In addition to correlation, the mean values of the absolute differences between self- and proxy-reports for total score and for all subscales of the Japanese-language version of the PedsQL Multidimensional Fatigue Scale were calculated as a further indicator of the level of agreement between child and parent responses. The directional differences can indicate bias of proxy-reported scores relative to those of self-reported scores [31]. The mean values of directional differences were standardized by relating a given score to its standard deviation to determine the statistical magnitude of any observed systematic bias. The size of bias of a standardized difference (d) was labeled as small when it was 0.2, medium when it was =0.5, and large when it was =0.8.

All statistical analyses were conducted using The Statistical Package for Social Sciences 14.0 for Windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Effects of citrin deficiency on PedsQL Multidimensional Fatigue Scale and Generic Core Scales

Table 1 shows the mean values of the child self-report scores and proxy-report scores in citrin-deficient patients on the PedsQL Scales. On the PedsQL Multidimensional Fatigue Scale, the total fatigue scores and all subscales scores of both child self-report and parent proxy-report for citrin-deficient patients were significantly lower than the fatigue scores of healthy children examined in our previous study [22]. On the PedsQL Generic Core Scales, the total scores of self-report and parent proxy-report for citrin-deficient patients were significantly lower than the control. In the subscale evaluation, significant decreases were recognized in physical functioning and school functioning in child self-report, whereas significant decreases were noted on all subscales in parent proxy-report.

Fig. 1 shows the distribution of the child self-report score in citrin-deficient patients based on the percentile of the scores in healthy children on the PedsQL Fatigue Scale and Generic Core Scales. The numbers of citrin-deficient patients with scores of 50 percentile or less of those of healthy children were 27 (67.5%) on the Fatigue Scale and 26 (68.4%) on the Generic Core Scales. The above results demonstrated enhanced fatigue and low QOL in citrin-deficient patients in silent period around school life. Parents also recognized patient's fatigue and QOL impairment.

3.2. Correlation between PedsQL Fatigue Scale and Generic Core Scales

We also evaluated the correlation between the PedsQL Fatigue Scale and PedsQL Generic Core Scales in citrin-deficient patients using the Spearman rank correlation coefficient (Table 2). In the child self-report, there was a moderate correlation ($r=0.56$) between the total score on the Fatigue Scale and total score on the Generic Core Scales. The total score on the Generic Core Scales showed the strongest correlation with general fatigue among the subscales of the Fatigue Scale. Meanwhile, the total score on the Fatigue Scale showed the strongest correlation with physical functioning among the subscales of the Generic Core Scale (r=0.59). In the parent proxy-report, there was a good correlation between total fatigue score and total generic core score (r=0.71).

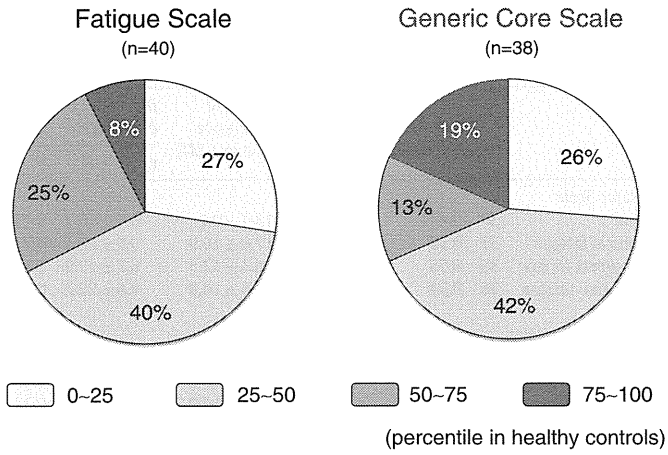

Fig. 1. PedsQL Multidimensional Fatigue Scale and Generic Core Scale of the child self-report in citrin-deficient patients, relative to those of the healthy control. A circle graph shows the number (%) of the citrin-deficient patients at the score of every 25 percentile of healthy controls on PedsQL Multidimensional Fatigue Scale and Generic Core Scales. For healthy controls, the score points of the Fatigue Scale for the 0–25, 26–50, 51–75, and 76–100 percentiles were 0–67.4, 67.5–80.9, 81–89.4, and 89.5–100, respectively, while those of the Generic Core Scale were 0–72.4, 72.5–83.2, 83.3–90, and 90.1–100, respectively.

3.3. Assessment of difference between citrin-deficient children and their parents

Table 3 summarizes the Spearman rank correlation coefficients (ρ) for child/parent assessment differences in fatigue and QOL recognition, ICC, absolute differences, and directional differences. The ICC of the child–parent agreement was moderate to good at 0.40–0.70 on the Fatigue Scale and fair to moderate at 0.36–0.49 on the Generic Core Scales. Large values in child–parent absolute differences and directional differences were noted in the cognitive fatigue on the Fatigue Scale and in the emotional functioning on the Generic Core Scales. These are items that are not easily shown in behaviors or daily living on the surface. Therefore, the above findings suggest

Table 1
Effects of citrin deficiency on PedsQL Multidimensional Fatigue Scale and Generic Core Scales.

	Child self-report		Parent proxy-report	
	Citrin deficiency mean ± SD	Control mean ± SD	Citrin deficiency mean ± SD	Control mean ± SD
Fatigue Scale	(n=40)		(n=52)	
Total	67.8 ± 14.3¶	77.6 ± 16.0	77.7 ± 17.8¶	85.6 ± 13.6
General	68.5 ± 22.5*	81.7 ± 18.3	78.0 ± 20.6¶	84.9 ± 15.6
Sleep/rest	68.9 ± 22.5¶	72.8 ± 18.6	76.5 ± 21.7¶	86.2 ± 14.8
Cognitive	65.9 ± 21.9¶	78.5 ± 20.4	77.7 ± 17.8*	85.8 ± 16.1
Generic Core Scales	(n=38)		(n=54)	
Total	77.3 ± 11.9*	80.9 ± 12.4	82.5 ± 14.5¶	88.7 ± 11.3
Physical	79.9 ± 14.9¶	86.6 ± 13.6	86.3 ± 17.6¶	90.0 ± 18.4
Emotional	69.9 ± 16.4	67.8 ± 21.5	78.6 ± 18.3¶	87.7 ± 13.1
Social	82.9 ± 14.4	84.8 ± 16.1	84.3 ± 20.0*	89.9 ± 13.2
School	75.0 ± 16.8*	84.9 ± 12.9	77.5 ± 16.9¶	87.3 ± 12.6

*$p<0.05$, ¶$p<0.01$, compared with the respective control (by Mann–Whitney U test).

Table 2
Correlation between PedsQL Multidimensional Fatigue Scale and Generic Core Scales.

	n	Fatigue Scale[a]			
		Total	General	Sleep/rest	Cognitive
Generic Core Scales					
Child self-report					
Total	38	0.56	0.45	0.22	0.27
Physical	38	0.59	0.60	0.15	0.26
Emotional	38	0.33	0.25	0.22	0.09
Social	38	0.39	0.27	0.06	0.40
School	38	0.37	0.17	0.40	0.05
Parent proxy-report					
Total	52	0.71	0.70	0.49	0.58
Physical	52	0.60	0.63	0.42	0.47
Emotional	52	0.60	0.60	0.44	0.46
Social	52	0.68	0.66	0.44	0.59
School	48	0.69	0.56	0.55	0.60

[a] Data are Spearman Rank Correlation Coefficient ρ.

Table 3
Agreement between child self-report and parent proxy-report on PedsQL Multidimensional Fatigue Scale and Generic Core Scales.

	n	Spearman rank correlation coefficient (ρ)	ICC	Absolute difference (mean ± SD)	Directional difference	
					mean ± SD	*D*
Fatigue Scale						
Total	36	0.47	0.56	13.4 ± 9.7	6.6 ± 15.3	0.43
General fatigue	37	0.30	0.40	17.6 ± 16.9	5.0 ± 24.1	0.21
Sleep/rest fatigue	36	0.55	0.70	14.3 ± 12.5	6.7 ± 17.9	0.37
Cognitive fatigue	36	0.54	0.54	18.5 ± 14.8	8.8 ± 22.2	0.40
Generic Core Scales						
Total	38	0.51	0.49	10.2 ± 9.3	3.6 ± 13.4	0.27
Physical	38	0.41	0.38	12.6 ± 13.9	6.3 ± 17.8	0.35
Emotional	38	0.42	0.36	15.6 ± 14.0	6.4 ± 20.1	0.32
Social	38	0.36	0.39	12.9 ± 11.7	−0.5 ± 17.5	0.03
School	38	0.47	0.48	11.7 ± 12.3	0.7 ± 17.1	0.04

ICC: intraclass correlation coefficient.

that it is difficult even for parents to grasp the internal mental state of their children, resulting in a large recognition difference. On the PedsQL Fatigue Scale, the directional differences showed a positive score difference, indicating that parents assessed the conditions of their children more favorably than the children themselves.

4. Discussion

Fatigue is one of the three important body alarms, in addition to pain and fever, and is one of the important signals for the body to maintain health and life. In healthy individuals, physiological fatigue is a state where performance is temporarily decreased due to the load imposed on the mind or body. It often accompanies a desire for rest. Fatigue is caused not only by excessive exercise or chronic fatigue syndrome but also by various diseases and their therapies. Fatigue is a symptom associated with pain in various diseases. The following diseases were reported on the PedsQL fatigue scale so far: diabetes (73.5) [32]; rheumatism (73.8) [33]; cancer (71.0) [34]; obesity (67.7) [35]; and brain tumor (69.7) [36]. The fatigue scale score of citrin-deficient patients during silent period in this study was 67.8, which is one of the lowest in the above disease groups. The fatigue scale scores of 67.5% patients were 50 percentile or less of the scores of healthy children. Thus, citrin deficiency is a disease accompanied by a strong fatigue.

Fatigue is largely associated with biological oxidation caused by excessive activities of cells in the body, such as muscle cells and nerve cells. Oxidative stress reduces cell functions and energy generation in mitochondria, inducing fatigue due to the lack of energy source. Therefore, oxidative stress is regarded as a marker of fatigue [37,38]. The metabolic state of citrin-deficient patients in the silent period includes: 1) high level of oxidative stress, including increased activities of antioxidant enzymes (SOD and catalase) in red blood cells and high levels of urinary oxidative stress-related substances of acrolein-lysine and 8-hydroxy-2′-deoxyguanosine and; 2) high lactic acid/pyruvic acid ratio, in association with high NADH concentration in the cytoplasm; 3) hypercitrullinemia resulting from suppressed urea cycle; and 4) hypercholesterolemia [18]. These changes/abnormalities hamper full metabolic adaptation and compensation in citrin-deficient patients during the silent period. Although citrin-deficient patients during the silent period show no marked pathological symptoms, the metabolic dysfunction is likely to cause fatigue in citrin-deficient patients.

One of the characteristic results of PedsQL Multidimensional Fatigue Scale and Gemeric Core Scale was that fatigue impaired QOL in citrin-deficient patients during the silent period. In the child self-report, the QOL subscale score of physical and school functioning, which is easy to be affected by fatigue, was significantly lower than those in the control (Table 1). The results also showed a moderate correlation between the total score in Fatigue Scale and the total score in Generic Core Scales (Table 2). Furthermore, in the parent proxy-report, both the total fatigue score and the total generic core score in the citrin-deficient patients were significantly lower than those in the control, (Table 1), with a strong and significant correlation between the two variables (Table 2). These results indicate that fatigue correlates with QOL, and that parents of citrin-deficient patients believe that fatigue strongly correlates with QOL and affects QOL.

Other characteristic result of the PedsQL Fatigue Scale and Gemeric Core Scales in citrin deficiency was the presence of few assessment differences between child self-report and parent proxy-report. That is, the agreement level between child self-report and parent proxy-report was moderate to good agreement (0.51–0.73) on the Fatigue Scale, and moderate agreement (0.42–0.53) on the Generic Core Scales. To our knowledge, there is little or no information in the literature on the agreement level between child self-report and parent proxy-report on the Fatigue Scale. Concerning type 1 diabetes and obesity, the ICC of patient–parent agreement was poor to fair [32,35]. We reported previously in a fair to moderate agreement between Japanese children with chronic disease (asthma, atopic dermatitis) and their parents, compared with poor to fair agreement in the healthy control [22].

Although the assessments of fatigue and QOL by citrin-deficient patients and their parents correlated well, the parents underestimated fatigue compared with the patients themselves. Thus, the severity of fatigue experienced by the children during the silent period, and impairment of QOL and pain associated with the fatigue, exceeded that estimated by their parents. An important factor in the consultation of medical institution for children is how parents evaluate the health condition of their child [39]. In the case of citrin-deficient children, active involvement of the parents and medical profession is important. Diet therapy and sodium pyruvate are proposed as therapies for CTLN2 [40], and both therapies are reported to be effective in school-age children with marked growth disturbances [41]. It is expected that diet therapy and sodium pyruvate provided during the silent period improve the defective metabolic adaptation and compensatory processes in citrin-deficient children.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; grants from the Ministry of Health, Labor and Welfare of Japan; and funds from Suyama Research Foundation.

