Japanese type 1 pediatric population in the near future.

In conclusion, the GA/A1C-SI ratio represented the G-gap better than did the GA/A1C-% ratio. We propose that mean GA/A1C-SI ratios obtained individually over time can be used as reference values in Japanese children with type 1 diabetes; 6.75 ± 0.60 (6.73: 6.31–7.17), means ± SD (median; interquartile range), easily obtainable in regular medical practice. The association of the GA/A1C-SI ratio with diabetic complications remains to be investigated in comparison with other study populations of various races, genders, ages, and disease types.

Acknowledgments

This study was supported by the Japan Diabetes Foundation.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

References

1. Diabetes Control and Complications Trial Research Group (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Eng J Med* 329: 977-986.
2. White NH, Cleary PA, Dahms W, Goldstein D, Malone J, et al. (2001) Beneficial effects of intensive therapy of diabetes during adolescence: outcomes after the conclusion of the Diabetes Control and Complications Trial (DCCT). *J Pediatr* 139: 804-812.
3. McCarter RJ, Hempe JM, Gomez R, Chalew SA (2004) Biological variation in HbA1c predicts risk of retinopathy and nephropathy in type 1 diabetes. *Diabetes Care* 27: 1259-1264.
4. Chalew SA, McCarter RJ, Hempe JM (2013) Biological Variation and Hemoglobin A1c: Relevance to Diabetes Management and Complications. *Pediatr Diabetes* 14: 391-398.
5. Cohen RM, Holmes YR, Chenier TC, Joiner CH (2003) Discordance between HbA1c and fructosamine: evidence for a glycosylation gap and its relation to diabetic nephropathy. *Diabetes Care* 26: 163-167.
6. Nayak AU, Holland MR, Macdonald DR, Nevill A, Singh BM (2011) Evidence for consistency of the glycation gap in diabetes. *Diabetes Care* 34: 1712-1716.
7. Rodríguez-Segade S, Rodríguez J, Cabezas-Agricola JM, Casanueva FF, Camiña F (2011) Progression of nephropathy in type 2 diabetes: the glycation gap is a significant predictor after adjustment for glycohemoglobin (Hb A1c). *Clin Chem* 57: 264-271.
8. Sacks DB, Nathan DM, Lachin JM (2011) Gaps in the glycation gap hypothesis. *Clin Chem* 57: 150-152.
9. Weykamp C, John WG, Mosca A, Hoshino T, Little R, et al. (2008) The IFCC Reference Measurement System for HbA1c: a 6-year progress report. *Clin Chem* 54: 240-248.
10. Sacks DB (2012) Measurement of hemoglobin A(1c): a new twist on the path to harmony. *Diabetes Care* 35: 2674-2680.
11. Hoelzel W, Weykamp C, Jeppsson JO, Miedema K, Barr JR, et al.(2004) IFCC reference system for measurement of hemoglobin A1c in human blood and the national standardization schemes in the United States, Japan, and Sweden: a method-comparison study. *Clin Chem* 50: 166-174.
12. Kaiser P, Akerboom T, Ohlendorf R, Reinauer H (2010) Liquid Chromatography-Isotope Dilution–Mass Spectrometry as a New Basis for the Reference Measurement Procedure for Hemoglobin A1c Determination. *Clin Chem* 56: 750-754.
13. Lorentz K (1998) Approved recommendation on IFCC methods for the measurement of catalytic concentration of enzymes. Part 9. IFCC method for alpha-amylase (1,4-alpha-D-glucan 4-glucanohydrolase, EC 3.2.1.1). International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). Committee on Enzymes. *Clin Chem Lab Med* 36: 185-203.
14. Koga M, Kasayama S (2010) Clinical impact of glycated albumin as another glycemic control marker. *Endocr J* 57: 751-762.
15. Selvin E, Francis LM, Ballantyne CM, Hoogeveen RC, Coresh J, et al.(2011) Nontraditional markers of glycemia: associations with microvascular conditions. *Diabetes Care* 34: 960-967.
16. Nathan DM, McGee P, Steffes MW, Lachin JM and the DCCT/EDIC Research Group (2014) Relationship of gycated albumin to blood glucose and glycated hemoglobin (HbA1c) values and to retinopathy, nephropathy and cardiovascular outcomes in the DCCT/EDIC study. *Diabetes* 63: 282-290.
17. Selvin E, Rawlings AM, Grams M, Klein R, Sharrett AR, et al. (2014) Fructosamine and glycated albumin for risk stratification and prediction of incident diabetes and microvascular complications: a prospective cohort analysis of the Atherosclerosis Risk in Communities (ARIC) study. *Lancet Diabetes Endocrinol* 2: 279-288.
18. Jones IR, Owens DR, Williams S, Ryder RE, Birtwell AJ, et al. (1983) Glycosylated serum albumin: an intermediate index of diabetic control. *Diabetes Care* 6: 501-503.
19. Koga M, Murai J, Saito H, Kasayama S (2010) Glycated albumin and glycated hemoglobin are influenced differently by endogenous insulin secretion in patients with type 2 diabetes. *Diabetes Care* 33: 270-272.
20. Koga M, Matsumoto S, Saito H, Kasayama S (2006) Body mass index negatively influences glycated albumin, but not glycated hemoglobin, in diabetic patients. *Endocr J* 53: 387-391.
21. Imai T, Oikawa Y, Shimada A (2007) Improved monitoring of the hyperglycemic state in type 1 diabetes patients by use of the glycoalbumin/HbA1c ratio. *Rev Diabet Stud* 4: 44-48.
22. Yoshiuchi K, Matsuhisa M, Katakami N, Nakatani Y, Sakamoto K, et al.(2008) Glycated albumin is a better indicator for glucose excursion than glycated hemoglobin in type 1 and type 2 diabetes. *Endocr J* 55: 503-507.
23. Koga M, Otsuki M, Matsumoto S, Saito H, Mukai M, et al. (2007) Negative association of obesity and its related chronic inflammation with serum glycated albumin but not glycated hemoglobin levels. *Clin Chim Acta* 378: 48-52.
24. Nishimura R, Kanda A, Sano H, Matsudaira T, Miyashita Y, et al. (2006) Glycated albumin is low in obese, non-diabetic children. *Diabetes Res Clin Pract* 71: 334-338.

25. Rodríguez-Segade S, Rodríguez J, García Lopez JM, Casanueva FF, Camiña F (2012) Estimation of the glycation gap in diabetic patients with stable glycemic control. *Diabetes Care* 35: 2447-2450.
26. Kato N, Takimoto H, Sudo N (2011) The cubic functions for spline smoothed L, S and M values for BMI reference data of Japanese children. *Clin Pediatr Endocrinol* 20: 47-49.
27. Kouzuma T, Uemastu Y, Usami T, Imamura S (2004) Study of glycated amino acid elimination reaction for an improved enzymatic glycated albumin measurement method. *Clin Chim Acta* 346: 135-143.
28. Kohzuma T, Koga M (2010) Lucica GA-L glycated albumin assay kit: a new diagnostic test for diabetes mellitus. *Mol Diagn Ther* 14: 49-51.
29. Kashiwagi A, Kasuga M, Araki E, Oka Y, Hanafusa T, et al.(2012) International clinical harmonization of glycated hemoglobin in Japan: From Japan Diabetes Society to National Glycohemoglobin Standardization Program values. *Diabetol Int* 3: 8-10.
30. Takei I, Hoshino T, Tominaga M, Nakayama T, Kuwa K, et al. (2008) JSCC recommended method for glycated albumin measurement in serum. *Rynsho Kagaku (Jpn J Clin Chem)* 37: 179-191 (In Japanese).
31. Macdonald DR, Hanson AM, Holland MR, Singh BM (2008) Clinical impact of variability in HbA1c as assessed by simultaneously measuring fructosamine and use of error grid analysis. *Ann Clin Biochem* 45: 421-425.
32. Cohen RM, Herman WH (2014) Are glycated serum proteins ready for prime time? *Lancet Diabetes Endocrinol* 2: 264-265.
33. Tahara Y, Shima K (1995) Kinetics of HbA1c, glycated albumin, and fructosamine and analysis of their weight functions against preceding plasma glucose level. *Diabetes Care* 18: 440-447.
34. Tahara Y, Shima K (1993) The response of GHb to stepwise plasma glucose change over time in diabetic patients. *Diabetes Care* 16: 1313-1314.
35. Saisho Y, Tanaka K, Abe T, Shimada A, Kawai T, et al. (2011) Glycated albumin to glycated hemoglobin ratio reflects postprandial glucose excursion and relates to beta cell function in both type 1 and type 2 diabetes. *Diabet Int* 2:146-153.
36. Ogawa A, Hayashi A, Kishihara E, Yoshino S, Takeuchi A, et al.(2012) New indecies for predicting glycaemic variability. *PLoS One* 7: e46517.
37. Bry L, Chen PC, Sacks DB (2001) Effects of hemoglobin variants and chemically modified derivatives on assays for glycohemoglobin. *Clin Chem* 47: 153-163.
38. Herman WH, Dungan KM, Wolffenbuttel BH, Buse JB, Fahrbach JL, et al.(2009) Racial and ethnic differences in mean plasma glucose, hemoglobin A1c, and 1,5-anhydroglucitol in over 2000 patients with type 2 diabetes. *J Clin Endocrinol Metab* 94: 1689-1694.
39. Rohlfing CL, Wiedmeyer HM, Little RR, England JD, Tennill A, et al.(2002) Defining the relationship between plasma glucose and HbA(1c): analysis of glucose profiles and HbA(1c) in the Diabetes Control and Complications Trial. *Diabetes Care* 25: 275-278.
40. Nathan DM, Kuenen J, Borg R, Zheng H, Schoenfeld D, et al.(2008) Translating the A1C assay into estimated average glucose values. *Diabetes Care* 31: 1473-1478.
41. Diabetes Research in Children Network (DirecNet) Study Group (2008) Relationship of A1C to glucose concentrations in Children with type1 diabetes. *Diabetes Care* 31: 381-385.
42. Hempe JM, Gomez R, McCarter RJ Jr, Chalew SA(2002) High and low hemoglobin glycation phenotypes in type 1 diabetes: a challenge for interpretation of glycemic control. *J Diabetes Complications* 16: 313-320.
43. Soros AA, Chalew SA, McCarter RJ, Shepard R, Hempe JM (2010) Hemoglobin glycation index: a robust measure of hemoglobin A1c bias in pediatric type 1 diabetes patients. *Pediatr Diabetes* 11: 455-461.

Journal of Human Genetics (2015) 60, 167–173

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REVIEW

SPTAN1 encephalopathy: distinct phenotypes and genotypes

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Recent progress in genetic analysis reveals that a significant proportion of cryptogenic epileptic encephalopathies are single-gene disorders. Mutations in numerous genes for early-onset epileptic encephalopathies have been rapidly identified, including in *SPTAN1*, which encodes α-II spectrin. The aim of this review is to delineate *SPTAN1* encephalopathy as a distinct clinical syndrome. To date, a total of seven epileptic patients with four different in-frame *SPTAN1* mutations have been identified. The major clinical features of *SPTAN1* mutations include epileptic encephalopathy with hypsarrhythmia, no visual attention, acquired microcephaly, spastic quadriplegia and severe intellectual disability. Brainstem and cerebellar atrophy and cerebral hypomyelination, as observed by magnetic resonance imaging, are specific hallmarks of this condition. A milder variant is characterized by generalized epilepsy with pontocerebellar atrophy. Only in-frame *SPTAN1* mutations in the last two spectrin repeats in the C-terminal region lead to dominant negative effects and these specific phenotypes. The last two spectrin repeats are required for α/β spectrin heterodimer associations and the mutations can alter heterodimer formation between the two spectrins. From these data we suggest that *SPTAN1* encephalopathy is a distinct clinical syndrome owing to specific *SPTAN1* mutations. It is important that this syndrome is recognized by pediatric neurologists to enable proper diagnostic work-up for patients.
Journal of Human Genetics (2015) **60,** 167–173; doi:10.1038/jhg.2015.5; published online 29 January 2015

INTRODUCTION

Early-onset epileptic encephalopathies (EOEEs) are neurological disorders in children characterized by frequent severe seizures and persistent abnormality of cortical function, which can be documented on electroencephalograms (EEGs). These features lead to impaired neurodevelopmental outcomes during neonatal or early infantile periods and beyond.[1,2] The clinical and EEG characteristics depend on the age at onset and may change over time according to the successive age range.[3] These serious conditions during the first 6 months after birth include early myoclonic encephalopathy, Ohtahara syndrome, West syndrome, migrating partial seizure in infancy and unclassified infantile epileptic encephalopathy. In patients with West syndrome and Ohtahara syndrome, structural brain malformations, inborn errors of metabolism and acquired brain insults are the major underlying causes. After the initial identification of mutations for cryptogenic West syndrome and Ohtahara syndrome, in the genes *ARX*, *CDKL5* and *STXBP1*,[4–8] numerous other mutated genes for West syndrome and Ohtahara syndrome have been found,[9–14] revealing that a significant proportion of cryptogenic EOEEs are single-gene disorders. Recent progress in DNA sequencing technologies has undoubtedly contributed to this progress. This enables the rapid detection of point mutations, and *de novo* mutations can be systematically identified by family based exome sequencing.[15] In the future, comprehensive genetic analysis will come into widespread use in clinics, which will contribute greatly for the further elucidation of EOEE genetics. However, at present, the genetic diagnosis of EOEEs remains challenging; therefore, more detailed studies of EOEEs are needed to establish efficient gene testing based on detailed clinical information of EOEEs.[2,16,17]

In 2010, we found *de novo* in-frame mutations in *SPTAN1* in two patients showing early-onset West syndrome with severe hypomyelination and developmental delay.[18] *SPTAN1* at 9q34.11 consists of 53 exons and encodes α-II spectrin, which is essential for proper myelination in zebrafish.[19] The phenotypes of patients with *SPTAN1* mutations are not well recognized because the number of identified patients is small despite extensive genetic testing of epileptic encephalopathy cases. This review aims to: (1) describe the electroclinical and neuroradiological features of patients with *SPTAN1* mutations; (2) delineate *SPTAN1* encephalopathy as a distinct clinical syndrome with specific genotypes and (3) lead clinicians towards the efficient and appropriate choice of genetic testing.

