Table 1
Spectrophotometric assays of respiratory chain enzyme activity

	NCCR	SCCR	COX
Patient 1	2.1	54.0	18.6
Patient 2	3.8	60.8	31.8
Controls ($n = 5$)	27.3 ± 11.6	76.6 ± 17.7	33.0 ± 16.1

The results of this assay are expressed as nanomoles per minute per milligram of mitochondrial protein. The control values are presented as mean ± 1 SD. Abbreviations are as follows: NCCR, rotenone-sensitive NADH-cytochrome-*c* reductase; SCCR, succinate-cytochrome-*c* reductase; COX, cytochrome-*c* oxidase.

abnormalities, a reduction in synthesis would be observed only when pyruvate/malate were used as substrates. Therefore, the findings of two independent assays for respiratory chain confirmed both patients have an isolated complex I deficiency in their muscle. However, the ATP synthesis of fibroblasts in patient 1 was normal. These findings suggest complex I deficiency exists in a tissue-specific manner.

3.3. Molecular genetic analysis

Total mtDNA sequence analysis in the patients revealed many polymorphisms (Table 2). No mtDNA rearrangements were detected by the long PCR method. The majorities of the polymorphisms were silent mutations or reported as normal polymorphisms according to a human mitochondrial genome database (MITOMAP) (Kogelnik et al., 1998).

Several homoplasmic mutations in coding regions such as T4216C in ND1, A4317G in tRNA-Ile, G8854A in ATP6, and T11394C in ND4 were not present in 100 normal Japanese adults (Table 2). Among the novel polymorphisms, we focused on a C-to-A mutation at np 11,777, which highly converted an arginine to a serine at codon 340 in the NADH dehydrogenase subunit 4 gene (Fig. 2). Using a PCR-RFLP method, we detected that this mutation was not present in 215 healthy subjects, parents of patient 1, or 98 clinically diagnosed LS patients. The DNA of the mother of patient 2 was not available. This method also revealed the heteroplasmic nature of this mutation in both patients (data not shown). For rapid and accurate quantification of mutant mtDNA, we devised a real-time PCR amplification method using two fluorogenic TaqMan™ probes in the same tube sharing common reagent. The percentages of mutant type mtDNA in patient 1 were 83% in muscle, 40% in blood, 78% in myoblasts and 57% in fibroblasts. The percentages of mutant type mtDNA in patient 2 were 76% in muscle, 52% in blood, and 76% in myoblasts (Table 3).

3.4. Cybrid study

To confirm the 11,777 mutation is pathogenic, we performed functional analysis of transmitochondrial cells (cybrids). Mitochondria from both patients with np 11,777 mutation and a LHON patient with np 11,778 mutation were separately introduced into a human osteosarcoma cell line (143B.TK- cells) lacking mtDNA (143B.TK-/rho-0 cells) and we obtained many clones with a different percentage of heteroplasmic np 11,777 mutation. Only cybrids with homoplasmic np 11,778 mutation were obtained because the patient had a homoplasmic mutation, which is common finding of np 11,778 mutation in LHON patients. ATP synthesis in digitonin permeabilized cells with glutamate and malate was correlated with the percentage of np 11,777 mutation ($R2 = 0.789$ in cybrids with mtDNA of patient 1, 0.769 in cybrids with mtDNA of patient 2; Pearson's correlation coefficient, Fig. 3a). No significant differences in correlation between the percentage of np 11,777 mutation and ATP synthesis was observed among cybrids with each patients' mtDNA. Moreover, the np 11,778 mutation had a milder effect than the np 11,777 mutation, when comparing cybrids with

Table 3
Quantification of the np 11,777 mutation using real-time PCR amplification methods

	Sample	Mutation percent value (%)
Patient 1	Muscle	83
	Blood	40
	Myoblasts	78
	Fibroblasts	57
	Father's blood	ND
	Mother's blood	ND
Patient 2	Muscle	76
	Blood	52
	Myoblasts	76

ND, not detected.

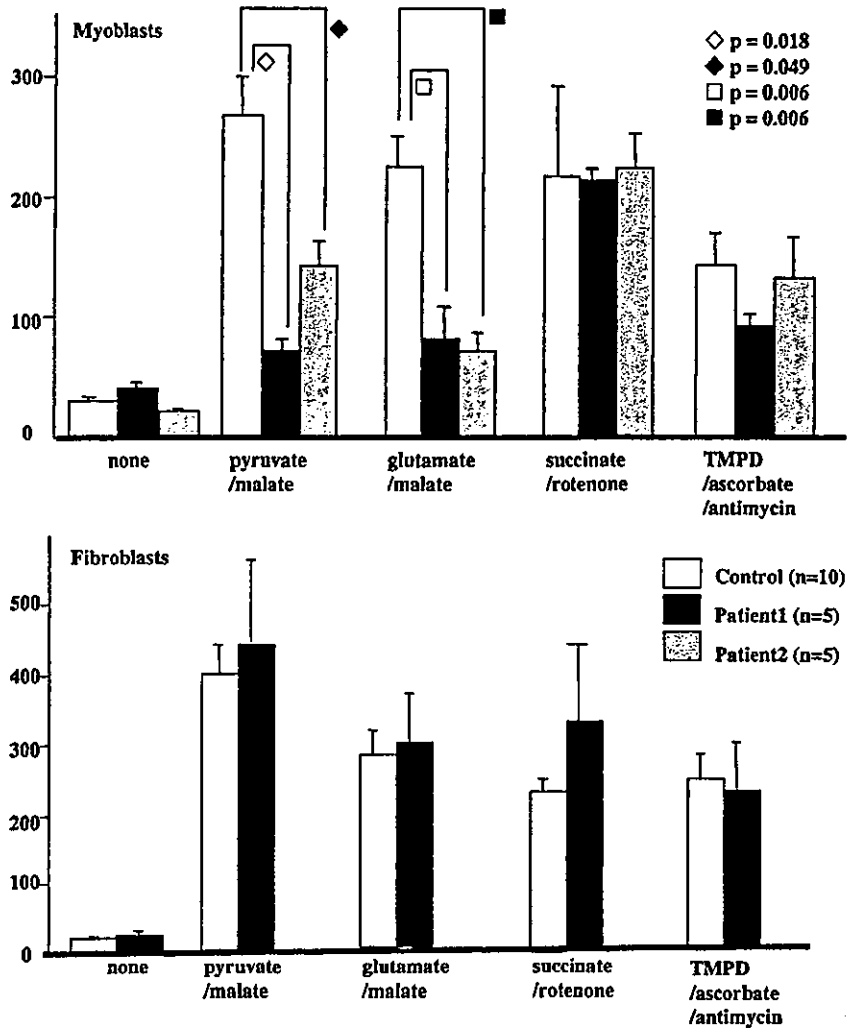

Fig. 1. ATP synthesis in digitonin-treated primary culture of myoblast and fibroblast. ATP synthesis was measured using the following combinations of substrates and specific inhibitors: pyruvate/malate, glutamate/malate, succinate/rotenone, and ascorbate/TMPD/antimycin. The results of this assay are expressed as nanomoles ATP per hour per milligram cell protein. The control values are presented as mean ± 1 SD.

the homoplasmic np 11,778 mutation to those with more than 90% of np 11,777 mutation (Fig. 3b).

4. Discussion

We identified a novel C-to-A mutation at np 11,777 in the ND4 subunit gene. This mutation is pathogenic for several reasons. First, it was not found in over 200 normal individuals and converted a highly evolutionary conserved arginine to a serine (i.e. a charged to an uncharged amino acid). Moreover, the fact that the np 11,778 mutation, the most frequent mutation in LHON patients, also converts the same arginine to a histidine strongly supports the assumption that this mutation is pathogenic (Wallace et al., 1988). Second, the mutation exists in a heteroplasmic condition, which is a common feature of pathogenic mtDNA mutation. Third, respiratory chain function assays in patient 1 indicated an isolated complex I deficiency with tissue specificity, which means that there are abnormalities in muscle and myoblasts, but not in

Table 2
mtDNA sequence data in two patients

Genes	Patient 1	Patient 2	Comment or frequency
12SrRNA		G709A	MITOMAP
	T1107C		MITOMAP
	A1438G	A1438G	MITOMAP
16SrRNA	A2706G		MITOMAP
	C3106del	C3106del	MITOMAP
ND1		A3426G	MITOMAP
	A4200T		MITOMAP
	T4216C (Tyr- > His)		None in 100 Japanese
tRNA- Ile	A4317G		None in 100 Japanese
ND2	A4769G	A4769G	MITOMAP
	A4833G	A4833G	MITOMAP
	G4985A	G4985A	MITOMAP
		T5108C	MITOMAP
	C5178A		MITOMAP
	A5301G		MITOMAP
	T5442C		MITOMAP
tRNA Trp	C5554T		MITOMAP
COI	C7028T	C7028T	MITOMAP
	A7129G		MITOMAP
COII		T7621C (Ala- > Ala)	Three in 100 Japanese
	C7669T		MITOMAP
ATP6	C8580T		MITOMAP
		A8701G	MITOMAP
		G8854A (Ala- > Thr)	None in 100 Japanese
	A8860G	A8860G	MITOMAP
COIII		T9540C	MITOMAP
ND3	A10397G		MITOMAP
	A10398G	A10398G	MITOMAP
	C10400T	C10400T	MITOMAP
ND4	T10873C	T10873C	MITOMAP
		T11335C	MITOMAP
	T11394C (Leu- > Pro)		None in 100 Japanese
	G11719A	G11719A	MITOMAP
	C11777A	C11777A	None in 100 Japanese
ND5	C12705T	C12705T	MITOMAP
	A12810G		MITOMAP
	C13984T		MITOMAP

(continued on next page)

fibroblasts. Tissue specificity is also a common feature of pathogenic mtDNA mutation. Direct enzymatic complex I measurement is hampered by contaminating non-mitochondrial NADH cytochrome-*c* reductase activity in cultured cells (Benit et al., 2001). Therefore, we performed assays of ATP synthesis and rotenone-sensitive NCCR for complex I function. Fourth, the functional analysis of cybrid revealed the significant decrease in respiratory chain function was observed in cells with a higher percentage of mutant mtDNA. Evidence is accumulating that mtDNA mutations can have addictive and even synergistic effects on phenotype (Lertrit et al., 1994; Vergani et al., 1995). Several polymorphisms found in each patient, also present in none of 100 normal controls might have some effects on cell dysfunction, but cybrid analysis using each patient's mtDNA revealed no significant difference in cell function among cybrids with each patients' mtDNA. Therefore, it seems reasonable to conclude that the np 11,777 mutation is the most dominant in these patients.