References

[1] K. Kobayashi, D.S. Sinasac, M. Iijima, A.P. Boright, L. Begum, J.R. Lee, T. Yasuda, S. Ikeda, R. Hirano, H. Terazono, M.A. Crackower, I. Kondo, L.C. Tsui, S.W. Scherer, T. Saheki, The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein, Nat. Genet. 22 (1999) 159–163.
[2] L. Palmieri, B. Pardo, F.M. Lasorsa, A. del Arco, K. Kobayashi, M. Iijima, M.J. Runswick, J.E. Walker, T. Saheki, J. Satrustegui, F. Palmieri, Citrin and aralar1 are Ca(2+) stimulated aspartate/glutamate transporters in mitochondria, EMBO J. 20 (2001) 5060–5069.
[3] F. Palmieri, Diseases caused by defects of mitochondrial carriers, Biochim. Biophys. Acta 1777 (2008) 564–578.
[4] T. Saheki, K. Kobayashi, Mitochondrial aspartate glutamate carrier (citrin) deficiency as the cause of adult-onset type II citrullinemia (CTLN2) and idiopathic neonatal hepatitis (NICCD), J. Hum. Genet. 47 (2002) 333–341.
[5] N. Yamaguchi, K. Kobayashi, T. Yasuda, I. Nishi, M. Iijima, M. Nakagawa, M. Osame, I. Kondo, T. Saheki, Screening of SLC25A13 mutations in early and late onset patients with citrin deficiency and in the Japanese population: identification of

two novel mutations and establishment of multiple DNA diagnosis method for nine mutations, Hum. Mutat. 19 (2002) 122–130.
[6] K. Kobayashi, N. Shaheen, R. Kumashiro, K. Tanikawa, W.E. O'Brien, A.L. Beaudet, T. Saheki, A search for the primary abnormality in adult-onset type II citrullinemia, Am. J. Hum. Genet. 53 (1993) 1024–1030.
[7] K. Kobayashi, Y. Bang Lu, M. Xian Li, I. Nishi, K.J. Hsiao, K. Choeh, Y. Yang, W.L. Hwu, J.K. Reichardt, F. Palmieri, Y. Okano, T. Saheki, Screening of nine SLC25A13 mutations: their frequency in patients with citrin deficiency and high carrier rates in Asian populations, Mol. Genet. Metab. 80 (2003) 356–359.
[8] K. Kobayashi, M. Iijima, T. Yasuda, D.S. Sinasac, N. Yamaguchi, L.C. Tsui, S.W. Scherer, T. Saheki, Type II citrullinemia (citrin deficiency): a mysterious disease caused by a defect of calcium-binding mitochondrial carrier protein, in: R. Pochet, R. Donato, J. Haiech, C. Heizmann, V. Gerke (Eds.), Calcium: The Molecular Basis of Calcium Action in Biology and Medicine, Kluwer Academic Publishers, New York, 2000, pp. 565–587.
[9] T. Saheki, K. Kobayashi, T. Miura, S. Hashimoto, Y. Ueno, T. Yamasaki, H. Araki, H. Nara, Y. Shiozaki, Y. Sameshima, M. Suzuki, Y. Yamauchi, Y. Sakazume, K. Akiyama, Y. Yamamura, Serum amino acid pattern of type II citrullinemic patients and effect of oral administration of citrulline, J. Clin. Biochem. Nutr. 1 (1986) 129–142.
[10] T. Ohura, K. Kobayashi, Y. Tazawa, I. Nishi, D. Abukawa, O. Sakamoto, K. Iinuma, T. Saheki, Neonatal presentation of adult-onset type II citrullinemia, Hum. Genet. 108 (2001) 87–90.
[11] Y. Tazawa, K. Kobayashi, T. Ohura, D. Abukawa, F. Nishinomiya, Y. Hosoda, M. Yamashita, I. Nagata, Y. Kono, T. Yasuda, N. Yamaguchi, T. Saheki, Infantile cholestatic jaundice associated with adult-onset type II citrullinemia, J. Pediatr. 138 (2001) 735–740.
[12] T. Tomomasa, K. Kobayashi, H. Kaneko, H. Shimura, T. Fukusato, N. Tabata, Y. Inoue, S. Ohwada, M. Kasahara, Y. Morishita, M. Kimura, T. Saheki, A. Morikawa, Possible clinical and histological manifestations of adult-onset type II citrullinemia in early infancy, J. Pediatr. 138 (2001) 741–743.
[13] A. Tamamori, Y. Okano, H. Ozaki, A. Fujimoto, M. Kajiwara, K. Fukuda, K. Kobayashi, T. Saheki, Y. Tagami, T. Yamano, Neonatal intrahepatic cholestasis caused by citrin deficiency: severe hepatic dysfunction in an infant requiring liver transplantation, Eur. J. Pediatr. 161 (2002) 609–613.
[14] A. Tamamori, A. Fujimoto, Y. Okano, K. Kobayashi, T. Saheki, Y. Tagami, H. Takei, Y. Shigematsu, I. Hata, H. Ozaki, D. Tokuhara, Y. Nishimura, T. Yorifuji, N. Igarashi, T. Ohura, T. Shimizu, K. Inui, N. Sakai, D. Abukawa, T. Miyakawa, M. Matsumori, K. Ban, H. Kaneko, T. Yamano, Effects of citrin deficiency in the perinatal period: feasibility of newborn mass screening for citrin deficiency, Pediatr. Res. 56 (2004) 608–614.
[15] T. Ohura, K. Kobayashi, Y. Tazawa, D. Abukawa, O. Sakamoto, S. Tsuchiya, T. Saheki, Clinical pictures of 75 patients with neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), J. Inherit. Metab. Dis. 30 (2007) 139–144.
[16] T. Shigeta, M. Kasahara, T. Kimura, A. Fukuda, K. Sasaki, K. Arai, A. Nakagawa, S. Nakagawa, K. Kobayashi, S. Soneda, H. Kitagawa, Liver transplantation for an infant with neonatal intrahepatic cholestasis caused by citrin deficiency using heterozygote living donor, Pediatr. Transplant. 14 (2010) E86–E88.
[17] T. Saheki, K. Kobayashi, M. Terashi, T. Ohura, Y. Yanagawa, Y. Okano, T. Hattori, H. Fujimoto, K. Mutoh, Z. Kizaki, A. Inui, Reduced carbohydrate intake in citrin-deficient subjects, J. Inherit. Metab. Dis. 31 (2008) 386–394.
[18] H. Nagasaka, Y. Okano, H. Tsukahara, Y. Shigematsu, T. Momoi, J. Yorifuji, T. Miida, T. Ohura, K. Kobayashi, T. Saheki, K. Hirano, M. Takayanagi, T. Yorifuji, Sustaining hypercitrullinemia, hypercholesterolemia and augmented oxidative stress in Japanese children with aspartate/glutamate carrier isoform 2-citrin-deficiency even during the silent period, Mol. Genet. Metab. 97 (2009) 21–26.
[19] J.W. Varni, M. Seid, C.A. Rode, The PedsQL™: measurement model for the pediatric quality of life inventory, Med. Care 37 (1999) 126–139.
[20] J.W. Varni, M. Seid, P.S. Kurtin, PedsQL™ 4.0: reliability and validity of the Pediatric Quality of Life Inventory™ version 4.0 generic core scales in healthy and patient populations, Med. Care 39 (2001) 800–812.
[21] K. Kobayashi, K. Kamibeppu, Measuring quality of life in Japanese children: development of the Japanese version of PedsQL™, Pediatr. Int. 52 (2010) 80–88.
[22] K. Kobayashi, Y. Okano, N. Hohashi, Reliability and validity of the PedsQL™ Multidimensional Fatigue Scale in Japan, Qual. Life Res. 20 (2011) 1091–1102.
[23] M.G. Swain, Fatigue in chronic disease, Clin. Sci. 99 (2000) 1–8.
[24] R.J. Bakker, E.M. van de Putte, W. Kuis, G. Sinnema, Risk factors for persistent fatigue with significant school absence in children and adolescents, Pediatrics 124 (2009) e89–e95.
[25] A. Sankey, C.M. Hill, J. Brown, L. Quinn, A. Fletcher, A follow-up study of chronic fatigue syndrome in children and adolescents: symptom persistence and school absenteeism, Clin. Child. Psychol. Psychiatry 11 (2006) 126–138.
[26] C.A. McHorney, J.E. Ware Jr., J.F. Lu, C.D. Sherbourne, The MOS 36-item Short-Form Health Survey (SF-36): III. Tests of data quality, scaling assumptions, and reliability across diverse patient groups, Med. Care 32 (1994) 40–66.
[27] J. Cohen, Statistical Power Analysis for the Behavioral Sciences, 2nd ed. Lawrence Erlbaum Associates, Hillsdale, 1988.
[28] K.O. McGraw, S.P. Wong, Forming inferences about some intraclass correlation coefficients, Psychol. Methods 1 (1996) 30–46.
[29] P.E. Shrout, Measurement reliability and agreement in psychiatry, Stat. Methods Med. Res. 7 (1998) 301–317.
[30] K.A. Wilson, A.J. Dowling, M. Abdolell, I.F. Tannock, Perception of quality of life by patients, partners and treating physicians, Qual. Life Res. 9 (2000) 1041–1052.
[31] K.C. Sneeuw, N.K. Aaronson, M.A. Sprangers, S.B. Detmar, L.D. Wever, J.H. Schornagel, Comparison of patient and proxy EORTC QLQ-C30 ratings in assessing the quality of life of cancer patients, J. Clin. Epidemiol. 51 (1998) 617–631.
[32] J.W. Varni, C.A. Limbers, W.P. Bryant, D.P. Wilson, The PedsQL Multidimensional Fatigue Scale in type 1 diabetes: feasibility, reliability, and validity, Pediatr. Diabetes 10 (2009) 321–328.
[33] J.W. Varni, T.M. Burwinkle, I.S. Szer, The PedsQL Multidimensional Fatigue Scale in pediatric rheumatology: reliability and validity, J. Rheumatol. 31 (2004) 2494–2500.
[34] J.W. Varni, T.M. Burwinkle, E.R. Katz, K. Meeske, P. Dickinson, The PedsQL in pediatric cancer: reliability and validity of the Pediatric Quality of Life Inventory ™ Generic Core Scales, Multidimensional Fatigue Scale, and Cancer Module, Cancer 94 (2002) 2090–2106.
[35] J.W. Varni, C.A. Limbers, W.P. Bryant, D.P. Wilson, The PedsQL Multidimensional Fatigue Scale in pediatric obesity: feasibility, reliability and validity, Int. J. Pediatr. Obes. 5 (2010) 34–42.
[36] S.N. Palmer, K.A. Meeske, E.R. Katz, T.M. Burwinkle, J.W. Varni, The PedsQL Brain Tumor Module: initial reliability and validity, Pediatr. Blood Cancer. 49 (2007) 287–293.
[37] K.B. Norheim, G. Jonsson, R. Omdal, Biological mechanisms of chronic fatigue, Rheumatology 50 (2011) 1009–1018.
[38] G.L. Nicolson, Metabolic syndrome and mitochondrial function: molecular replacement and antioxidant supplements to prevent membrane peroxidation and restore mitochondrial function, J. Cell. Biochem. 100 (2007) 1352–1369.
[39] D.M. Janicke, J.W. Finney, A.W. Riley, Children's health care use: a prospective investigation of factors related to care-seeking, Med. Care 39 (2001) 990–1001.
[40] T. Saheki, K. Inoue, A. Tushima, K. Mutoh, K. Kobayashi, Citrin deficiency and current treatment concepts, Mol. Genet. Metab. 100 (Suppl. 1) (2010) S59–S64.
[41] K. Mutoh, K. Kurokawa, K. Kobayashi, T. Saheki, Treatment of a citrin-deficient patient at the early stage of adult-onset type II citrullinaemia with arginine and sodium pyruvate, J. Inherit. Metab. Dis. 31 (Suppl. 2) (2008) S343–S347.

Reye 症候群はどこへ行った？

高柳正樹*

はじめに

インフルエンザ感染はミトコンドリアの機能障害をきたすことはよく知られている。インフルエンザ感染後発症する脳症はインフルエンザ脳症として精力的にその病態が検討されている。

Reye 症候群はミトコンドリア機能障害が認められ，インフルエンザ感染後の発症も多く報告されていたので，Reye 症候群とインフルエンザ感染との関連についても多くの報告がされている。

最近の若い小児科医では，Reye 症候群という症例を経験をしたことのない人が多くなってきている。筆者が小児科医になった35年ぐらい前は，Reye 症候群の患者が連続して運び込まれるような経験をしたことがある。

医学中央雑誌の和文の論文検索システムにより，「ライ症候群（Reye 症候群）」というキーワードで検索すると，1983～1987の5年間で検索された論文数は295件であり，2008～2012の5年では96件と明らかにその数の減少をみている。

さて，本当に「Reye 症候群はどこへ行った？」のだろうか。

Ⅰ．Reye 症候群とは，さらにはアスピリンとの関係は？

Reye 症候群は1963年に Reye らが初めて報告した症候群で[1]，日本では1967年に第1例が報告されている。

インフルエンザ，水痘および各種ウイルス性疾患に罹患後数日後に，意識障害，嘔吐，けいれん，発熱などで発症し，臨床像は多臓器不全を呈し生命的予後や神経学的な予後のきわめて悪い疾患であった。その病理学的な特徴は，全身臓器とくに肝臓に，Reye 症候群型といってもよいほど特有な脂肪変性とミトコンドリアの変化がみられることである。

さらに，注目すべきことは Reye 症候群とアセチルサルチル酸（アスピリン）との関係である。米国におけるアスピリン投与と Reye 症候群との関連に関する綿密な疫学的研究により[2]，その関連性が指摘され，米国さらには日本においても，アスピリンの使用をインフルエンザ，水痘罹患時には控えるように勧告がなされている。この勧告後にアスピリンの使用頻度の減少に伴い，Reye 症候群の発生が減少してきているという報告もある。この件については筆者の総説を参照されたい[3]。

それでは，最近の Reye 症候群の減少はアスピリンの使用制限だけによるものなのだろうか？

Ⅱ．Reye 症候群の診断における問題点

筆者は先天代謝異常症を専門としているが，最初に先天代謝異常症にふれたのが，この Reye 症候群である。1週間に何人もの患者が Reye 症候群で亡くなったこともあったことから，当時はこの病気の診断法，治療法の確立が急がれていた。

Reye 症候群の診断においては，ミトコンドリアの機能異常をきたす各種の代謝性疾患を除外することが必要であることは，この疾患が初めて報告されたころから重要な問題であった。しかし，先天代謝異常症の診断は測定機器の開発や普及，さらには各種先天代謝異常疾患の一般小児科医へ