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Received 22 August 2014; revised 26 December 2014; accepted 6 January 2015; published online 29 January 2015

IDENTIFICATION OF *DE NOVO SPTAN1* MUTATIONS

In 2008, we reported four patients showing early-onset West syndrome with cerebral hypomyelination and reduction of cerebral white matter, as observed by magnetic resonance imaging (MRI).[20] Among these patients, we identified a *de novo* microdeletion at 9q33.3–q34.11 involving *STXBP1* and *SPTAN1* in one patient and *de novo* in-frame mutations in *SPTAN1* in two patients,[18] including an in-frame 3 bp deletion (c.6619_6621 del) leading to p.E2207 del in the continuous helix region between the last two spectrin repeats and an in-frame 6 bp duplication (c.6923_6928 dup, p.R2308_M2309 dup) within the last spectrin repeat. An additional four patients with *de novo* in-frame *SPTAN1* mutations within the last spectrin repeat were subsequently identified in Slovenian,[21] Japanese[22] and Malaysian patients with West syndrome (c.6619_6621 del; p.E2207 del in the Slovenian patient, and c.6908_6916 dup; p.D2303_L2305 dup in two Japanese patients and one Malaysian patient). The Malaysian patient (subject 5) and one Japanese patient (subject 6) are presented here for the first time. Three patients (subjects 3, 4 and 6) were suspected as having *SPTAN1* mutations based on the clinical features consistent with the initial report. Whole-exome sequencing (WES) unexpectedly revealed a *SPTAN1* mutation in subject 5, in whom a *SPTAN1* mutation was never suspected. In addition to West syndrome, Hamdan *et al.*[23] identified an in-frame *de novo SPTAN1* mutation (c.6605_6607 del; p.Q2202 del) within the continuous helix region between the last two spectrin repeats in a patient with generalized epilepsy, intellectual disability (ID) and pontocerebellar atrophy. A total of seven epileptic patients with four different in-frame *SPTAN1* mutations have been identified to date. Interestingly, Hamdan *et al.*[23] also reported a *de novo* p.R566P mutation in a patient with mild nonsyndromic ID without epilepsy. The pathological significance of this missense mutation is unclear because the patient's sister, who has similar clinical features, does not carry the *SPTAN1* mutation.[23] Therefore, in this review, we are focusing on patients with in-frame *SPTAN1* mutations, who showed epileptic encephalopathy or epilepsy.

It is notable that no *SPTAN1* mutations were detected through genetic screening by WES or whole-genome sequencing in epileptic encephalopathy cases[24–27] or in patients with ID.[28] This suggests that patients with *SPTAN1* mutations are quite rare. Alternatively, it is possible that in-frame mutations were not considered as pathogenic in the routine WES workflow. The recognition of clinical and neuroradiological features characteristic of in-frame *SPTAN1* mutations means that such in-frame mutations should not be overlooked when carrying out WES analysis for epileptic encephalopathy.

CLINICAL FEATURES

Among the seven individuals with *SPTAN1* mutations, six patients showed West syndrome symptoms since early infancy and one patient showed generalized epilepsy during childhood. With respect to the six patients with West syndrome, all patients were born at term without asphyxia. Their body weight, height and head circumference at birth were normal, but postnatal microcephaly was noted in all six patients. The range of microcephaly was under the 3rd centile at their respective follow-up ages (Table 1). Three patients showed spastic tetraplegia and the other three showed hypotonia during the neonatal period, which evolved to spastic tetraplegia with generalized hypotonia. All patients were blind, and one patient had coloboma-like optic discs. No apparent abnormality of visceral organs was observed except for atrial septal defect in one patient. At an age of 2 years, they were bedridden and needed tube feeding, except for the youngest patient. All had profound ID and spoke no meaningful words. Two patients passed away at 2 and 3 years of age.

Seizures were characterized by epileptic spasms, which appeared from 3 weeks to 3 months of age. Two patients suffered myoclonic seizures, three patients tonic seizures and one patient asymmetric tonic seizures. In addition, one patient showed neonatal seizures. These seizures were refractory to various antiepileptic and hormonal therapies. In two patients, adrenocorticotrophic hormonal therapy was partially effective, and another patient partially responded to a ketogenic diet.

In contrast, one male patient with generalized epilepsy showed a much milder phenotype. Although he had severe ID and spoke no meaningful words, he could sit alone without support. He did not show postnatal microcephaly. His seizure that developed at 2 years of age consisted of generalized tonic–clonic seizures and absence seizures, which were well controlled by valproic acid.

Detailed clinical information of individuals is summarized in Table 1.

ELECTROENCEPHALOGRAM AND LABORATORY FINDINGS

In all six patients with West syndrome, EEGs showed hypsarrhythmia or modified hypsarrhythmia. EEGs in two patients initially showed multifocal spikes that evolved to hypsarrhythmia. No patient showed a suppression-burst pattern, even though the seizure onset was at early infancy. In contrast, interictal EEG was normal in the one milder variant.

No specific laboratory findings were determined in relation to this disease. Routine hematological and chemical examinations were normal in all patients. Cerebral spinal fluid analysis showed no abnormality, including amino-acid and lactate analysis. Metabolic screening tests were negative, including analysis of plasma amino acids, urine organic acids, and blood lactate and pyruvate. Chromosome analysis of peripheral blood lymphocytes showed normal karyotypes.

NEURORADIOLOGICAL FINDINGS

Initially, we noted that hypomyelination and reduced volume of white matter are characteristic features of this syndrome. By taking account of the neuroradiological findings of additional patients, it turned out that atrophy/hypoplasia of the brainstem and cerebellum is also the hallmark of patients with *SPTAN1* mutations.

All six patients with West syndrome showed severe hypomyelination, hypoplasia of the corpus callosum, cortical atrophy, and atrophy of the brainstem and cerebellum. In addition, five patients showed marked attenuation of white matter volume in combination with hypomyelination, especially in the frontal lobes. These findings were sometimes misdiagnosed as influences of severe hypoxia. Follow-up study in four patients was able to demonstrate that atrophy of the brain was progressive and myelination of the hemispheric white matter had never commenced. No migrational abnormality was observed. The patient with generalized epilepsy showed only atrophy of the brainstem and cerebellum without involvement of the cerebrum. Calcification was not observed by computed tomography.

Characteristic MRI pictures of individuals are presented in Figure 1.

ROLE OF SPECTRINS

Spectrins are considered to be membrane skeletons involved in the stabilization of membrane proteins and activation of membrane channels, receptors and transporters.[29–31] The spectrin repertoire in humans includes two α subunits and five β subunits. Spectrins are long flexible molecules consisting of α and β subunits, which are assembled in an antiparallel side-by-side manner into heterodimers.[29,30] Heterodimers form by end-to-end tetramers

Table 1 Summary of clinical features in seven individuals

Subject	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Sex	F	M	F	M	F	M	M
Nationality	Japanese	Japanese	Slovenian	Japanese	Malaysian	Japanese	Canadian
Mutation	c.6619_6621 del p.E2207 del *De novo*	c.6923_6928 dup p.R2308_M2309 dup *De novo*	c.6619_6621 del p.E2207 del *De novo*	c.6908_6916 dup p.D2303_L2305 dup *De novo*	c.6908_6916 dup p.D2303_L2305 dup *De novo*	c.6908_6916 dup p.D2303_L2305 dup *De novo*	c.6605_6607 del p.Q2202 del *De novo*
Dx	West syndrome	West syndrome	West syndrome	West syndrome	West syndrome	West syndrome	Generalized epilepsy
Age at onset	2 months	3 months	1 month	1 month	3 weeks	1 day	2 years
Initial symptoms	No visual contact	No visual contact	No visual contact	Failure to thrive	Seizure	Seizure, apnea	Seizure
Seizure type	Spasms, tonic sz	Spasms, tonic sz	Myoclonic sz, spasms, tonic sz	Spasms	Spasms	Spasms, myoclonic sz, asymmetric tonic seizure	Generalized tonic–clonic sz, absence
Age at onset of seizure/spasms	3 months	3 months	2 months	3 months	3 weeks	4 months (spasms)	2 years
Initial EEG	Hypsarrhythmia	Hypsarrhythmia	Focal spikes	Hypsarrhythmia	Multifocal spike	NA	Normal
Age at onset hypsarrhythmia	3 months	4 months	4 months	3 months	2 years[a]	4 months	—
Response to therapy	Intractable, daily	Intractable, daily	Intractable, daily	Intractable, daily	Intractable, daily	Intractable, daily	Good
Effective treatment	—	Clobazam partially effective	Topiramate and ketogenic diet partially effective	ACTH partially effective	—	ACTH partially effective	Valproic acid
Development	No head control, no social contact	No head control, no social contact	No head control, no social contact	No head control, no social contact	No head control, no social contact	No head control, no social contact	Sitting, no word
Neurologic examination	Profound ID, spastic quadriplegia	Profound ID, spastic quadriplegia	Profound ID, spastic quadriplegia with hypotonia	Profound ID, spastic quadriplegia, dystonia	Profound ID, spastic quadriplegia with hypotonia, dystonia	Profound ID, spastic quadriplegia with hypotonia	Profound ID, hypotonia
OFC at birth (cm)	31.5 (10th centile)	33 .0 (25–50th centile)	34.0 (25th centile)	31.5 (10th centile)	34.0 (75th centile)	32.4 (10–25th centile)	NA
Postnatal OFC (cm)	46.5 (3rd centile>) at 143 months	42.4 (3rd centile >) at 36months	44.5 (2nd centile>) at 40 months	41.5 (3rd centile>) at 12 months	42.0 (3rd centile>) at 24 months	44.8 (3rd centile>) at 70 months	56.0 (90–97th centile) at 11 years
Others	—	Myocarditis	Coloboma	Atrial septal defect	—	—	—
Magnetic resonance imaging	Cortical atrophy, thin CC, hypomyelination, atrophy of cbll and brainstem (at 8 years old)	Cortical atrophy, thin CC, hypomyelination, atrophy of cbll and brainstem (at 3 years old)	Cortical atrophy, thin CC, hypomyelination, atrophy of cbll and brainstem (at 3 months old)	Cortical atrophy, thin CC, hypomyelination, atrophy of cbll and brainstem (at 2 years old)	Cortical atrophy, thin CC, hypomyelination, atrophy of cbll and brainstem (at 5 months old)	Brain atrophy, thin CC, hypomyelination, atrophy of cbll and brainstem (at 5 years old)	Atrophy of cbll and brainstem (at 11 years old)
Last follow-up age	11 years old	Died at 3 years	3 years old	Died at 2 years	2 years old	6 years old	11 years old
Reference	Saitsu *et al.*[18]	Saitsu *et al.*[18]	Writzl *et al.*[21]	Nonoda *et al.*[22]	This paper	This paper	Hamdan *et al.*[23]

Abbreviations: ACTH, adrenocorticotrphic hormone; cbll, cerebellum; CC, corpus callosum; Dx, diagnosis; EEG, electroencephalogram; F, female; ID, intellectual disability; M, male; NA, not applicable; OFC, occipitofrontal circumference.
[a]Subject 5 recored EEG only twice at 5 months and 2 years of age.