A comparison of the polymorphisms especially in the D-loop region between the two patients showed many nucleotide differences, indicating this mutation occurred independently (Table 2). Usually, pathogenic mtDNA point mutations are maternally transmitted including np 11,778 mutation with LHON patients, however, we could not confirm that the np 11,777 mutation was maternally transmitted, because only one mother's blood sample was available and the mutant level in blood could be far too low to detect

Table 2 (*continued*)

Genes	Patient 1	Patient 2	Comment or frequency
ND6		G14569A	MITOMAP
Cyt b		T14766C	MITOMAP
	T14783C		MITOMAP
	A14927G		MITOMAP
	G15043A	G15043A	MITOMAP
	G15301A	G15301A	MITOMAP
	A15326G	A15326G	MITOMAP
		A15746G (Ile- > Val)	Four in 100 Japanese
	T15622C		MITOMAP
	G15737A		MITOMAP
D-loop	C16184ins		MITOMAP
	C16190del		MITOMAP
	C16233T	C16223T	MITOMAP
		G16274A	MITOMAP
	C16291T		MITOMAP
	T16311C		MITOMAP
	A16316G		MITOMAP
	T16362C	T16362C	MITOMAP
	A73G	A73G	MITOMAP
		G143A	MITOMAP
	C150T		MITOMAP
	C151T		MITOMAP
		T152C	MITOMAP
		T204C	MITOMAP
	A263G	A263G	MITOMAP
	C303del		MITOMAP
	C310ins		MITOMAP
	T489C	T489C	MITOMAP
	C527G		Three in 100 Japanese

MITOMAP means that any polymorphisms have been listed in the database (Kogelnik et al., 1998).

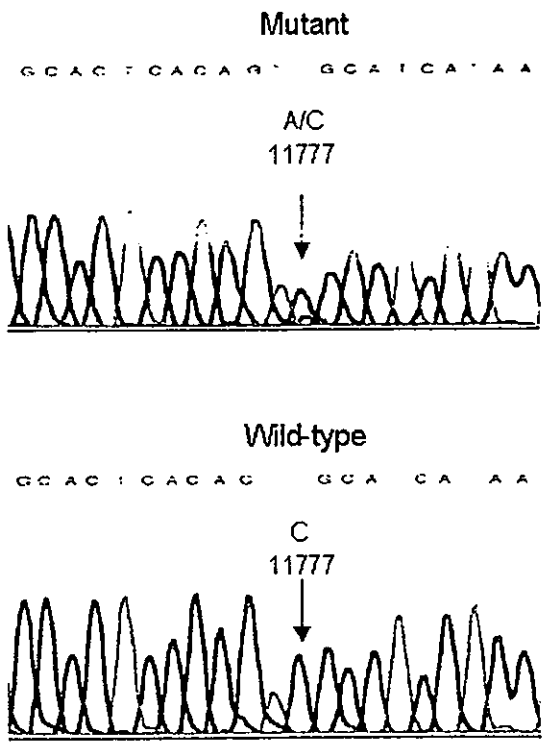

Fig. 2. A heteroplasmic mutation in ND4 gene. Partial nucleotide sequence of ND4 amplified from patient 1 (top) and a control subject (bottom). The mutant sequence exhibits a C-to-A transition at np 11,777, resulting in substitution from arginine to serine at codon 340.

with the PCR technique. In the present study, we screened 100 DNA samples from clinically diagnosed LS patients for this np 11,777 mutation, and discovered it in only these two patients. So far, we have found ten patients with T-to-G mutation at np 8993, three with T-to-C at np 8993, and five with T-to-C at np 9176 (Makino et al., 2000). These findings suggest this np 11,777 mutation is a less common mutation than the mutations in the subunit 6 gene of ATP synthase in Japan.

In the majority of LS patients, the onset is in infancy, and the prognosis is usually poor because of brainstem dysfunction in an early stage of the disease. In the present patients, the relatively high lactate levels compared to pyruvate and neuroradiological findings are compatible with LS. However, the clinical courses are relatively mild, in that, there is no obvious brainstem dysfunction. This symptomatology may be due to the nature of this mutation. As mentioned above, the np 11,777 mutation converts an arginine to a serine at codon 340 in the ND4 gene. Interestingly, this amino acid change also occurs with a np 11,778 mutation, the most common mutation in patients with LHON, but amino acid replacement was different (R340S vs. R340H). There were no signs of optic neuropathy in detailed examinations by ophthalmologists in either patient. Moreover, there are usually no central nervous system effects of np 11,778 mutation, or mild effects including dystonia, which is the prominent symptom in patient 2. Therefore, it is interesting that the phenotype of LHON patients is quite different from that of the present cases despite the same amino acid substitution. Since the mean onset of LHON patients is in their 20s, we suppose the np 11,777 mutation may produce optic neuropathy in later life.

A few functional studies infer that ND4 may be involved in the binding of the ubisemiquinone intermediate that is also formed in the oxidation of NADH by complex I (Fearnley and Walker, 1992; Degli Esposti et al., 1993, 1994; Gray et al., 1994). According to a model made by multiple sequence analysis, an arginine of codon 340 is located at the beginning of the transmembrane helix near the matrix side of the membrane (Fearnley and Walker, 1992; Degli Esposti et al., 1994) and protein-bound ubisemiquinone anions are stabilized by positively charged amino acids such as arginine (Gray et al., 1994). The conversion of arginine to a weakly charged histidine by the np 11,778 mutation, or an uncharged serine by the np 11,777 mutation, may cause instability of the ubisemiquinone bound to complex I, resulting in defective electron transport. Our data using the cybrids cells with both np 11,777 and 11,778 mutation demonstrated the defects in complex I function, and the np 11,777 mutation caused a more severe defect in ATP synthesis than the np 11,778 mutation. The cause of the differences in phenotype between the np 11,777 and 11,778 mutation is unclear, however, the difference in the ubisemiquinone binding property produced by each amino acid substitution may affect the differences in phenotypic severity. Further studies on the difference between the np 11,777 and 11,778 mutations are needed to provide key information for the roles of

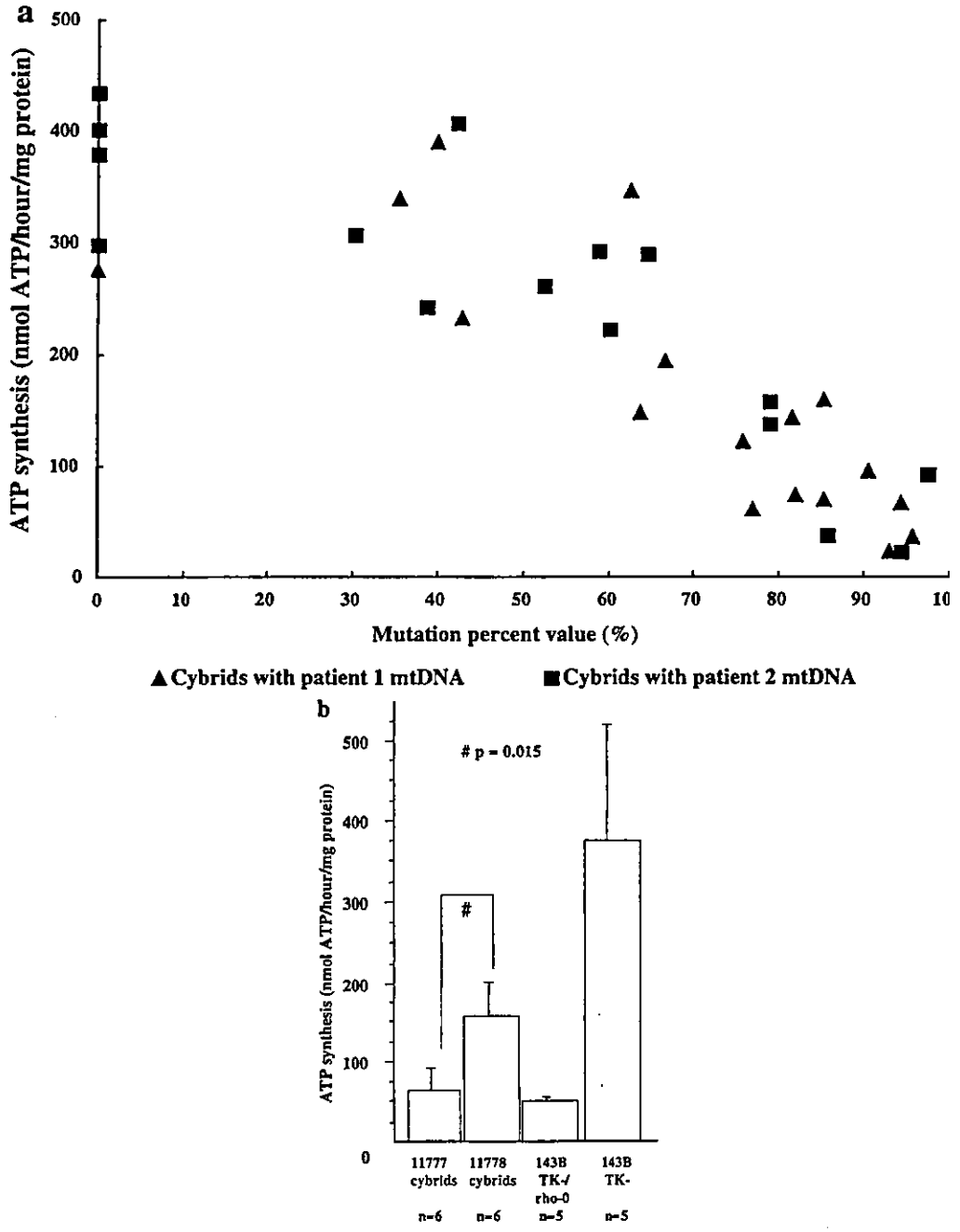

Fig. 3. ATP synthesis in cybrids. (a) Glutamate + malate as substrates in 143B-derived cybrid clones with various percentages of the np 11,777 mutation from each patient were used. (b) The reduction in ATP synthesis of the np 11,777 mutation with over 90% mutant was more marked than that of the homoplasmic np 11778 mutation. The values of 143B original and their derived rho-0 cells stand for normal control and baseline, respectively.

