Table 2. Frequency of RANTES −403/−28/In1.1 haplotypes in infants with severe respiratory syncytial virus (RSV) bronchiolitis and control subjects

Haplotype of RANTES −403/−28/In1.1		Infants with severe RSV bronchiolitis (118 Alleles)	Control subject (402 Alleles)	P[1]
H1	G-C-T	0.719	0.619	0.0443
H2	A-C-C	0.161	0.214	0.2081
H3	A-G-C	0.067	0.154	0.0156*

[1]: Significant P value after Bonferroni correction for three haplotypes is 0.0167 (0.05/3).
*Statistically significant.

significantly lower in patients than in control subjects, suggesting that these RANTES polymorphisms are associated with the risk of developing RSV bronchiolitis. The present results differ from those of several previous studies (12,20,21), and the reasons for these differences are not clear but may include the following. First, the patients in the present study may differ from those in other studies. We applied strict selection criteria for RSV bronchiolitis; in order to exclude preexisting asthma as much as possible, we recruited only patients who had experienced their first wheezing episode during the RSV infection. Second, our case-control data were adjusted for risk factors (sex, birth weight, the presence of older siblings, breast-feeding, day care attendance during infancy, and parental smoking during infancy).

To clarify the functional significance of the SNPs in RANTES, several studies have compared the transcriptional activity of different alleles by luciferase assay (19,20,22,23). Taking into consideration our results and those of previous studies, the relationship between RSV bronchiolitis and RANTES polymorphisms may be as follows. If we take into account only the RANTES promoter region, −403A reported by Tian et al. (20) and −28G reported by Liu et al. (23) are associated with increased promoter activity. Because our patients showed a lower frequency of −403A and −28G, we can speculate that individuals with higher RANTES production may be less susceptible to severe RSV bronchiolitis. In fact, there are a few studies that agree with this hypothesis (24,25).

If we also take the intron sequence into consideration, based on the results of An et al. (19) and Tian et al. (20), the A-C-T and A-G-T haplotypes are thought to be associated with higher promoter activity than the other haplotypes. However, the estimated frequencies of A-C-T and A-G-T haplotypes were very low in our subjects; therefore, any relationship between RSV bronchiolitis and these two haplotypes can be neglected. In our study, the frequency of the C allele (lower transcriptional activity) in In1.1T/C was lower in patients than in control subjects. In other words, the frequencies of the A-G-C and A-C-C haplotypes, which correspond to lower transcriptional activity, were lower in patients than in control subjects. This suggested that individuals with lower RANTES production might be less susceptible to severe RSV bronchiolitis. There are many studies that are compatible with this hypothesis (26–28). It is generally accepted that RANTES recruits memory T cells, monocytes, eosinophils, and basophils and is implicated in airway inflammation (29,30). Considering these data

and the expression data of An et al. (19), our results support the notion that individuals with higher RANTES expression are more susceptible to severe RSV bronchiolitis. However, there are few reports focusing on the relationship between disease severity and RANTES concentration in airway tissues (25). In addition to RANTES expression, there are other factors that are important in the pathophysiology of severe RSV bronchiolitis, including the antigenicity of RSV and host immune conditions, among others (31). A study evaluating the relative importance of RANTES expression and the interaction between RANTES expression and the aforementioned factors would be an interesting next step.

In conclusion, our results show an association of RANTES gene polymorphisms with risk for severe RSV bronchiolitis. Because the high-risk alleles in this study differ from those in previous studies, further analyses are needed to clarify the relationship between RANTES polymorphisms and RSV bronchiolitis.

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Original Article

Neonatal lactic acidosis with methylmalonic aciduria due to novel mutations in the *SUCLG1* gene

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Abstract   *Background*: Succinyl-coenzyme A ligase (SUCL) is a mitochondrial enzyme that catalyses the reversible conversion of succinyl-coenzyme A to succinate. SUCL consists of an α subunit, encoded by *SUCLG1*, and a β subunit, encoded by either *SUCLA2* or *SUCLG2*. Recently, mutations in *SUCLG1* or *SUCLA2* have been identified in patients with infantile lactic acidosis showing elevated urinary excretion of methylmalonate, mitochondrial respiratory chain (MRC) deficiency, and mitochondrial DNA depletion.
Methods: Case description of a Japanese female patient who manifested a neonatal-onset lactic acidosis with urinary excretion of methylmalonic acid. Enzymatic analyses (MRC enzyme assay and Western blotting) and direct sequencing analysis of *SUCLA2* and *SUCLG1* were performed.
Results: MRC enzyme assay and Western blotting showed that MRC complex I was deficient. *SUCLG1* mutation analysis showed that the patient was a compound heterozygote for disease-causing mutations (p.M14T and p.S200F).
Conclusion: For patients showing neonatal lactic acidosis and prolonged mild methylmalonic aciduria, MRC activities and mutations of *SUCLG1* or *SUCLA2* should be screened for.