TAKAYANAGI Masaki
* 千葉県こども病院小児救急総合診療科
〔〒266-0007 千葉市緑区辺田町579-1〕
TEL 043-292-2111

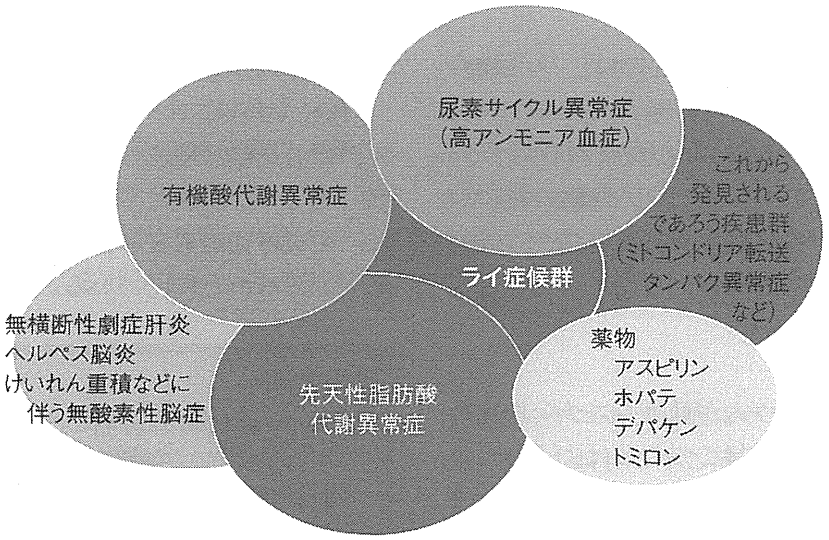

図　Reye 症候群はどこへ行った—Reye 症候群およびその周辺疾患

の教育，啓蒙が不十分であったため非常に難しく，1980 年代後半までは非常に少数の症例にのみ正しい診断がつけられていたという状況であった。昭和という年号がついていたころには，脂肪酸代謝異常症は日本には存在しないとされていたが，現在，拡大新生児マススクリーニングで数多くの脂肪酸代謝異常症が次々と発見されている。つまり，アミノ酸自動分析機，ガスクロマトグラフィー質量分析機，さらにはタンデム質量分析機などが，日常診療に容易に使用できるようになってきた 1990 年代後半の年代になってはじめて，Reye 症候群の正確な鑑別診断が可能になってきたと考えられる。つまり，以前は尿素サイクル異常症，有機酸代謝異常症，脂肪酸代謝異常症，ミトコンドリア呼吸鎖異常症などが，Reye 症候群と誤って診断されていたわけである。

筆者が 1995 年ころに作成した Reye 症候群の周辺に存在する各種疾患の関係を**図**に示したものである。今からみてもこの図は，よくできているのではと筆者は思っている。歴史的なことをいえば尿素サイクル異常症から左回りに，それぞれの疾患の研究が順次進んできた経緯がある。

1．尿素サイクル異常症

この疾患は高アンモニア血症，意識障害，けいれんなどを起こし，無黄疸性の肝不全となるので Reye 症候群として診断されていた症例は数多い。アミノ酸分析などで容易に診断がつくので，現在この疾患が Reye 症候群とされることはないと思われる。

2．有機酸代謝異常症

この疾患は意識障害，けいれんなどが起きるので急性脳症として Reye 症候群とされることがある。ガスクロマトグラフィー質量分析機による尿有機酸分析で容易に診断がつくので，鑑別疾患として有機酸代謝異常症を上げ，有機酸分析をオーダーできれば診断を間違えることはない。

3．脂肪酸代謝異常症

この疾患も急性脳症として発症するので Reye 症候群との鑑別が重要である。脂肪酸代謝異常症は肝臓に脂肪の蓄積があるが，その病理学的な特徴は Reye 症候群とは異なりやや粗大である。タンデム質量分析機による血中アシルカルニチンプロフィール分析が診断に有用である。

4．ミトコンドリア病

最後の輪として書かれている「これから発見されるであろう疾患群」としては，最近急速な病態の解明が進んでいる，ミトコンドリア呼吸鎖異常症があげられる。ミトコンドリア病はわが国ではミトコンドリア遺伝子の病気という考えが強いが，実際はミトコンドリアの機能をつかさどっているのは核にコードされた多くのたんぱく質であり，その生化学的機能や病態の解明は驚くほどの

早さで進んでいる。ことに最近では，リポ酸や鉄・硫黄クラスター，ビタミンB_2などのミトコンドリアコファクター異常症などが注目されている[4]。これら最近のミトコンドリア機能の新たな解明により，新しい疾患概念がさらに次々と確立されていくものと思われる。

5．各種薬剤

バルブロン酸ナトリウムが低カルニチン血症と高アンモニア血症をひき起こすことはよく知られている[5]。バルブロン酸ナトリウム服用によりReye症候群がひき起こされたという報告もある[6]が，先天代謝異常症の検討は十分ではない。ホパテン酸カルシウムによるReye症候群の報告もあるが，これも先天代謝異常症の検討は十分ではない[7]。

Ⅲ．Reye症候群は消えゆく病名である！

図に示されているようにReye症候群は，その周辺の各種疾患により覆い隠されつつある。問題は今後さらにミトコンドリア機能の解明が進んでいったときに，本当にReye症候群として独立した疾患特異性をもった疾患が残るかどうかである。Reye症候群というのは名前のとおり症候群であり，最終的にはそれぞれのきちんとした診断名がつけられる時代が，近いうちにくるものと筆者は考えている。つまり，Reye症候群という診断名は消えていくであろうと考えている。これまで述べたように，かつてReye症候群とよばれていた病態の解明は進んでいるが，その治療法に関しては決して十分ではなく，とくにミトコンドリア病とよばれている疾患群にかんしては，有効な治療法の開発が待たれるところである。

Ⅳ．インフルエンザ脳症も消えゆく病名か？

インフルエンザ脳症と診断された症例を精査し，オルニチントランスカルバミラーゼ欠損症，カルニチンパルミトイルトランスカルバミラーゼ2型やミトコンドリア呼吸鎖異常症という診断となった報告が散見される[8,9]。

今後，さらにミトコンドリアの機能の解明が進み，新しい疾患概念が確立されてくれば，さらに多くのインフルエンザ脳症症例の発症機序として，先天性代謝異常の役割が重要視される時代が必ずくるものと考えている。

Key Points

❶ Reye症候群の診断においては，尿素サイクル異常症，有機酸代謝異常症，脂肪酸代謝異常症，ミトコンドリア呼吸鎖異常症などを十分検討する必要がある。

❷ Reye症候群は消えゆく病名である！ 問題は今後さらにミトコンドリア機能の解明が進んでいったときに，本当にReye症候群として独立した疾患特異性をもった疾患が残るかどうかである。

❸ インフルエンザ脳症の診断においても，十分すぎるほどの先天代謝異常症の鑑別疾患が必要である。

文献

1) Reye RDK, Morgan G, Baral J, et al：Encephalopathy and fatty degeneration of the viscera：A disease entity in childhood. Lancet **2**：749-752, 1963
2) Hurtiza E, Barret MJ, Bregmen D, et al：Public healthservice study of Reye's syndrome and medications. Report of the main study. JAMA **257**：1905-1911, 1987
3) 高柳正樹：薬物とライ症候群．トキシコロギーフォーラム **11**：161-169，1988
4) Lill R, Hoffmann B, Molik S, et al：The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism. Biochim Biophys Acta **1823**：1491-508, 2012
5) 松田一郎，大谷宣伸：バルブロン酸によるライ様症候群の病態．小児科診療 **49**：1020-1026，1986
6) 杉本健郎，西田直樹，安原昭博，他：バルブロン酸投与中におこった，Reye-lile syndromeの1例．脳と発達 **14**：437-439，1982
7) 杉本健郎，安原昭博，西田直樹，他：ホパテン酸カルシウム投与中におこった，急性脳症の3例．脳と発達 **15**：258-259，1983
8) Arakawa C, Endo A, Kohira R, et al：Liver-specific mitochondrial respiratory chain complex I deficiency in fatal influenza encephalopathy. Brain & Development **34**：115-117, 2012
9) Purevsuren J, Hasegawa Y, Kobayashi H, et al：Urinary organic metabolite screening of children with influenza-associated encephalopathy for inborn errors of metabolism using GC/MS. Brain & Development **30**：520-526, 2008

PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society

Pediatrics International (2014) **56**, 180–187 doi: 10.1111/ped.12249

Original Article

Molecular diagnosis of mitochondrial respiratory chain disorders in Japan: Focusing on mitochondrial DNA depletion syndrome

Taro Yamazaki,[1,6] Kei Murayama,[3] Alison G Compton,[6] Canny Sugiana,[6]* Hiroko Harashima,[1] Shin Amemiya,[1] Masami Ajima,[3] Tomoko Tsuruoka,[3] Ayako Fujinami,[3] Emi Kawachi,[3] Yoshiko Kurashige,[4] Kenshi Matsushita,[4] Hiroshi Wakiguchi,[4] Masato Mori,[5] Hiroyasu Iwasa,[2] Yasushi Okazaki,[2] David R Thorburn[6] and Akira Ohtake[1]

[1]*Department of Pediatrics, Faculty of Medicine and* [2]*Translational Research Center, International Medical Center, Saitama Medical University, Saitama,* [3]*Department of Metabolism, Chiba Children's Hospital, Chiba,* [4]*Department of Pediatrics, Kochi Medical School Kochi University, Kochi,* [5]*Department of Pediatrics, Jichi Medical University, Tochigi, Japan and* [6]*Murdoch Childrens Research Institute, Royal Children's Hospital and Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia*

Abstract ***Background***: Although mitochondrial respiratory chain disorders (MRCD) are one of the most common congenital metabolic diseases, there is no cumulative data on enzymatic diagnosis and clinical manifestation for MRCD in Japan and Asia.

Methods: We evaluated 675 Japanese patients having profound lactic acidemia, or patients having symptoms or signs of multiple-organ origin simultaneously without lactic acidemia on respiratory chain enzyme activity assay and blue native polyacrylamide gel electrophoresis. Quantitative polymerase chain reaction was used to diagnose mitochondrial DNA depletion syndrome (MTDPS). Mutation analysis of several genes responsible for MTDPS was also performed.

Results: A total of 232 patients were diagnosed with a probable or definite MRCD. MRCD are common, afflicting one in every several thousand people in Japan. More than one in 10 of the patients diagnosed lacked lactic acidemia. A subsequent analysis of the causative genes of MTDPS identified novel mutations in six of the patients. A 335 bp deletion in deoxyguanosine kinase (*DGUOK*; g.11692_12026del335 (p.A48fsX90)) was noted in two unrelated families, and may therefore be a common mutation in Japanese people. The proportion of all patients with MTDPS, and particularly those with recessive *DNA polymerase γ* (*POLG*) mutations, appears to be lower in Japan than in other studies. This is most likely due to the relatively high prevalence of ancient European *POLG* mutations in Caucasian populations. No other significant differences were identified in a comparison of the enzymatic diagnoses, disease classifications or prognoses in Japanese and Caucasian patients with MRCD.

Conclusion: MTDPS and other MRCD are common, but serious, diseases that occur across all races.

Key words *DGUOK* deletion mutation, enzymatic diagnosis, mitochondrial DNA depletion syndrome, mitochondrial respiratory chain disorder, racial difference.

Mitochondrial respiratory chain disorders (MRCD) are disorders of the oxidative phosphorylation system, which is responsible for ATP production. MRCD are the most common congenital metabolic diseases, afflicting at least 1 in 5000 persons.[1] Mitochondrial DNA depletion syndrome (MTDPS), in which mitochondrial DNA (mtDNA) level is lower than normal, is one of the major MRCD. A number of responsible genes of MTDPS have been identified, and the pathophysiology of this disease is partially characterized at the molecular level.[2–5] We have previously diagnosed and characterized MRCD cases in Japan using respiratory chain enzyme analysis.[6–9] Having recently analyzed the molecular diagnoses and clinical manifestations of MRCD in Japanese patients, and analyzing several genes responsible for hepatocerebral MTDPS, we herein discuss and compare the collected data to those reported for MRCD outside of Japan.

Correspondence: Akira Ohtake, MD PhD, Department of Pediatrics, Faculty of Medicine, Saitama Medical University, 38 Morohongo, Moroyama, Iruma-gun, Saitama 350-0495, Japan. Email: akira_oh@saitama-med.ac.jp

*Present address: Monash IVF PTY Ltd, Melbourne, Victoria, Australia.

Received 11 July 2013; revised 19 August 2013; accepted 21 October 2013.

Methods

Patients and samples

The subjects consisted of patients clinically suspected of having MRCD. We measured respiratory chain enzyme activity and quantity for patients with profound lactic acidemia, or patients with symptoms or signs of multiple-organ origin simultaneously without lactic acidemia. Other metabolic disorders were excluded on plasma tandem mass spectrometry and urine organic acid analysis. Approximately half of candidates were <1 year old,

and nearly 90% were <10 years old. In total, 1051 samples from 675 patients in 657 families were analyzed. Of the samples, 479 were cultured skin fibroblast cells, 239 were liver samples, 208 were muscle samples, 84 were myocardial samples, and 41 were other samples (including 25 kidney and seven brain samples).

Respiratory chain enzyme analysis

Both an *in vitro* respiratory chain enzyme activity assay[10] and blue native polyacrylamide gel electrophoresis (BN-PAGE)[11–13] were used to quantify the activity and amount of respiratory chain enzyme complexes. A diagnosis of MRCD was made when the results from the enzyme activity or BN-PAGE raised the diagnostic criteria assessment to definite or probable for MRCD according to the diagnostic criteria of Bernier *et al.*[14]

Entire mtDNA analysis

DNA was purified according to standard methods. The mitoSEQr™ system (Applied Biosystems, Foster City, CA, USA) was used for entire mtDNA analysis in each patient diagnosed with MRCD.

