる年代において発症しうる。また、その遺伝的二重支配から、母系遺伝だけでなく、常染色体遺伝、性染色体遺伝のすべての遺伝形式をとる。

臨床病型としては、代表的な MELAS や Leigh 脳症、ミトコンドリア糖尿病や難聴のほか、小児分野においては、ミトコンドリア肝症、ミトコンドリア心筋症、新生児ミトコンドリア病といった病型も認識されるようになっている。

病因分類としては、

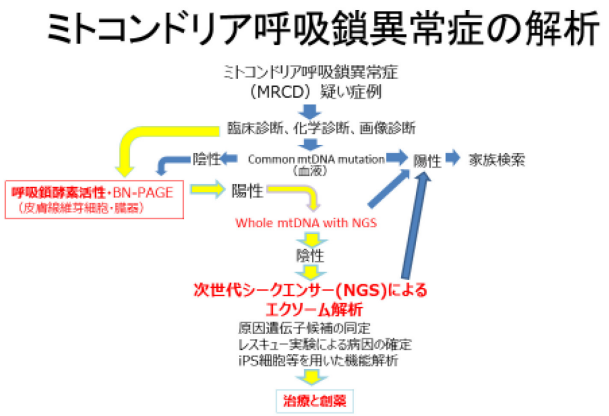
- 各呼吸鎖複合体（I～V）欠損症
- 複数の複合体の活性が低下する複合型欠損症（ミトコンドリア DNA の翻訳、呼吸鎖の生合成、ミトコンドリア蛋白の輸送、ミトコンドリア膜の動力学にかかわる遺伝子の異常等による。）
- ミトコンドリア DNA 枯渇症候群

がある。例えば Leigh 脳症は、すべての病因から発症しうるが、呼吸鎖複合体 I 欠損症がもっとも多い。逆に、呼吸鎖複合体 I 欠損症は、Leigh 脳症のほかに、MELAS や致死型乳児ミトコンドリア病など様々な臨床病型をとりうる。生化学的な理論に依拠した治療法は、病因によりその有効性が異なると考えられるため（例えば、高脂肪・低炭水化物栄養療法は、

複合体 I 欠損症であって、ほかの複合体の活性が比較的保たれている症例において有効性が期待される等)、臨床病型診断にとどまらず、病因を明らかにすることが重要になってくる。

各病因の背後にはその原因となる遺伝子異常がある。ミトコンドリア呼吸鎖に関連する遺伝子は、現在 250 以上あるといわれており、ミトコンドリア病を起こすことが確認されている遺伝子として 200 程度が知られている。

現在、ミトコンドリア遺伝子解析のプロジェクトが各国で進められており、日本においては、特に小児のミトコンドリア呼吸鎖異常症について、埼玉医科大学と千葉県こども病院の共同研究として「ミトコンドリア呼吸鎖異常症の酵素診断および責任遺伝子解析に関する研究」が進められている。この研究のスキームは右図に示すとおり、全国から提供される細胞や組織検体の酵素活性を測定し、活性低下を示すものについてその原因遺伝子を同定し、創薬や治療法の開発につなげるものである。診断の付いていない小児症例が多いため、この研究で解析している症例は、Leigh 脳症を初めとする神経変性疾患、ミトコンドリア呼吸鎖異常が関与していると思われる肝症や心筋症、非常に重篤な致死型乳児ミトコンドリア病、そして乳幼児突然死症候群として法医学教室から依頼されてくるものなどが多い。




【本研究の目的】

生化学的な分析技術や遺伝子解析技術の進歩により、ミトコンドリア病の診断において、酵素活性測定や遺伝子解析はいまやスタンダードとなっている。小児ミトコンドリア病の代表的な病型である Leigh 脳症についても、Leigh 脳症における呼吸鎖複合体欠損症について論じた文献や、Leigh 脳症を呈する新たな遺伝子異常の報告は枚挙にいとまがない。その一方で、呼吸鎖酵素活性の低下や原因遺伝子が特定されていない Leigh 脳症について論じた文献はほとんどない。そのため、これらの診断技術の有用性、すなわち、臨床診断された Leigh 脳症患者の何割において現在の生化学的、分子生物学的手法がミトコンドリア呼吸鎖異常を確定できるのかは、明らかになっていない。