Key words   lactic acidosis, methylmalonic acid, mitochondrial respiratory chain, *SUCLA2*, *SUCLG1*.

Urinary excretion of methylmalonic acid is caused by a defect in the isomerization of L-methylmalonyl-coenzyme A to succinyl-coenzyme A. The reaction is catalyzed by L-methylmalonyl-coenzyme A mutase (MCM), an enzyme that requires adenosylcobalamin as a cofactor.[1] Methylmalonic acidemia/aciduria is mainly classified into two types: one resulting from a defect in the MCM apoenzyme and another resulting from a defect in the steps leading to adenosylcobalamin synthesis. In some cases, other causes of methylmalonic acidemia/aciduria have been reported. Recently, deficiency of the succinyl-coenzyme A ligase (SUCL) has been reported in cases of infantile lactic acidosis with mild urinary excretion of methylmalonic acid.[2]

Succinyl-coenzyme A ligase is a mitochondrial enzyme associated with the Krebs cycle, catalyzing the reversible conversion of succinyl-coenzyme A to succinate. The enzyme consists of two subunits. The substrate specificity for guanosine diphosphate (GDP) or adenosine diphosphate (ADP) is determined by the β subunit. The α subunit is encoded by the *SUCLG1* gene, whereas the β subunit is encoded by *SUCLA2* for the ADP-specific

subunit and by *SUCLG2* for the GDP-specific subunit. *SUCLG1* is ubiquitously expressed, but its expression is particularly high in the heart, brain, kidney, and liver. The SUCLA2 protein is primarily present in the brain, skeletal muscle, and heart, and the SUCLG2 protein is present in the liver and kidney. More than 20 cases of deficiency in the α subunit (mutation in *SUCLG1*) or ADP-forming β subunit (mutation in *SUCLA2*) have been reported.[3–5] These patients have mitochondrial respiratory chain (MRC) deficiency, mitochondrial DNA (mtDNA) depletion, encephalomyopathy, and mild methylmalonic aciduria.[6–9]

Here, we describe the case of a Japanese female patient who presented with neonatal-onset lactic acidosis with urinary excretion of methylmalonic acid. *SUCLG1* mutation analysis showed that the patient was a compound heterozygote for disease-causing mutations.

Case report

In 1993 a female infant was born at 38 weeks gestation (birth-weight, 2640 g; birth length, 47.3 cm). Her Apgar scores were normal. On the day after birth, she developed problems. Her blood sugar was lower than 1.1 mmol/L, and hence, continuous glucose infusion was started. Mechanical ventilation and peritoneal dialysis were started when the infant was 2 days old because of cyanosis, severe metabolic acidosis (pH, 6.638, base excess,

Table 1 Laboratory data

	2 days	4 days	4 months
WBC (/μL)	46 500	19 400	
RBC (×10⁶/μL)	4.50	4.59	
Hb (g/dL)	18.0	18.0	
Ht (%)	59.0	51.5	
Plt (×10³/μL)		105	
Total bilirubin (mg/dL)		8.8	
γ-GTP (IU/L)		136	
AST (IU/L)	607	217	
ALT (IU/L)	125	128	
LDH (IU/L)	5400	3860	
CK (IU/L)		6370	
CK-MB (IU/L)		216	
Na (mBq/L)		140	
K (mBq/L)		3.0	
TP (mg/dL)		4.7	
BUN (mg/dL)	23	24	
Cr (mg/dL)		1.2	
pH	6.638	7.477	
HCO₃⁻ (mEq/L)		14.5	
Base excess	−26.8	−4.9	
NH₃ (mmol/L)	191	45	
Lactate (mmol/L)	11	8.1	
Pyruvate (mmol/L)		0.41	
BS (mg/dL)		101	
Urine (organic acids excretion)		High, lactate, pyruvate; Moderate, methylmalonate, methylcitrate; Slight, glutarate, fumarate, succinate, 3-methylglutaconate	
Acylcarnitine (dried blood spots)			increase in C3 and C4DC
Methylmalonic acid (serum)			13 μmol/L (control, not detected MCM-deficient patients, 220–2900)
Methylmalonic acid (urine)			321 mmol/molCr (control, mean [SD], 2.0 [1.2])
¹⁴C-propionate fixation (cultured fibroblasts)			8% of control