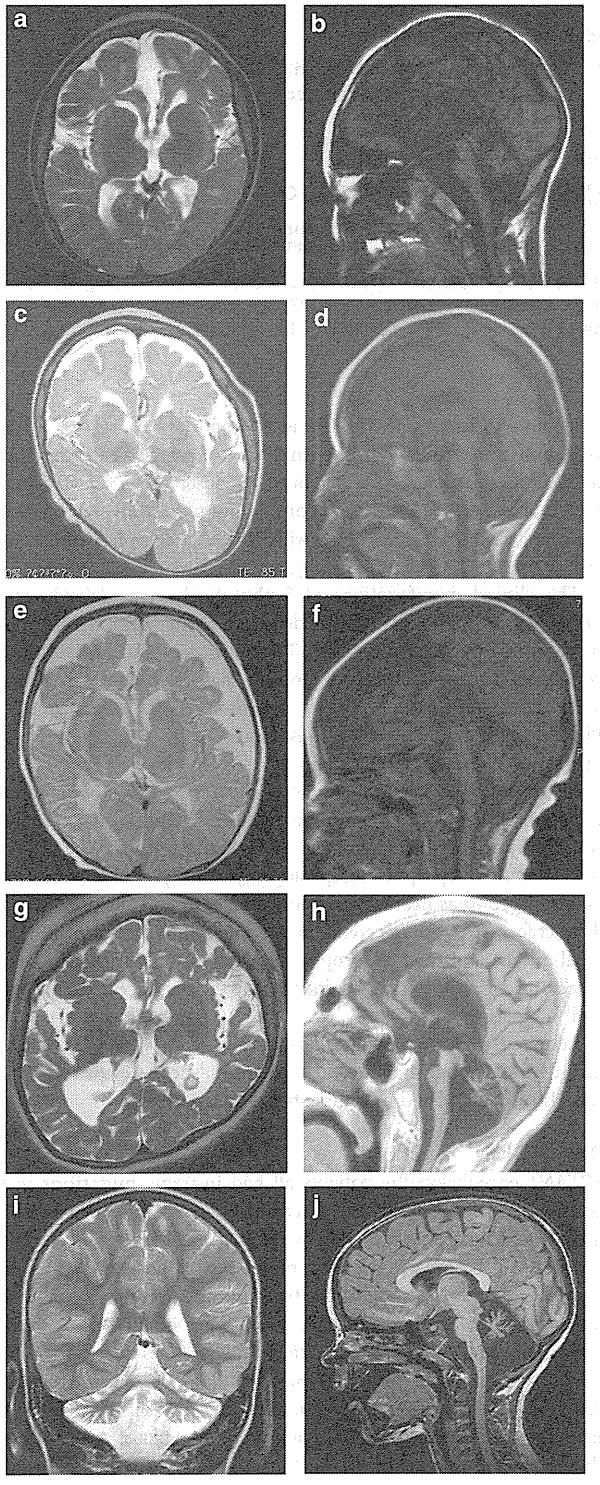

Figure 1 Brain MRI of subjects with *SPTAN1* mutations. T2-weighted axial and T1-weighted mid-sagittal images of subject 1 (**a**, **b**), subject 2 (**c**, **d**), subject 5 (**e**, **f**), subject 6 (**g**, **h**) and T2-weighted coronal and T1-weighted mid-sagittal images of subject 7 (**i**, **j**). Severe hypomyelination, hypoplasia of corpus callosum, cortical atrophy, and atrophy of brainstem and cerebellum are evident in subject 1, 2, 5 and 6. Only atrophy of brainstem and cerebellum are observed in subject 7.[23] Images of **i** and **j** are adapted from ref. 23.

integrating into the membrane cytoskeleton.[29,30] Defects of erythroid α-I and β-I spectrins and neuronal β-III spectrin are associated with hereditary spherocytosis (SPH3 and SPH2 [MIM#270970 and +182870]) and spinocerebellar ataxia type 5 (SCA5 [MIM #600224]), respectively.[29,32,33] α-II spectrin is considered as the major α spectrin expressed in nonerythroid cells, and α-II/β-II spectrin heterodimers are the predominant species in these cells.[29,34] Abnormal node of Ranvier development and destabilization of nascent voltage-gated sodium channel clusters were observed in zebrafish α-II spectrin mutants harboring a nonsense mutation. These mutants also showed impaired myelination in motor nerves and in the dorsal spinal cord, suggesting that α-II spectrin has important roles in the maintenance of the integrity of myelinated axons.[19]

DOMINANT NEGATIVE EFFECT OF *SPTAN1* MUTATIONS

The effect of *SPTAN1* mutations on the function of spectrin is important in elucidating pathogenesis of *SPTAN1* encephalopathy. What is interesting is that all the in-frame mutations found are located at the last two spectrin repeats in the C-terminal region (Figure 2). The last two spectrin repeats are required for α/β spectrin heterodimer associations;[29] therefore, these mutations could alter heterodimer formation between the spectrins. In fact, we previously showed that two mutant α-II spectrins (p.E2207 del and p.R2308_M2309 dup) caused aggregation, predominantly in the cell bodies and axons.[18] Double immunostaining revealed that these aggregations were colocalized with β-II and β-III spectrins, indicating that unstable α-II/β-II and α-II/β-III spectrin heterodimers were involved in the aggregation.[18] In contrast, the p.Q2202 del mutation showed a similar pattern of expression to that of the wild-type α-II spectrin in N2A cells. Furthermore, in primary neuronal cultures, the p.Q2202 del mutation showed a similar aggregation profile, but in a lower proportion of cells.[23] These expression data suggest that the degree of aggregation involving α-II/β-II and α-II/β-III spectrin heterodimers could correlate with the severity of clinical symptoms. It is also notable that two patients (subjects 2 and 4) with a duplication mutation in the last spectrin repeat passed away early in childhood. In contrast to these patients, one patient (subject 1) with a p.E2207 del mutation within the continuous helix region between the last two spectrin repeats has survived longer despite severe motor impairment. In addition, among the four in-frame mutations, the p.Q2202 del mutation, in the patient with generalized epilepsy (milder than West syndrome), is located in the N-terminal region. Therefore, it is possible that the location of in-frame mutations within the last two spectrin repeats may correlate with phenotype severity; C-terminally located mutations cause more severe phenotypes, including West syndrome, and are associated with the characteristic MRI findings. However, analysis of additional patients with *SPTAN1* mutations will be needed to validate this hypothesis.

Whether other types of *SPTAN1* mutation cause epileptic encephalopathy is an interesting issue. Apart from the patients with exonic deletion of *STXBP1*, 13 patients having 9q34.11 microdeletion (where *STXBP1* and *SPTAN1* are located) have been reported in the literature.[8,35–40] Three patients had *STXBP1* deletion but intact *SPTAN1*.[35,36] Among these patients, one patient revealed infantile spasms without infratentorial involvement on MRI, another exhibited nonsyndromic infantile epilepsy with severe ID and the other suffered from moderate ID, which are within the clinical spectrum of *STXBP1* mutations.[35,41,42] Six patients showed deletion of both *SPTAN1* and *STXBP1*,[8,36–39] and clinical features closely resemble those of *STXBP1*-mutated patients with Ohtahara syndrome or West syndrome.[8,37,43] In contrast, three patients had *SPTAN1* deletion but intact *STXBP1*.[36,40]

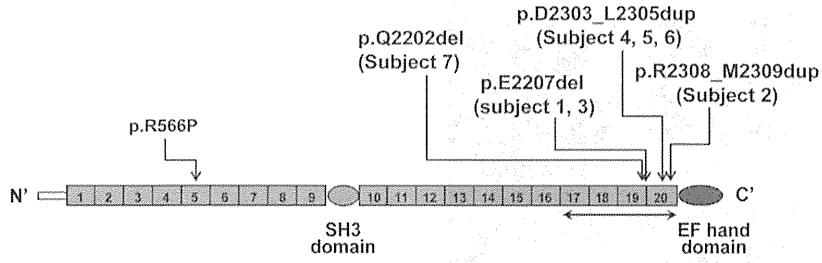

Figure 2 *De novo* mutations identified in *SPTAN1*. Schematic representation of the SPTAN1 protein[23] consisting of 22 domains, including 20 spectrin repeats, an SH3 domain and an EF hand domain. All identified mutations including p.Q2202 del, p.E2207 del, p.D2303_L2305 dup and p.R2308_M2309 dup are shown. The last four spectrin repeats, which are required for α/β heterodimer association, are indicated by a bidirectional arrow. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Clinical phenotypes of these three patients varied and their phenotypic inconsistency might depend on the content of other deleted genes. It is notable that the patient with deletion of only *SPTAN1* had no epileptic encephalopathy.[36,40] In addition, one patient had complete *SPTAN1* deletion with partial deletion of *STXBP1* (exons 16–20), and this patient also showed only profound ID with normal brain MRI.[36]

Collectively, these results suggest that *SPTAN1* haploinsufficiency alone does not cause epileptic encephalopathy or brainstem and cerebellar lesions, and that only in-frame *SPTAN1* mutations at specific positions can cause specific phenotypes (*SPTAN1* encephalopathy) in a dominant negative manner. The p.Q2202 del mutation may cause a milder form of *SPTAN1* encephalopathy.

DIFFERENTIAL DIAGNOSIS

SPTAN1 encephalopathy may be differentiated from other diseases associated with West syndrome and brainstem and cerebellar hypoplasia/atrophy or cerebral hypomyelination by MRI.

Clinical features in *SPTAN1* encephalopathy are mostly shared with progressive encephalopathy with edema, hypsarrhythmia and optic atrophy (PEHO) syndrome.[44] Patients with PEHO syndrome exhibit progressive encephalopathy, microcephaly, edema of the extremities, infantile spasms, severe hypotonia and optic atrophy. Psychomotor development ceases in early infancy and patients show profound mental retardation. Poor or absent visual fixation from the first months of life is also observed. Brain CT or MRI in PEHO syndrome shows progressive generalized atrophy, more in cerebellar and brainstem areas than in supratentorial regions. Underlying causes of PEHO syndrome remain elusive. Patients suspected of PEHO syndrome are good candidates for *SPTAN1* testing.

Pontocerebellar hypoplasia (PCH) is sometimes accompanied by intractable epilepsy. PCH type 6 is caused by recessive mutations in the gene encoding mitochondrial arginyl-tRNA synthetase (*RARS2*). Some PEHO-like features were reported in a nonconsanguineous British female patient with PCH type 6.[45] The patient suffered myoclonic seizures from the neonatal period onwards and her MRI showed generalized hypoplasia, but hypsarrhythmia was absent. Lactate levels in the plasma and cerebrospinal fluid were elevated, implying mitochondrial dysfunction.

Recently, *CASK* aberrations were reported in two patients with Ohtahara syndrome and cerebellar hypoplasia.[46] Both patients showed epileptic encephalopathies with severe cerebellar hypoplasia along with other congenital anomalies. Originally, *CASK* mutations were found in four female patients with X-linked ID, microcephaly and PCH,[47] and their phenotypes expanded to epileptic encephalopathy. In patients with *CASK* mutations, the supratentorial region is not usually involved according to MRI.

Haploinsufficiency of *STXBP1* is an important cause of Ohtahara syndrome and West syndrome. In patients with *STXBP1* mutations, delayed myelination in the cerebrum is observed.[8,43,48] MRI shows that patients with *STXBP1* mutations do not usually involve brainstem and cerebellum, whereas those with *SPTAN1* encephalopathy show brainstem and cerebellum atrophy.

The clinical manifestations of 3-phosphoglycerate dehydrogenase deficiency, which results from a defect of serine biosynthesis, mimic those of *SPTAN1* mutations.[49] This disorder is characterized by congenital microcephaly, profound mental retardation, hypertonia and intractable seizures, and occasional West syndrome. 3-Phosphoglycerate dehydrogenase is diagnosed by amino-acid analysis of plasma and cerebrospinal fluid.

Molybdenum cofactor deficiency, which is due to the combined enzymatic deficiency of xanthine oxidase and sulfite oxidase, presents with epileptic encephalopathy and PCH with retrocerebellar cyst.[50] In this condition, epileptic encephalopathy usually occurs during the neonatal period as neonatal seizures emerge. Brain MRI demonstrates distinct features, such as cerebral infarction, symmetric involvement of basal ganglia and cerebral atrophy, in addition to PCH. Molybdenum cofactor deficiency can be screened for by the presence of urine sulfite and low plasma urate.

Acquired white matter disorders during infancy, hypoxic-ischemic disorders and congenital infectious disorders, such as cytomegalovirus, can also be considered as differential diagnoses.