Quantitative polymerase chain reaction for diagnosis of MTDPS

Quantitative polymerase chain reaction (qPCR)[15] was used to determine whether mtDNA depletion was present in patients with decreased activity level of multiple respiratory chain enzymes (the mtDNA gene *MT-ND1* was compared against a nuclear gene, *CFTR* exon 24). A diagnosis of MTDPS was made when the relative copy number of mtDNA to nuclear DNA was <35% of that in healthy control tissue using four independent experiments.

Mutation analysis of genes responsible for MTDPS

Mutation analysis was performed on the genomic DNA using primers designed to amplify the coding exons and the exon–intron boundaries of DNA polymerase γ (*POLG*; NM_002693.2), *deoxyguanosine kinase* (*DGUOK*; NM_080916.1 and NM_080918.1), and *MPV17* (NM_002437.4).[16] Fragments were analyzed by direct sequencing using ABI 3130XL (Applied Biosystems, Melbourne, Vic., Australia). Long-range PCR encompassing the 335 bp deletion was performed using primers shown in Figure 1(a).

DNA from healthy Japanese controls

A PSC Cell Line Purified DNA 100 set (Japan Health Sciences Foundation, Tokyo, Japan) was used as control DNA for healthy Japanese.

Statistical analysis

The log-rank test and Gehan-Breslow-Wilcoxon test were used to test for statistically significant differences.

Ethics

This study was approved by the Institutional Review Board in Saitama Medical University.

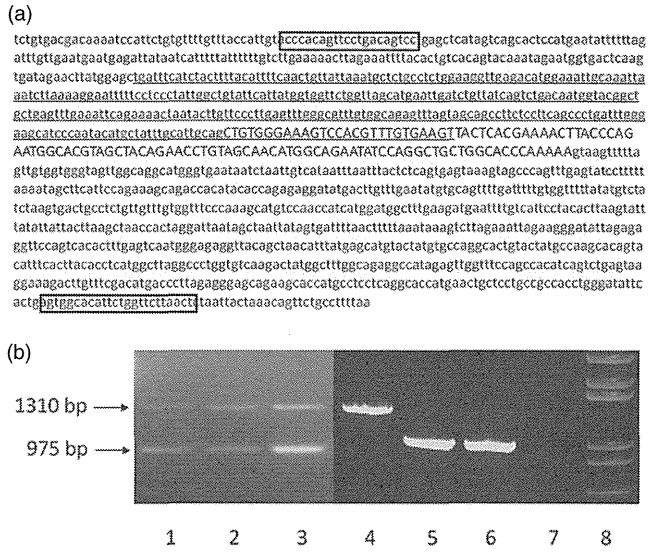

Fig. 1 Genomic sequence determination of 335 bp deoxyguanosine kinase (*DGUOK*) deletion in the family of patients 1 and 2. (a) Capitalization, sequence of exon 2; two rectangles, long-range polymerase chain reaction (PCR) primer sets; underline, 335 bp deletion. The large 335 bp deletion encompassing from the end of intron 1 to the beginning of exon 2 causes the complete skipping of exon 2, and the resultant mRNA has a premature termination codon (p.A48fsX90). (b) Lane 1, father; lane 2, mother; lane 3, middle healthy sister; lane 4, normal control; lane 5, patient 1; lane 6, patient 2; lane 7, no sample; lane 8, molecular weight marker. The 1310 bp band represents the normal sized PCR product. The 975 bp band represents the PCR product with 335 bp *DGUOK* deletion in this family.

Case reports: DGUOK deficiency in three Japanese patients

Patient 1

This Japanese girl was the first child to unrelated healthy parents and was born without any complications at 40 weeks of gestational age, weighing 2510 g. At 3 months of age, she was referred to hospital because of failure to thrive, nystagmus and incomplete head control. Laboratory tests showed mild liver dysfunction of unknown etiology. She was suspected to have hereditary tyrosinemia because her blood tyrosine level was 800 nmol/mL (cut-off, 500 nmol/mL), but urinary succinylacetone was not detected. At the age of 18 months, her liver dysfunction deteriorated to the level of liver failure with prolonged coagulation time (hepaplastin time 39%), and she underwent a liver transplantation, but died of cardiac tamponade at 19 months of age. Liver respiratory chain enzyme assay showed low activity of complexes I, III, and IV (0%, 9%, and 28% of normal control, respectively). In contrast, complex II activity was normal and citrate synthase was moderately increased (74% and 308%, respectively). On BN-PAGE analysis, the band corresponding to assembled complex I was invisible and those of complex III and IV were strikingly weak (data not shown). On qPCR, liver mtDNA was markedly decreased (3%), confirming a diagnosis of hepatocerebral MTDPS.

Patient 2

A healthy sister of patient 1 was born 2 years after her elder sister died. A third girl was born 4 years after her eldest sister died, without any complications at 40 weeks of gestation, with a weight of 2750 g. At 2 days of age, she was referred to hospital due to tachypnea, hypoglycemia, and metabolic acidosis. After that, mild liver dysfunction was found (total bilirubin, 4.2 mg/dL; direct bilirubin, 1.4 mg/dL; aspartate aminotransferase, 215 IU/L; alanine aminotransferase, 49 IU/L; γ-glutamyl transpeptidase, 842 IU/L) with hyperammonemia (180 μg/dL). Blood lactate and pyruvate were 20.9 mmol/L, and 0.27 mmol/L, respectively. Because of her eldest sister's course, she did not undergo liver transplantation and she died of liver failure at 9 months of age. The liver showed low activity of complexes I, III, and IV (0%, 6%, and 17% of normal control, respectively). In contrast, complex II activity was normal and citrate synthase was moderately increased (105% and 281%, respectively), as for the eldest sister. On qPCR, liver mtDNA was markedly decreased (6%) and she was diagnosed with hepatocerebral MTDPS.

Patient 3

A Japanese girl, unrelated to patients 1 and 2, was born as the third child to unrelated healthy parents at 37 weeks of gestational age weighing 1688 g. Symmetrical intrauterine growth retardation was noted from 30 weeks gestation. Her eldest brother died at 1 year 4 months with a hepatic disorder of unknown origin. Her elder sister was healthy. At 8 days of age, she was suffering from feeding difficulty with liver dysfunction and nystagmus. Developmental delay and failure to thrive gradually progressed. At the age of 8 months, her liver dysfunction deteriorated to the level of liver failure, and she underwent liver transplantation, but died at 18 months of age. Liver respiratory chain enzyme assay showed low activity of complexes I, III, and IV (12%, 12%, and 16% of normal control, respectively). In contrast, complex II and citrate synthase activity were normal (68% and 106%, respectively). On qPCR, liver mtDNA was markedly decreased (2%) and she was diagnosed with hepatocerebral MTDPS.

Results

Characteristics of Japanese children diagnosed with MRCD

In total, we diagnosed MRCD in 232 patients; these patients comprised 34% of the study group. The age distribution of these patients is as follows; nearly 40% before 1 month of age, three-fourths by age 1 year, and >90% by age 7 years. One hundred and twenty patients (52%) were male, and approximately half of the diagnosed patients were deceased. Diverse clinical diagnoses are shown in Figure 2. Eighty-seven patients (38%) had neurological disorders consisting of Leigh syndrome, neurodegenerative disorders, and so-called mitochondrial cytopathy. Fifty-nine (25%) had a lethal or non-lethal infantile mitochondrial disorder. Twenty-nine (13%) had mitochondrial hepatopathy, and 17 (7%) had mitochondrial cardiomyopathy. Among all MRCD, 28 patients (12%) lacked lactic acidemia, a feature that traditionally prompts suspicion of MRCD. The entire mitochondrial DNA

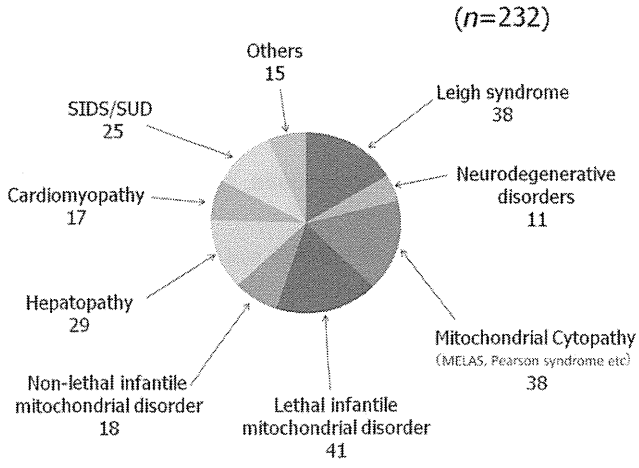

Fig. 2 Clinical diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan. Neurodegenerative disorders, neurodegenerative disorders unclassified to specific diseases. Patients with non-lethal infantile mitochondrial disorder started with symptoms such as lethal infantile mitochondrial disorder but survived beyond 1 year old. SIDS, sudden infant death syndrome; SUD, sudden unexplained death.

sequence was determined for 139 patients, but a causative genetic abnormality was found in only 34 (24%) of these patients (data not shown); indicating that, in most cases, the causative gene or genes may be present in nuclear DNA.

The enzymatic diagnoses were compared with Australian data (Fig. 3).[17] In Japanese patients, a respiratory chain complex I abnormality was most common (105 patients, 45%), followed, in decreasing order of prevalence, by respiratory chain abnormalities in multiple complexes (80 patients, 34%), a complex IV abnormality (33 patients, 13%), and a complex III abnormality (10 patients, 4%). No patient was given a probable or definitive

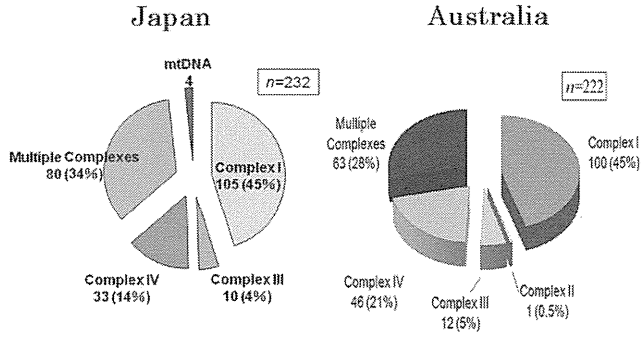

Fig. 3 Percentage distribution of enzymatic diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan and those reported previously in Australia. The enzymatic diagnosis of MRCD showed similar trends in prevalence between the Japanese and Australian patients,[17] with respiratory chain complex I being the most common type of MRCD, followed by abnormalities in multiple complexes, complex IV abnormalities, and complex III abnormalities. Complex II abnormalities were very rare among the two populations.

diagnosis of a complex II abnormality. Similarly, according to the Australian data, the most common abnormality was in complex I (45%), followed by abnormalities in multiple complexes (28%), complex IV (21%), and complex III (5%); only one patient had a complex II abnormality.

Manifestations, genetic diagnoses, and prognoses of MTDPS

A qPCR-based diagnosis of MTDPS was made for 16 of the 80 patients with an enzymatic diagnosis of a multiple complex abnormality, and for seven of the 105 patients with an enzymatic diagnosis of a respiratory chain complex I abnormality. Three of these 23 patients died due to sudden infant death syndrome and thus had no available records of clinical findings; the clinical findings from the remaining 20 patients were further analyzed.

The disease types among these 20 patients were compared with those reported by Sarzi *et al.*[4] (Fig. 4). Among the Japanese patients, 13 (65%) had acute hepatocerebral MTDPS, two (10%) had Alpers-like syndrome (delayed-onset hepatocerebral MTDPS), and five (25%) had encephalomyopathic MTDPS. This distribution is similar to that reported by Sarzi *et al.* We must note here that "Alpers-like" refers simply to delayed-onset hepatocerebral MTDPS. This is because no true case of Alpers syndrome has yet been identified in Japan. The results of analyses of the three main genes responsible for MTDPS are shown in Figure 5. Causative genetic anomalies were identified in six of the 20 Japanese patients (30%). No abnormality was identified in the three genes of the remaining 14 patients (70%). The responsible genes were *DGUOK* in three patients whose clinical reports are described in the previous section, *MPV17* in two patients,[7] and *POLG* in one patient whose clinical report will be published elsewhere. The individual genetic abnormalities are listed with the clinical findings in Table 1. Although three of the patients

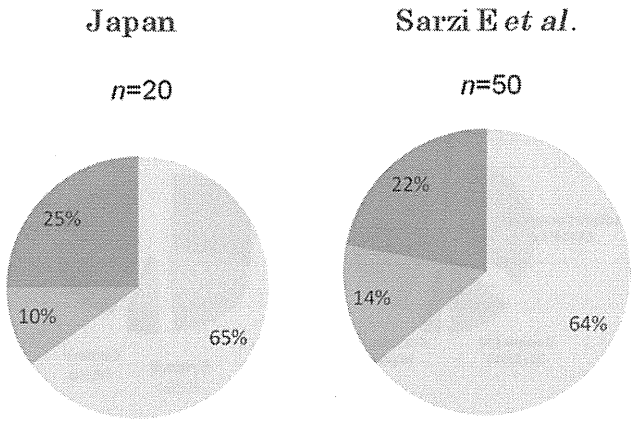

Fig. 4 Percentage distribution of disease types of mitochondrial DNA depletion syndrome (MTDPS) in Japan and those reported by Sarzi *et al.* "Alpers-like" refers simply to delayed-onset hepatocerebral MTDPS, because no true case of Alpers syndrome has yet been identified in Japan. The distribution of disease types in the present study did not differ from that reported by Sarzi *et al.*[4]. (▫) Hepatocerebral, (▪) Alpers–like syndrome, (■) Encephalomyopathic.