ALT, alanine aminotransferase; AST. aspartate aminotransferase; BS, blood sugar; BUN, blood urea nitrogen; CK, creatine kinase; γ-GTP, γ-glutamyltransferase; Hb, hemoglobin; Ht, hematocrit; LDH, lactate dehydrogenase; MCM, L-methylmalonyl-coenzyme A mutase; Plt, platelets; RBC, red blood cells; TP, total protein; WBC, white blood cells.

−26.8), lactic acidemia (11 mmol/L), and hyperammonemia (191 μmol/L; Table 1). She was transferred to Tohoku University Hospital at 4 days old.

Upon admission there was a swelling in the liver 4 cm below the costal margin. The lactate and pyruvate levels were 8.1 mmol/L and 0.41 mmol/L, respectively (L/P ratio, 20). Gas chromatography and mass spectrometry of urinary organic acid showed high levels of lactate and pyruvate excretion; moderate methylmalonate and methylcitrate excretion; and slight glutarate, fumarate, succinate, and 3-methylglutaconate excretion.

Acidosis improved on the following day, and mechanical ventilation and peritoneal dialysis were stopped. She developed prolonged hypotonia. At 1 month of age, auditory brainstem response was absent, and severe hearing impairment was noted. Head computed tomography showed diffuse atrophy. At 4 months of age, mild cardiac hypertrophy was seen on echocardiogram. The patient could not balance her head.

Lactic acidemia (4–9 mmol/L) with an elevated L/P ratio (20–25) and mild urinary excretion of methylmalonic acid persisted.

An acylcarnitine profile of dried blood spots showed an increase in C3 (propionylcarnitine) and C4DC (isomers of methylmalonyl carnitine and succinylcarnitine). The serum level of methylmalonic acid was 13 μmol/L (control, not detected; MCM-deficient patients, 220–2900 μmol/L). The urinary levels of methylmalonic acid and methylcitrate were 321 mmol/molCr and 81.7 mmol/molCr, respectively (control, mean ± SD, 2.0 ± 1.2 mmol/molCr and 2.0 ± 0.9 mmol/molCr, respectively). A ¹⁴C-propionate fixation assay using cultured fibroblasts showed that propionate fixation in the patient was 8% of that in the control. Enzymatic analyses of the pyruvate dehydrogenase complex and pyruvate carboxylase were normal.

Histology of a liver biopsy specimen indicated moderate macrovesicular and microvesicular steatosis in the hepatic parenchyma. There was no active inflammation or fibrosis. On electron microscopy hepatocytes containing lipid droplets were seen. Mitochondrial abnormalities and other specific findings were not apparent morphologically. Muscle biopsy samples were stained with hematoxylin and eosin, reduced nicotinamide adenine

dinucleotide tetrazolium reductase, modified Gomori-Trichrome, succinate dehydrogenase, periodic acid–Schiff, and cytochrome oxidase. No particular abnormalities were noted in the muscle biopsy specimens.

At 6 months of age, the patient was discharged from hospital. She was able to follow objects with her eyes. Because of feeding difficulty, a naso-gastric tube was used. She developed a social smile at 13 months of age but did not have head control. At 20 months of age, she suddenly died at home. Autopsy was not performed.

Because her clinical course was similar to that of previously reported SUCL-deficient patients,[3,4] we restarted diagnostic analysis using fibroblasts and biopsied muscle samples that had been stored for 16 years in liquid nitrogen.

Methods

Blue native polyacrylamide gel electrophoresis and Western blotting

Expression levels of the MRC complex (Co) I, II, III, and IV proteins in cultured fibroblasts were assessed on Western blotting using blue native polyacrylamide gel electrophoresis (BN-PAGE) according to previously described methods.[10] Immunostaining was performed using monoclonal antibodies specific for the 39 kD subunit of Co I, 70 kD subunit of Co II, core 1 subunit of Co III, and subunit 1 of Co IV (Invitrogen, Camarillo, CA, USA).