そこで、本研究では、Leigh 脳症における生化学的、分子生物学的解析の現時点での有用性を明らかにすることを目的に、106 例の Leigh 脳症及び Leigh 様症候群（以下 Leigh 脳症等）患者の酵素活性測定結果と遺伝子解析結果を分析した。また、従来からミトコンドリア病では筋生検がスタンダードとされてきたが、Leigh 脳症等において材料間の診断率を比

較した研究はないため、材料による診断率について検討した。さらに、酵素活性低下を認めない Leigh 脳症等におけるマイクロスケールオキシグラフィーの有用性を明らかにした報告はないため、酵素活性低下を認めない症例について酸素消費量の測定と遺伝子パネルでの解析を行い、酸素消費量測定の有用性について検討した。

Leigh 脳症は、小児期ミトコンドリア病として MELAS とともに多く見られる病型であり、中枢神経系のエネルギー産生障害により、精神運動発達遅滞や退行を示す、乳幼児期に発症する難治性の慢性進行性疾患である。1951 年に Leigh が剖検例の脳病理において特徴的な左右対称性の壊死性病変を指摘したことに端を発しており、当初は死後において剖検でのみ診断される病理学的診断名であった。現在は、CT や MRI により、病理学的変化に対応する画像変化をとらえることができるようになったため、生存中に診断がされるようになり、典型的な臨床経過、血液・髄液検査所見、および画像所見から臨床的に診断されている。有病率は、40000 人に一人程度と考えられている。

【対象と方法】

＜対象＞

2007 年から 2015 年の間に「ミトコンドリア呼吸鎖異常症の酵素診断と責任遺伝子解析に関する研究」に解析依頼のあった症例のうち、Leigh 脳症等と考えられる症例を抽出し、そのうち、Rahman の診断基準（表 1）に照らして Leigh 脳症等と診断できるものを対象とした。

表 1　Rahman の診断基準 [1)]

① 精神運動発達の退行を伴った進行性疾患
② 不随意運動、哺乳嚥下障害、呼吸障害、眼球運動障害、運動失調などの脳幹・大脳基底核症状を伴う
③ 血中・髄液中の乳酸値の上昇
④ 次のうちの一つ以上
- 画像上の対称性基底核・脳幹病変
- 典型的神経病理学的変性（海綿状壊死）
- 同様症状の同胞の存在

以上のすべてを満たすものを Leigh 脳症とする。
臨床所見は Leigh 脳症を強く示唆するものの、非典型的な病理所見、画像所見が不存在、画像所見が正常または非典型的、典型的な画像所見だが乳酸値が正常のものを ”Leigh 様”とする。

＜方法＞

各症例について、発症年齢、現況、主な症状、血中・髄液中の乳酸／ピルビン酸比、酵素活性測定の結果、遺伝子解析の結果を収集・分析した。酵素活性低下が認められない症例については、酸素消費量を測定するとともに、遺伝子パネルで遺伝子変異のスクリーニングを行った。

＜酵素活性の測定＞

皮膚由来の線維芽細胞または組織検体を用いて、各呼吸鎖複合体の酵素反応の基質または生成物の増減を分光光度計で測定した。Bernier らの診断基準(表2)に照らして、培養細胞で 40%未満、組織検体で 30%未満の場合活性低下とした。