ND4 in complex I biogenesis and the genotype–phenotype relationship.

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References

Akanuma, J., Muraki, K., Komaki, H., Nonaka, I., Goto, Y., 2000. Two pathogenic point mutations exist in the authentic mitochondrial genome, not in the nuclear pseudogene. J. Hum. Genet. 45, 337–341.

Benit, P., Chretien, D., Kadhom, N., et al., 2001. Large-scale deletion and point mutations of the nuclear NDUFV1 and NDUFS1 genes in mitochondrial complex I deficiency. Am. J. Hum. Genet. 68, 1344–1352.

Bookelman, H., Trijbels, J.M.F., Sengers, R.C.A., Janssen, A.J.M., 1978. Measurement of cytochromes in human skeletal muscle mitochondria isolated from fresh and frozen stored muscle specimens. Biochem. Med. 19, 366–373.

Bourgeron, T., Rustin, P., Chretien, D., et al., 1995. Mutation of a nuclear succinate dehydrogenase gene results in mitochondrial respiratory chain deficiency. Nat. Genet. 11, 144–148.

Carrozzo, R., Tessa, A., Vazquez-Memije, M.E., et al., 2001. The T9176G mtDNA mutation severely affects ATP production and results in Leigh syndrome. Neurology 56, 687–690.

Degli Esposti, M.D., De Vries, S., Crimi, M., Ghelli, A., Patarnello, T., Meyer, A., 1993. Mitochondrial cytochrome *b*: evolution and structure of the protein. Biochim. Biophys. Acta 1143, 243–271.

Degli Esposti, M., Carelli, V., Ghelli, A., et al., 1994. Functional alterations of the mitochondrially encoded ND4 subunit associated with Leber's hereditary optic neuropathy. FEBS Lett. 352, 375–379.

de Vries, D.D., van Engelen, B.G., Gabreels, F.J., Ruitenbeek, W., van Oost, B.A., 1993. A second missense mutation in the mitochondrial ATPase 6 gene in Leigh's syndrome. Ann. Neurol. 34, 410–412.

DiMauro, S., Bonilla, E., De Vivo, D.C., 1999. Does the patient have a mitochondrial encephalomyopathy? J. Child Neurol. 14, S23–S35.

Endo, H., Hasegawa, K., Narisawa, K., Tada, K., Kagawa, Y., Ohta, S., 1989. Defective gene in lactic acidosis: abnormal pyruvate dehydrogenase E1 alpha-subunit caused by a frame shift. Am. J. Hum. Genet. 44, 358–364.

Fearnley, I.M., Walker, J.E., 1992. Conservation of sequences of subunits of mitochondrial complex I and their relationships with other proteins. Biochim. Biophys. Acta 1140, 105–134.

Gray, K.A., Dutton, P.L., Daldal, F., 1994. Requirement of histidine 217 for ubiquinone reductase activity (Qi site) in the cytochrome *bc*1 complex. Biochemistry 33, 723–733.

King, M.P., Attardi, G.M., 1989. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science 246, 500–503.

Kirby, D.M., Kahler, S.G., Freckmann, M.L., Reddihough, D., Thorburn, D.R., 2000. Leigh disease caused by the mitochondrial DNA G14459A mutation in unrelated families. Ann. Neurol. 48, 102–104.

Koga, Y., Nonaka, I., Sunohara, N., Yamanaka, R., Kumagai, K., 1988. Variability in the activity in of respiratory chain enzymes in mitochondrial myopathy. Acta Neuropathol. (Berl) 76, 135–141.

Kogelnik, A.M., Lott, M.T., Brown, M.D., Navathe, S.B., Wallace, D.C., 1998. MITOMAP: a human mitochondrial genome database – 1998 update. Nucleic Acids Res. 26, 112–115.

Leigh, D.,, 1951. Subacute necrotizing encephalopathy in an infant. J. Neurol. Neurosurg. Psychiatry 14, 216–221.

Lertrit, P., Kapsa, R.M.I., Jean-Francois, M.J.B., et al., 1994. Mitochondrial DNA polymorphism in disease: a possible contributor to respiratory dysfunction. Hum. Mol. Genet. 11, 1973–1981.

Loeffen, J., Smeitink, J., Triepels, R., et al., 1998. The first nuclear-encoded complex I mutation in a patient with Leigh syndrome. Am. J. Hum. Genet. 63, 1598–1608.

Loeffen, J.L., Smeitink, J.A., Trijbels, J.M., et al., 2000. Isolated complex I deficiency in children: clinical, biochemical and genetic aspects. Hum. Mutat. 15, 123–134.

Makino, M., Horai, S., Goto, Y., Nonaka, I., 2000. Mitochondrial DNA mutations in Leigh syndrome and their phylogenetic implications. J. Hum. Genet. 45, 69–75.

Miranda, A.F., 1994. Diseased muscle in tissue culture. In: Engel, A.G., Franzini-Armstrong, C. (Eds.), Myology, McGraw Hill/USA, New York, NY, pp. 1046–1070.

Richler, C., Yaffe, D., 1970. The in vitro cultivation and differentiation capacities of myogenic cell lines. Dev. Biol. 23, 1–22.

Robinson, B.H., 1996. Use of fibroblast and lymphoblast cultures for detection of respiratory chain defects. In: Attardi, G.M., Chomyn, A. (Eds.), Methods in Enzymology No. 264, Academic Press/USA, San Diego, CA, pp. 454–463.

Shoubridge, E.A., 2001. Nuclear genetic defects of oxidative phosphorylation. Hum. Mol. Genet. 10, 2277–2284.

Silvestri, G., Ciafaloni, E., Santorelli, F.M., et al., 1993. Clinical features associated with the A-- > G transition at nucleotide 8344 of mtDNA ('MERRF mutation'). Neurology 43, 1200–1206.

Sue, C.M., Bruno, C., Andreu, A.L., et al., 1999. Infantile encephalopathy associated with the MELAS A3243G mutation. J. Pediatr. 134, 696–700.

Tatuch, Y., Christodoulou, J., Feigenbaum, A., et al., 1992. Heteroplasmic mtDNA mutation (T-G) at 8993 can cause

Leigh disease when the percentage of abnormal mtDNA is high. Am. J. Hum. Genet. 50, 852–858.

Thyagarajan, D., Shanske, S., Vazquez-Memije, M., De Vivo, D.C., DiMauro, S., 1995. A novel mitochondrial ATPase 6 point mutation in familial bilateral striatal necrosis. Ann. Neurol. 38, 468–472.

Triepels, R.H., van den Heuvel, L.P., Loeffen, J.L.C.M., et al., 1999. Leigh syndrome associated with a mutation in the NDUF7 (PSST) nuclear encoded subunit of complex I. Ann. Neurol. 45, 787–790.

van den Heuvel, L., Ruitenbeek, W., Smeets, R., et al., 1998. Demonstration of a new pathogenic mutation in human complex I deficiency: a 5-bp duplication in the nuclear gene encoding the 18 kD (AQDQ) subunit. Am. J. Hum. Genet. 62, 262–268.

Vergani, L., Martinuzzi, A., Carelli, V., et al., 1995. MtDNA mutations associated with Leber's hereditary optic neuropathy: studies on cytoplasmic hybrid (cybrid) cells. Biochem. Biophys. Res. Commun. 210, 880–888.

Wallace, D.C., Singh, G., Lott, M.T., et al., 1988. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242, 1427–1430.

Zhu, Z., Yao, J., Johns, T., et al., 1998. SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. Nat. Genet. 20, 337–343.

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ORIGINAL ARTICLE

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Leigh syndrome caused by mitochondrial DNA G13513A mutation: frequency and clinical features in Japan

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Abstract The mitochondrial DNA (mtDNA) G13513A mutation in the ND5 subunit gene has been recently reported as a common cause of some phenotypes of mitochondrial myopathy. Until now, the prevalence and characteristics of this mutation in Leigh syndrome (LS) has not been determined. We screened 84 patients with Leigh syndrome (LS) and found the mutation in six (7%) of them. The proportions of mutant mtDNA in muscles were relatively low (42–70%). The onset of symptoms for patients with this mutation was from 9 months to 5 years. It should be noted that five patients had cardiac conduction abnormalities, particularly Wolff-Parkinson-White (WPW) syndrome (three patients). This study suggests that G13513A mutation is a frequent cause of LS and that patients with this mutation may have a characteristic clinical course.

