A systematic mutation screen of 10 nuclear and 25 mitochondrial candidate genes in 21 patients with cytochrome c oxidase (COX) deficiency shows tRNA^{Ser(UCN)} mutations in a subgroup with syndromal encephalopathy

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Abstract

COX deficiency is believed to be the most common defect in neonates and infants with mitochondrial diseases. To explore the causes of this group of disorders, we examined 25 mitochondrial genes (three COX subunit genes and 22 tRNA genes) and 10 nuclear COX subunit genes for disease associated mutations using PCR-SSCP and direct sequencing of polymorphic SSCP fragments. DNA from one patient with severe COX deficiency and with consanguineous parents was entirely sequenced. The patient population consisted of 21 unrelated index patients with mitochondrial disorders and predominant (n=7) or isolated (n=14) COX deficiency.

We detected two distinct tRNA^{Ser(UCN)} mutations, which have been recently described in single kindreds, in a subgroup of four patients with COX deficiency, deafness, myoclonic epilepsy, ataxia, and mental retardation. Besides a number of nucleotide variants, a single novel missense mutation, which may contribute to the disease phenotype, was found in the mitochondrial encoded COX 1 gene (G6480A). Mutations in nuclear encoded COX subunit genes were not detected in this study.

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made up of 13 subunits, three encoded by mitochondrial (mt) DNA and 10 of nuclear (n) origin. The mitochondrial encoded subunits are evolutionarily highly conserved and form the catalytic core of the enzyme, and the less conserved nuclear encoded subunits are thought to modulate enzyme activity.^{8,9}

Since components of COX are encoded by mtDNA and nDNA, deficiency may be caused by either a mitochondrial or nuclear mutation. The 13 COX subunits are obvious candidate genes for isolated COX deficiencies; however, only a single mutation in mtDNA encoded COX subunit III has so far been reported in this disorder.¹⁰ Genes involved in import, assembly, and regulation of mitochondrial subunits are further candidates for COX deficiency related diseases. A number of pathogenic mutations in mitochondrial tRNA genes have already been described, which are predicted to decrease mitochondrial protein synthesis and thus the levels of those RC complexes partly encoded by the mtDNA (complexes I, III, and IV).¹¹⁻¹⁵

In an attempt to find the more common genetic defects in mitochondrial disorders with COX deficiency, we systematically screened both nuclear and mitochondrial candidate genes in 21 patients assigned to different clinical subgroups. Besides a number of nucleotide variants, we found two pathogenic mtDNA tRNA mutations in four unrelated patients and one missense mutation in the COX 1 gene. No nuclear mutations were detected.

Patients and methods

PATIENTS

Twenty-one unrelated patients presenting with different mitochondrial disorders (table 1) were included in the study.

MEASUREMENT OF RC ACTIVITIES IN SKELETAL MUSCLE

Rotenone sensitive NADH-ubiquinone oxidoreductase (complex I), succinate-cytochrome c oxidoreductase (complexes II and III), and COX (complex IV) activities were determined spectrophotometrically according to the methods described by Fischer.¹⁶ In one patient (patient 15), measurements were performed according to the description of

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Cytochrome c oxidase (COX) is the terminal enzyme (complex IV) of the respiratory chain (RC). It is embedded in the inner mitochondrial membrane and catalyses the transfer of reducing equivalents from cytochrome c to molecular oxygen, thereby sustaining a pH gradient and membrane potential. COX deficiency most often presents in infancy as a severe multisystem disorder, with predominant features of encephalopathy and myopathy.^{1,2} Diagnoses associated with COX deficiency include fatal infantile myopathy,^{3,4} benign reversible infantile myopathy,⁵ subacute necrotising encephalomyelopathy or Leigh's disease,⁶ and a variety of syndromic mitochondrialopathies.⁷ The COX enzyme is

Table 1 Clinical and morphological characteristics of patients with COX deficiency