表 2 Bernier らのミトコンドリア呼吸鎖異常症の診断基準 [2)]

| |
|---|
| 大基準（抜粋） |
| In vitro 呼吸鎖酵素活性 |
| 1 つの臓器で 20%以下または 2 つ以上の臓器にまたがって 30%以下 |
| 1つの培養細胞で 30%以下 |
| 小基準（抜粋） |
| In vitro 呼吸鎖酵素活性 |
| 1つの臓器で 20–30%または 2 つ以上の臓器にまたがって 30–40% |
| 1つの培養細胞で 30–40% |
| Definite:大基準 2 つ 又は 大基準1つ＋小基準 2 つ |
| Probable:大基準 1 つ＋小基準 1 つ 又は 小基準 3 つ |
| Possible:大基準 1 つ 又は 小基準の I(臨床症状：略)＋他の小基準 1 つ |

＜酸素消費量の測定＞

皮膚線維芽細胞を用いて、細胞外フラックスアナライザーで測定した。基礎呼吸を測定後、オリゴマイシン、脱共役薬、ロテノン（ミトコンドリア呼吸鎖複合体阻害薬）を順次添加し、その都度酸素消費量を測定した。脱共役薬添加後の最大酸素消費量からロテノン添加後の酸素消費量を引いた差分を MRR（最大酸素消費量）とした。酸素消費量の測定にあたっては、毎回１ないし２の正常検体を同一プレートで測定した。すべての正常検体について 5 回測定を行い、正常検体の測定値の分布から 5%ile 未満となる値(71.6%)を下回るものを低下とした。

＜遺伝子解析＞

酵素活性低下が認められたものは、ミトコンドリア DNA の全周囲解析を行った。ミトコンドリア DNA で変異が確認されないものは次世代シークエンサーで核遺伝子の変異を検索した。酵素活性が正常のものは、Mayr(2015)に記載する 251 のミトコンドリア関連遺伝子を搭載した遺伝子パネルでスクリーニングを行った [3)]。

本研究外で主治医により他の研究施設、検査機関等において遺伝子解析が行われ、高頻度変異等が同定された症例については、遺伝子解析の診断率を明らかにするためその結果を本研究に盛り込んだ。

【結果】

- 酵素活性測定の診断率は 71%(75/106)、遺伝子解析の診断率は 51%(53/104)(内訳：ミトコンドリア遺伝子 29%(30/103)、核遺伝子 30%(23/76))だった。生化学的手法と遺伝子解析を組み合わせると、診断率は 82%(87/106)に向上した。（別表 1）
- 一般的な呼吸鎖酵素活性では測定しない複合体 V の欠損による症例が少なからず存在し、それらではミトコンドリア DNA の高頻度変異を多く認めた。（別表 2）
- 複合体 V 欠損以外にも、Leigh 脳症関連の遺伝子に変異があるにもかかわらず、呼吸

鎖複合体 I～IV の活性が正常なケースが複数確認された。(別表 2,3)

- 酵素学的にミトコンドリア呼吸鎖異常が確かめられているが、エクソーム解析をもってしても遺伝子変異が確認できていない症例が 34 例存在した。
- 酵素活性と遺伝子解析の結果により、106 例の Leigh 脳症等を、次の四群に分類し、予後、発症年齢、新生児発症の頻度、患者年齢、主な症状や乳酸／ピルビン酸比等を比較したが、四群間に有意な差は認めなかった。(別表 4)
  - ① 酵素活性が低下し、遺伝子変異も同定されたもの　41 例（核 DNA 変異 19 例、mtDNA 変異 22
  - ② 酵素活性の低下はあるものの、遺伝子変異が同定されなかったもの　34 例
  - ③ 酵素活性は正常だったが、遺伝子変異が同定されたもの　12 例
  - ④ 酵素活性が正常で、遺伝子変異も同定されなかったもの　19 例
- 検体種別酵素活性低下検出率は、皮膚線維芽細胞で 59%、骨格筋で 56%であり、この二者間に有意差は認めなかった。一種類の検体（例：骨格筋生検検体）で酵素活性の低下を認めない症例においても、異なる検体（例：皮膚培養細胞）で活性低下を認めるものがあることが確認された。
- 酵素活性低下のない症例のうち、皮膚線維芽細胞が提出されていた 19 例で酸素消費量を測定したところ、10 例で有意な酸素消費量の低下を認めた。このうち 2 例は遺伝子パネル解析で核遺伝子の変異を認め、家系解析にて確定された。(別表 5)
- 酵素活性測定、遺伝子解析、酸素消費量の測定すべてを合わせると、Leigh 脳症等におけるミトコンドリア異常症の診断率は 90%（95/106）となった。