DISCUSSION

SPTAN1 encephalopathy patients all had in-frame mutations in the last two spectrin repeats of the C-terminal region. However, it remains unclear which types of mutation are pathogenic. Three answers to this question can be considered. First, mutations cluster at the C-terminal region only by chance, and other types of mutation may result in the same clinical phenotype. Second, other types of mutation, such as missense mutations, may cause different phenotypes or no phenotype. A *de novo* missense mutation was found in a nonsyndromic ID patient,[23] and no *SPTAN1* mutations have been found despite extensive genetic screening of epilepsy or ID patients. As mentioned above, patients with *SPTAN1* haploinsufficiency showed no epileptic encephalopathy. Therefore, it is most likely that missense mutations or haploinsufficiency of *SPTAN1* results in different phenotypes. Third, mutation in the N-terminal region could severely affect spectrin function and result in lethality. It would be difficult to prove this possibility through patient screening. Animal models harboring various types of *Sptan1* mutation may provide further evidence for impaired spectrin functions.

Regardless of severe neurological features caused by in-frame *SPTAN1* mutations, no apparent visceral organ involvement is known. Only atrial septal defect in one patient and acquired myocarditis in another are seen. Exceptionally, one patient developed coloboma-like optic discs. It is not conclusive whether these complications were coincidence or can be explained by incomplete penetrance and variable expressivity of *STPAN1* mutational effects. Identification and characterization of more patients with *STPAN1* mutations would be required to investigate the phenotype spectrum and involvement of other organs.

The onset of epilepsy ranged from 3 weeks to 3 months of age in patients with West syndrome. In addition, one patient showed neonatal seizure that disappeared without specific treatment. Epileptic seizures were refractory to various antiepileptic drugs in all cases and to adrenocorticotrophic hormone or hydrocortisone in one case. Among the various antiepileptic drugs, clobazam, valproic acid and topiramate were partially effective in some patients. In addition to epileptic seizures, two patients showed dystonia or dystonic movement. Recently, choreoballistic movement and generalized tremor were reported in patients with *STXBP1* mutations,[35,48,51] and *ARX* and *FOXG1* mutations are also associated with movement disorders.[52,53] In-frame *SPTAN1* mutations may also be considered for epileptic encephalopathy and involuntary movements.

CONCLUSION

SPTAN1 encephalopathy has distinct clinical and neuroradiological phenotypes. Brainstem and cerebellar atrophy and cerebral hypomyelination, identified by MRI, are specific hallmarks of this disorder. These phenotypes are caused by in-frame *SPTAN1* mutations in the C-terminal region that act in a dominant negative manner. Knowledge of clinical profiles and MRI features in *SPTAN1* encephalopathy will help clinicians to perform efficient genetic testing and to identify *SPTAN1* mutations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the patients and their parents for their valuable contributions. We are grateful to Dr Masumi Inagaki for critical advice; Dr Masayuki Sasaki for his continuous support; and Dr Noriyuki Akasaka, Dr Yutaka Nonoda, Dr Barbara G.Stražišar and Dr Karin Writzl for providing patients' information. This work was supported by the Ministry of Health, Labour and Welfare of Japan; the Japan Society for the Promotion of Science (a Grant-in-Aid for Scientific Research (B), and a Grant-in-Aid for Scientific Research (A)); the Takeda Science Foundation; the fund for Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program in the Project for Developing Innovation Systems; the Strategic Research Program for Brain Sciences; and a Grant-in-Aid for Scientific Research on Innovative Areas (Transcription cycle) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

1 Berg, A. T., Berkovic, S. F., Brodie, M. J., Buchhalter, J., Cross, J. H. & van Emde Boas, W. *et al.* Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. *Epilepsia* **51**, 676–685 (2010).
2 Sharma, S. & Prasad, A. N. Genetic testing of epileptic encephalopathies of infancy: an approach. *Can. J. Neurol. Sci.* **40**, 10–16 (2013).
3 Dulac, O. Epileptic encephalopathy. *Epilepsia* **42**, 23–26 (2001).
4 Strømme, P., Mangelsdorf, M. E., Scheffer, I. E. & Gécz, J. Infantile spasms, dystonia, and other X-linked phenotypes caused by mutations in Aristaless related homeobox gene, *ARX*. *Brain Dev.* **24**, 266–268 (2002).
5 Kato, M., Das, S., Petras, K., Sawaishi, Y. & Dobyns, W. B. Polyalanine expansion of *ARX* associated with cryptogenic West syndrome. *Neurology* **61**, 267–276 (2003).
6 Kalscheuer, V. M., Tao, J., Donnelly, A., Hollway, G., Schwinger, E. & Kübart, S. *et al.* Disruption of the serine/threonine kinase 9 gene causes severe X-linked infantile spasms and mental retardation. *Am. J. Hum. Genet.* **72**, 1401–1411 (2003).
7 Kato, M., Saitoh, S., Kamei, A., Shiraishi, H., Ueda, Y. & Akasaka, M. *et al.* A longer polyalanine expansion mutation in the *ARX* gene causes early infantile epileptic encephalopathy with suppression-burst pattern (Ohtahara syndrome). *Am. J. Hum. Genet.* **81**, 361–366 (2007).
8 Saitsu, H., Kato, M., Mizuguchi, T., Hamada, K., Osaka, H. & Tohyama, J. *et al. De novo* mutations in the gene encoding STXBP1 (MUNC18-1) cause early infantile epileptic encephalopathy. *Nat. Genet.* **40**, 782–788 (2008).
9 Molinari, F., Kaminska, A., Fiermonte, G., Boddaert, N., Raas-Rothschild, A. & Plouin, P. *et al.* Mutations in the mitochondrial glutamate carrier *SLC25A22* in neonatal epileptic encephalopathy with suppression bursts. *Clin. Genet.* **76**, 188–194 (2009).
10 Paciorkowski, A. R., Thio, L. L. & Dobyns, W. B. Genetic and biologic classification of infantile spasms. *Pediatr. Neurol.* **45**, 355–367 (2011).
11 Nakamura, K., Kato, M., Osaka, H., Yamashita, S., Nakagawa, E. & Haginoya, K. *et al.* Clinical spectrum of *SCN2A* mutations expanding to Ohtahara syndrome. *Neurology* **81**, 992–998 (2013).
12 Nakamura, K., Kodera, H., Akita, T., Shiina, M., Kato, M. & Hoshino, H. *et al. De Novo* mutations in *GNAO1*, encoding a $G\alpha_o$ subunit of heterotrimeric G proteins, cause epileptic encephalopathy. *Am. J. Hum. Genet.* **93**, 496–505 (2013).
13 Kodera, H., Nakamura, K., Osaka, H., Maegaki, Y., Haginoya, K. & Mizumoto, S. *et al. De novo* mutations in *SLC35A2* encoding a UPD-galactose transporter cause early-onset epileptic encephalopathy. *Hum. Mutat.* **34**, 1708–1714 (2013).
14 Ng, B. G., Buckingham, K. J., Raymond, K., Kircher, M., Turner, E. H. & He, M. *et al.* Mosaicism of the UDP-galactose transporter *SLC35A2* causes a congenital disorder of glycosylation. *Am. J. Hum. Genet.* **92**, 632–636 (2013).
15 Vissers, L. E. L. M., de Ligt, J., Gillissen, C., Janssen, I., Steehouwer, M. & de Vries, P. *et al.* A *de novo* paradigm for mental retardation. *Nat. Genet.* **42**, 1109–1112 (2010).
16 Kamien, B. A., Cardamone, M., Lawson, J. A. & Sachdev, R. A genetic diagnostic approach to infantile epileptic encephalopathies. *J. Clin. Neurosci.* **19**, 934–941 (2012).
17 Mastrangelo, M. & Leuzzi, V. Genes of early-onset epileptic encephalopathies: from genotype to phenotype. *Pediatr. Neurol.* **46**, 24–31 (2012).
18 Saitsu, H., Tohyama, J., Kumada, T., Egawa, K., Hamada, K. & Okada, I. *et al.* Dominant-negative mutations in α-II spectrin cause West syndrome with severe cerebral hypomyelination, spastic quadripkegia, and developmental delay. *Am. J. Hum. Genet.* **86**, 881–891 (2010).
19 Voas, M. G., Lyons, D. A., Naylor, S. G., Arana, N., Rasband, M. N. & Talbot, W. S. αII-spectrin is essential for assembly of the nodes of Ranvier in myelinated axons. *Curr. Biol.* **17**, 562–568 (2007).
20 Tohyama, J., Akasaka, N., Osaka, H., Maegaki, Y., Kato, M. & Saito, N. *et al.* Early onset West syndrome with cerebral hypomyelination and reduced cerebral white matter. *Brain Dev.* **30**, 349–355 (2008).
21 Writzl, K., Primec, Z. R., Strazisar, B. G., Osredkar, D., Pecaric-Meglic, N. & Kranjc, B. S. *et al.* Early onset West syndrome with severe hypomyelination and coloboma-like optic discs in a girl with *SPTAN1* mutation. *Epilepsia* **53**, e106–e110 (2012).
22 Nonoda, Y., Saito, Y., Nagai, S., Sasaki, M., Iwasaki, T. & Matsumoto, N. *et al.* Progressive diffuse brain atrophy in West syndrome with marked hypomyalination due to *SPTAN1* gene mutation. *Brain Dev.* **35**, 280–283 (2013).
23 Hamdan, F. F., Saitsu, H., Nishiyama, K., Gauthier, J., Dobrzeniecka, S. & Spingelman, D. *et al.* Identification of a novel in-frame *de novo* mutation in *SPTAN1* in intellectual disability and pontocerebellar atrophy. *Eur. J. Hum. Genet.* **20**, 796–800 (2012).
24 Lemke, J. R., Riesch, E., Scheurenbrand, T., Schubach, M., Wilhelm, C. & Steiner, I. *et al.* Targeted next generation sequencing as a diagnostic tool in epileptic disorders. *Epilepsia* **53**, 1378–1398 (2012).
25 Allen, A. S., Berkovic, S.F., Cossette, P., Delanty, N., Dlugos, D. & Eichler, E. E. *et al. De novo* mutations in epileptic encephalopathies. *Nature* **501**, 217–221 (2013).
26 Veeramah, K. R., Johnstone, L., Karafet, T. M., Wolf, D., Sprissler, R. & Salogiannis, J. *et al.* Exome sequencing reveals new causal mutations in children with epileptic encephalopathies. *Epilepsia* **54**, 1270–1281 (2013).
27 Martin, H. C., Kim, G. E., Pagnamenta, A. T., Murakami, Y., Carvill, G. L. & Meyer., E. *et al.* Clinical whole-genome sequencing in severe early-onset epilepsy reveals new genes and improves molecular diagnosis. *Hum. Mol. Genet.* **23**, 3200–3211 (2014).
28 de Ligt, J., Willemsen, M. H., van Bon, B. W. M., Kleefstra, T., Yntema, H. G. & Kroes, T. *et al.* Diagnostic exome sequencing in persons with severe intellectual disability. *N. Engl. J. Med.* **367**, 1921–1929 (2012).
29 Bennett, V. & Baines, A. J. Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues. *Physiol. Rev.* **81**, 1353–1392 (2001).
30 Bennett, V. & Healy, J. Organizing the fluid membrane bilayer: diseases linked to spectrin and ankyrin. *Trends Mol. Med.* **14**, 28–36 (2008).
31 Machnicka, B., Czogalla, A., Hryniewicz-Jankowska, A., Bogusławska, D. M., Grochowalska, R. & Heger, E. *et al.* Spectrins: a structural platform for stabilization and activation of membrane channels, receptors and transporters. *Biochim. Biophys. Acta* **1838**, 620–634 (2014).
32 Ikeda, Y., Dick, K. A., Weatherspoon, M. R., Gincel, D., Armbrust, K. R. & Dalton, J. C. *et al.* Spectrin mutations cause spinocerebellar ataxia type 5. *Nat. Genet.* **38**, 184–190 (2006).