Patient No	Age of onset/ (death)	Sex	Clinical phenotype	Morphology	COX rest-activity (% from lowest ref value)	Family history
1	Birth (1 mth)	M	Subgroup 1: fatal hypertrophic cardiomyopathy (HCMP) and muscular hypotonia	HCMP, musc hypotonia	16	Parental consanguinity
2	Birth (1 mth)	M		HCMP, musc hypotonia	0	Single offspring
3	Birth (5 mth)	F		HCMP, musc hypotonia	64	One affected, one healthy sister
			Subgroup 2: Leigh's disease (subacute necrotising encephalomyelopathy)			
4	8 mth	M	Leigh	ND	30	Single offspring
5	1 y (4 y)	F	Leigh	Normal	51	Single offspring
6	4 y	F	Leigh	Normal	27	Single offspring
			Subgroup 3a: fatal infantile myopathy			
7	Birth (1 mth)	M	Musc hypotonia, resp insuff	ND	21	Parental consanguinity
8	Birth (4 mth)	F	Musc hypotonia, resp insuff, sepsis	Mitochondrial proliferation	53	Parental consanguinity
			Subgroup 3b: slowly progressive generalised muscular hypotonia as predominant feature			
9	5 mth	F	Musc hypotonia, psychomotor retard	Normal	11	Single offspring
10	5 mth	F	Musc hypotonia	Normal	24	Single offspring
11	1 y	M	Musc hypotonia	Mitochondrial proliferation	50	Single offspring
12	1 y	F	Musc hypotonia, psychomotor retard	Mitochondrial proliferation	17	Single offspring
13	2 y	M	Musc hypotonia, psychomotor retard	ND	36	Single offspring
14	6 y	M	Musc hypotonia, resp insuff	Ragged red fibres	9	Single offspring
			Subgroup 4: myoclonic epilepsy, ataxia, and sensorineural hearing loss			
15	1 y	M	Myocl epilepsy, ataxia, SHL, ment ret	Normal	29	Single case
16	3 y	F	Myocl epilepsy, ataxia, SHL, ment ret	Normal	39	Brother, mother affected
17	8 y (14 y)	F	Myocl epilepsy, ataxia, SHL, ment ret	Normal	24	Single case
18	14 y (36 y)	F	Myocl epilepsy, ataxia, SHL, ment ret	Normal	73	Sister, mother affected
			Not classified in subgroups 1–4			
19	Birth (5 mth)	F	Recurrent infections, neurol deficits	Normal	31	One affected, one healthy sister
20	Birth (5 mth)	F	Cerebral atrophy, musc hypotonia	Normal	12	Single offspring
21	2 y	M	Ataxia, small for dates	Lipidosis	17	Single offspring

SHL=sensorineural hearing loss, ND=not done.

Krähenbühl *et al.*¹⁷ A detailed genotype-phenotype correlation for index patients 15-18 has been described elsewhere.¹⁸

DNA ANALYSIS

Total DNA from leucocytes or from small amounts of tissue (muscle, fibroblasts) was obtained using standard extraction procedures (Qiagen). All DNA analyses of index patients were performed on skeletal muscle DNA or RNA, except for patient 5, where fibroblast DNA/RNA was used. The polymerase chain reaction (PCR) and Southern blot analysis were performed as previously described.¹⁹ For PCR single strand conformation polymorphism (SSCP) analysis, electrophoresis was performed as previously described.²⁰ MtDNA-RFLP analysis of first degree relatives of index patients 15, 17, and 18 was performed with leucocyte DNA. MtDNA-RFLP analysis in first degree relatives of index patient 16 was performed on skeletal muscle DNA. To encompass the COX 1-COX 3 genes, we selected 14 primer pairs (upstream and downstream primers possess sequences specific for the light (L) and the heavy (H) strands of the mtDNA²¹): L5873 - 5903, H 6139 - 6162, L 6029 - 6050, H 6139 - 6162, L 6325 - 6351, H 6667 - 6639, L 6464 - 6489, H 6824 - 6801, L 6703 - 6730, H 7044 - 7020, L 7019 - 7044, H 7400 - 7378, L 7405 - 7428, H 7666 - 7642, L 7458 - 7487, H 7817 - 7788, L 7760 - 7787, H 8073 - 8049, L 7890 - 7915, H 8280 - 8257, L 7959 - 7982, H 8253 - 8230, L 9138 - 9258, H 9405 - 9382, L 9281 - 9300, H 9630 - 9605, L 9561 - 9588, H 9869 - 9842.