【結論】

以上から、

1．Leigh 脳症等では、生化学的または分子生物学的手法の一方のみでは Leigh 脳症等のミトコンドリア機能異常をすべて確定することはできず、これらの手法を組み合わせることで診断率が向上すること

2．原因遺伝子がミトコンドリア遺伝子か核遺伝子かにかかわらず、皮膚線維芽細胞と骨格筋検体で酵素活性低下の検出率に有意差がないこと、また、一種類の材料で活性低下がなくとも、ほかの臓器から採取した材料で活性低下を認めることがあり、複数の臓器から検体を採取することで診断率が向上すること

が明らかになった。

【考察】

本研究にはいくつかの限界がある。

本研究は、対象患者の選定基準をあらかじめ定めた研究ではなく、酵素診断および遺伝子解析目的に集められた母集団から Leigh 脳症等を取り出して検討している。Leigh 脳症ま

たは Leigh 様症候群の定義を満たしているもののみを対象としてはいるが、年齢や発症からの時間経過は様々である。酵素活性は、年齢や発症からの時間、また検体の保存状態によっても結果に差が生じるため、酵素活性の測定結果がこれらの要素の影響を受けた可能性は否定できない。また、8 年にわたり蓄積されたデータを対象としているため、この間の小児分野におけるミトコンドリア病の認知度の高まりにより、集まってくる症例の性質（蓋然性、重症度、発症からの期間等）が時とともに変化している可能性も否定できない。さらに、同一時点でみても、きわめて重症の症例から、年長まで生存している比較的軽症といえる症例まで、重症度が様々であり、酵素活性や遺伝子変異の種類に影響を与えている可能性がある。

ミトコンドリア病では、同一の遺伝子異常が、異なる臨床病型を呈することが観察される。ミトコンドリア遺伝子異常では、その背景に、ミトコンドリアの fusion、fission、変異の偏り（heteroplasmy）、臓器・組織による heteroplasmy の閾値の違いなどが発症に関与していると考えられるが、詳しいメカニズムについては依然として不明なことが多く、今後の研究課題である。核遺伝子変異例においても、病型や重症度の多様性が認められ、これは heteroplasmy では説明できない。なぜ重症度や症状の発現する臓器が異なるのか、今後さらなる研究がすすむことが期待される。逆に、同一遺伝子変異や同一の複合体欠損が原因であるならば、臨床病型にかかわらず同一治療法が有効である可能性があり、今後の治療法の開発に際し、臨床病型にとらわれない姿勢も求められるであろう。

本研究でミトコンドリア機能異常を確認できなかった 18%(19/106)の症例について、ミトコンドリア機能異常を確認できなかった理由は複数考えられる。酵素活性測定については、発症から時間が経過すると活性低下を捉えられない可能性があることや、細胞や組織の状態により活性低下を捕捉できない場合があることはすでに述べたとおりである。遺伝子変異については、われわれはいまだ Leigh 脳症等の原因となる遺伝子変異の全容を把握しているわけではなく、エクソーム解析でもわからないイントロンの深い部分での変異や、エクソーム解析では捉えられない大きな欠失などもあり得る。また、候補遺伝子は挙がっているが、両親の検体提供がないため家系解析ができず、その結果病因として特定できていないものも含まれている。さらには、原則として他の代謝疾患は除外しているが、二次性のミトコンドリア機能異常を捉えている可能性は完全には排除できず、その場合は、個々の呼吸鎖複合体の活性低下やミトコンドリア関連遺伝子には変異が同定されない。つまり、Rahman の診断基準に基づく Leigh 脳症等には、primary なミトコンドリア呼吸鎖異常以外の二次的なミトコンドリア機能低下による Leigh 脳症等が含まれている可能性も否定できない。