33 Perrotta, S., Gallagher, P. G. & Mohandas, N. Hereditary spherocytosis. *Lancet* **372**, 1411–1426 (2008).
34 Meary, F., Metral, S., Ferreira, C., Eladari, D., Colin, Y. & Lecomte, M. C. *et al.* A mutant αII-spectrin designed to resist calpain and caspase cleavage questions the functional importance of this process *in vivo*. *J. Biol. Chem.* **282**, 14226–14237 (2007).
35 Mignot, C., Moutard, M. L., Trouillard, O., Gourfinkel-An, I., Jacquette, A. & Arveiler, B. *et al.* STXBP1-related encephalopathy presenting as infantile spasms and generalized tremor in three patients. *Epilepsia* **52**, 1820–1827 (2011).
36 Campbell, I. M., Yatsenko, S. A., Hixson, P., Reimschisel, T., Thomas, M. & Wilson, W. *et al.* Novel 9q34.11 gene deletions encompassing combinations of four Mendelian disease genes: *STXBP1*, *SPTAN1*, *ENG*, and *TOR1A*. *Genet. Med.* **14**, 868–876 (2012).
37 Barcia, G., Chemaly, N., Gobin, S., Milh, M., Van Bogaert, P. & Barnerias, C. *et al.* Early epileptic encephalopathies associated with *STXBP1* mutations: could we better delineate the phenotype? *Eur. J. Med. Genet.* **57**, 15–20 (2014).
38 Saitsu, H., Kato, M., Shimono, M., Senju, A., Tanabe, S. & Kimura, T. *et al.* Association of genomic deletions in the *STXBP1* gene with Ohtahara syndrome. *Clin. Genet.* **81**, 399–402 (2012).
39 Mastrangelo, M., Peron, A., Spaccini, L., Novara, F., Scelsa, B. & Introvini, P. *et al.* Neonatal suppression-burst without epileptic sezures: expanding the electroclinical phenotype of STXBP1-related, early-onset encephalopathy. *Epileptic Disord.* **15**, 55–61 (2013).
40 Tzschach, A., Grasshoff, U., Schäferhoff, K., Bonin, M., Dufke, A. & Wolff, M. *et al.* Interstitial 9q34.11-q34.13 deletion in a patient with severe intellectual disability, hydrocephalus, and cleft lip/palate. *Am. J. Med. Genet. A* **158A**, 1709–1712 (2012).
41 Hamdan, F. F., Piton, A., Gauthier, J., Lortie, A., Dubeau, F. & Dobrzeniecka, S. *et al.* *De novo STXBP1* mutations in mental retardation and nonsyndromic epilepsy. *Ann. Neurol.* **65**, 748–753 (2009).
42 Hamdan, F.F., Gauthier, J., Dobrzeniecka, S., Lortie, A., Mottron, L. & Vanasse, M. *et al.* Intellectual disability without epilepsy associated with *STXBP1* disruption. *Eur. J. Hum. Genet.* **19**, 607–609 (2011).
43 Saitsu, H., Kato, M., Okada, I., Orii, K.E., Higuchi, T. & Hoshino, H. *et al.* *STXBP1* mutations in early infantile epileptic encephalopathy with suppression-burst pattern. *Epilepsia* **51**, 2397–2405 (2010).
44 Salonen, R., Somer, M., Haltia, M., Lorentz, M. & Norio, R. Progressive encephalopathy with edema, hypsarrhythmia, and optic atrophy (PEHO syndrome). *Clin. Genet.* **39**, 287–293 (1991).
45 Rankin, J., Brown, R., Dobyns, W.B., Harington, J., Patel, J. & Quinn, M. *et al.* Pontocerebellar hypoplasia type 6: a British case with PEHO-like features. *Am. J. Med. Genet. A* **152A**, 2079–2084 (2010).
46 Saitsu, H., Kato, M., Osaka, H., Moriyama, N., Horita, H. & Nishiyama, K. *et al.* *CASK* aberrations in male patients with Ohtahara syndrome and cerebellar hypoplasia. *Epilepsia* **53**, 1441–1449 (2012).
47 Najm, J., Horn, D., Wimplinger, I., Golden, J. A., Chizhikov, V. V. & Sudi, J. *et al.* Mutations of *CASK* cause an X-linked brain malformation phenotype with microcephaly and hypoplasia of the brainstem and cerebellum. *Nat. Genet.* **40**, 1065–1067 (2008).
48 Deprez, L., Weckhuysen, S., Holmgren, P., Suls, A., Van Dyck, T. & Goossens, D. *et al.* Clinical spectrum of early-onset epileptic encephalopaties associated with *STXBP1* mutations. *Neurology* **75**, 1159–1165 (2010).
49 Jaeken, J., Detheux, M., Van Maldergem, L., Foulon, M., Carchon, H. & Van Schaftingen, E. 3-Phosphoglycerate dehydrogenase deficiency: an inborn error of serine biosynthesis. *Arch. Dis. Child* **74**, 542–545 (1996).
50 Vijayakumar, K., Gunny, R., Grunewald, S., Carr, L., Chong, K. W. & DeVile, C. *et al.* Clinical neuroimaging features and outcome in molybdenum cofactor deficiency. *Pediatr. Neurol.* **45**, 246–252 (2011).
51 Kanazawa, K., Kumada, S., Kato, M., Saitsu, H., Kurihara, E. & Matsumoto, N. Choreo-ballistic movement in a case carrying a missense mutation in syntaxin binding protein 1 gene. *Mov. Disord.* **25**, 2265–2267 (2010).
52 Guerrini, R., Moro, F., Kato, M., Barkovich, A. J., Shiihara, T. & McShane, M. A. *et al.* Expansion of the first PolyA tract of *ARX* causes infantile spasms and status dystonicus. *Neurology* **69**, 427–433 (2007).
53 Guerrini, R. & Parrini, E. Epilepsy in Rett syndrome, and *CDKL5*- and *FOXG1*-gene-related encephalopathies. *Epilepsia* **53**, 2067–2078 (2012).

Molecular Genetics and Metabolism Reports xxx (2014) xxx–xxx

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A rapid screening with direct sequencing from blood samples for the diagnosis of Leigh syndrome

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ARTICLE INFO

Article history:
Received 12 February 2014
Accepted 12 February 2014
Available online xxxx

Keyword:
Leigh syndrome
Complex I deficiency
Heteroplasmy
mDNA mutation

ABSTRACT

Large numbers of genes are responsible for Leigh syndrome (LS), making genetic confirmation of LS difficult. We screened our patients with LS using a limited set of 21 primers encompassing the frequently reported gene for the respiratory chain complexes I (ND1–ND6, and ND4L), IV(SURF1), and V(ATP6) and the pyruvate dehydrogenase E1α-subunit. Of 18 LS patients, we identified mutations in 11 patients, including 7 in mDNA (two with ATP6), 4 in nuclear (three with SURF1). Overall, we identified mutations in 61% of LS patients (11/18 individuals) in this cohort. Sanger sequencing with our limited set of primers allowed us a rapid genetic confirmation of more than half of the LS patients and it appears to be efficient as a primary genetic screening in this cohort.

1. Introduction

Leigh syndrome (LS) (OMIM 256000) is an early onset, devastating neurodegenerative disease of the central nervous system (CNS) characterized by symmetrical necrotic lesions in the brainstem, basal

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http://dx.doi.org/10.1016/j.ymgmr.2014.02.006

ganglia and thalamus [1,2]. The symptoms of LS include psychomotor retardation, respiratory difficulties, nystagmus, hypotonia, seizures, myoclonus, ataxia, dystonia, ptosis, ophthalmoplegia and high lactate levels in the blood and cerebrospinal fluid. Mutations in both mitochondrial DNA (mDNA) and nuclear DNA cause LS [3].

LS arises from a deficiency in the enzymes relating to energy production in the mitochondria, such as the respiratory chain complexes I–V, and the pyruvate dehydrogenase complex. Among the enzymes, isolated complex I deficiency is the most frequent oxidative phosphorylation (OXPHOS) defect in children with LS [4,5], followed by a deficiency of complex IV (cytochrome C oxidase) and complex V (ATP synthase). Complex I is composed of seven mDNA encoded NADH dehydrogenase (ND) subunits (ND1–6, ND4L) and at least 38 nuclear DNA subunits [4]. An isolated generalized defect of complex IV is the second most common biochemical abnormalities found in patients with Leigh syndrome [6,7]. *SURF1* mutations, which encode the putative assembly protein of complex IV, have been repeatedly reported [6].

Since a large number of genes are reportedly related to LS, molecular diagnosis appears challenging. However, emerging drugs for LS demand prompt diagnostic confirmation of LS. Although exome sequencing is a powerful method of suspected mitochondrial disorders, it is time and cost consuming, and impractical to be applied to all patients with LS. Based on the reported mutation information, we designed a small set of 21 primers that cover the gene in which LS mutations have been frequently reported [3]. In this study, we have examined the efficacy of our Sanger sequencing method as a genetic screening for LS in 18 unrelated LS cases from one children's hospital. We identified 7 patients with point mutations in mDNA including 2 cases in the *ATP6* gene and five in the *ND* genes. We also elucidated 4 mutations in the nuclear encoded gene, including 3 patients with a mutation in *SURF1* and 1 patient with a mutation in *PDHA1* (pyruvate dehydrogenase E1α-subunit). Our data suggest that Sanger screening using limited sets of primers is useful as first line screening for LS.

2. Methods

We identified 18 patients from 16 families that met the criteria of LS at our institution (2005–2012). Diagnoses of LS were defined as presenting progressive neurologic disease with signs and symptoms of brain stem and/or basal ganglia abnormalities revealed on MR images. The clinical courses are summarized in Table 1 and Supplementary text. We have designed 7 sets of primers encoding mitochondrial derived subunits for complex I (*ND1-6*, *ND4L*) [3]. Primers were also designed on frequently reported gene *SURF 1* from complex IV [7] and *ATP synthase* from complex V [8]. If the blood lactate/pyruvate ratio is less than 10, we first sequenced the *PDHA1* gene (Suppl. Fig. 1) [8]. Methods of genetic analysis, enzyme assays and determination of heteroplasmic rate and associated references are available in the online version of the paper (Suppl. text).

3. Results (Table 1, Suppl. Fig. 2)

Of 18 LS patients, we identified gene mutations in 11 patients from 11 families. mDNA mutations were identified in 7 patients. An *ND1* mutation of complex I (m3697G>A, p.Gly131Ser) was identified in 2 individuals with homoplasmy. Mutations in *ND3* (m10158T>C, p.Ser34Pro; mutant rate 90% in white blood cell), *ND5* (m13513G>A, p.Asp393Asn; mutant rate 50% in white blood cell) and *ND6* (m14459G>A, p.Ala71Val, homoplasmic state) were identified in a single patient, respectively. One severe patient died at 1 year, and carried a mutation in *ATP6* (m8993T>G, p.Leu156Arg) of complex V of OXPHOS as a homoplasmic state. Instead of T>G, T>C mutation of the same nucleotide, m8993T>C p.Leu156Pro, was observed with homoplasmy in a milder case.

Four patients were identified with mutations in nuclear DNA. *SURF1* mutations were identified in 3 cases, including 2 cases that were compound heterozygous (c.49+1G>T/c.752_753delAG) and (c.574C>T, p.Arg192Trp and c.743C>A, p.Ala248Asp) and 1 case that was homozygous (c.743C>A, p.Ala248Asp). One male patient was identified with a hemizygous mutation (c.121T>C, p.Cys41 Arg) in *PDHA1*. Overall, we identified mutations in 61% of LS patients (11/18 individuals) in this cohort.

Please cite this article as: H. Shimbo, et al., A rapid screening with direct sequencing from blood samples for..., Molecular Genetics and Metabolism Reports (2014), http://dx.doi.org/10.1016/j.ymgmr.2014.02.006

4. Discussion

Molecular elucidation of LS at the DNA level is challenging. LS has been associated with a variety of genes in either mitochondrial or nuclear encoded DNA [3]. Surprisingly, we could reveal mutations in 61% of LS patients (11/18 individuals).

We disclosed 7 patients with mDNA mutations. From mitochondrial *ND1*, we identified an m3697G>A mutation in 2 unrelated patients, which has been reported previously in association with mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes (MELAS) [9] and Leber's hereditary optic neuropathy (LHON) [10]. To our knowledge, this is the first report of the m3697G>A/*ND1* gene mutation causing Leigh syndrome. The heteroplasmy rate is reportedly 80% in patients with MELAS (skeletal muscle) and was 56% with LOHN [9,10]. A high mutation load (100%), found in the blood of Patients 1 and 2 may be associated to severe phenotype in our patients [11]. Low level of m3697G>A mutation (~40%) was found in the blood from an asymptomatic mother of Patient 1 (Suppl. Figs. 3 and 4).

For ND3, we found a mutation of m10158T>C with 90% of heteroplasmic rate in one patient showing an early onset and very rapid progress. Severe clinical course and high mutant loads are consistent with reported cases with rapid progression and lethal consequences at early childhood [12]. A mutation of m10158T>C was not detected in the mother of Patient 3 in several tissues examined.

We found one patient with ND5 mutation, m13513G>A which has been described as causing MELAS, LS or overlapping features of the two syndromes [13–15]. We also found one LS patient with m14459G>A/*ND6* mutation that was reported in patients with LHON, dystonia [16] and LS [17]. So far, the phenotype of these two patients is LS without MELAS, LHON.

We found two patients with *ATPase6* mDNA mutations, m8993T>G and T>C, that are frequently reported in the literature [8]. A patient with a T>G mutation usually exhibits earlier onset and more rapid progression compared to T>C mutation at m8993 that was compatible with our patients (Table 1).