Keywords Leigh syndrome · Mitochondrial DNA · ND5 subunit gene · G13513A · WPW syndrome

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Introduction

Leigh syndrome (LS) (MIM 256000) is a progressive neurodegenerative disorder characterized by bilaterally symmetrical lesions in the brainstem and/or basal ganglia in infancy and childhood (Leigh 1951; Pincus 1972; van Erven et al. 1987). LS is associated with defects in mitochondrial energy production. Though the protein subunits of the mitochondrial respiratory chain enzymes are predominantly encoded by nuclear genes, some mitochondrial DNA (mtDNA) mutations are pathogenic in LS, mainly T → G or T → C point mutations occurring at nucleotide position (np) 8993 (8993 mutation) (Tatuch et al. 1992; Santorelli et al. 1993; de Vries et al. 1993) and T → C or T → G at np9176 (Thyagarajan et al. 1995; Campos et al. 1997; Makino et al. 1998). Rare mtDNA mutations such as A3243G, A8344G, and C11777A have also been reported (Koga et al. 2000; Berkovic et al. 1991; Komaki et al. 2003).

The mtDNA G13513A mutation was first reported in patients with MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) by Santorelli et al. (1997). This G13513A mutation was discovered to be a more frequent cause of MELAS than previously recognized (Pulkes et al. 1999; Penisson-Besnier et al. 2000; Corona et al. 2001). Moreover, the G13513A mutation was also seen in Leigh-like syndrome (Chol et al. 2003).

In this study, we screened 84 Japanese patients with LS for this mutation and detected it in six of them. We report on the LS patients with the G13513A mutation and discuss their phenotypes.

Patients and methods

We used the following diagnostic criteria for LS in this study, referring to the previous reports (Rahman S, et al. 1996; Makino M, et al. 2000; Arii J, et al. 2000): (1) progressive neurologic disease with motor and/or intellectual developmental delay, (2) elevated lactate levels (> 20 mg/dl) in the blood and/or CSF, and (3) char-

acteristic features on neuroimaging (i.e., hyperintense lesions on T2-weighted MRI or hypointense lesions on CT scans in the bilateral brainstem and/or basal ganglia). One hundred Japanese patients fulfilled these criteria, but 16 were excluded because of stroke-like episodes and the presence of ragged red fibers (RRF) or strongly succinate dehydrogenase-reactive blood vessels (SSV) in their muscles (Hasegawa et al. 1991).

Muscle samples were obtained by open biopsy. The samples were divided into three portions for histochemical, biochemical, and mtDNA analyses. Serial frozen sections were stained with various histochemical methods, including Gomori trichrome and cytochrome c oxidase (COX). Respiratory chain enzyme activities in skeletal muscles were assayed by slight modification of the published methods (Koga et al. 1988).

Total DNA was extracted from muscle biopsies and/or blood samples by conventional methods. We first screened deletions and well-known point mutations in mtDNA at np3243, 8344, 8993, and 9176 by the method previously described (Goto et al. 1990; Makino et al. 2000). To detect a G → A mutation at np13513, we amplified 170-bp fragments using oligonucleotide primers corresponding to np13369–13394 and np13514–13538 with a G-to-A mismatch at np13515 in 25 cycles of denaturation (94°C, 30 s), annealing (50°C, 30 s), and extension (72°C, 30 s). The products after digestion with *Mbo*I were electrophoresed on a 4% agarose gel and visualized with ethidium bromide under a UV transilluminator (Alpha Imager 2000; Alpha Innotech). The mismatch-containing primer introduced a restriction site for *Mbo*I only in the wild type mtDNA, which was cleaved into two fragments of 144 and 26 bp (Fig. 1). The amount of digested versus undigested DNA fragments were measured with a densitometer. To correctly determine the proportion of the mutant mtDNA, we constructed plasmids containing wild or mutant DNA using a TOPO TA cloning kit according to the manufacturer's instructions. A mixture-template standard curve was used to revise the percentage of mutant mtDNA. We performed total mtDNA sequencing for selected cases, such as those with diffuse COX deficiency in their muscle biopsy, by the method previously described (Akanuma et al. 2000).

We obtained informed consent for muscle pathology and genetic analysis from all patients, and in the case of minors, their parents, using the form approved by the IRB of our institute.

Results

Of the 84 patients, 19 showed diffuse COX deficiency in their muscle biopsy, but their total sequences of mtDNA had no significant mutation (data not shown). These findings indicated that all these cases were caused by nuclear gene mutations such as SURF-1 or others (Zhu et al. 1998; Tiranti et al. 1998). Of the remaining 65 patients, point mutation in mtDNA was detected in 19.

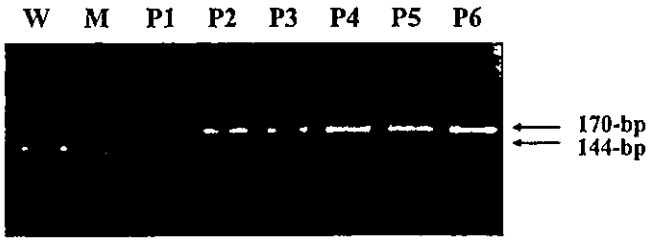

Fig. 1 Restriction fragment length polymorphism analysis of the G13513A mutation. The PCR products after digestion with *Mbo*I were electrophoresed on a 4% agarose gel. The amplified 170-bp fragment was cleaved into two fragments 144 bp and 26 bp in size (the 26-bp fragment is not shown here). *W* 100% wild type, *M* 100% mutant, *P1*~*P6* patients' muscles

Six patients had the G13513A mutation (Fig. 1). The same number of patients had the 8993 mutation (four with T-to-G and two with T-to-C mutations). The T9176C, C11777A, and A8344G mutations were found in four, two, and one patients (Makino et al. 2000; Komaki et al. 2003). All patients with the G13513A mutation had neither other mutation at np3243, 8344, 8993, or 9176 nor deletion. None of the 150 healthy Japanese controls or 120 patients with the A3243G mutation harbored the G13513A mutation.

Clinical features and laboratory data of the six patients with G13513A mutation are summarized in 1. The onset of neurological symptoms other than developmental delay varied from 9 months to 5 years (mean ± SD; 28.8 ± 21.1 months). In comparison, onset for patients with the 8993 mutation was 4.6 ± 4.6 months. Ptosis was present in five out of the six patients. It should also be noted that five patients had cardiac conduction abnormalities, especially Wolff-Parkinson-White (WPW) syndrome (three patients).

In muscle pathology, there were nonspecific myopathic changes, while one patient (patient 1) had an equivocal SSV (Table 2). The mean percentage of mutant mtDNA in muscles was 59 ± 10% (range 42–70%). No clear correlation was found between the percentage of mutant mtDNA and onset or severity of the disease. The activities of the mitochondrial respiratory chain enzyme were within normal limits in all four patients examined.

Discussion

The G13513A mutation was first reported in patients with MELAS (Santorelli et al. 1997). Its pathogenicity has been confirmed with several lines of evidence as follows: The mutant was detected in heteroplasmic fashion in patients, resulted in amino acid substitution at a highly conserved position in the ND5 subunit, and was absent in healthy controls (Santorelli et al. 1997; Pulkes et al. 1999). Single-fiber polymerase chain reaction studies demonstrated a significantly higher amount of mutant mtDNA in RRFs compared with non-RRFs (Pulkes et al. 1999). Furthermore, Corona et al. (2001) reported two MELAS patients with a 13514 A → G mutation, which hits the same codon affected by the G13513A mutation. The report suggested that the amino acid position of this codon was crucial for the function of complex I. In mouse mitochondria, respiration was tightly controlled by the NADH dehydrogenase ND5 subunit gene (Bai et al. 2000). Therefore, ND5 might also play an important role in human mitochondria. Some recent reports have suggested the validity of this theory (Kirby et al. 2000; Taylor et al. 2002; Liolitsa et al. 2003; Crimi et al. 2003).

The lesions of LS affect brainstem and medulla oblongata, particularly midbrain and pons, and most commonly the tegmentum, the periaqueductal gray, and the posterior colliculi. Other lesions are found in the

Table 1 Clinical data. Normal value of blood and CSF lactate = 3.3–14.9 mg/dl. *WPW* Wolff-Parkinson-White syndrome, *PSVT* paroxysmal supraventricular tachycardia, *ICRBBB* incomplete right bundle branch block

Patients	1	2	3	4	5	6
Age at muscule biopsy	7 years 8 months	1 year 1 month	2 years 6 months	1 year 2 months	7 years 8 months	6 years 8 months
Gender	F	M	M	M	F	F
Body height	(−3.2 SD)	(−1.3 SD)	(−2.0 SD)	(−0.7 SD)	(−2.7 SD)	(−1.0 SD)
Body weight	(−3.2 SD)	(−1.2 SD)	(−2.7 SD)	(−4.0 SD)	(−2.6 SD)	(−0.1 SD)
Age at onset	1 year 7 months	9 months	2 years 4 months	9 months	5 years	4 years
Mental retardation	(+)	Borderline	Normal	?	(+)	Borderline
Motor developmental delay	(+)	(+)	(+)	(+)	(+)	(−)
Sitting alone	8 months	11 months	7–8 months	Impossible	?	?
Unaided walking	1 year 6 months	Impossible	11 months	Impossible	?	1 year 2 months
Hypotonia	(+)	(+)	(+)	(+)	(+)	(+)
Optic atrophy	(+)	(−)	(−)	?	?	(+)
Ptosis	(+)[a]	(+)[a]	(−)	(+)[a]	(+)[a]	(+)
Strabismus	(+)	(−)	(−)	(+)[a]	(−)	(+)[a]
Facial palsy	(+)	(−)	(−)	(+)	(−)	(+)
Seizure	(−)	(+)[a]	(+)[a]	(−)	(−)	(−)
Respiratory disturbance	(−)	(−)	(+)	(−)	(+)	(−)
Ataxia	(+)	(+)	(−)	(−)	(+)	(−)
Other neurological signs	Deafness		Tremor		Depression	Clumsy
ECG	WPW	WPW	PSVT	ICRBBB	Ophthalmoplegia WPW,PSVT,AV block	Normal
Blood lactate (mg/dl)	13.9	22.2	68.9	38.1	31.5	24.3
CSF lactate (mg/dl)	31.2	25.2	45.9	38.6	85.4	29.6
T2-weighted hyperintensity lesions on MRI						
In basal ganglia	(+)	(−)	(−)	(−)	(+)	(+)
In brainstem	(+)	(+)	(+)	(+)	(+)	(+)
In others		Thalamus	Thalamus		Cerebral lesion	