逆に、ミトコンドリア呼吸鎖異常症の確定診断が現時点の技術でできないことをもって、ミトコンドリア呼吸鎖異常症でないとは言い切れないため、本研究で Rahman の基準の特異度を論ずることはできないと考える。

【結語】

Leigh 脳症では、ミトコンドリア呼吸鎖の機能低下をもたらす要因は様々であり、その背景にある遺伝子変異も現時点で 75~85 種類といわれている heterogeneous な疾患である。生化学的、分子生物学的技術の進展に伴い、今後は、Leigh 脳症の原因となっているミトコンドリア異常を遺伝子レベルまで解明することを前提に、病態を踏まえた治療法の開発等が進むことが期待される。そのような時代にあって、本研究は、現時点における生化学的、分子生物学的診断の有用性を明らかにし、一人でも多くの Leigh 脳症等患者でミトコンドリア機能異常が確定されるよう、診断の道筋を示すことができた。本研究の提示した道筋に沿って効率的に診断が行われ、継続的にデータが蓄積されることで、迅速な診断と病因を踏まえた治療法の開発につながっていくことが期待される。

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なお、本論文を学位論文として使用することについては、全ての共著者から文書にて承諾をいただいております。

＜文献＞

1) Rahman S et al (1996) Leigh syndrome: clinical features and biochemical and DNA abnormalities. Ann Neurol 39(3):343-351.
2) Bernier FP et al (2002) Diagnostic criteria for respiratory chain disorders in adults and children. Neurology 59(9):1406-1411.
3) Mayr JA et al (2015) Spectrum of combined respiratory chain defects. J Inherit Metab Dis 38(4): 629-640.

別表 1　酵素活性測定及び遺伝子解析の診断率

| Method | Analyzed | Defect/ mutation detected | Detection rate |
|---|---|---|---|
| Spectrophotometric enzyme assay | 106 | 75 | 71% |
| Mitochondrial gene analysis | 103 | 30 | 29% |
| Nuclear gene analysis | 76 | 23 | 30% |
| Total | 106 | 87 | 82% |

別表 2　ミトコンドリア遺伝子変異

| Patient | Gene | Mutation | Enzyme assay |
|---|---|---|---|
| Pt416 | *MT-ND1* | m.3697G>A:p.G131S | CI |
| Pt619 | *MT-ND1* | m.3946G>A:p.E214K | CC(I,IV) |
| Pt179 | *MT-ATP6* | m.8993T>G:pL156R | Ns |
| Pt274 | *MT-ATP6* | m.8993T>C:p.L156P | CC(I,III) |
| Pt453 | *MT-ATP6* | m.8993T>G:p.L156R | CC(I,IV) |
| Pt341 | *MT-ATP6* | m.8993T>C:p.L156R | Ns |
| Pt720 | *MT-ATP6* | m.8993T>G:p.L156R | Ns |
| Pt772 | *MT-ATP6* | m.8993T>G:p.L156R | Ns |
| Pt968 | *MT-ATP6* | m.8993T>G/C:p.L156R/P | Ns |
| Pt400 | *MT-ATP6* | m.9176T>C:p.L217P | Ns |
| Pt698 | *MT-ATP6* | m.9176T>C:p.L217P | CIV |
| Pt127 | *MT-ATP6* | m.9185T>C:p.L220P | Ns |
| Pt728 | *MT-ND3* | m.10158T>C:p.S34P | CI |
| Pt994 | *MT-ND3* | m.10158T>C:p.S34P | CI |
| Pt43 | *MT-ND3* | m.10191T>C:pS45P | CI |
| Pt44 | *MT-ND3* | m.10191T>C:pS45P | CI |
| Pt58 | *MT-ND3* | m.10191T>C:pS45P | CI |
| Pt83 | *MT-ND3* | m.10191T>C:pS45P | CI |
| Pt108 | *MT-ND3* | m.10191T>C:p.S45P | CI |
| Pt965 | *MT-ND3* | m.10197G>C:pA47P (VUS) | CC(I,III,IV) |
| Pt190 | *MT-ND4* | m.11246G>A:pA163T (VUS) | CC(I,IV) |
| Pt153 | *MT-ND5* | m.13094T>C:pV253A | CC(I,IV) |
| Pt467 | *MT-ND5* | m.13513G>A:p.D393N | CI |
| Pt744 | *MT-ND5* | m.13513G>A:p.D393N | CC(I,IV) |
| Pt377 | *MT-ND6* | m.14439G>A:pP79S | CI |
| Pt28 | *MT-ND6* | m.14459G>A:pA72V | CI |
| Pt593 | *MT-ND6* | m.14459G>A:p.A72V | CI |
| Pt224 | *MT-ND6* | m.14487T>C:p.M63V | CI |
| Pt1063 | *MT-ND6* | m.14487T>C:p.M63V | Ns |
| Pt396 | *tRNAGlu* | m.14687A>G | CI |