Determination of enzyme activities

Activities of MRC Co I, II, III, and IV were assayed.[10] The activity of each complex was presented as a percentage of the mean value obtained from 20 controls. The percentages of Co I, II, III, and IV activities relative to that of citrate synthase (CS) as a mitochondrial enzyme marker or Co II activity were calculated. Deficiency of each complex is confirmed when either the CS ratio and/or the Co II ratio is <45% (fibroblasts) or 35% (muscle).

Quantitative polymerase chain reaction

The mtDNA was quantitatively estimated on real-time amplification of ND1 fragments in the mtDNA genome, as described previously.[10] To determine the overall abundance of mtDNA, the real-time amplification result of ND1 was compared with that of exon 24 of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, as nuclear DNA (nDNA).

Direct sequencing of the SUCLG1 and SUCLA2 genes

Genomic DNA was extracted from cultured fibroblasts using a Sepa Gene Kit (Sanko Junyaku, Tokyo, Japan). All coding exons, including flanking introns, in SUCLG1 and the SUCLA2 genes were amplified using polymerase chain reaction (PCR). To facilitate cycle sequencing analysis, M13 universal and reverse primer sequences were attached to the 5′ ends of sense primers and antisense primers, respectively. PCR products were directly sequenced using a Big Dye Primer Cycle Sequencing kit and an ABI 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA).

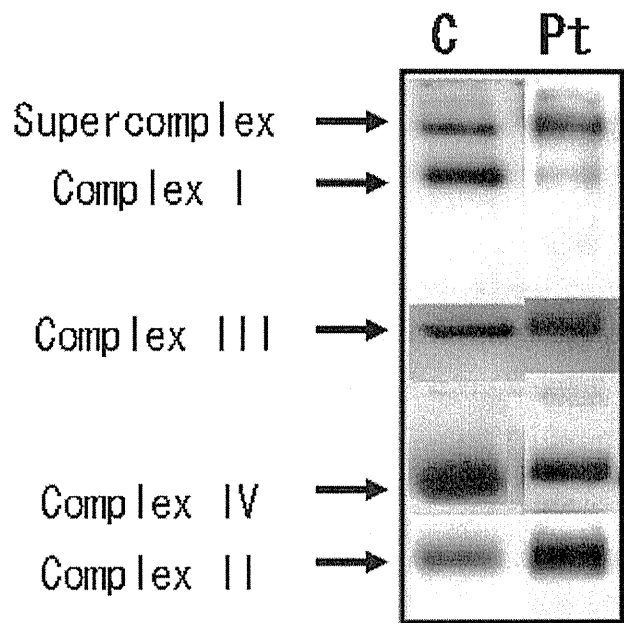The Ethics Committee of the Tohoku University School of Medicine approved the present study.

Fig. 1 Blue native polyacrylamide gel electrophoresis and subsequent Western blot analysis of mitochondrial respiratory chain complexes. The amount of assembled complex I was decreased. Complex I, anti-39 kDa subunit; complex II, anti-70 kDa subunit; complex III, anti-core 1 subunit; complex IV, anti-subunit 1.

Results

The amount of respiratory-chain complex in fibroblasts was determined on BN-PAGE Western blot. The intensity of the band corresponding to the assembled Co I of fibroblasts was decreased (Fig. 1). The intensity of the bands corresponding to Co II, III, and IV remained unchanged.

In fibroblasts, the enzyme activities of Co I and Co IV relative to that of Co II were decreased (<45%; Table 2). Even in the muscle biopsy samples, the ratios of (Co II + Co III)/CS, Co IV/CS, Co I/Co II, (Co II + Co III)/Co II, Co III/Co II, and CoIV/Co II were decreased.

Quantitative PCR showed that the ratio of mtDNA/nDNA of the fibroblasts did not decrease (72.9%; control, 76.4%). The ratio in the muscle biopsy specimen was also not decreased (270.1%).

Mutation analysis showed a heterozygous T-to-C substitution at position 41 in exon 1 of SUCLG1 (c.41T > C; Fig. 2). This c.41T > C mutation changes the Met at position 14 to a Thr (p.M14T). Additionally, in exon 6, a heterozygous C-to-T substitution at position 599 in exon 1 of SUCLG1 was found (c.599C > T). This mutation changes the Ser at position 200 to Phe (p.S200F). The p.M14T mutation was transmitted to the child from her mother; the other mutation (p.S200F) was transmitted to the child from her father (data not shown). Both substitutions were absent in the 100 alleles screened from healthy volunteers. No substitution was found in SUCLA2.