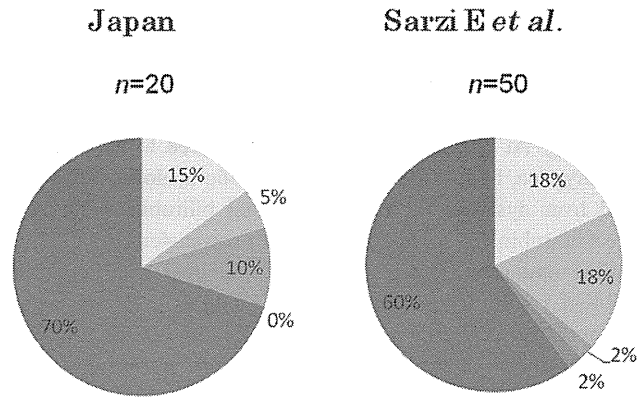

Fig. 5 Percentage distribution of responsible genes for mitochondrial DNA depletion syndrome (MTDPS) in Japan and those reported by Sarzi *et al.* The causative gene was not identified in the majority of patients in each population. Four genes, *DGUOK*, *POLG*, *MPV17*, and *TK2*, contained 40% of the causative genetic abnormalities identified by Sarzi *et al.*,[4] while three genes, *DGUOK*, *POLG*, and *MPV17*, contained 30% of the abnormalities found in the Japanese patients. (▫) *DGUOK*, (▫) *POLG*, (▪) *MPV17*, (▪) *TK2*, (▪) *unknown*. DGUOK, deoxyguanosine kinase; POLG, DNA polymerase γ.

underwent liver transplantation during infancy, five of them died before 2 years of age. Patient 5 lived longer than the others because of dietary and pharmaceutical treatment targeting the mitochondrial respiratory chain complex II.[7]

The *DGUOK*-related patients were two sisters, with a homozygous 335 bp deletion (Fig. 1a; g.11692_12026del335; encompassing 308 bp of intron 1 and 27 bp at the start of exon 2), and a compound heterozygote patient, genetically unrelated to these sisters, with the same deletion and a c.743T>C (p.L248P) missense mutation. The large 335 bp deletion encompassing from intron 1 to exon 2 causes the complete skipping of exon 2, and the resultant mRNA has a premature termination codon (p.A48fsX90). Each parent and healthy sister is heterozygous for this mutation (Fig. 1b). The p.L248P variation is not listed as a polymorphism in the ensembl_mart_47 database (martdb.ensembl.org) and has not been reported as a disease-causing mutation. Moreover, the alignment shows that Leu248 is absolutely conserved in all species (Fig. 6).[18]

The *MPV17* patients were previously reported compound heterozygote siblings.[7] The *POLG* patient was a compound heterozygote. The genetic mutations noted in these six patients were confirmed to be absent in DNA of 100 healthy Japanese controls (data not shown).

Like Sarzi *et al.*, who did not find the responsible gene or genes in 60% of the patients, we were unable to identify the responsible gene or genes in a majority of the cases. We sequenced the whole exome of all the MTDPS patients to identify the underlying nuclear disease genes using next-generation sequencing system (data not shown). This did not identify pathogenic mutations in any of the known genes associated with MTDPS (*TK2, SUCLA2, RRM2B, SUCLG1, MGME1, C10orf2, TYMP, and AGK*) in the present MTDPS patients.

Table 1 Clinical and molecular characteristics for Japanese hepatocerebral MTDPS patients

Patient	Sex	Initial symptoms (age)	Outcome (age)	Clinical diagnosis	Complications	Liver transplantation	Blood lactate/ pyruvate (mmol/L)	%mtDNA in liver	Identified mutations	Ref
1	F	Failure to thrive (3 months)	Dead (1 year)	Hereditary tyrosinemia	Developmental delay	Done	Not available	3	*DGUOK* (g.11692_12026del335 (p.A48fsX90) homozygote)	
2	F	Tachypnea (2 days)	Dead (9 months)	Mitochondrial hepatopathy	Hypoglycemia	Not done	20.9/0.27	6	*DGUOK* (g.11692_12026del335 (p.A48fsX90) homozygote)	
3	F	Feeding difficulty (8 days)	Dead (1 year)	Mitochondrial hepatopathy	Developmental delay, failure to thrive	Done	2.9/0.14	2	*DGUOK* (g.11692_12026del335 (p.A48fsX90) / c.743T>C (p.L248P))	
4	M	Failure to thrive, acholic stool (3 months)	Dead (1 year)	Hepatic failure	Developmental delay	Done	Not available	8	*MPV17* (c.451insC (p.L151fsX189)/ c.509C>T (p.S170F))	7
5	M	Failure to thrive, vomiting (8 months)	Dead (6 years)	Hepatic failure	Developmental delay, gastroesophageal reflux, respiratory failure	Done (at 6 years)	Normal	7	*MPV17* (c.451insC (p.L151fsX189)/ c.509C>T (p.S170F))	7
6	F	Failure to thrive (4 months)	Dead (7 months)	Hepatic failure	Hypotonia	Not done	1.76/0.1	3	*POLG* (c.2869G>C (p.A957V) / c.3354T>C (p.I1185T))	

Shaded columns, two pairs of siblings. MTDPS, mitochondrial DNA depletion syndrome.

```
Human          241   -----ALMNIPVLVLDV--NDDFSEEVTKQEDLMREVNTFVKNL------   277
Pan Trog       241   -----ALMNIFVLVLDV--NDDFSEEVTKQEDLMREVNTFVKNL------   277
Canis          241   -----ALLNIPVLVLDV--NDDFSEEVTKQEELMKKVNIFVKNL------   277
Bos            241   -----ALLNIPVLVLDV--NDDFSEEVTIQEELMRRVNTFVKNL------   277
Mus            241   -----ALQHVPVLVLDV--TEDFSENAARQEELMGQVNTFMRNL------   277
Rat            241   -----ALRHVPVLVLNI--SEDFSENAAKQEELMGQVNTFMRNL------   277
Danio          233   -----QLMKVPVLVLDA--EVAFEQNPEVQDCLLSKVRDFLSQL------   269
Arabidopsis    483   NHMHSSIQKVPALVLDCEPNIDFSRDIEAKTQYARQVAEFFEFVKKKQET   532
Oryza          408   DHMHSSIQKVPALVLDCEHDIDFNKDIEAKRQ------------------   439
```

Fig. 6 ClustalW multiple sequence alignment of deoxyguanosine kinase (*DGUOK*) orthologs. The alignment shows that amino acid 248Leu mutated in the patient is absolutely conserved in all species. URLs: HomoloGene, http://www.ncbi.nlm.nih.gov/homologene (for the DGUOK ortholog amino acid sequences of human [accession no. NP_550438.1], Pan [accession no. XP_001153473.1], Canis [accession no. XP_533001.2], Bos [accession no. NP_001014888.2], Mus [accession no. XP_001107072.1], Rat [accession no. NP_001100072.1], Danio [accession no. XP_001093561.1], Arabidopsis [accession no. NP_565032.2], Oryza [accession no. NP_001044956.1]). ClustalW, http://www.ebi.ac.uk/Tools/clustalw/.[18]

Of the genetic mutations identified, *POLG* mutations were less prevalent than in Caucasian subjects. Only one of the present 15 cases of Alpers syndrome or hepatocerebral MTDPS were caused by recessive *POLG* mutations, compared with eight of 39 such cases diagnosed in France.

Sixteen of the 20 Japanese MTDPS patients were deceased. Sarzi *et al.* reported that 29 of the 50 MTDPS patients they analyzed were deceased. The data of the deceased patients were plotted to obtain curves of the ages of death (in months) in the two groups for comparison (Fig. 7). MTDPS patients had a short life in both study groups; many died during or before reaching early childhood. On log-rank test and Gehan-Breslow-Wilcoxon test no significant differences were seen between the survival data.

Discussion

We started an enzyme diagnosis referral service for children suspected of MRCD in 2007 and have diagnosed MRCD in 30–40 patients from around Japan annually since then. In the last year we have made >100 new MRCD diagnoses. Approximately half of the diagnoses are for neonates. There are approximately one million births in Japan annually. Under the assumption that the patients referred for enzyme diagnosis represent approximately half of all Japanese MRCD patients, the prevalence of neonatal-onset MRCD becomes $50 \times 2/1\,000\,000 = 1/10\,000$. When patients with juvenile-onset and adult-onset mitochondrial disease are factored in, the prevalence of these diseases in Japan becomes one in several thousand, which is comparable to the prevalence in Western countries.[1]

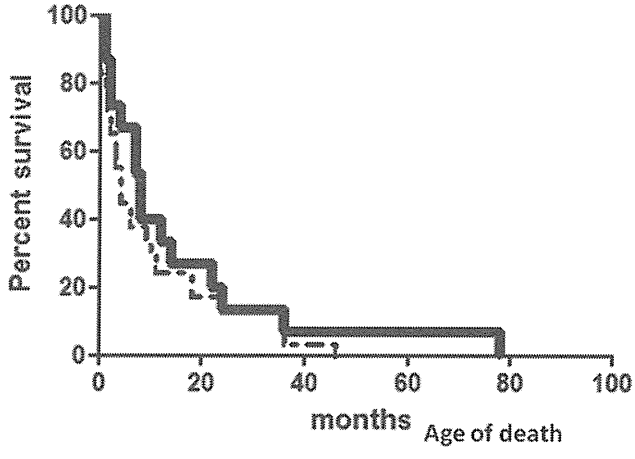

Fig. 7 Comparison of the ages of death (in months) in the two studies. A commonality between the Japanese patients and the Sarzi *et al.* patients[4] was observed. No significant difference in disease severity was identified (log-rank test, $P = 0.3637$; Gehan-Breslow-Wilcoxon test, $P = 0.2667$). (▬) Japanese, $n = 16/20$; (▪▪▪) Sarzi *et al.*, $n = 29/50$.

It is noteworthy that >10% of the patients lacked lactic acidemia, which many physicians still regard as synonymous with mitochondrial disease. Hence, mitochondrial disease must also be considered in lactic acidemia-free patients with unexplained signs and symptoms in multiple organs.

The enzymatic diagnosis of MRCD showed similar trends in prevalence between Japanese and Australian patients, with respiratory chain complex I being the most common type of MRCD, followed by abnormalities in multiple complexes, complex IV abnormalities, and complex III abnormalities. Complex II abnormalities were very rare in both populations.

Twenty percent of the patients with multiple respiratory chain disorders in the present study and 50% of the patients in the Sarzi *et al.* study[4] had MTDPS. Although MTDPS was the leading cause of MRCD in both groups, MTDPS represented a smaller proportion of the MRCD in Japan. According to the Online Mendelian Inheritance in Man database, MTDPS can be classified as encephalomyopathic, hepatocerebral, or specific (a classification that includes mitochondrial neurogastrointestinal encephalopathy [MNGIE] and Sengers syndrome). Encephalomyopathic MTDPS features respiratory failure and myopathy. Hepatocerebral MTDPS is characterized by liver disorders, growth disorders, and hypoglycemia. The distribution of the disease type classifications of the Japanese patients did not differ from the distribution reported by Sarzi *et al.*

Four genes, *DGUOK*, *POLG*, *MPV17*, and *TK2*, contained 40% of the causative genetic abnormalities in the Sarzi *et al.* study, while three genes, *DGUOK*, *POLG*, and *MPV17*, contained 30% of the abnormalities found in the Japanese patients. The causative gene, however, was not identified in the majority of patients in each study. The six Japanese hepatocerebral MTDPS patients in whom the responsible gene was identified are listed in Table 1. The serious nature of this disease is evident, given that all six experienced onset as neonates or infants and died during or before reaching early childhood.

Deoxyguanosine kinase deficiency was originally described as the cause of infantile-onset hepatocerebral mitochondrial disease, typically featuring hepatic failure, nystagmus and hypotonia.[19] Recently it has been shown that patients with *DGUOK* mutation may present with neonatal hemochromatosis[20] or adult-onset myopathy and mitochondrial DNA multiple deletions, with or without liver involvement.[21,22] We found two novel *DGUOK* mutations in two apparently unrelated Japanese families. Three patients in two families had typical signs and symptoms of hepatocerebral MTDPS, and both parents in each family were

heterozygous for these mutations. A 335 bp deletion in *DGUOK* was found in both families, and may therefore be a common mutation in the Japanese population.

The present analysis of MTDPS patients concludes with a comparison of the ages of death (in months) in the two groups (Fig. 7). A commonality between the Japanese patients and the Sarzi *et al.* patients was the early age of death: most patients died during or before reaching early childhood. *DGUOK* deficiency was most serious in both studies. Likewise, many patients in each study experienced onset as neonates or infants. No significant difference in disease severity was identified between the two studies.

The present results indicate a lower prevalence of *POLG* mutations in the Japanese population, which is likely attributable to several common mutations found in Caucasian people that appear to be ancient European founder mutations (p.A467T, p.G848S, and p.W748S).[23] In children with recessive *POLG* mutations, these three mutations represented seven of 16 mutant alleles reported by Sarzi *et al.*[4] A recent study collated the prevalence of these three mutations in 10 studies reporting a total of 249 POLG patients and found that they represented 49% of mutant alleles in predominantly Caucasian patients.[24] Most Caucasian POLG patients will thus have at least one allele carrying one of these three founder mutations, and Hakonen *et al.* suggested that they may have been spread during Viking times.[23] The carrier frequency of these mutations is as high as 2% in some European countries. Their expected absence in Asian patients likely explains a lower prevalence of recessive POLG disease in Asian populations.

Conclusion

Mitochondrial DNA depletion syndrome and other mitochondrial respiratory chain disorders are common, but serious, diseases that occur across all races.

Acknowledgments

This work was supported in part by a grant for Innovative Cell Biology by Innovative Technology (Cell Innovation Program) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and Support Project and a grant of Strategic Research Center in Private Universities from the MEXT, Japan to Saitama Medical University Research Center for Genomic Medicine, and by Grants-in-Aid of the Research on Intractable Diseases (Mitochondrial Disorder) from the Ministry of Health, Labour and Welfare of Japan. Dr Murayama was supported by Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics. Dr Thorburn was supported by a Principal Research Fellowship and grants from the National Health and Medical Research Council (NHMRC) of Australia. The authors thank Dr Ri at Kumamoto University, Dr Kaji at Tsuyama Central Hospital and Dr Maegaki at Tottori University for referral of patient materials.