CI, isolated complex I deficiency; CIV, isolated complex IV deficiency; CC, combined complex deficiency; VUS, variant of unknown significance; ns, not significant

別表 3　核遺伝子変異

| Patient | Gene | Mutation | Enzyme assay |
|---|---|---|---|
| Pt27 | *SURF1* (NM_003172.2) | c.743C>A:p.A248D, c.743C>A:p.A248D | CIV |
| Pt756 | *SURF1* (NM_003172.2) | c.367_368delAG:p.R123Gfs, c.54+1G>T | CIV |
| Pt981 | *SURF1* (NM_003172.2) | c.743C>A:p.A248D, c.54+1G>T | CIV |
| Pt1066 | *SURF1* (NM_003172.2) | c.367_368delAG:p.R123Gfs, c.867G>A:p.W289X | CIV |
| Pt1143 | *SURF1* (NM_003172.2) | c.743C>A:p.A248D, c.826_827ins18:p.V276_T277ins6 | CIV |
| Pt312 | *NDUFA1* (NM_004541) | c.55C>T:p.P19S | CI |
| Pt286 | *BOLA3* (NM_212552) | c.287A>G:p.H96R, c.287A>G:p.H96R | CC(I,II) |
| Pt376 | *ECHS1* (NM_004092) | c.98T>C:p.F33S, c.176A>G:p.N59S | CIV |
| Pt536 | *ECHS1*(ENST00000368547) | c.5C>T:p.A2V, c.1A>G:p.M1V | ns |
| Pt1038 | *ECHS1*(NM_004092) | c.5C>T:p.A2V, c.176A>G:p.N59S | ns |
| Pt1135 | *ECHS1*(NM_004092) | c.5C>T:p.A2V, c.176A>G:p.N59S | CI |
| Pt101 | *NDUFAF6* (NM_152416) | c.371T>C:p.I124T, c.805C>G:p.H269D | CI |
| Pt330 | *NDUFAF6* (NM_152416) | c.820A>G:p.R274G, c.820A>G:p.R274G | CI |
| Pt512 | *NDUFAF6* (NM_152416) | c.226T>C:p.S76P, c.805C>G:p.H269D | CI |
| Pt598 | *NDUFAF6* (NM_152416) | c.206A>T:p.D69V, c.371T>C:p.I124T | CI |
| Pt866 | *NDUFAF6* (NM_152416) | c.371T>C:p.I124T, c.805C>G:p.H269D | CI |
| Pt711 | *NDUFS4* (NM_002495) | c.340T>C:p.W114R, c.340T>C:p.W114R | CI |
| Pt1087 | *NDUFS6* (NM_004553) | c.309+5G>A, c.343T>C:p.C115R | CC(I,IV) |
| Pt1177 | *NDUFV2* (NM_021074) | c.427C>T:p.R143X, c.580G>A:p.E194K | CI |
| Pt628 | *SCO2* (NM_001169109) | c.577G>A:p.G193S, c.773T>C:p.M258T | CC(I,IV) |
| Pt751 | *GTPBP3* (NM_032620) | c.8G>T:p.R3L, c.923-947del:p.E309Rfs*8 | CC(I,IV) |
| Pt156 | *SLC19A3* (NM_025243) | c.372C>G:p.Y124X, c.265A>C:p.S89R | ns |

CI, isolated complex I deficiency; CIV, isolated complex IV deficiency; CC, combined complex deficiency; VUS, variant of unknown significance; ns, not significant.