[a] Initial symptom

Table 2 Clinical data. Normal value of blood and CSF lactate = 3.3–14.9 mg/dl. *WPW* Wolff-Parkinson-White syndrome, *PSVT* paroxysmal supraventricular tachycardia, *ICRBBB* incomplete right bundle branch block

	Patients					
	1	2	3	4	5	6
Muscle pathology						
RRF or SSV	One SSV	(−)	(−)	(−)	(−)	(−)
Type-2 fiber atrophy	(+)	(−)	(+)	(+)	(+)	(−)
Respiratory chain enzyme activity						
Complex I + III (normal range: 27.3 + 11.6) (% of activity)	ND	ND	24.4 (89)	57.2 (210)	93.9 (344)	28.9 (106)
Complex II + III (normal range: 76.6 + 17.7) (% of activity)	ND	ND	69.1 (90)	162.8 (213)	106.4 (139)	120.1 (157)
Complex IV (normal range: 33.0 + 16.1) (% of activity)	ND	ND	28.4 (87)	44.5 (135)	29.2 (88)	37.7 (114)
Mitochondrial DNA mutation						
nt13513	(+)	(+)	(+)	(+)	(+)	(+)
(% of mutant in muscle)	70	63	42	64	58	58
nt3243	(−)	(−)	(−)	(−)	(−)	(−)
nt8993	(−)	(−)	(−)	(−)	(−)	(−)
nt9176	(−)	(−)	(−)	(−)	(−)	(−)
nt8344	ND	(−)	(−)	(−)	(−)	(−)
Deletion	(−)	(−)	ND	(−)	(−)	(−)

basal ganglia, spinal cord, cerebellum, and cerebral cortex (Leigh 1951; Pincus 1972; van Erven et al. 1987). Chol et al. (2003) reported that three patients with the G13513A mutation exhibited a selective brainstem involvement in whom they referred to Leigh-like syndrome. We think it unnecessary to use the term Leigh-like because the brainstem lesion is essential for LS. A recent report also used Leigh disease for three patients with this mutation, two of whom had no radiological involvement of basal ganglia (Kirby et al. 2003). Three of our six patients with the G13513A mutation showed involvement of both brainstem and basal ganglia, and the remaining three showed a selective brainstem involvement.

In this study, we found the G13513A mutation and confirmed its relatively high incidence (approximately 7%) in LS patients. However, the frequency was not as high as that of Chol's report (2003). This mutation was found in the same number of patients as was 8993 in this study, but it seems to be the second most common mtDNA mutation in LS because the 8993 mutation has been reported to account for as many as 15–24% of cases (Santorelli et al. 1993; Rahman et al. 1996).

We also found that onset in patients with this mutation was delayed compared with the typical infantile form, or 8993 mutation. Moreover, the clinical signs were characteristic because ptosis and cardiac conduction abnormalities were frequently seen (83%). Although these symptoms could often be recognized in Kearns-Sayre syndrome (KSS), our patients did not have apparent ophthalmoplegia, retinitis pigmentosa, or mtDNA deletion. Interestingly, three patients had WPW syndrome. There are few reports on the association between WPW syndrome and mtDNA mutation. In an examination of the surgical resected accessory atrioventricular pathway in a patient with WPW syndrome, highly abnormal cardiomyocytes with abnormal mitochondria were characterized (Peters et al. 1994). Further study on cardiac tissue specimens, especially the conduction system, using a combined method of morphology, histochemistry, and molecular genetics, is necessary to explain the association between this mutation and cardiac conduction abnormalities.

Contrary to the previous reports (Santorelli et al. 1997; Pulkes et al. 1999; Chol et al. 2003), our patients had no significant respiratory enzyme deficiency in their muscle biopsies; instead, they were affected with LS. One reason for this might be that relatively low heteroplasmy in muscles could not induce an enzyme deficiency in our patients. In mice experiments, it has been shown that tissue with mtDNA abnormality can show normal enzyme activity until the mtDNA abnormality accumulates to a critical level (Nakada et al. 2001). A severely damaged region may have more mutant mtDNA and hence profound enzyme deficiency. Very recently, Kirby et al. (2003) reported that mutant loads of approximately 50% or less induced the defect in complex I amount and activity in skeletal muscle, liver, and fibroblasts. We could not detect apparent enzyme deficiency in the skeletal muscles with the mutant proportion from 42 to 64%. Factors other than the average mutant load should be considered, such as uneven distribution of the individual cells with different mutant load resulting in normal enzyme activity.

It remains unclear how the 13513 mutation may be a cause of both MELAS and LS. Our preliminary results suggested that RRF and/or SSV were present in almost all MELAS patients with this mutation, but they were absent in the LS patients in this study (data not shown). We could not attribute variability of this phenotype solely to the proportion of mutant mtDNA; there must be other factors relating to cell or tissue specificity. Alternatively, we should suppose a possibility that our patients will show the symptoms compatible to MELAS or KSS later in life.

In conclusion, this mutation should be considered as possibly existing in LS patients. Understanding this mutation would provide us more insight into the complex genotype-phenotype relationships in mitochondrial diseases.

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References

Akanuma J, Muraki K, Komaki H, Nonaka I, Goto Y (2000) Two pathogenic point mutations exist in the authentic mitochondrial genome, not in the nuclear pseudogene. J Hum Genet 45: 337–341

Arii J, Tanabe Y (2000) Leigh syndrome: serial MR imaging and clinical follow-up. Am J Neuroradiol 21:1502–1509

Berkovic SF, Shoubridge EA, Andermann F, Andermann E, Carpenter S, Karpati G (1991) Clinical spectrum of mitochondrial DNA mutation at base pair 8344. Lancet 338:457

Bai Y, Shakeley RM, Attardi G (2000) Tight control of respiration by NADH dehydrogenase ND5 subunit gene expression in mouse mitochondria. Mol Cell Biol 20:905–815

Campos Y, Martin MA, Rubio JC, Solana LG, Garcia-Benayas C, Terradas JL, Arenas J (1997) Leigh syndrome associated with the T9176C mutation in the ATPase 6 gene of mitochondrial DNA. Neurology 49:595–597

Chol M, Lebon S, Benit P, Chretien D, Lonlay P, Goldenberg A, Odent S, Hertz-Pannier L, Vincent-Delorme C, Cormier-Daire V, Rustin P, Rötig A, Munnich A (2003) The mitochondrial DNA G13513A MELAS mutation in the NADH dehydrogenase 5 gene is a frequent cause of Leigh-like syndrome with isolated complex I deficiency. J Med Genet 40:188–191

Corona P, Antozzi C, Carrara F, D'Incerti L, Lama tea E, Tiranti V, Zeviani M (2001) A novel mtDNA mutation in the ND5 subunit of complex I in two MELAS patients. Ann Neurol 49: 106–110

Crimi M, Galbiati S, Moroni I, Bordoni A, Perini MP, Lamantea E, Sciacco M, Zeviani M, Biunno I, Moggio M, Scarlato G, Comi GP (2003) A missense mutation in the mitochondrial ND5 gene associated with a Leigh-MELAS overlap syndrome. Neurology 60:1857–1861

Erven PMM van, Cillessen JPM, Eekhoff EMW, Gabreels FJM, Doesburg WAJG, Lemmens WAJG, Sloff JL, Renier WO, Ruitenbeek W (1987) Leigh syndrome, a mitochondrial encephalo(myo)pathy. Clin Neurol Neurosurg 89:217–230

Goto Y, Nonaka I, Horai S (1990) A mutation in the tRNA-Leu(UUR) gene association with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348:651–653

Hasegawa H, Matsuoka T, Goto Y, Nonaka I (1991) Strongly succinate dehydrogenase-reactive blood vessels in muscles from patients with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. Ann Neurol 1991:601–605

Kirby DM, Kahler SG, Freckmann ML. Reddihough D, Thorburn DR (2000) Leigh disease caused by the mitochondrial DNA G14459A mutation in unrelated families. Ann Neurol 48: 102–104

Kirby DM, Boneh A, Chow CW, Ohtake A, Ryan MT, Thyagarajan D, Thorborn DR (2003) Low mutant load of mitochondrial DNA G13513A mutation can cause Leigh's disease. Ann Neurol 54:473–478

Koga Y, Nonaka I, Sunohara N, Yamanaka R, Kumagai K (1988) Variability in the activity of respiratory chain enzymes in mitochondrial myopathies. Acta Neuropathol 76:135–141

Koga Y, Akita Y, Takane N, Sato Y, Kato H (2000) Heterogenous presentation in A3243G mutation in the mitochondrial tRNA-Leu(UUR) gene. Arch Dis Child 82:407–411

Komaki H, Akanuma J, Iwata H, Takahashi T, Mashima Y, Nonaka I, Goto Y (2003) A novel mtDNA C11777A mutation in Leigh syndrome. Mitochondrion 2:293–304

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

Liolitsa D, Rahman S, Benton S, Carr LJ, Hanna MG (2003) Is the mitochondrial complex I ND5 gene a hot-spot for MELAS causing mutation? Ann Neurol 53:128–132

Makino M, Horai S, Goto Y, Nonaka I (1998) Confirmation that a T-to-C mutation at 9176 in mitochondrial DNA is an additional candidate for Leigh's syndrome. Neuromuscul Disord 8:149–151

Makino M, Horai S, Goto Y, Nonaka I (2000) Mitochondrial DNA mutations in Leigh syndrome and their phylogenetic implications. J Hum Genet 45:69–75

Nakada K, Inoue K, Ono T, Isobe K, Ogura A, Goto YI, Nonaka I, Hayashi JI (2001) Inter-mitochondrial complementation: Mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA. Nature Med 7: 934–940