Table 2 Respiratory chain enzyme assay of the present patient

%	Co I	Co II	Co II + III	Co III	Co IV	CS
Fibroblasts						
% of normal	73	236	378	140	60	71
CS ratio	100	326	515	190	85	–
Co II ratio	**30**	–	158	58	**26**	–
Muscle						
% of normal	89	291	40	76	17	197
CS ratio	44	147	**20**	39	**8**	–
Co II ratio	**30**	–	**13**	**26**	**6**	–

Enzyme activities are expressed as % of mean normal control activity relative to protein, relative to CS, and relative to Co II. **Bold**, deficiency of the respective complex: <45% (fibroblasts) or 35% (muscle) of either CS ratio and/or Co II ratio. Reference range, fibroblasts 45–170; muscle 35–160.

Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthase.

Discussion

The patient was identified to have a compound heterozygote mutation in *SUCLG1* (p.M14T and p.S200F). Clinical manifestations such as infantile lactic acidosis, mild methylmalonic aciduria, hypotonia, and hearing loss were compatible with symptoms previously reported in patients with *SUCLG1* or *SUCLA2* mutations.[3,6] The p.M14T and p.S200F mutations have not been reported previously. These substitutions were not found in the 100 alleles from healthy volunteers. p.M14 is located within the mitochondrial targeting sequence. Van Hove *et al.*

reported a patient with a mutation at the same methionine (p.M14L) and speculated that the substitution of p.M14 would prevent proper translation initiation.[11] p.S200 is conserved across several species (Fig. 2). These data suggest that p.M14T and p.S200F are not polymorphisms but disease-causing mutations.

The amount of MRC complex I was decreased on BN-PAGE and Western blotting using fibroblasts, and multiple MRC defects were detected on enzyme assay. The ratios of mtDNA/nDNA of fibroblasts and muscle, however, did not decrease. Valayannopoulos *et al.* also reported that mtDNA depletion was not observed in two patients.[6] It is suggested that not all SUCL-deficient

(a)

(b)

(c)