We found 4 patients carrying nuclear encoded gene mutations. SURF1 deficiency is the most frequent cause of LS with complex IV (cytochrome C oxidase) deficiency [7]. We identified 3 patients with the *SURF1* mutations [18]. Pyruvate dehydrogenase deficiency (PDH) is a common cause of primary congenital lactic acidosis. The biochemical features of PDH deficiency is elevated blood lactate and pyruvate levels with a normal lactate/pyruvate ratio [19]. According to the genetic screening flowchart for Leigh syndrome (Suppl. Fig. 1), we confirmed 1 patient with a hemizygous mutation in the *PDHA1* gene with 7 sets of primers.

Recently, new drugs such as EPI-743 have been shown to improve neurological and neuromuscular symptoms in LS [20,21]. Rapid genetic confirmation of mitochondrial disease may help initiate such treatment early. Next gene sequencing is revealing a wide range of dual mutations both mitochondrial and nuclear gene from patients with mitochondrial disorders [22–24]. However, it is costly and time consuming. Aiming to elucidate genetic basis of LS patients, we screened with our limited set of primers. Surprisingly, it allowed us confirmation for more than half of the patients. Therefore, this method appears to be efficient as a primary genetic screening. Our data also implicates that LS consisted of few "common" causative genes and a large number of "rare" genes. We are now undertaking whole mDNA and exome sequencing for negative cases of this method [22–24]. These data, together with increasing data of mutations, would help us improve our screening method.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgmr.2014.02.006.

Conflict of interest statement

We have no conflict of interest to disclose.

Acknowledgments

This work was supported in part by a grant of the Innovative Cell Biology by Innovative Technology (Cell Innovation Program) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, by Grants-in-Aid of the Research on Intractable Diseases (Mitochondrial Disorder) from the Ministry of Health, Labour and Welfare of Japan, and by Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics.

Please cite this article as: H. Shimbo, et al., A rapid screening with direct sequencing from blood samples for..., Molecular Genetics and Metabolism Reports (2014), http://dx.doi.org/10.1016/j.ymgmr.2014.02.006

Table 1
Genetically determined Leigh syndrome in our institution (2005–2012).

Patient	1	2	3	4	5	6	7	8	9	10	11
Age, gender	6 y, M	0 y, F	9 m, F	7 y, M	11 y, M	1 y†, M	2 y, M	4 y, F	9 y, M	25 y†, M	17 y, M
Type of gene	Mito	Mito	Mito	Mito	Mito	Mito	Mito	Nuclear	Nuclear	Nuclear	Nuclear
Gene	*ND1*	*ND1*	*ND3*	*ND5*	*ND6*	*ATPase6*	*ATPase6*	*SURF1*	*SURF1*	*SURF1*	*PDHA1*
Complex	I	I	I	I	I	V	V	IV	IV	IV	
Mutations	m3697G>A (p.G131S) Homo (b)	m3697G>A (p.G131S) Homo (b,s,h,n)	m10158T>C (p.S34P) Hetero (90%) (b)	m13513G>A (p.D393N) Hetero (50%) (b)	m14459G>A (p.A71V) Homo (b)	m8993T>G (p.L156R) Homo (b)	m8993T>C (p.L156P) Homo (b)	c.49+1G>T c.752–753delAG	c.743 C> A p.A248D c.743C> A p.A248D	c.574C>T p.R192 W c.743C>A p.A248D	c.121T>C p.C41R
Consanguinity	N	N	N	N	N	N	N	N	Y	N	N
Inheritance	Maternal* hetero:40%	N.A.	De novo	N.A.	N.A.	N.A.	N.A.	Maternal/ paternal	Maternal/ paternal	N.A.	N.A.
Age at onset	3 y 9 m	3 y 0 m	0 y 5 m	1 y 6 m	2 y 0 m	6 m	1 y 0 m	1 y 7 m	1 y 9 m	2 y	1 y 0 m
Initial Symptoms	Hypertonia Walk regre	Ataxic gait Walk regre Tremor	Hypotonia Strabismus	Dev. delay	Fever → lethargy	Dev. delay/ seizure Hypotonia/ nystagmus	Fever → lethargy	Ataxic gait	Ataxic gait	Dev. delay Ataxia	Dev. delay
Status	Walk Normal class	Wheelchair Special class	Tracheo Mech. venti	Walk	Wheelchair Normal class	(Respiratory failure)	No sitting	Tracheo Mech. venti	Tracheo Mech. venti	(Respiratory failure)	Walk Special school
RC enzymes ↓	I, IV (m)	I, III, IV (m)	I (f)	Normal (m/f)	I, III (m)	I, IV (m)	N.A.	N.A.	IV (f)	IV (m)	N.A.
Morphological findings in muscle	No RRF	No RRF	N.A.	No RRF	RRF	N.A.	N.A.	N.A.	N.A.	RRF	N.A.

MRI											
Basal ganglia hyperintensities	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Brainstem hyperintensities	N	Y	Y	N	N	N	Y	Y	Y	Y	N
Cerebellar atrophy	N	N	N	Y	N	N	N	N	N	Y	Y
Symptoms											
Dysmorphisms	N	N	N	N	N	N	N	Y	Y	N	N
Developmental delay	N	N	Y	Y	N	N	Y	Y	Y	Y	N
Regression	Y	Y	Y	N	N	Y	Y	Y	Y	Y	N
Feeding problems	N	N	Y	N	N	Y	N	N	N	N	N
Ptosis	N	N	N	N	N	N	N	Y	N	N	N
Ophthalmople	N	N	Y	N	N	N	N	Y	N	Y	N
Pyramidal symptoms	Y	Y	Y	Y	Y	N	N	Y	Y	N	Y
Extrapyramidal symptoms	Y	Y	Y	Y	N	Y	N	Y	Y	N	Y
Dystonia	Y	Y	Y	N	N	N	N	Y	Y	N	Y
Hypotonia	N	N	Y	Y	N	Y	Y	Y	Y	N	Y
Ataxia	Y	Y	Y	Y	N	N	N	Y	Y	Y	Y
Neuropathy	N	N	N	N	N	N	N	Y	Y	Y	Y
Others				WPW syndrome		West syndrome					Nystagmus

y: year, m: month, M: male, F: female, mito: mitochondria, Complex: complex in oxidative phosphorylation, b: blood, s: saliva, h: hair, n: nail, RC: respiratory chain, m: muscle, f: fibroblast, RRF: ragged red fibers, N.A.: not analyzed/not determined, N: no, negative, Y: yes, positive, regre: regression, Dev. delay: Developmental delay, Mech.venti: Mechanically ventilated, Ophthalmople: Ophthalmoplegia, *: asymptomatic.

Please cite this article as: H. Shimbo, et al., A rapid screening with direct sequencing from blood samples for..., Molecular Genetics and Metabolism Reports (2014), http://dx.doi.org/10.1016/j.ymgmr.2014.02.006

References

[1] D. Leigh, Subacute necrotizing encephalomyelopathy in an infant, J. Neurol. Neurosurg. Psychiatry 14 (1951) 216–221.
[2] S. Rahman, R.B. Blok, H.H. Dahl, D.M. Danks, D.M. Kirby, C.W. Chow, J. Christodoulou, D.R. Thorburn, Leigh syndrome: clinical features and biochemical and DNA abnormalities, Ann. Neurol. 39 (1996) 343–351.
[3] J. Finsterer, Leigh and Leigh-like syndrome in children and adults, Pediatr. Neurol. 39 (2008) 223–235.
[4] E. Fassone, S. Rahman, Complex I deficiency: clinical features, biochemistry and molecular genetics, J. Med. Genet. 49 (2012) 578–590.
[5] S. Koene, R.J. Rodenburg, M.S. van der Knaap, M.A. Willemsen, W. Sperl, V. Laugel, E. Ostergaard, M. Tarnopolsky, M.A. Martin, V. Nesbitt, J. Fletcher, S. Edvardson, V. Procaccio, A. Slama, L.P. van den Heuvel, J.A. Smeitink, Natural disease course and genotype–phenotype correlations in complex I deficiency caused by nuclear gene defects: what we learned from 130 cases, J. Inherit. Metab. Dis. 35 (2012) 737–747.
[6] Z. Zhu, J. Yao, T. Johns, K. Fu, I. De Bie, C. Macmillan, A.P. Cuthbert, R.F. Newbold, J. Wang, M. Chevrette, G.K. Brown, R.M. Brown, E.A. Shoubridge, SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome, Nat. Genet. 20 (1998) 337–343.
[7] Y. Wedatilake, R. Brown, R. McFarland, J. Yaplito-Lee, A.A. Morris, M. Champion, P.E. Jardine, A. Clarke, D.R. Thorburn, R.W. Taylor, J.M. Land, K. Forrest, A. Dobbie, L. Simmons, E.T. Aasheim, D. Ketteridge, D. Hanrahan, A. Chakrapani, G.K. Brown, S. Rahman, SURF1 deficiency: a multi-centre natural history study, Orphanet J. Rare Dis. 8 (2013) 96.
[8] M. Makino, S. Horai, Y. Goto, I. Nonaka, Mitochondrial DNA mutations in Leigh syndrome and their phylogenetic implications, J. Hum. Genet. 45 (2000) 69–75.
[9] D.M. Kirby, R. McFarland, A. Ohtake, C. Dunning, M.T. Ryan, C. Wilson, D. Ketteridge, D.M. Turnbull, D.R. Thorburn, R.W. Taylor, Mutations of the mitochondrial ND1 gene as a cause of MELAS, J. Med. Genet. 41 (2004) 784–789.
[10] L. Spruijt, H.J. Smeets, A. Hendrickx, M.W. Bettink-Remeijer, A. Maat-Kievit, K.C. Schoonderwoerd, W. Sluiter, I.F. de Coo, R.Q. Hintzen, A MELAS-associated ND1 mutation causing leber hereditary optic neuropathy and spastic dystonia, Arch. Neurol. 64 (2007) 890–893.
[11] A.L. Mitchell, J.L. Elson, N. Howell, R.W. Taylor, D.M. Turnbull, Sequence variation in mitochondrial complex I genes: mutation or polymorphism? J. Med. Genet. 43 (2006) 175–179.
[12] R. McFarland, D.M. Kirby, K.J. Fowler, A. Ohtake, M.T. Ryan, D.J. Amor, J.M. Fletcher, J.W. Dixon, F.A. Collins, D.M. Turnbull, R.W. Taylor, D.R. Thorburn, De novo mutations in the mitochondrial ND3 gene as a cause of infantile mitochondrial encephalopathy and complex I deficiency, Ann. Neurol. 55 (2004) 58–64.
[13] A. Sudo, S. Honzawa, I. Nonaka, Y. Goto, Leigh syndrome caused by mitochondrial DNA G13513A mutation: frequency and clinical features in Japan, J. Hum. Genet. 49 (2004) 92–96.
[14] M. Crimi, S. Galbiati, I. Moroni, A. Bordoni, M.P. Perini, E. Lamantea, M. Sciacco, M. Zeviani, I. Biunno, M. Moggio, G. Scarlato, G.P. Comi, A missense mutation in the mitochondrial ND5 gene associated with a Leigh–MELAS overlap syndrome, Neurology 60 (2003) 1857–1861.
[15] A. Brautbar, J. Wang, J.E. Abdenur, R.C. Chang, J.A. Thomas, T.A. Grebe, C. Lim, S.W. Weng, B.H. Graham, L.J. Wong, The mitochondrial 13513G>A mutation is associated with Leigh disease phenotypes independent of complex I deficiency in muscle, Mol. Genet. Metab. 94 (2008) 485–490.
[16] A. Gropman, T.J. Chen, C.L. Perng, D. Krasnewich, E. Chernoff, C. Tifft, L.J. Wong, Variable clinical manifestation of homoplasmic G14459A mitochondrial DNA mutation, Am. J. Med. Genet. A 124A (2004) 377–382.
[17] D.M. Kirby, S.G. Kahler, M.L. Freckmann, D. Reddihough, D.R. Thorburn, Leigh disease caused by the mitochondrial DNA G14459A mutation in unrelated families, Ann. Neurol. 48 (2000) 102–104.
[18] J. Tanigawa, K. Kaneko, M. Honda, H. Harashima, K. Murayama, T. Wada, K. Takano, M. Iai, S. Yamashita, H. Shimbo, N. Aida, A. Ohtake, H. Osaka, Two Japanese patients with Leigh syndrome caused by novel SURF1 mutations, Brain Dev. 34 (2012) 861–865.
[19] L. De Meirleir, Defects of pyruvate metabolism and the Krebs cycle, J. Child Neurol. 17 (Suppl. 3) (2002) 3S26-33 (discussion 23S33-24).
[20] A.A. Sadun, C.F. Chicani, F.N. Ross-Cisneros, P. Barboni, M. Thoolen, W.D. Shrader, K. Kubis, V. Carelli, G. Miller, Effect of EPI-743 on the clinical course of the mitochondrial disease Leber hereditary optic neuropathy, Arch. Neurol. 69 (2012) 331–338.
[21] D. Martinelli, M. Catteruccia, F. Piemonte, A. Pastore, G. Tozzi, C. Dionisi-Vici, G. Pontrelli, T. Corsetti, S. Livadiotti, V. Kheifets, A. Hinman, W.D. Shrader, M. Thoolen, M.B. Klein, E. Bertini, G. Miller, EPI-743 reverses the progression of the pediatric mitochondrial disease—genetically defined Leigh syndrome, Mol. Genet. Metab. 107 (2012) 383–388.
[22] D.S. Lieber, S.E. Calvo, K. Shanahan, N.G. Slate, S. Liu, S.G. Hershman, N.B. Gold, B.A. Chapman, D.R. Thorburn, G.T. Berry, J.D. Schmahmann, M.L. Borowsky, D.M. Mueller, K.B. Sims, V.K. Mootha, Targeted exome sequencing of suspected mitochondrial disorders, Neurology 80 (2013) 1762–1770.
[23] J.T. Dare, V. Vasta, J. Penn, N.T. Tran, S.H. Hahn, Targeted exome sequencing for mitochondrial disorders reveals high genetic heterogeneity, BMC Med. Genet. 14 (2013) 118–126.
[24] D.L. Dinwiddie, L.D. Smith, N.A. Miller, A.M. Atherton, E.G. Farrow, M.E. Strenk, S.E. Soden, C.J. Saunders, S.F. Kingsmore, Diagnosis of mitochondrial disorders by concomitant next-generation sequencing of the exome and mitochondrial genome, Genomics 102 (2013) 148–156