Penisson-Besnier I, Reynier P, Asfar P, PharmD OD, Sortais A, Dubas F, Emile J, Malthiery Y (2000) Recurrent brain hematomas in MELAS associated with an *ND5* gene mitochondrial mutation. Neurology 55:317–318

Peters NS, Rowland E, Bennet JG, Green CR, Anderson RH, Severs NJ (1994) The Wolff-Parkinson-White syndrome: the cellular substrate for conduction in the accessory atrioventricular pathway. Eur Heart J 15:981–987

Pincus JH (1972) Subacute necrotizing encephalomyelopathy (Leigh's disease): a consideration of clinical features and etiology. Dev Med Child Neurol 14:87–101

Pulkes T, Eunson L, Patterson V, Siddiqui A, Wood NW, Nelson IP, Morgan-Hughes JA, Hanna MG (1999) The mitochondrial DNA G13513A transition in ND5 is associated with a LHON/MELAS overlap syndrome and may be a frequent cause of MELAS. Ann Neurol 46:916–919

Rahman S, Blok RB, Dahl HHM, Danks DM, Kirby DM, Chow CW, Christodoulou J, Thorburn DR (1996) Leigh syndrome: clinical features and biochemical and DNA abnormalities. Ann Neurol 39:343–351

Santorelli FM, Shanske S, Macaya A, Devivo DC, DiMauro S (1993) The mutation at nt 8993 of mitochondrial DNA is a common cause of Leigh's syndrome. Ann Neurol 34:827–834

Santorelli FM, Tanji K, Kulikova R, Shanske S, Vilarinho L, Hays AP, DiMauro S (1997) Identification of a novel mutation in the mtDNA ND5 gene associated with MELAS. Baiochem Biophys Res Commun 238:326–328

Tatuch Y, Christodoulou J, Feigenbaum A, Clarke JTR, Wherret J, Smith C, Rudd N, Petrova-Benedict R, Robinson BH (1992) Heteroplasmic mtDNA mutation (T-to-G) at 8993 can cause Leigh disease when the percentage of abnormal mtDNA is high. Am J Hum Genet 50:852–858

Taylor RW, Morris AAM, Hutchinson M, Turnbull DM (2002) Leigh disease associated with a novel mitochondrial DNA ND5 mutation. Eur J Hum Genet 10:141–144

Thyagarajan D, Shanske S, Vazquez-Memije M, De Vivo D, DiMauro S (1995) A novel mitochondrial ATPase 6 point mutation in familial bilateral striatal necrosis. Ann Neurol 38:468–472

Tiranti V, Hoertnagel K, Carrozzo R, Galimberti C, Munaro M, Grantiero M, Zelante L, Gasparini P, Marzella R, Rocchi M, Bayona-Bafaluy MP, Enriquez J-A, Uziel G, Bertini E, Dionisi-Vici C, Franco B, Meitinger T, Zeviani M (1998) Mutations of SURF-1 in Leigh disease associated with cytochrome c oxidase deficiency. Am J Hum Genet 63:1609–1621

Vries DD de, Engelen BGM van, Gabreels FJM, Ruitenbeek W, Oost BA van (1993) A second missense mutation in the mitochondrial ATPase 6 gene in Leigh's syndrome. Ann Neurol 34:410–412

Zhu Z, Yao J, Johns T, Fu K, De Bie I, Macmillan C, Cuthbert AP, Newbold RF, Wang J-C, Chevrette M, Brown GK, Brown RM, Shoubridge EA (1998) SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. Nat Genet 20:337–343

核遺伝子変異によるミトコンドリア異常症

後藤雄一*

抄　録　核DNA上にある遺伝子変異によっておこるミトコンドリア異常症のうち，電子伝達系酵素欠損症とミトコンドリアDNAの複製・維持に関わる病態を解説した。ミトコンドリアDNAの異常によっておきる数々の病態との関係から，核DNAとミトコンドリアDNAの複雑な相互関係が徐々にあきらかにされつつある。ミトコンドリアの持つ機能が，エネルギー代謝に限らず，アポトーシス，活性酸素産生，カルシウムイオンの保持などに及び，病態を多面的に理解する必要性がでてきた。

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key words : electron transfer complex, mitochondrial DNA, multiple deletion, mitochondrial DNA depletion

Ⅰ．はじめに

ミトコンドリアの機能は細胞内のエネルギー産生が主体である。したがって，ミトコンドリア病とはそのエネルギー産生が低下することで細胞機能が障害され，臨床症状が現れている疾患である。ミトコンドリア内には，多くのエネルギー代謝に関わる酵素が存在していることから，これらの酵素の活性低下がミトコンドリア病の原因になり得る。しかしながら関連する酵素は多数存在しその欠損症すべてをここで網羅することは不可能である。そこで，ミトコンドリアDNAと核DNAの両者にコードされているサブユニットから構成されている電子伝達系酵素複合体とミトコンドリアDNAの維持や複製に関連する核遺伝子変異による病態を解説する。さらに，ミトコンドリア内で発現する核DNAにコードされた蛋白の異常による疾患の発見が相次いでおり，それらについても簡単に触れることにする。

Mitochondrial diseases caused by nuclear gene mutation.

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Ⅱ．電子伝達系酵素複合体に関わる核遺伝子

1．複合体Ⅰ

複合体Ⅰは，TCA回路やβ酸化で生成されるNADH（reduced form of nicotinamide adenine dinucleotide）から電子を受け取り，その電子をコエンザイムQ（CoQ）に渡すまでの反応を担っており，NADH-CoQ還元酵素（NADH-CoQ reductase）とも呼ばれる。この複合体は，電子伝達系酵素複合体の中で最大であり，その構成はきわめて複雑である。牛の心筋を用いた研究では，少なくとも41個の異なるタンパクサブユニットからなり，Flavin mononucleotide：FMN，鉄-イオウ蛋白，CoQ類似物質などで構成されているが，その全貌を理解できるまでには到っていない。41個のサブユニットのうち，ミトコンドリアDNAにコードされているのは7個であり，それ以外はすべて核DNAにコードされている。

表2に核DNAにコードされた複合体Ⅰサブユ

表1　電子伝達系酵素複合体の構成

複合体名	核DNA由来サブユニット	mtDNA由来サブユニット
複合体Ⅰ (NADH-CoQ oxidoreductase)	34	7
複合体Ⅱ (succinate-CoQ oxidoreductase)	4	0
複合体Ⅲ (CoQ-cytochrome c oxidoreductase)	10	1
複合体Ⅳ (cytochrome *c* oxidase)	10	3
複合体Ⅴ (ATP synthase)	10～16	2

ニットで，ヒトの病気で変異が確認されたものを掲げた。その臨床症状は，乳児期発症の脳症，脳筋症，Leigh脳症であり，ほとんどは早期に死亡する重症の経過をとっている[2,11,12,20,23,27]。複合体Ⅰ欠損症の生化学的診断は容易ではなく，また多数のサブユニットについての遺伝子診断も容易でなく，確定診断がされていない症例が多数存在している可能性が高い。

2. 複合体Ⅱ

電子伝達系酵素複合体のうちミトコンドリアDNAでコードされているサブユニットを持たない唯一の複合体が，複合体Ⅱ (succinate-ubiquinone oxidoreductase) である。この複合体は，フラボプロテインであるFpサブユニット (*SDHA*)，鉄-イオウ蛋白であるIpサブユニット (*SDHB*)，これらを内膜につなげるアンカーとしてはたらき，ヘムを有している2つのサブユニットCybLとCybS (*SDHC* と *SDHD*) で構成されている。

病気との関連では，Fp遺伝子の変異が視神経萎縮と運動失調を示した患者とLeigh脳症患者(2家系) で報告されている[4,5,17]。また *SDHC* と *SDHD* の変異がそれぞれ家族性傍神経節腫感受性領域 (*PGL3* と *PGL1*) に対応することが報告され，Ip遺伝子 (*SDHB*) 変異が家族性褐色細胞種および傍神経節腫の感受性と相関のあることが示されている。

3. 複合体Ⅲ

複合体Ⅲは，複合体Ⅰあるいは複合体ⅡからCoQに伝えられた電子をチトクロームcに受け渡す働きがあり，その際CoQを酸化しチトクロームcを還元するので，CoQ-チトクロームc酸化還元酵素とも呼ばれる。この酵素複合体は，2個のチトクロームb，チトクロームc1，鉄-イオウ蛋白，Q結合蛋白，コア蛋白などを含む11個のサブユニットからなる。ミトコンドリアDNAでコードされているのは，チトクロームbのみであり，残りは核DNAにコードされている。

サブユニット自体の遺伝子変異はミトコンドリアDNA以外には報告されていないが，複合体Ⅲの集合に関わる因子であるBCS1Lの遺伝子変異が報告された[29]。その症状は，成長障害 (growth retardation)，アミノ酸尿 (aminoaciduria)，胆汁うっ滞 (cholestasis)，鉄過剰 (iron overload)，乳酸アシドーシス (lactic acidosis)，早期死亡 (early death) であり，これらの頭文字をとってGRACILE症候群と称される。また，トルコから報告されたBCS1Lの変異を持つ症例では，複合体Ⅲ欠損による尿細管機能異常，脳症，肝不全の症状を示した[6]。

4. 複合体Ⅳ

複合体Ⅳは，電子伝達系酵素反応の最終段階を受け持ち，チトクロームcを酸化し，二酸化炭素と水を生成するため，チトクロームc酸化酵素 (COX) と呼ばれる。13個のサブユニットからなり，3個がミトコンドリアDNAで，10個が核DNAにコードされている。COX活性は生化学的にも，また組織化学的にも証明できるので，COX欠損症はミトコンドリア病のなかではもっとも研究の進んでいる疾患である。