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Homo sapiens (human)                      CKIGIMPGHIHKKGRIGIV S RSGTLTYEAVHQTTQVGLGQSLCVGIGGDPFNGTDFIDCL
Pan troglodytes (chimpanzee)              CKIGIMPGHIHKKGRIGIV S RSGTLTYEAVHQTTQVGLGQSLCVGIGGDPFNGTDFTDCL
Canis familiaris (dog)                    CKIGIMPGHIHKKGRIGIV S RSGTLTYEAVHQTTQVGLGQSLCVGIGGDPFNGTDFIDCL
Bos taurus (cow)                          CKIGIMPGHIHKKGRVGIV S RSGTLTYEAVHQTTQVGLGQSLCVGIGGDPFNGTDFTDCL
Mus musculus (mouse)                      CKIGIMPGHIHKKGRIGIV S RSGTLTYEAVHQTTQVGLGQSLCIGIGGDPFNGTDFIDCL
Rattus norvegicus (rat)                   CKIGIMPGHIHKKGRIGIV S RSGTLTYEAVHQTTQVGLGQSLCIGIGGDPFNGTNFIDCL
Gallus gallus (chicken)                   CKIGIMPGHIHKKGRIGIV S RSGTLTYEAVHQTTQVGLGQSFCVGIGGDPFNGTNFIDCL
Xenopus tropicalis (western clawed frog)  CKIGIMPGHIHKKGRIGIV S RSGTLTYEAVHQTTQVGLGQSLCVGIGGDPFNGTNFIDCL
Danio rerio (zebrafish)                   CKIGIMPGHIHKKGRIGIV S RSGTLTYEAVHQTTQVGLGQSLCIGIGGDPFNGTNFIDCL
                                          *****************.*** * *********************.*.*********.*.***
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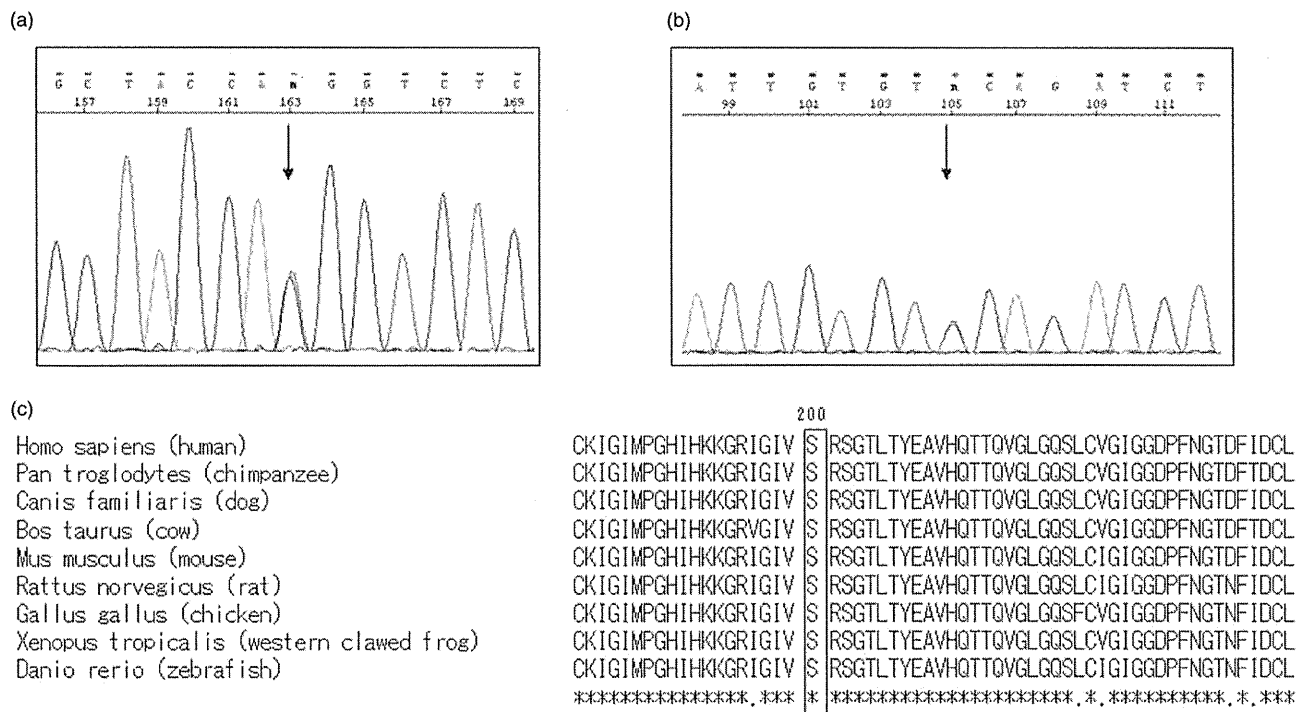
Fig. 2 (a) Heterozygous T-to-C substitution detected at c.41 in exon 1 of *SUCLG1*. This c.41T > C mutation changes the Met at position 14 to Thr (p.M14T). (b) Heterozygous C-to-T substitution detected at c.599 in exon 6 of *SUCLG1*. The c.599C > T substitution changes the Ser at position 200 to Phe (p.S200F). (c) Comparison of succinyl-coenzyme A ligase (SUCL) α subunits from several species. Serine at p.200 was conserved across all the species tested.

patients have mtDNA depletion, and that some mechanisms other than mtDNA depletion might participate in the multiple MRC deficiency observed in these patients.

In the present case, serum methylmalonic acid accumulation and low ^{14}C-propionate fixation capacity suggested disturbance of methylmalonic acid metabolism. Elevated methylmalonic acid may result from the accumulation of succinyl-coenzyme A under the assumption that accumulated succinyl-CoA inhibits the reaction catalyzed by MCM or causes an equilibrium shift, leading to the accumulation of methylmalonyl-coenzyme A, which is converted to methylmalonic acid. As usual, increased levels of C4DC are detected in patients with severe MCM deficiency during acute crises. It is suggested that the C4DC of the present patient was associated with an increased level of succinylcanritine due to accumulated succinyl-coenzyme A.

In conclusion, we identified two novel *SUCLG1* mutations in a Japanese female patient with neonatal lactic acidosis and prolonged mild methylmalonic aciduria. For patients showing these combined manifestations, MRC activities and mutations of *SUCLG1* or *SUCLA2* should be screened for.

Acknowledgments

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