References

1 Skladal D, Halliday J, Thorburn DR. Minimum birth prevalence of mitochondrial respiratory chain disorders in children. *Brain* 2003; **126**: 1905–12.
2 DiMauro S, Hirano M. Mitochondrial DNA Deletion Syndromes. In: Pagon RA, Adam MP, Bird TD, *et al.* (eds). *GeneReviews*™ [Internet]. University of Washington, Seattle; 1993–2013. [Initial posting: 17 December 2003; Last update: 3 May 2011.]
3 Spinazzola A, Invernizzi F, Carrara F *et al.* Clinical and molecular features of mitochondrial DNA depletion syndromes. *J. Inherit. Metab. Dis.* 2009; **32**: 143–58.
4 Sarzi E, Bourdon A, Chretien D *et al.* Mitochondrial DNA depletion is a prevalent cause of multiple respiratory chain deficiency in childhood. *J. Pediatr.* 2007; **150**: 531–4. 34 e1–6.
5 Copeland WC. Inherited mitochondrial diseases of DNA replication. *Annu. Rev. Med.* 2008; **59**: 131–46.
6 Yamamoto T, Emoto Y, Murayama K *et al.* Metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy: Diagnosis of mitochondrial respiratory chain disorders. *Mol. Genet. Metab.* 2012; **106**: 474–7.
7 Kaji S, Murayama K, Nagata I *et al.* Fluctuating liver functions in siblings with MPV17 mutations and possible improvement associated with dietary and pharmaceutical treatments targeting respiratory chain complex II. *Mol. Genet. Metab.* 2009; **97**: 292–6.
8 Sakamoto O, Ohura T, Murayama K *et al.* Neonatal lactic acidosis with methylmalonic aciduria due to novel mutations in the SUCLG1 gene. *Pediatr. Int.* 2011; **53**: 921–5.
9 Murayama K, Nagasaka H, Tsuruoka T *et al.* Intractable secretory diarrhea in a Japanese boy with mitochondrial respiratory chain complex I deficiency. *Eur. J. Pediatr.* 2009; **168**: 297–302.
10 Kirby DM, Crawford M, Cleary MA, Dahl HH, Dennett X, Thorburn DR. Respiratory chain complex I deficiency: An underdiagnosed energy generation disorder. *Neurology* 1999; **52**: 1255–64.
11 Schagger H, von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* 1991; **199**: 223–31.
12 Kirby DM, Salemi R, Sugiana C *et al.* NDUFS6 mutations are a novel cause of lethal neonatal mitochondrial complex I deficiency. *J. Clin. Invest.* 2004; **114**: 837–45.
13 Dabbeni-Sala F, Di Santo S, Franceschini D, Skaper SD, Giusti P. Melatonin protects against 6-OHDA-induced neurotoxicity in rats: A role for mitochondrial complex I activity. *FASEB J.* 2001; **15**: 164–70.
14 Bernier FP, Boneh A, Dennett X, Chow CW, Cleary MA, Thorburn DR. Diagnostic criteria for respiratory chain disorders in adults and children. *Neurology* 2002; **59**: 1406–11.
15 Pagnamenta AT, Taanman JW, Wilson CJ *et al.* Dominant inheritance of premature ovarian failure associated with mutant mitochondrial DNA polymerase gamma. *Hum. Reprod.* 2006; **21**: 2467–73.
16 Compton AG, Troedson C, Wilson M *et al.* Application of oligonucleotide array CGH in the detection of a large intragenic deletion in POLG associated with Alpers Syndrome. *Mitochondrion* 2011; **11**: 104–7.
17 Thorburn DR, Chow CW, Kirby DM. Respiratory chain enzyme analysis in muscle and liver. *Mitochondrion* 2004; **4**: 363–75.
18 Thompson JD, Higgins DG, Gibson TJ. ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994; **22**: 4673–80.
19 Mandel H, Szargel R, Labay V *et al.* The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. *Nat. Genet.* 2001; **29**: 337–41.
20 Hanchard NA, Shchelochkov OA, Roy A *et al.* Deoxyguanosine kinase deficiency presenting as neonatal hemochromatosis. *Mol. Genet. Metab.* 2011; **103**: 262–7.
21 Buchaklian AH, Helbling D, Ware SM, Dimmock DP. Recessive deoxyguanosine kinase deficiency causes juvenile onset mitochondrial myopathy. *Mol. Genet. Metab.* 2012; **107**: 92–4.

22 Ronchi D, Garone C, Bordoni A *et al.* Next-generation sequencing reveals DGUOK mutations in adult patients with mitochondrial DNA multiple deletions. *Brain* 2012; **135**: 3404–15.
23 Hakonen AH, Davidzon G, Salemi R *et al.* Abundance of the POLG disease mutations in Europe, Australia, New Zealand, and the United States explained by single ancient European founders. *Eur. J. Hum. Genet.* 2007; **15**: 779–83.
24 Tang S, Wang J, Lee NC *et al.* Mitochondrial DNA polymerase gamma mutations: An ever expanding molecular and clinical spectrum. *J. Med. Genet.* 2011; **48**: 669–81.

Biochimica et Biophysica Acta 1840 (2014) 1355–1359

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Diagnosis and molecular basis of mitochondrial respiratory chain disorders: Exome sequencing for disease gene identification☆,☆☆

A. Ohtake [a,*], K. Murayama [b], M. Mori [c], H. Harashima [a], T. Yamazaki [a], S. Tamaru [d], Y. Yamashita [d], Y. Kishita [d], Y. Nakachi [d], M. Kohda [d], Y. Tokuzawa [d], Y. Mizuno [d], Y. Moriyama [d], H. Kato [d], Y. Okazaki [d]

[a] Department of Pediatrics, Faculty of Medicine, Saitama Medical University, Saitama 350-0495, Japan
[b] Department of Metabolism, Chiba Children's Hospital, Chiba 266-0007, Japan
[c] Department of Pediatrics, Jichi Medical University, Tochigi 329-0498, Japan
[d] Research Center for Genomic Medicine, Saitama Medical University, Saitama 350-0495, Japan

ARTICLE INFO

Article history:
Received 30 September 2013
Received in revised form 13 January 2014
Accepted 14 January 2014
Available online 24 January 2014

Keywords:
Mitochondrial respiratory chain disorder
Blue native polyacrylamide gel
Electrophoresis
Exome sequencing
Narrowing down protocol

ABSTRACT

Mitochondrial disorders have the highest incidence among congenital metabolic diseases, and are thought to occur at a rate of 1 in 5000 births. About 25% of the diseases diagnosed as mitochondrial disorders in the field of pediatrics have mitochondrial DNA abnormalities, while the rest occur due to defects in genes encoded in the nucleus. The most important function of the mitochondria is biosynthesis of ATP. Mitochondrial disorders are nearly synonymous with mitochondrial respiratory chain disorder, as respiratory chain complexes serve a central role in ATP biosynthesis. By next-generation sequencing of the exome, we analyzed 104 patients with mitochondrial respiratory chain disorders. The results of analysis to date were 18 patients with novel variants in genes previously reported to be disease-causing, and 27 patients with mutations in genes suggested to be associated in some way with mitochondria, and it is likely that they are new disease-causing genes in mitochondrial disorders. This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

1. Introduction

1.1. Mitochondrial disorders

Mitochondrial disorders have the highest incidence among congenital metabolic disorders, and are thought to occur at a rate of 1 in 5000 births [1]. The common view of mitochondrial disorders is that they include mitochondrial encephalopathy and myopathy, with onset due to mitochondrial DNA defects inherited through the maternal line. In fact, however, only about 25% of the diseases diagnosed as mitochondrial disorders in the field of pediatrics have mitochondrial DNA abnormalities [2,3], while the rest occur due to defects in genes encoded in the nucleus. Most cases are sporadic (do not have a clear genetic association), and a majority of cases resulting from nuclear gene abnormalities are autosomal recessive. Mitochondrial DNA has a circular structure with a length of 16.6 kbp, and encodes only 13 proteins [4]. These 13 proteins are part of the structural composition of complex I (7 proteins), complex III (1 protein), complex IV (3 proteins) and complex V (2 proteins) in the respiratory chain. They do not include any complex II structural proteins. The remaining genes encoded in mitochondrial DNA are 22 tRNAs and two ribosomal RNAs, and mitochondrial disorders due to defects in these RNAs have also been reported. Meanwhile, a certain amount of the gene products encoded in the nucleus exists in the mitochondria, and roughly 1500 are thought to serve important roles in mitochondrial function [5]. In this analysis, we focused on mitochondrial disorders thought to occur due to defects in genes encoded in the nucleus. Mitochondria have many functions, one of the most important being biosynthesis of energy (ATP), and we assume for the following discussion that mitochondrial disorders are nearly synonymous with mitochondrial respiratory chain disorders (MRCD), as respiratory chain complexes [6] serve a central role in ATP biosynthesis.

1.2. Mitochondrial disorders of nuclear origin

As stated above, of the approximately 1500 genes encoded in the nucleus that are thought to be involved in biosynthesis and mitochondrial function, more than 100 have been reported to be causes of mitochondrial disorders [7–9] (Table 1). Among these, about 90% of genes have an autosomal recessive inheritance pattern, and only a small portion

Abbreviations: MRCD, mitochondrial respiratory chain disorder; BN-PAGE, blue native polyacrylamide gel electrophoresis; iPS, induced pluripotent stem cells; LIMD, lethal infantile mitochondrial disease; LCSH, Long Contiguous Stretch of Homozygosity

☆☆ This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

* Corresponding author. Fax: +81 49 276 1790.
E-mail address: akira_oh@saitama-med.ac.jp (A. Ohtake).

0304-4165/$ – see front matter © 2014 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2014.01.025

Table 1
The genetic basis of MRCD.

mtDNA mutations: 35/37 genes	
tRNAs, subunits, rRNAs, and deletions & duplications	
Nuclear mutations: 117 genes	
Nuclear-encoded subunits: 27/~80 genes	**mtDNA replication: 5 genes**
Complex I: *NDUFV1, 2, NDUFB3, 9*	*POLG, POLG2, C10orf2, MPV17, AGK*
NDUFA1, 2, 9, 10, 11, 12, NDUFS1, 2, 3, 4, 6, 7, 8	**mtDNA expression: 24 genes**
Complex II: *SDHA, SDHB, SDHC, SDHD*	*LRPPRC, TACO1, MTPAP, MRPS16, MRPS22,MRPL3,*
Complex III: *UQCRB, UQCRQ*	*GFM1,TSFM,TUFM,TRMU,C12orf65,MTFMT, DARS2,*
Complex IV: *COX6B1, COX4I2, COX7B*	*RARS2,YARS2,SARS2,AARS2,HARS2,MARS2,EARS2,*
Complex V: *ATP5E*	*RMND1, MTO1, FARS2, GFM2*
Import, processing, assembly: 38 genes	**Nucleotide transport, synthesis: 9 genes**
Complex I: *C8orf38, C20orf7, NDUFAF1, F2, F3, F4,*	*SLC25A4, SLC25A3, TYMP, DGUOK, TK2, PUS1,*
FOXRED1, NUBPL, ACAD9, AIFM1	*SUCLA2, SUCLG1, RRM2B*
Complex II:*SDHAF1, SDHAF2*	**Membrane composition: 14 genes**
Complex III:*BCS1L, HCCS, TTC19*	*COQ2, COQ6, COQ9, PDSS1, PDSS2, CABC1,*
Complex IV:*SURF1, SCO2, SCO1, COX10, COX15,*	*SERAC1, MPC1, NMT, TAZ, CYCS, OPA1, MFN2, DNM1L*
ETHE1, FASTKD2, C2orf64, C12orf62	
Complex V:*ATPAF2, TMEM70*	
Multiple:*TIMM8A, SPG7, HSPD1, AFG3L2, DNAJC19, GFER*	
Iron/FeS: *FXN, ISCU, GLRX5, ABCB7, NFU1, BOLA3*	
117 nuclear gene defects	Categories are based on D.R Thorburn's paper[7]

95: autosomal recessive·
10: autosomal dominant·
5: recessive or dominant·
7: X-linked·

have a dominant inheritance pattern [10]. There have also been seven reported cases of mitochondrial disorders from defects in genes encoded by the X chromosome. By function, these include genes involved in the structural composition of the complexes and mitochondrial biosynthesis, genes involved in membrane composition, genes involved in the synthesis and transport of nucleic acids, genes involved in regulating the expression of mitochondrial DNA, and genes involved in mitochondrial DNA replication.

We have actively analyzed the exomes of patients with MRCD in order to identify the cause. Here, we briefly describe our project and discuss the results of exome analyses performed to date, touching on some of the problems that have been encountered.

2. Outline of exome analysis project for MRCD patients

Fig. 1 outlines our current project. It is supported by the Ministry of Education, Culture, Sports, Science and Technology's Research Program of Innovative Cell Biology by Innovative Technology (Cell Innovation) (http://www.cell-innovation.org/english/html/program/theme_010_okazaki.html). First, analyses of enzyme activity [11], quantity and size were performed using fibroblasts from patient skin or biopsy specimens from diseased organs of patients suspected of having MRCD in clinical practice [12]. Quantity and size were analyzed using blue native polyacrylamide gel electrophoresis (BN-PAGE) [13]. Next, among patients in whom decreased enzyme activity or complex formation abnormalities were seen biochemically, whole exome analysis was performed in those with no known mitochondrial DNA abnormalities, and the obtained candidate causal genes were confirmed at the cellular level by rescue experiment or other methods, such as siRNA experiment. Many patients with mitochondrial disorders have primary symptoms in the central nervous system, but brain biopsy in these patients is untenable. Therefore, induced pluripotent stem (iPS) cells were created using fibroblasts from the skin of patients from whom informed consent was obtained. These iPS cells were then differentiated into neurons and glia cells to reproduce the pathology of mitochondrial dysfunction that occurs specifically in the nervous system, based on the notion that this may lead to treatment at the cellular level and ultimately to treatment in humans.