Please cite this article as: H. Shimbo, et al., A rapid screening with direct sequencing from blood samples for..., Molecular Genetics and Metabolism Reports (2014), http://dx.doi.org/10.1016/j.ymgmr.2014.02.006

RESEARCH ARTICLE

Human Mutation

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ECHS1 Mutations Cause Combined Respiratory Chain Deficiency Resulting in Leigh Syndrome

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Communicated by David Rosenblatt

Received 4 September 2014; accepted revised manuscript 5 November 2014.
Published online 13 November 2014 in Wiley Online Library (www.wiley.com/humanmutation). DOI: 10.1002/humu.22730

ABSTRACT: The human *ECHS1* gene encodes the short-chain enoyl coenzyme A hydratase, the enzyme that catalyzes the second step of β-oxidation of fatty acids in the mitochondrial matrix. We report on a boy with ECHS1 deficiency who was diagnosed with Leigh syndrome at 21 months of age. The patient presented with hypotonia, metabolic acidosis, and developmental delay. A combined respiratory chain deficiency was also observed. Targeted exome sequencing of 776 mitochondria-associated genes encoded by nuclear DNA identified compound heterozygous mutations in *ECHS1*. ECHS1 protein expression was severely depleted in the patient's skeletal muscle and patient-derived myoblasts; a marked decrease in enzyme activity was also evident in patient-derived myoblasts. Immortalized patient-derived myoblasts that expressed exogenous wild-type ECHS1 exhibited the recovery of the ECHS1 activity, indicating that the gene defect was pathogenic. Mitochondrial respiratory complex activity was also mostly restored in these cells, suggesting that there was an unidentified link between deficiency of ECHS1 and respiratory chain. Here, we describe the patient with ECHS1 deficiency; these findings will advance our understanding not only the pathology of mitochondrial fatty acid β-oxidation disorders, but also the regulation of mitochondrial metabolism.
Hum Mutat 36:232–239, 2015. © 2014 Wiley Periodicals, Inc.

KEY WORDS: combined respiratory chain deficiency; Leigh syndrome; ECHS1; fatty acid β-oxidation disorder

Introduction

Mitochondrial fatty acid β-oxidation provides carbon substrates for gluconeogenesis during the fasting state and contributes electrons to the respiratory chain for energy production. Once a fatty acid is activated to the acyl-coenzyme A (CoA) form and enters the mitochondrial fatty acid β-oxidation pathway, it undergoes the four following enzymatically catalyzed reaction steps during each β-oxidation cycle (Supp. Table S1): (1) dehydrogenation, (2) hydration, (3) a second dehydrogenation step, and finally (4) a thiolytic cleavage that generates one acetyl-CoA or, in certain cases, one propionyl-CoA and an acyl-CoA that is two carbons shorter than the acyl-CoA precursor. Each individual step involves specific enzymes encoded by different genes with different substrate preferences (Supp. Table S1). The first dehydrogenation reaction is catalyzed mainly by four enzymes—short-, medium-, long-, and very long chain acyl-CoA dehydrogenases (SCAD, MCAD, LCAD, and VLCAD)—with substrate optima of C4, C8, C12, and C16 acyl-CoA esters, respectively, still each dehydrogenase can utilize other suboptimal substrates [Ikeda et al., 1983, 1985a, 1985b; Ensenauer et al., 2005]. The short-chain enoyl-CoA hydratase (ECHS1) catalyzes the next step and has substrate optima of C4 2-trans-enoyl-CoA, also called crotonyl-CoA. Although ECHS1 also catalyzes hydration of medium chain substrates, longer acyl chains (e.g., C16-intermediates) are hydrated by mitochondrial trifunctional protein (MTP) [Uchida et al., 1992; Kamijo et al., 1993]. MTP consists of an alpha-subunit with long-chain enoyl-CoA hydratase and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) activities and a beta-subunit with long-chain 3-ketothiolase activity.

Mitochondrial fatty acid β-oxidation disorders generally cause impaired energy production and accumulation of partially oxidized fatty acid metabolites. They are clinically characterized by hypoglycemic seizures, hypotonia, cardiomyopathy, metabolic acidosis, and liver dysfunction [Kompare and Rizzo, 2008]. The most common genetic defect in MTP is LCHAD deficiency [MIM #609016]; deficiency involving reduced activity of all three MTP enzymes [MIM #609015] is reported much less frequently and is often associated with infantile mortality secondary to severe cardiomyopathy [Spiekerkoetter et al., 2004]. Deficiency of SCAD [MIM #201470], which catalyzes the first dehydrogenation reaction and has similar substrate optima with regard to carbon chain as ECHS1, have been studied for years, and the range of associated phenotypes includes failure to thrive, metabolic acidosis, ketotic hypoglycemia, developmental delay, seizures, and neuromuscular symptoms such as myopathy and hypotonia [Jethva et al., 2008].

Additional Supporting Information may be found in the online version of this article.

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Contract grant sponsor(s): Grants-in-Aid for Research on Intractable Diseases (Mitochondrial Disease) from the Ministry of Health, Labor and welfare of Japan; Research Grant for Nervous and Mental Disorders from the National Center of Neurology and Psychiatry (21A-6, 24-8) and JSPS KAKENHI (25670275).

Here, we describe a patient with ECHS1 deficiency who presented with Leigh syndrome [MIM #256000] accompanied by hypotonia, metabolic acidosis, and developmental delay. Additionally, the patient presented with combined respiratory chain deficiency, which is not commonly described in most clinical reports of mitochondrial fatty acid β-oxidation disorders. Finally, we discuss the pathology of ECHS1 deficiency and possible interactions between mitochondrial fatty acid β-oxidation and the respiratory chain, which are two important pathways in mitochondrial energy metabolism.

Materials and Methods

This study was approved by the ethical committee of National Center of Neurology and Psychiatry. All the samples in this study were taken and used with informed consent from the family.

Whole-mtDNA Genome Sequence Analysis

Long and accurate PCR amplification of mtDNA followed by direct sequencing was performed according to the previous publication with a slight modification [Matsunaga et al., 2005].

Targeted Exome Sequencing

Almost all exonic regions of 776 nuclear genes (Supp. Table S2), in total 7,368 regions, were sequenced using the Target Enrichment System for next-generation sequencing (HaloPlex; Agilent Technologies, Santa Clara, California, USA) and MiSeq platform (Illumina, San Diego, California, USA). Sequence read alignment was performed with a Burrows–Wheeler Aligner (version 0.6.1) to the human reference genome (version hg19). Realignment and recalibration of base quality scores was performed with the Genome Analysis Toolkit (version 1.6.13). Variants were detected and annotated against dbSNP 135 and 1000 Genomes data (February 2012 release) by Quickannotator.

Sanger Sequencing

Sanger sequencing of candidate genes was performed with the BigDye Terminators v1.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as per manufacturer's protocol. Details of primers and conditions are available upon request. DNA sequences from the patients were compared against the RefSeq sequence and the sequences of a healthy control or parents those were sequenced in parallel.

Cell Culture

The patient-derived primary myoblasts were established from the biopsy of patient's skeletal muscle and cultured in DMEM/F-12 (Thermo Fisher Scientific) supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific). DLD-1 (human colon carcinoma) cells were provided by Taiho pharmaceutical company (Tokyo, Japan) and cells were cultured in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated FBS (Thermo Fisher Scientific). All cells were cultured in 5% CO_2 at 37°C.

Preparation of Mitochondrial Fraction

Mitochondrial fractions from patient's skeletal muscle and patient-derived myoblasts were prepared according to the literature with a slight modification [Frezza et al., 2007].

Immunoblotting

Mitochondrial fraction and protein lysates were prepared from patient's skeletal muscle and patient-derived Myoblasts. Thirty micrograms of protein of mitochondrial fraction or 50 micrograms of protein lysate was separated on 4%–12% Bis-Tris gradient gels (Thermo Fisher Scientific) and transferred to polyvinylidene fluoride membranes. Primary antibodies used were against ECHS1 (Sigma-Aldrich, St. Louis, Missouri, USA), complex II 70 kDa subunit (Abcam, Cambridge, England), β-actin (Santa Cruz, Biotechnology, Dallas, Texas, USA), HA (Wako, Tokyo, Japan), and AcGFP (Thermo Fisher Scientific).

Enzyme Assays

Enzyme activities of mitochondrial respiratory complexes I–V and citrate synthase (CS) were measured in mitochondrial fraction prepared from patient's specimens. The assays for complexes I–IV and CS were performed as described previously [Shimazaki et al., 2012]. The assay for complex V was carried out following the method by Morava and his colleagues with modifications [Morava et al., 2006]. The enoyl-CoA hydratase activity was assayed by the hydration of crotonyl-CoA by a slight modification of the procedure described earlier [Steinman and Hill, 1975]. Five micrograms of protein of the mitochondrial fraction prepared from patient-derived myoblasts was added to 0.3 M Tris–HC1, pH 7.4, containing 5 mM EDTA (Ethylenediaminetetraacetic acid). The reaction was started by the addition of 200 μM crotonyl-CoA and the decrease in absorbance at 280 nm was monitored at 30°C.

Construction of the Immortalized Patient-Derived Myoblasts

The patient-derived myoblasts and control myoblasts were transfected with pEF321-T vector (A kind gift from Dr. Sumio Sugano, University of Tokyo) and the cells were cultured serially for more than ten population doublings until the morphological alteration was observed [Kim et al., 1990].

Expression Vector Preparation and Transfection

For construction of a mammalian expression vector, full-length *ECHS1* (GenBank accession number NM_004092.3) was amplified from a cDNA prepared from control subject using PrimeSTAR GXL DNA polymerase (TaKaRa, Tokyo, Japan). The PCR product was cloned into pEBMulti-Pur (Wako) and the clone was verified by Sanger sequencing. The empty expression vector or an ECHS1 expression vector was transfected into immortalized patient-derived myoblasts using Lipofectamine LTX Reagent (Thermo Fisher Scientific). Each of the two missense variants, c.2T>G; p.M1R and c.5C>T; p.A2V, was independently introduced into the clone by PCR-based site-directed mutagenesis. Each insert with C-terminal HA tag was cloned into pIRES2-AcGFP1 (Clontech Laboratories, Mountain View, California, USA) and the clones were verified by Sanger sequencing. WT and mutant ECHS1 expression vector were transfected into DLD-1 cells using Lipofectamine LTX Reagent (Thermo Fisher Scientific). Twenty-four hours later, the cell lysate was subjected to immunoblotting.