別表 4　臨床的な特徴

| | Defect and mut | Mut only | Defect only | No defect, no validated mut | Total |
|---|---|---|---|---|---|
| N | 41 | 12 | 34 | 19 | 106 |
| Leigh-like | 6 | 4 | 10 | 4 | 24 |
| Living* | 71%(20/28) | 78%(7/9) | 62%(16/26) | 83%(10/12) | 71%(53/75) |
| Age of living patients** (median (range)) | 9y (3y-17y) | 8y (3y-15y) | 9.5y (3y-38y) | 8.5y (6y-20y) | 8y (3y-38y) |
| Age at onset (median (range)) | 10.5m (0m-8y) | 9m (0m-5y) | 5.5m (0m-6y) | 10m (0m-2y) | 9m (0m-8y) |
| Neonatal onset | 2(5%) | 2(17%) | 7(21%) | 1(5%) | 12(11%) |
| Seizure | 20% | 33% | 41% | 42% | 32% |
| Involuntary movement | 10% | 25% | 18% | 16% | 15% |
| Hypotonia | 24% | 42% | 9% | 32% | 23% |
| Nystagmus/ Ophthalmoplegia | 17% | 33% | 26% | 11% | 21% |
| Dysphagia | 10% | 17% | 29% | 32% | 21% |
| Respiratory distress | 24% | 17% | 41% | 37% | 31% |
| Serum L/P(mean±SD) | 26.4±16.4 | 22.9±14.9 | 21.4±9.9 | 18.2±5.7 | 23.0±13.2 |
| (number of data) | 36 | 10 | 27 | 16 | 89 |
| CSF L/P(mean±SD) | 27.2±28.0 | 20.7±4.5 | 25.0±9.6 | 18.7±7.1 | 24.1±18.9 |
| (number of data) | 30 | 9 | 20 | 14 | 73 |

*,**As of Nov 2016. Mut, mutations in mitochondrial and nuclear DNA; L/P, lactate to pyruvate ratio; CSF, cerebrospinal fluid.

別表 5 酵素活性正常例における酸素消費量

| Patient | Enzyme analysis | MRR(%) | Mut |
|---|---|---|---|
| Pt139 | ns (Fb) | 136 | |
| Pt156 | ns (Fb) | 69 | *SLC19A3* |
| Pt161 | ns (Fb) | 36 | |
| Pt207 | ns (M, Fb) | 90 | |
| Pt216 | ns (M, Fb) | 94 | |
| Pt394 | ns (Fb) | 94 | |
| Pt430 | ns (M, Fb) | 62 | |
| Pt536 | ns (M, Fb) | 96 | *ECHS1* |
| Pt545 | ns (M, Fb) | 53 | |
| Pt668 | ns (M, Fb) | 62 | |
| Pt696 | ns (Fb) | 127 | |
| Pt701 | ns (Fb) | 61 | |
| Pt703 | ns (M, Fb) | 81 | |
| Pt794 | ns (Fb) | 48 | |
| Pt822 | ns (M, Fb) | 108 | |
| Pt840 | ns (Fb) | 43 | |
| Pt1038 | ns (Fb) | 51 | *ECHS1* |
| Pt1065 | ns (M, Fb) | 78 | |
| Pt1120 | ns (Fb) | 51 | |

MRR reduction to <71.6% of normal control value was considered to indicate mitochondrial impairment. MRR, maximal respiration rate; Mut, mutations in mitochondrial or nuclear DNA; ns, not significant; F, fibroblast; M, skeletal muscle.