3. Clinical diagnosis of MRCD

Mitochondria exist in all tissues, and symptoms are presented in various organs and/or pathological entities. In pediatric MRCD, symptoms are broadly divided into: (1) encephalomyopathy symptoms; (2) gastrointestinal/hepatic symptoms; and (3) myocardial symptoms [14]. So-called "mitochondrial encephalomyopathy," which has traditionally been considered the main form of mitochondrial disease, belongs among the relatively mild mitochondrial diseases and occurs mostly in older people. Fig. 2 shows a breakdown of clinical diagnoses of mitochondrial disorders in our institute as of January 2013 [15]. Patients with the traditionally described nerve and muscle symptoms numbered 111 in total, including 50 with Leigh syndrome, 11 with neurodegenerative disorders for which no clear cause could be identified, and 50 with so-called "mitochondrial encephalomyopathy." These 111 patients accounted for 40% of the total of 275 patients. Conversely, other forms accounted for two-thirds of cases, among which were 49 cases of lethal infantile mitochondrial disease (LIMD). Together with non-lethal infantile mitochondrial disease (NLIMD), which follows the same course but in which patients survive beyond 1 year of age, the number reached 71, and was by far the most common clinical diagnosis. LIMD encompasses hyperlactacidemia occurring in the neonatal period together with multiple organ failure. Most cases have poor outcomes, and it is thought that most of these patients died with the cause remaining unknown and no diagnosis established. Next were mitochondrial disorders showing single organ dysfunction only, such as mitochondrial hepatopathy (12%) and cardiomyopathy (7%).

4. Exome analysis of MRCD patients

As most mitochondrial diseases occur sporadically with only a few cases discovered in one family line, linkage analysis using a large pedigree cannot be applied, thus suggesting that we cannot use information on chromosomal localization for causal gene identification. When identifying disease-causing genes using bioinformatics analysis for exome data, knowledge of the inheritance patterns is very important [16]. As approximately 90% of MRCD-causing genes show a recessive mode of

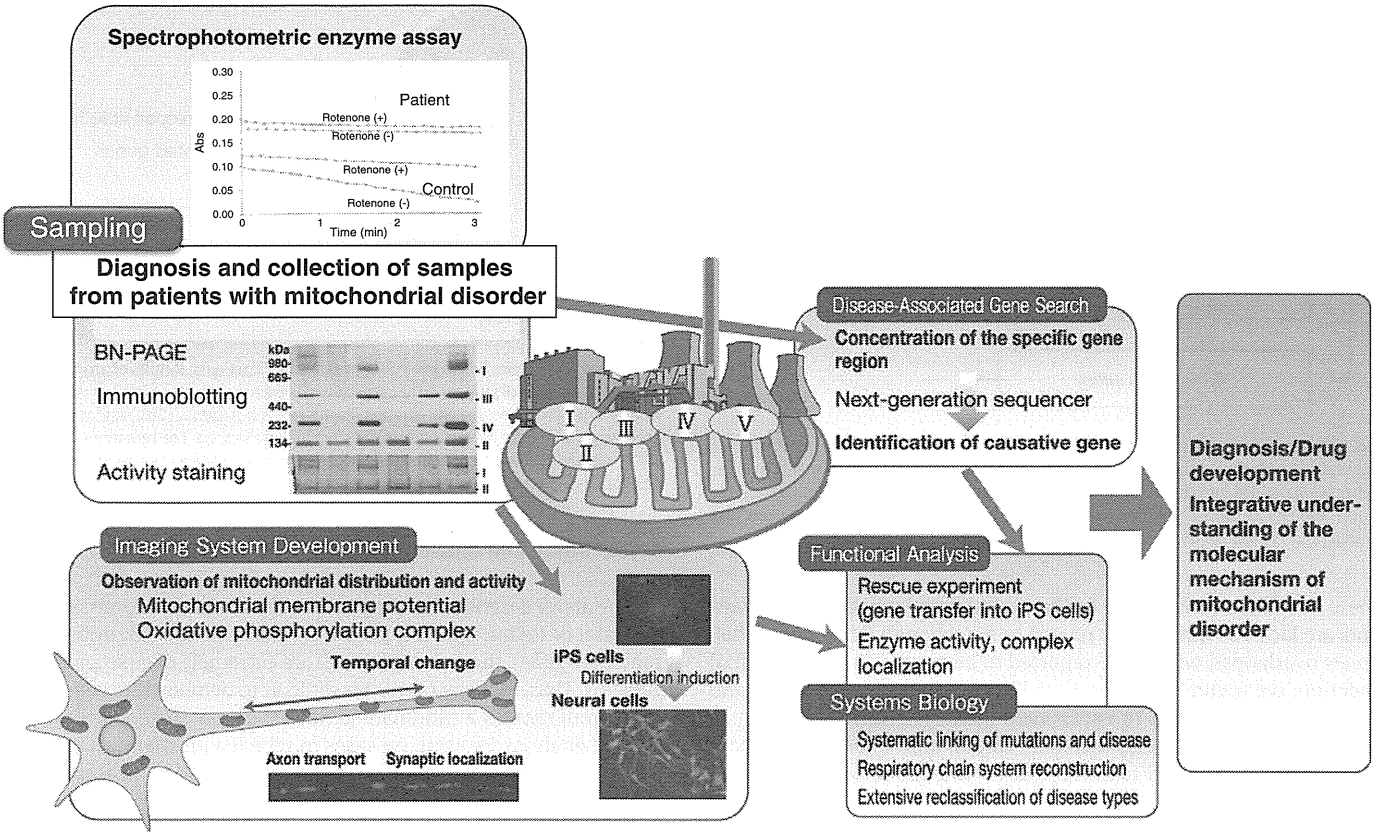

Fig. 1. Outline of exome analysis project for MRCD patients. The first step is 'Sampling', which refers to diagnosis and collection of samples from patients with mitochondrial disorders using both spectrophotometric enzyme assay [11] and BN-PAGE [13]. The next step is 'Disease-Associated Gene Search' using exome analysis. In 'Functional Analysis' and 'System Biology', candidate causal genes are confirmed at the cellular level by rescue experiment or other means. In 'Imaging System Development', induced pluripotent stem cells are created using fibroblasts and differentiated into neurons and glia cells to reproduce the pathology of mitochondrial dysfunction. The final purpose of our project is integrative understanding of the molecular mechanisms of mitochondrial disorders.

inheritance (as shown in Table 1), we prioritized such genes as harboring rare variants in a homozygous or compound heterozygous fashion. Low priority is given to the analysis of genes showing mutation in only one allele because patients and healthy control individuals harbored a comparable number of rare heterozygous alleles; we were unable to prioritize dominant-acting genes.

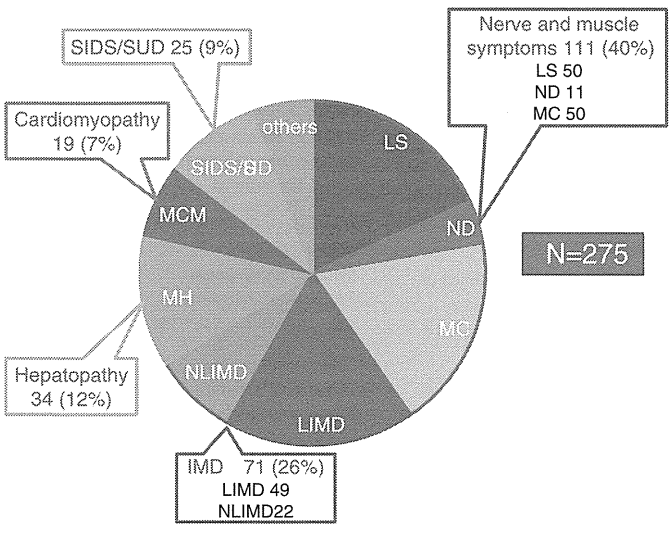

Fig. 2. Breakdown of clinical diagnoses of mitochondrial disorders in our institute as of January 2013. LS, Leigh syndrome; ND, neurodegenerative disorder; MC, mitochondrial cytopathy; IMD, infantile mitochondrial disease (lethal and non-lethal); MH, mitochondrial hepatopathy; MCM, mitochondrial cardiomyopathy; SIDS, sudden infant death syndrome; SUD, sudden unexpected death.

Our current bioinformatics analysis pipeline is as follows: read alignment was performed with a Burrows–Wheeler Aligner (BWA, version 0.7.0) [17] using the 1000 Genomes project phase II reference genome (hs37d5.fa). PCR duplicate reads were removed using Picard (version 1.89) (http://picard.sourceforge.net) and non-mappable reads were removed using SAMtools (version 0.1.19) [18]. After filtering out these reads, the Genome Analysis Toolkit (GATK) version 2.4-9-nightly-2013-04-12-g3fc5478 [19] was used to realign insertions and deletions, and for quality recalibration and variant calling (UnifiedGenotyper). Detected variants were annotated using ANNOVAR (version 2013Feb21) [20] and custom ruby scripts. The effect of the mutations on protein function was assessed by SIFT and GERP using dbNSFP [21]. The positions of mutations were based on RefSeq transcript sequences. Variants were assessed by comparing allele frequencies in the dbSNP135, Exome Sequencing Project (ESP5400) data set, and 1000 Genomes Projects (based on phase 1 release v3 called from 20101123 alignments). As mitochondrial disorders are rare, we excluded variants present in dbSNP with a frequency > 0.1%. After filtering out these variants, the VAAST program [22] was used to create a candidate gene list in each patient showing recessive characteristics.

As stated above, because mitochondrial disease patients have very high heterogeneity, the number of patients sharing the same gene mutation is quite low. Hence, attention should be directed towards removing these mutations from the disease candidates when the same amino acid substitutions are shared among multiple patients in our study, because these variants are highly likely to be SNPs unique to the Japanese population. Using these criteria, we are able to narrow down the number of variants to a mean of several genes for each patient. After listing

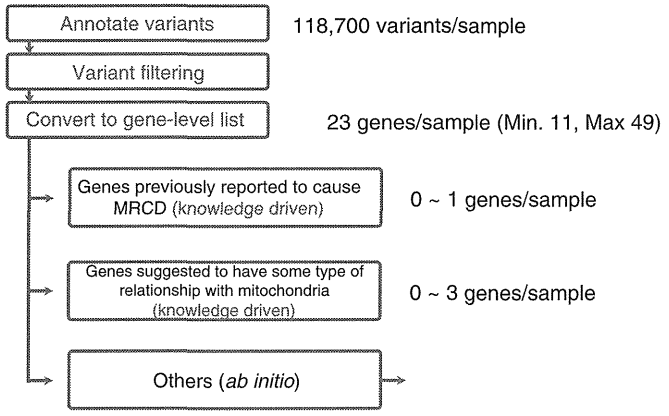

Fig. 3. Narrowing down of gene mutations discovered by exome analysis. After filtering out variants with the methods described in the 'Exome analysis of MRCD patients' section, genes were divided into three categories: (1) those that have previously been reported to cause MRCD; (2) those for which some relationship with mitochondria has been suggested; and (3) others (*ab initio*).

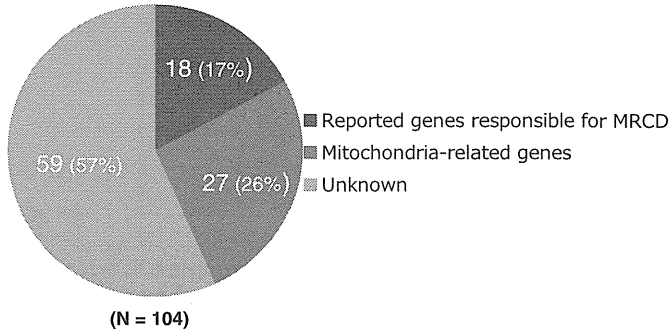

Fig. 4. Candidate genes with exome analysis for MRCD patients. Results of analysis for 104 patients to date (as of January 2013) are shown. Eighteen patients (17%) had variants previously reported to be disease-causing. Twenty-seven patients (26%) had mutations in genes suggested to be associated somehow with mitochondria. The remaining 59 patients (57%) are highly likely to contain completely novel disease-causing mutations for which no data have been obtained to date.

these candidate variants, we further investigated whether these variants are located within genes related to mitochondrial function. When genes overlapped with those reported to be related to mitochondrial function, we found that they were likely to be causative genes and were further subjected to experimental analysis such as haplotype phasing or functional assay including rescue experiments. To prepare a list of genes reported to be related to mitochondria, we included genes annotated as somehow related to mitochondria in the UniProt (http://www.uniprot.org/) [23] database, as well as the MitoCarta database (http://www.broadinstitute.org/pubs/MitoCarta/index.html) [24], which includes approximately 1000 gene products listed with the use of shotgun proteomics and mitochondrial localization analysis.

We also investigated whether there is Long Contiguous Stretch of Homozygosity (LCSH) using Affymetrix SNP arrays in a majority of patients. Although no cases of consanguineous marriage were reported in the interviews with the primary physician, about 5% of cases harbor LCSH proven by SNP arrays. When homozygous mutations are localized in these LCSH regions, the mutations are highly likely to be causative of disease.

5. Results of exome analysis for MRCD patients

The variants (mutations) found in the process of narrowing down the gene mutations discovered to date are shown in Fig. 3. These genes were narrowed down to the final candidate genes and divided into three categories: (1) those that have previously been reported to cause MRCD; (2) those for which some relationship with mitochondria has been suggested; and (3) others (*ab initio*). The results of analysis of 104 patients to date (as of January 2013) are shown in Fig. 4. Eighteen patients (17%) had variants previously reported to be disease-causing. Among these 18 patients, one had a homozygote of a previously reported mutation and two had a compound heterozygote of a reported and a novel mutation (data not shown). All other mutations found in this study were new. Twenty-seven patients (26%) had mutations in genes suggested to be associated somehow with mitochondria, and it is likely that they are novel disease-causing genes in mitochondrial disorders. Table 2 lists the functions of the genes in these 27 cases. For the remaining 59 cases, each patient has about 20 gene variants that are unique to each patient, and it is necessary to confirm whether any of these mutations can actually cause the disease. These 59 patients are highly likely to contain completely novel disease-causing mutations for which no clues have been obtained to date. The biggest issue we currently face is how to confirm the disease-causing gene from these 20 gene variants for each patient.