ミトコンドリアDNAがコードしている3個のサブユニットは，ミトコンドリアDNAのいろいろな変異 (欠失，転移RNA内の点変異，サブユ

表2 ミトコンドリア病の病因となる核DNA遺伝子

病因遺伝子	臨床表現型
複合体Iサブユニット	
NDUFV1	脳筋症
NDUFS4	Leigh脳症
NDUFS7	Leigh脳症
NDUFS8	心筋症を伴うLeigh脳症
NDUFS2	心筋症を伴う脳筋症
NDUFS1	脳筋症，Leigh脳症
複合体IIサブユニット	
SDHA	Leigh脳症，視神経萎縮＋運動失調
SDHB	家族性褐色細胞腫，遺伝性傍神経節腫
SDHC, SDHD	遺伝性傍神経節腫
複合体IIIに関係する因子	
BCS1L	GRACILE症候群 腎尿細管障害＋肝障害を伴う筋症
複合体IVに関係する因子	
SURF-1	Leigh脳症
SCO2	心筋症を伴う脳筋症
SCO1	肝不全を伴う脳筋症
COX10	脳筋症
COX15	心筋症を伴う脳筋症
その他	
Thymidine phosphorylase	MNGIE
ANT-1	進行性外眼筋麻痺症候群
Twinkle	進行性外眼筋麻痺症候群
Mitochondrial polymerase γ	進行性外眼筋麻痺症候群
Thymidine kinase-2	ミトコンドリアDNA欠乏症候群
Deoxyguanosine kinase	ミトコンドリアDNA欠乏症候群

ニット内の点変異で異常を来していると考えられる症例が報告されている。しかし，核DNAでコードされている10個のサブユニットについてはその責任遺伝子に変異が見つかった症例の報告はない。しかし，COXの集合に関わる遺伝子である，SURF1[22,31]，SCO1[25]，SCO2[16]，COX10[26]，COX15[1]に遺伝子変異が同定されている。

SURF1変異は，COX欠損を伴うLeigh脳症患者の一部で認められる。患者の骨格筋をCOX染色すると，骨格筋ばかりでなく，血管，筋紡錘などの細胞もCOX活性を持たない。この所見は皮膚生検でも確認できる。すでに多くのミス変異，ナンセンス変異，スプライス変異が報告されており，遺伝子診断にはすべてのエクソンとイントロン-エクソン境界の検査が必要になる。時に，一つのアレルにしか変異が同定されないことがあり，その意味付けは分かっていない。

SCO2とCOX15の変異は，重篤な心筋症を伴う乳児型のCOX欠損症症例で見いだされた。またSCO1変異は，ケトアシドーシス，肝不全を伴う乳児期発症の症例で，COX10変異は脳筋症患者で報告された。これらは，いずれもまだ日本人での報告例はない。

Ⅲ．ミトコンドリアDNAの複製・維持に関わる核遺伝子

1. adenine nucleotide translocator(ANT) 1

ANTはミトコンドリア内膜に局在し，マトリクス内のATPと細胞外のADPを交換輸送する

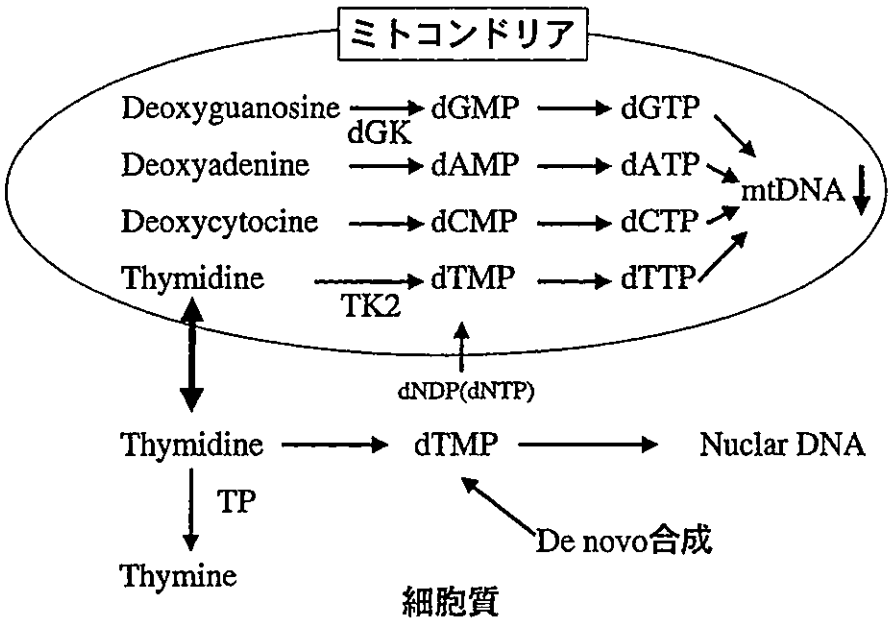


図1　ミトコンドリア内 dNTP プールの調節とその破綻
細胞内のチミジンは TP によって分解されチミンになるか，リン酸化されて DNA 合成に利用される。TP 活性のない MNGIE 患者ではチミンに分解されない結果として細胞内のチミジン量が高く，骨格筋のような非分裂細胞ではミトコンドリア内の TK 2のみ発現しているので，ミトコンドリア内に入ってリン酸化される経路が活性化され，dNTP プールの量的アンバランスが起きると考えられる。一方，dGK と TK2の機能低下は，それぞれ dGTP, dTTP の低下を来し，TP 活性低下の場合と同様に dNTP プールのアンバランスを起こすと予想される。

機能を持つ。この交換では，ATP の方がより負の電荷を持つために，電荷の移動も起き，膜電位の保持にも関わっている。さらに，ANT は Permeability transition pore の構成要素の一つとしてアポトーシスにも関与している。ヒトでは3つのアイソフォームがあり，骨格筋と心筋に主に発現しているものが ANT1である。

常染色体優性遺伝形式の進行性外眼筋麻痺症候群の家系の連鎖解析から4q33-35の領域に責任領域がマップされたあと，その中に存在する ANT1 遺伝子の塩基配列を調べたところ，変異が見いだされたのが最初である[9]。日本の症例でも，ANT1 変異が同定されている[10]。ANT1変異があると，ミトコンドリア DNA の多重欠失が認められるが，その詳細な機序は不明である。

2. DNA polymerase γ (POLG), Twinkle

POLG はミトコンドリアに存在する DNA ポリメラーゼであり，ANT1同様に，連鎖解析により15q22-26にマップできたミトコンドリア DNA の多重欠失を持つ大家系を用いて，POLG 遺伝子内にヘテロ接合体を見いだされた[28]。さらに別の劣性遺伝の家系から，複合ヘテロ接合体が見いだされた。優性遺伝か劣性遺伝かは，変異の種類とその障害程度によるものであろうと推測されている。

Twinkle は，10q24に連鎖する家系における病因遺伝子の検索から同定された C10orff2という新規遺伝子が，バクテリオファージの T7プライマーゼ・ヘリカーゼ gene 4 protein (gp4) と相同性が高いところから名付けられたものである (Twinkle: T7 gp4-like protein with intramitochondrial nucleoid localization)[21]。Twinkle は，アミノ末端にミトコンドリア局在シグナルを有し，ヌクレオイド (ミトコンドリア DNA-蛋白複合体) に一致した分布を示した。さらに培養細胞に Twinkle 遺伝子を導入し強発現を行ったところ，ミトコンドリア分画でのヘリカーゼ活性の

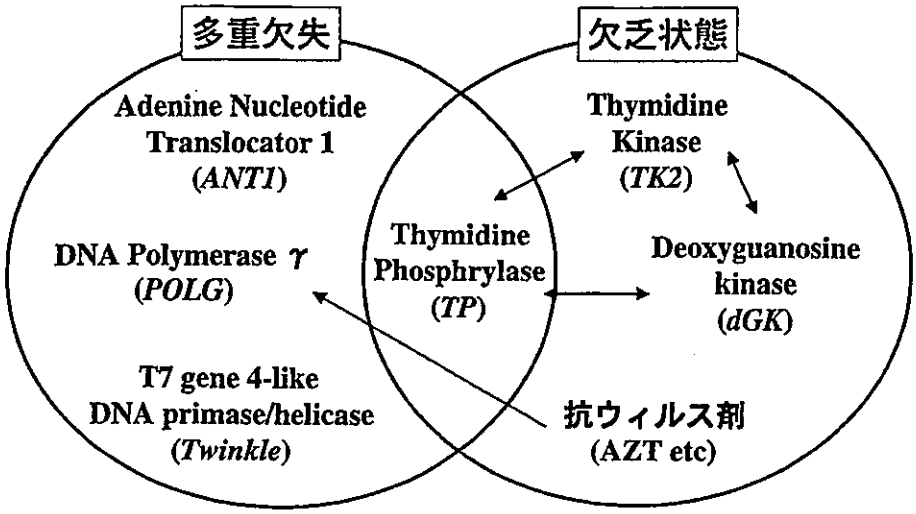


図2 多重欠失と欠乏状態に関連する遺伝子
ミトコンドリアDNAの複製や維持に関わる遺伝子の変異は，ミトコンドリアDNAの多重欠失と欠乏状態を引き起こす。TP，TK2，dGKはいずれもミトコンドリア内のdNTPプールのアンバランスを引き起こし，主に欠乏状態を引き起こすが，ANT1，POLG，Twinkleは多重欠失を起こす。しかしながら，AIDSの治療薬であるAZTなどの抗ウィルス剤は，ミトコンドリアDNAの欠乏状態を引き起こすが，AZTはPOLGの活性を低下させることが知られている。多重欠失と欠乏状態の関係は密接である。

上昇を認めた。ヘリカーゼは，二本鎖のポリヌクレオチドを一本鎖にする酵素のことで，DNA複製や転写活性に重要な働きをしている。最近の研究で，従来信じられてきた二つの複製点から時間差で複製されるというstrand-asynchronous replicationではなく，実はDループから同時に両方向の複製が行われるstrand-coupled replicationが優位であるが判明した(一口メモ参照)[7,30]。このような複製機構とTwinkleの機能との関係，ミトコンドリアDNAの多重欠失の起きるメカニズムの解明は，今後の研究課題である。