Results

The patient reported here was a boy born to unrelated, healthy parents after a 40-week pregnancy (weight 3,300 g, length 52 cm,

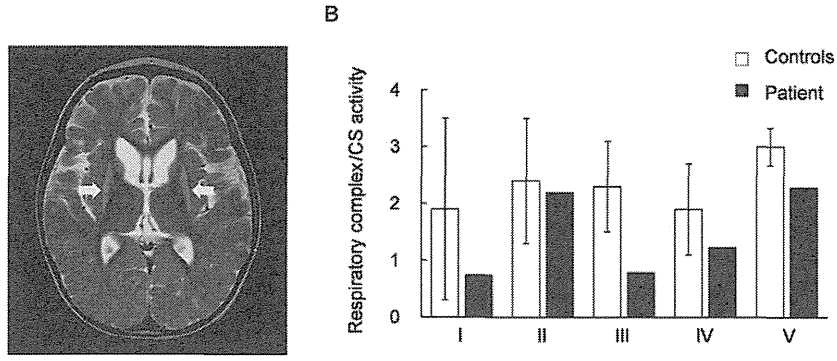

Figure 1. T2-weighted magnetic resonance scan image and enzyme activities of mitochondrial respiratory complexes. **A**: T2-weighted magnetic resonance scan image (MRI) shows bilaterally symmetrical hyperintensities in the putamen (arrows in the image); these are characteristic of Leigh syndrome. **B**: Enzymatic activities of five mitochondrial respiratory complexes (I, II, III, IV, and V) were measured in mitochondrial fractions prepared from the patient's skeletal muscle. Respiratory complexes activities were normalized to citrate synthase activity. Black bars show patient values and white bars show control values. Control values were mean values obtained from five healthy individuals. Patient activity values for complexes I, III, and IV were 39%, 34%, and 64% of the control values, respectively. Error bars represent standard deviations.

Table 1. Urinary Organic Acid Profiling

	Patient RPA (%)	Controls RPA (%)
TCA cycle intermediates		
α-Ketoglutarate	4.52	3.00–102.90
Aconitate	20.37	15.10–86.10
Isocitrate	8.98	8.30–29.00
Other metabolites		
Lactate	11.83[a]	<4.70
Pyruvate	3.18	<24.10
3-Hydroxyisobutyric acid	1.95	<9.00
Methylcitric acid	0.14[a]	Less than trace amount
p-Hydroxy-phenyllactic acid	40.05[a]	<7.00
Glyoxylate	37.71[a]	<6.10

[a]Values outside the normal range.
RPA(%), relative peak area to the area of internal standard (heptadecanoic acid, HDA).

occipitofrontal circumference (OFC) 34.5 cm). Auditory screening test at 2 months of age revealed hearing impairment, and he began to use a hearing aid at 6 months of age. Psychomotor developmental delay was noted at 5 months of age; he could not sit alone, or speak a meaningful word as of 4 years of age. Nystagmus was noted at 10 months of age. Muscle hypotonia, spasticity, and athetotic trunk movement became prominent after 1 year of age. His plasma (20.2 mg/dl) and a cerebrospinal fluid lactate were elevated (25.3 mg/dl, control below 15 mg/dl). Urinary organic acid profiling reveals significantly elevated excretion of glyoxylate (Table 1). Analysis of blood acylcarnitines showed no abnormalities. Brain magnetic resonance scan image showed bilateral T2 hyperintensity of the putamen, typical for Leigh syndrome (Fig. 1A). Because Leigh syndrome is generally caused by defects in the mitochondrial respiratory chain or the pyruvate dehydrogenase complex, we performed a muscle biopsy to measure enzyme activities of mitochondrial respiratory complexes in the patient. Mitochondrial fractions prepared from patient or control specimens were used for all activity measurements. Activity of each respiratory complex was normalized relative to CS activity; normalized values for complexes I, III, and IV activity were decreased to 39%, 34%, and 64% of control values, respectively (Fig. 1B). Moreover, we performed blue native PAGE (BN-PAGE) to examine if the assembly of respiratory complexes were altered in the patient. As a result, there were no clear difference between the patient and the control (Supp. Fig. S1).

Mitochondrial respiratory chain defects can be due to pathogenic mutations in mitochondrial DNA (mtDNA) or nuclear DNA (nDNA) coding for mitochondrial components. Initially, long and accurate PCR amplification of mtDNA followed by direct sequencing was performed and no mutations known to be associated with Leigh syndrome were identified, but previously reported polymorphisms were found (Supp. Table S3). Therefore, to identify the responsible mutations in nDNA, targeted exome sequencing was performed. Coverage was at least 10× for 86.2% of the target regions, and 30× or more for 73.4%. In all, 5,640 potential variants were identified; these included 811 splice-site or nonsynonymous variants. Among those 811 variants, 562 were on the mismapping reads that contained multiple apparent mismatches to the reference DNA sequence. Of the remaining 249 variants, nine that were on target regions with less than 10× coverage were eliminated because data reliability was low. Filtering against dbSNP 135 and 1000 Genomes data, this number was reduced to 13 including compound heterozygous variants in the *ECHS1* [MIM #602292] and 11 heterozygous variants in 11 separate genes (Supp. Table S4). Those variants have been submitted to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). Because most mitochondrial diseases caused by known nDNA mutations are inherited in an autosomal recessive manner, we focused on the compound heterozygous variants in *ECHS1*—c.2T>G; p.M1R and c.5C>T; p.A2V—as primary candidates.

To confirm the targeted exome sequencing results, we performed Sanger sequencing of genomic *ECHS1* DNA and *ECHS1* cDNA from the patient and his parents. We identified both variants, c.2T>G and c.5C>T, and the respective normal alleles in genomic DNA and cDNA from the patient (Fig. 2A and B) and no other *ECHS1* variants were detected except for common SNPs in the open reading frame. Analysis of genomic DNA from the patient's parents showed that patient's father was heterozygous for only one variant, c.2T>G, and the patient's mother for only the other variant, c.5C>T (Fig. 2A). These results indicated that the patient inherited each variant separately and that both mutant alleles were expressed in the patient (Fig. 2B). Each variant was nonsynonymous and in the region encoding the mitochondrial transit peptide (1–27 amino acids) of ECHS1 [Hochstrasser et al., 1992]; moreover, c.2T>G; p.M1R was a start codon variant (Fig. 2C).

Next, immunoblotting with primary antibodies against ECHS1 was performed to assess protein expression. Mitochondrial

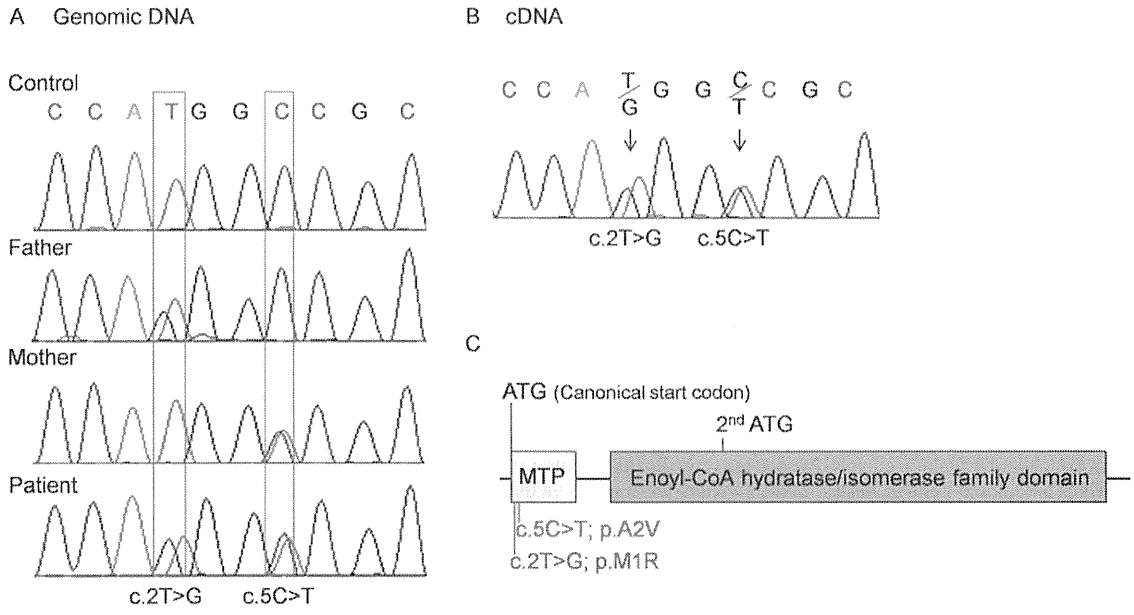

Figure 2. *ECHS1* Sanger sequencing analysis and ECHS1 functional domains. **A**: Sequence chromatograms from part of exon 1 of *ECHS1* were generated by Sanger sequencing of genomic DNA. Each parent had one wild-type allele; the patient's father also harbored a c.2T>G variant, and the patient's mother a c.5C>T variant. The patient inherited each variant allele and was a compound heterozygote. **B**: Sequence chromatograms from part of *ECHS1* exon 1 obtained by Sanger sequencing of cDNA prepared from patient mRNA. The same variants seen in genomic DNA were observed in the cDNA. **C**: A schematic diagram of the functional domains in ECHS1 and the locations of the mutations. MTP, mitochondrial transit peptide.

fractions prepared from patient and control skeletal muscle were used; whole-cell lysates or mitochondrial fractions prepared from patient-derived or control myoblasts were also used. All experiments using these specimens showed that the expression level of ECHS1 protein of the patient was too low to detect by immunoblotting even though the expression level of SDHA was almost the same as controls (Fig. 3A–C). These findings indicated that c.2T>G; p.M1R and c.5C>T; p.A2V mutations caused a remarkable reduction in ECHS1 protein expression. Notably, patient-derived and control myoblasts were similar with regard to *ECHS1* mRNA expression (Fig. 3D), indicating that the mutations apparently affected ECHS1 protein expression directly. Next, we measured ECHS1 enzyme activity in mitochondrial fractions prepared from patient-derived and control myoblasts. ECHS1 activity was normalized to CS activity, and activity in patient-derived myoblasts was 13% of that in control myoblasts (Fig. 3E). Therefore, the mutations caused a severe depletion of ECHS1 protein expression thereby decreasing ECHS1 enzyme activity.

To examine the stability of each mutated protein, we constructed three pIRES2-AcGFP1 expression plasmids, each expressed a different HA-tagged protein: wild-type, M1R-mutant, or A2V-mutant ECHS1. The expression of AcGFP was used as a transfection control. After the transfection into DLD-1 cells, immunoblotting of whole-cell lysate with anti-HA and GFP antibodies showed markedly higher expression of wild-type ECHS1 than of either mutant protein; all ECHS1 expression was normalized to AcGFP expression (Fig. 4, Supp. Fig. S2). This result indicated that ECHS1 protein expression was significantly reduced in the patient because of each mutation.

To confirm that the patient had ECHS1 deficiency, we performed a cellular complementation experiment. Patient-derived myoblasts had to be immortalized for these experiments because nonimmortalized cells exhibited poor growth and finite proliferation. The patient-derived myoblasts and control myoblasts were transfected with pEF321-T vector (a kind gift from Dr. Sumio Sugano, University of Tokyo). We then ascertained that ECHS1 protein expression and activity were lower in immortalized patient-derived myoblasts than in controls (Fig. 5A and B). We then transduced an empty expression vector, pEBMulti-Pur (Wako), or a pEBMulti-Pur construct containing a full-length, wild-type *ECHS1* cDNA into the immortalized patient-derived myoblasts; cells with the vector only or the ECHS1-expression construct are hereafter called vector-only and rescued myoblasts, respectively. ECHS1 protein expression level and enzyme activity were analyzed in mitochondrial fractions prepared from rescued myoblasts. Relative expression level of ECHS1 in rescued myoblasts was 11 times higher than that in vector-only myoblasts (Fig. 5A), and ECHS1 activity normalized to CS activity in rescued myoblasts was 49 times higher than that in vector-only myoblasts (Fig. 5B). From these cellular complementation experiments, we concluded the patient had ECHS1 deficiency.

Since the patient showed the combined mitochondrial respiratory chain deficiency in the skeletal muscle as mentioned above, we used a cellular complementation experiment to determine whether wild-type ECHS1 rescued the respiratory chain defect in patient-derived myoblasts. First, we measured enzyme activities of each mitochondrial respiratory complex in mitochondrial fractions prepared from immortalized patient-derived myoblasts. CS activity normalized values for complexes I, IV, and V activity in immortalized patient-derived myoblasts were decreased to 17%, 39%, and 43% of the mean values of immortalized control myoblasts (Fig. 5C). Then, we measured enzyme activity in mitochondrial fractions prepared from rescued myoblasts and found that each activity of complexes I, IV, and V was mostly restored relative to that in vector-only myoblasts. In rescued myoblasts, CS activity normalized values of complexes I, IV, and V were 3.5, 1.3, and 2.2 times higher than those in vector-only myoblasts (Fig. 5C). Mitochondrial respiratory complex activity was mostly restored in rescued myoblasts, suggesting that there was an unidentified link between deficiency of ECHS1 and respiratory chain.