6. Conclusion and future prospects

The above describes the progress we have made in exome analysis of neonatal or infantile MRCD patients. While we have identified many candidate genes, the causes of MRCD are extremely diverse and heterogeneous. Thus, in many cases, it is difficult to demonstrate conclusively that a mutation in a candidate gene is the true cause. We have performed analyses focusing on cases in which a biochemical diagnosis was established at the cellular level in addition to clinical symptoms such as enzyme activity and complex formation abnormalities. Nonetheless, confirmation of the causal genes with rescue experiments or other means is difficult. In the future, it will be necessary to increase the case number or search for patients with similar symptoms and similar gene mutations in collaboration with researchers throughout the world. We are currently conducting analyses of pediatric patients with a focus on MRCD, and gene mutations (amino acid substitutions) harbored by patients of the childhood onset type are probably variants conferring major damage on enzyme activity or protein function. Onset is also thought to occur in adulthood rather than in childhood in some cases of milder (hypomorphic: partial loss of function) variants with the same gene defect. As these are thought to include nerve diseases,

Table 2
Functions of new disease-causing candidate genes for MRCD.

MtoX#1	Non-receptor tyrosine kinase
MtoX#2	Acyl-CoA thioesterase
MtoX#3	Fatty acid β oxidation
MtoX#4	tRNA synthetase
MtoX#5	ABC transporter superfamily
MtoX#6	ATR-dependent AMP-binding enzyme family
MtoX#7	Heme biosynthesis
MtoX#8	AAA ATPase family
MtoX#9	Pre-mRNA splicing factor
MtoX#10	Creatine kinase
MtoX#11	Synaptic transmission
MtoX#12	Synthesis of Coenzyme Q
MtoX#13	Heme biosynthetic process
MtoX#14	Citrate synthase family.
MtoX#15	Cholesterol metabolism
MtoX#16	Mitochondrial fission
MtoX#17	Muscle organ development
MtoX#18	Cholesterol biosynthetic process
MtoX#19	Ribosomal protein
MtoX#20	Tumor suppressor
MtoX#21	A component of complex I
MtoX#22	A protease, located in inner membrane
MtoX#23	Regulation of PDH
MtoX#24	Mitochondrial translation
MtoX#25	Queuosine biosynthetic process
MtoX#26	Mitochondrial carrier family
MtoX#27	Methyltransferase superfamilya

mental disorders, and diabetes or other metabolic diseases of unknown cause, we plan to conduct research based on the assumption that such cases include those caused by abnormalities in genes identified in MRCD patients.

Acknowledgements

This work was supported in part by a grant for Innovative Cell Biology by Innovative Technology (Cell Innovation Program) and Support Project from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, by a grant for Strategic Research Centers in Private Universities from MEXT, Japan to Saitama Medical University Research Center for Genomic Medicine, and by Grants-in-Aid for Research on Intractable Diseases (Mitochondrial Disorder) from the Ministry of Health, Labor and Welfare of Japan. Dr. Murayama was supported by the Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics. The authors would also like to thank Dr. Ayako Fujinami, Dr. Kaori Muta, Dr. Emi Kawachi, Dr. Takuya Fushimi, Dr. Keiko Ichimoto, Dr. Tomoko Tsuruoka, Ms. Keio Baba and Ms. Masami Ajima at Chiba Children's Hospital for their support.

References

[1] D. Skladal, J. Halliday, D.R. Thorburn, Minimum birth prevalence of mitochondrial respiratory chain disorders in children, Brain 126 (2003) 1905–1912.
[2] S. Dimauro, G. Davidzon, Mitochondrial DNA and disease, Ann. Med. 37 (2005) 222–232.
[3] D.M. Kirby, D.R. Thorburn, Approaches to finding the molecular basis of mitochondrial oxidative phosphorylation disorders, Twin Res. Hum. Genet. 11 (2008) 395–411.
[4] D.C. Wallace, Mitochondria and cancer, Nat. Rev. Cancer 12 (2012) 685–698.
[5] M.F. Lopez, B.S. Kristal, E. Chernokalskaya, A. Lazarev, A.I. Shestopalov, A. Bogdanova, M. Robinson, High-throughput profiling of the mitochondrial proteome using affinity fractionation and automation, Electrophoresis 21 (2000) 3427–3440.
[6] H. Schägger, K. Pfeiffer, The ratio of oxidative phosphorylation complexes I–V in bovine heart mitochondria and the composition of respiratory chain supercomplexes, J. Biol. Chem. 276 (2001) 37861–37867.
[7] D.R. Thorburn, Mitochondrial disorders: prevalence, myths and advances, J. Inherit. Metab. Dis. 27 (2004) 349–362.
[8] W.J. Koopman, P.H. Willems, J.A. Smeitink, Monogenic mitochondrial disorders, N. Engl. J. Med. 366 (2012) 1132–1141.
[9] S.B. Vafai, V.K. Mootha, Mitochondrial disorders as windows into an ancient organelle, Nature 491 (2012) 374–383.
[10] E.J. Tucker, A.G. Compton, D.R. Thorburn, Recent advances in the genetics of mitochondrial encephalopathies, Curr. Neurol. Neurosci. Rep. 10 (2010) 277–285.
[11] D.M. Kirby, M. Crawford, M.A. Cleary, H.H. Dahl, X. Dennett, D.R. Thorburn, Respiratory chain complex I deficiency: an underdiagnosed energy generation disorder, Neurology 52 (1999) 1255–1264.
[12] F.P. Bernier, A. Boneh, X. Dennett, C.W. Chow, M.A. Cleary, D.R. Thorburn, Diagnostic criteria for respiratory chain disorders in adults and children, Neurology 59 (2002) 1406–1411.
[13] H. Schägger, G. von Jagow, Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form, Anal. Biochem. 199 (1991) 223–231.
[14] K. Gibson, J.L. Halliday, D.M. Kirby, J. Yaplito-Lee, D.R. Thorburn, A. Boneh, Mitochondrial oxidative phosphorylation disorders presenting in neonates: clinical manifestations and enzymatic and molecular diagnoses, Pediatrics 122 (2008) 1003–1008.
[15] T. Yamazaki, K. Murayama, A.G. Compton, C. Sugiana, H. Harashima, S. Amemiya, M. Ajima, T. Tsuruoka, A. Fujinami, E. Kawachi, Y. Kurashige, K. Matsushita, H. Wakiguchi, M. Mori, H. Iwasa, Y. Okazaki, D.R. Thorburn, A. Ohtake, Molecular diagnosis of mitochondrial respiratory chain disorders in Japan: focusing on mitochondrial DNA depletion syndrome, Pediatr. Int. 56 (2014)(in press).
[16] C. Gilissen, A. Hoischen, H.G. Brunner, J.A. Veltman, Disease gene identification strategies for exome sequencing, Eur. J. Hum. Genet. 20 (2012) 490–497.
[17] H. Li, R. Durbin, Fast and accurate short read alignment with Burrows–Wheeler transform, Bioinformatics 25 (2009) 1754–1760.
[18] S.B. Ng, E.H. Turner, P.D. Robertson, S.D. Flygare, A.W. Bigham, C. Lee, T. Shaffer, M. Wong, A. Bhattacharjee, E.E. Eichler, M. Bamshad, D.A. Nickerson, J. Shendure, Targeted capture and massively parallel sequencing of 12 human exomes, Nature 461 (2009) 272–276.
[19] A. McKenna, M. Hanna, E. Banks, A. Sivachenko, K. Cibulskis, A. Kernytsky, K. Garimella, D. Altshuler, S. Gabriel, M. Daly, M.A. DePristo, The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data, Genome Res. 20 (2010) 1297–1303.
[20] K. Wang, M. Li, H. Hakonarson, ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data, Nucleic Acids Res. 38 (2010) e164.
[21] X. Liu, X. Jian, E. Boerwinkle, dbNSFP: a lightweight database of human nonsynonymous SNPs and their functional predictions, Hum. Mutat. 32 (2011) 894–899.
[22] M. Yandell, C. Huff, H. Hu, M. Singleton, B. Moore, J. Xing, L.B. Jorde, M.G. Reese, A probabilistic disease-gene finder for personal genomes, Genome Res. 21 (2011) 1529–1542.
[23] The UniProt Consortium, Update on activities at the Universal Protein Resource (UniProt) in 2013, Nucleic Acids Res. 41 (2013) D43–D47.
[24] D.J. Pagliarini, S.E. Calvo, B. Chang, S.A. Sheth, S.B. Vafai, S.E. Ong, G.A. Walford, C. Sugiana, A. Boneh, W.K. Chen, D.E. Hill, M. Vidal, J.G. Evans, D.R. Thorburn, S.A. Carr, V.K. Mootha, A mitochondrial protein compendium elucidates complex I disease biology, Cell 134 (2008) 112–123.

PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society

Pediatrics International (2013) **55**, e103–e106 doi: 10.1111/ped.12098

Patient Report

Case of an infant with hepatic cirrhosis caused by mitochondrial respiratory chain disorder

Shigehiro Enkai,[1] Sachi Koinuma,[2] Reiko Ito,[2] Junko Igaki,[3] Yukihiro Hasegawa,[3] Kei Murayama[4] and Akira Ohtake[5]

[1]*Department of Pediatrics, Fussa Hospital,* [2]*Division of Gastroenterology, National Center for Child Health and Development,* [3]*Division of Endocrinology and Metabolism, Tokyo Metropolitan Children's Medical Center, Tokyo,* [4]*Division of Metabolism, Chiba Children's Hospital, Chiba, and* [5]*Department of Pediatrics, Faculty of Medicine, Saitama Medical University, Saitama, Japan*

Abstract The patient had hepatomegaly with liver dysfunction at the age of 1 month. Magnetic resonance imaging performed at the age of 1 year showed multiple nodules of varying size in his liver. We were able to examine the mitochondrial respiratory chain function in the liver biopsy samples because all other differential diagnoses for hepatic cirrhosis had been ruled out. Complex I and IV activities were below the normal level (<30%) of the citrate synthase (CS) ratio. Liver blue native polyacrylamide gel electrophoresis showed an extremely weak complex I and IV band. Liver respiratory chain complexes I and IV were found to be deficient in this patient. The histologic findings were highly suggestive of mitochondrial respiratory chain disorder. Findings of progressive liver cirrhosis changes were observed in magnetic resonance imaging at the age of 5 years. An examination of the mitochondrial respiratory chain function should be performed along with a liver biopsy if mitochondrial respiratory chain disorder is suspected as a possible differential diagnosis of idiopathic hepatitis.

Key words chronic hepatitis, infant, liver cirrhosis, mitochondrial respiratory chain complex I and IV deficiency, mitochondrial respiratory chain disorder.

Mitochondrial respiratory chain disorder (MRCD), which is caused by the loss of one or more enzyme activities in respiratory chain complexes I–IV, has many clinical manifestations in various organs and is a known cause of mitochondrial encephalomyopathy, idiopathic hepatitis and idiopathic muscle weakness. Although MRCD is one of the differential diagnoses for hepatic disorder, it is not actively diagnosed. The early diagnosis of MRCD in the liver is important because some patients will subsequently develop liver cirrhosis or liver failure.[1,2] This report is based on a boy with chronic hepatic disorder and cirrhosis who was found to have mitochondrial respiratory chain complex I and IV deficiencies during his infant period.

Case Report

The patient was a Japanese boy born at term and weighing 3296 g; he was the second child of healthy parents with consanguinity. His elder sister (3 years old) is presently in good health. The mother's brother (31 years old) was found to have hepatic dysfunction during his infant period and his condition progressed to cirrhosis during adulthood. The proband's weight gain after birth was good. Jaundice and hepatomegaly were observed at the age of 1 month and he was admitted to our hospital. Upon admission (32 days after birth), he exhibited conjunctival icterus, his liver was palpable 5 cm below the right costal margin, he had normal muscle tone and no external malformations were noted. His laboratory data on admission showed cholestatic hepatitis. Tandem mass spectrometry, urine organic acid and bile acid analysis were normal. The following differential diagnoses were ruled out: autoimmune disease, infectious disease, disorder of organic acid metabolism and fatty acid oxidation, alpha 1-antitrypsin deficiency, tyrosinemia, galactosemia, and citrin deficiency. Furthermore, respiratory disorder, abnormal findings on skin or bone, and susceptibility to infection, which are the main symptoms of Langerhans cell histiocytosis and cystic fibrosis, are not present in this patient at the current age of 6 years. Imaging studies did not reveal any congenital portal venous or portal biliary tract malformations. The patient's transaminase (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) levels were 78–477 IU/l (AST) and 13–181 IU/l (ALT) and fluctuated with his physical condition. The patient's γ-GTP levels decreased to a normal range before the age of 6 months. Throughout the clinical course, the patient's blood lactate and pyruvic acid levels were almost always normal. Hypoglycemia was not observed during follow-up examinations. He exhibited normal growth and development. An abdominal magnetic resonance imaging (MRI) examination performed at the age of 2 months was normal except for hepatomegaly. However, an abdominal MRI performed at 1 year and 4 months showed multiple nodules of varying size in his liver, which appeared

Correspondence: Shigehiro Enkai, MD, Department of Pediatrics, Fussa Hospital, 1-6-1 Kamidaira, Fussa, Tokyo 197-0012, Japan. Email: enkai@fussahp.jp

Received 5 January 2012; revised 17 January 2013; accepted 19 February 2013.