3. dNTPプールに関わる遺伝子

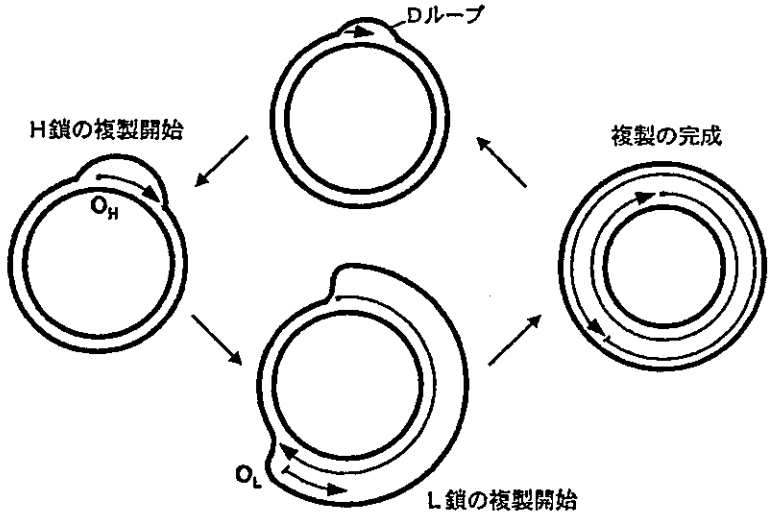
常染色体劣性遺伝形式をとり，消化管運動障害，末梢神経障害，白質脳症，ミオパチーを呈する成人で発症するミトコンドリア病であるMNGIE (mitochondrial neuro-gastro-intestinal encephalomyopathy) において，患者骨格筋ではミトコンドリアDNAの多重欠失や欠乏を認める。この疾患の責任遺伝子がthymidine phosphorylase (TP1) であることが証明された[15]。

実はこの遺伝子産物はミトコンドリア内にはなく，なぜミトコンドリアDNAの異常が引き起こされるか明らかではない。しかし，TP活性がなくなると細胞質内でのチミンの合成がなくなり，過剰なチミジンはサルベージ回路であるDNA合成系に用いられる。その際，非分裂細胞である骨格筋などはミトコンドリア内のTK2活性のみが存在するため，ミトコンドリア内のdTTPが増加し，dNTPプールのアンバランスが生じ，結果としてミトコンドリアDNAの合成が低下するものと考えられている。このモデルは，同様にミトコンドリアDNAの欠乏状態を引き起こすTK2やdGKの遺伝子異常が確認されたことから，その妥当性が高まっている[13,18]。ただし，dNTPプールのアンバランスが実際に確認されてはいない(図1)。

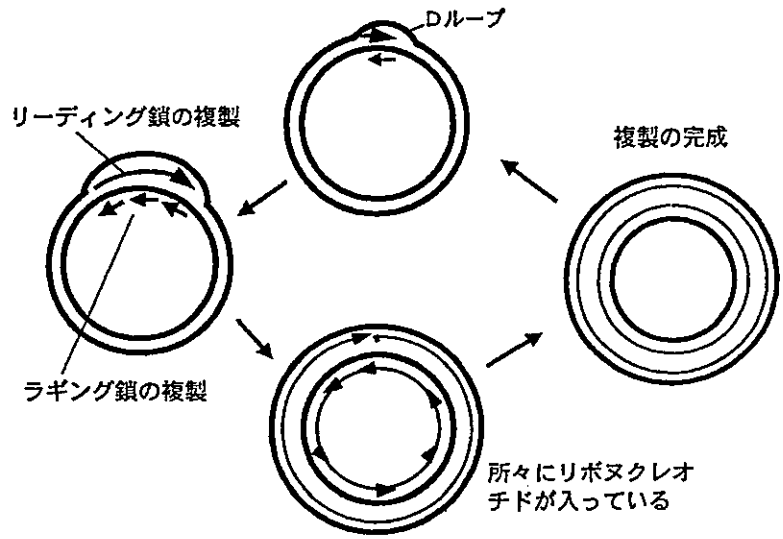
また，これらdNTPプールに影響すると考えられている遺伝子変異と，多重欠失を引き起こす遺伝子変異の関係を図2に示す。おそらく共通の，もしくは類似した機序で，多重欠失と欠乏が生じていると推察される。

一口メモ

ミトコンドリアDNAの複製機構



メモ図1



メモ図2

ミトコンドリアDNAの複製は，D-loopと呼ばれる調節領域にあるH鎖複製開始点（O_H）からH鎖のみの複製が起こり，全周の3分の2くらい進んだところで，L鎖複製開始点（O_L）から，逆方向の複製が始まるというモデル（strand-asynchronous replication）が信じられてきた（メモ図1）。しかし，最近の研究で，通常の核DNAの複製と同様に，D-loopからH鎖（リーディング鎖）の複製とL鎖（ラギング鎖）の複製が同時に起きること（strand-coupled replication）が明らかになってきた。そして，H鎖にもL鎖にも所々にリボヌクレオチドが入り込んでおり，それらが後になってデオキシリボヌクレオチドに置き換わるモデルが優勢になりつつある（メモ図2）。

Ⅳ．その他の核遺伝子変異による ミトコンドリア病

ミトコンドリアに蛋白が輸送される時に，多くのサブユニットからなる輸送装置が使われる。外膜に結合するTOM蛋白と内膜に結合しているTIM蛋白のうち，酵母のTIM8蛋白に類似したDDP1遺伝子に変異がある時に，ジストニアと難聴を呈するX連鎖性のMohr-Tranebjaerg症候群が起きる[8]。日本人でも報告がある[24]。

別なX連鎖性の疾患であるBarth症候群は，新生児や乳児期発症の拡張性心筋症，好中球減少症，ミオパチー，低身長，易感染性を呈する疾患であり，種々の電子伝達系酵素活性低下を伴う。責任遺伝子はG4.5，別名Tafazzin（TAZ）遺伝子である[29]。その機能はよく分かっていなかったが，最近になりリン脂質の合成やリモルディングに関わるアシル転移酵素との相同性が判明し，ミトコンドリア膜のリン脂質の研究が行われた。その結果，代表的なミトコンドリア膜のリン脂質であるカルジオリピン（CL）が患者では低下しており，さらにCLや他のリン脂質のリモルディングが障害されていることが示されている[19]。これらリン脂質の異常とミトコンドリア異常との関連は今後の研究課題であるが，新しいミトコンドリア障害の機序として興味深い。

Ⅴ．今後の展望

ミトコンドリアの機能は，エネルギー産生だけにとどまらず，アポトーシス，カルシウムイオンの保管，活性酸素の発生など細胞機能にいろいろな影響を与える[14]。これらの機能に関連して，パーキンソン病，アルツハイマー病，ハンチントン病などをはじめ，数多くの疾患と関連している。今後は，このような多くの疾患において，ミトコンドリアの持つどのような機能が病態と関っているかという研究が重要であり，あらたな治療や予防に役立つ知見が得られる可能性が高いと考える。ミトコンドリアの研究は，新しい時代を迎えている。

文　献

1) Antonicka, H., Mattman, A., Carlson, C.G. et al.: Mutations in *COX15* produce a defect in the mitochondrial heme biosynthetic pathway, causing early-onset fatal hypertrophic cardiomyopathy. Am. J. Hum. Genet., 72: 101-114, 2003.
2) Benit, P., Chretien, D., Kadhom, N. et al.: Large-scale deletion and point mutations of the nuclear NDUFV1 and NDUFS1 genes in mitochondrial complex I deficiency. Am. J. Hum. Genet., 68: 1344-1352, 2001.
3) Bione, S., D'Adamo, P., Maestrini, E. et al.: A novel X-linked gene, G4.5, is responsible for Barth syndrome. Nat. Genet., 12: 385-389, 1996.
4) Birch-Machin, M.A., Taylor, R.W., Cochran, B. et al.: Late-onset optic atrophy, ataxia, and myopathy associated with a mutation of a complex II. Ann. Neurol., 48: 330-335, 2000.
5) Bourgeron, T., Rustin, P., Chretien, D. et al.: Mutation of a nuclear succinate dehydrogenase gene results in mitochondrial respiratory chain deficiency. Nat. Genet., 11: 144-149, 1995.
6) de Lonlay, P., Valnot, I., Barrientos, A. et al.: A mutant mitochondrial respiratory chain assembly protein causes complex III deficiency in patients with tubulopathy, encephalopathy and liver failure. Nat. Genet., 29: 57-60, 2001.
7) Holt, I.J., Lorimer, H.E., Jacobs, H.T.: Coupled leading-and lagging-strand synthesis of mammalian mitochondrial DNA. Cell, 100: 515-524, 2000.
8) Jin, H., May, M., Tranebjaerg, L. et al.: A novel X-linked gene, DDP, shows mutations in families with deafness (DFN-1), dystonia, mental deficiency and blindness. Nat. Genet., 14: 177-180, 1996.
9) Kaukonen, J., Juselius, J.K., Tiranti, V. et al.: Role of adenine nucleotide translocator 1 in mtDNA maintenance. Science, 289: 782-785, 2000.
10) Komaki, H., Fukazawa, T., Houzen, H. et al.: A novel D104G mutation in the adenine nucleotide translocator 1 gene in autosomal dominant progressive external ophthalmoplegia patients with mitochondrial DNA with multiple deletions. Ann. Neurol., 51: 645-648, 2002.
11) Loeffen, J., Smeitink, J., Triepels, R. et al.: The first nuclear-encoded complex I mutation in a patient with Leigh syndrome. Am. J. Hum. Genet., 63: 1598-1608, 1998.
12) Loeffen, J., Elpeleg, O., Smeitink, J. et al.: Mutations in the complex I NDUFS2 gene of patients with car-