The mitochondrial tRNA genes were amplified using the following primer pairs: tRNA^{Phe} L: 516 - 536, H: 768 - 748; tRNA^{Val} L: 1484 - 1501, H: 1877 - 1760; tRNA^{Leu(UUR)} L: 3153 - 3172, H: 3423 - 3404; tRNA^{Ile, Glu, Met} L: 4237 - 4259, H: 4505 - 4482; tRNA^{Trp, Ala, Asn, Cys, Tyr} L:

5424 - 5447, H: 5920 - 5900; tRNA^{Ser(UCN), Asp} L: 7405 - 7428, H: 7666 - 7642; tRNA^{Lys} L: 8129 - 8151, H: 8423 - 8395; tRNA^{Gly} L: 9839 - 9867, H: 10288 - 10264; tRNA^{Arg} L: 10289 - 10314, H: 10628 - 10601; tRNA^{His} L: 11997 - 12019, H: 12209 - 12185; tRNA^{Ser(AGY), Leu(CUN)} L: 12210 - 12237, H: 12477 - 12450; tRNA^{Glu} L: 14456 - 14483, H: 14862 - 14842; tRNA^{Thr, Pro} L: 15806 - 15633, H: 16075 - 16052.

For sequencing of mtDNA, PCR amplified DNA was gel purified (Qiagen). Cycle sequencing of mtDNA gene fragments was performed using the Thermosequenase kit (USB) and [³⁵S]dATP incorporation (ICN).

The PCR amplification of cDNA of nuclear encoded COX subunits was performed on total RNA extracts from skeletal muscle by a conventional guanidine isothiocyanate/phenol isolation method (TRIZOL Reagent, Gibco) and reverse transcription method using the 1st strand cDNA synthesis kit (Boehringer Mannheim). COX 4, COX 5a, COX 6AM, COX 6b, COX 6c, COX 7AM, COX 7b, COX 7c, and COX 8 cDNAs were amplified using 5' and 3' primers according to the published cDNA sequences (EMBL accession number m34600, m22760, m83308, x13923, x13238, m83186, x16560, and J04823) to produce fragments encompassing the coding region sequence. COX Vb exons were amplified directly from genomic DNA (EMBL accession number m59250). PCR was performed using *Taq* polymerase (Perkin-Elmer) in the presence of 5% DMSO. The cycle conditions were five minutes at 94°C, then 35 cycles for 30 seconds at 94°C, one minute at 60°C, and one minute 30 seconds at 72°C.

The following primers were used: COX 4: forward (FW): 27 - 48, FW: 271 - 296, reverse (RV): 325 - 304, RV: 598 - 578. COX 5a: FW: 7 - 26, FW: 209 - 232, RV: 291 - 266, RV: 510 - 487. COX 5b: FW: 10 - 32, FW 930 - 952, FW: 1216 - 1240, FW: 1898 - 1929, RV: 273 -

Table 2 Respiratory chain activities of skeletal muscle in patients with COX deficiency

Patients	Complex I	Complex II+III	Complex IV	Citrate synthase
Subgroup 1				
1	7.1 (0.09)	8.1 (0.10)	14.3 (0.18)	79.4
2	* 14.2 (0.11)	7.6 (0.06)	0.0 (0)	124.0
3	* 15.0 (0.23)	6.2 (0.10)	58.8 (0.93)	63.0
Subgroup 2				
4	* 38.9 (0.25)	6.3 (0.04)	27.0 (0.18)	153.0
5	ND	ND	2.6†	28.0†
6	* 13.2 (0.19)	8.6 (0.12)	24.0 (0.34)	70.0
Subgroup 3a				
7	5.6 (0.05)	19.4 (0.16)	18.7 (0.15)	123.0
8	* 14.7 (0.20)	12.8 (0.18)	47.6 (0.65)	73.0
Subgroup 3b				
9	8.8 (0.12)	6.3 (0.08)	9.7 (0.13)	76.1
10	15.7 (0.15)	5.0 (0.05)	22.0 (0.21)	107.0
11	* 25.2 (0.22)	11.5 (0.10)	44.9 (0.39)	114.0
12	20.9 (0.19)	5.1 (0.04)	15.3 (0.14)	110.0
13	* 41.3 (0.29)	18.8 (0.13)	32.7 (0.23)	143.0
14	* 24.9 (0.10)	8.9 (0.03)	7.9 (0.03)	256.0
Subgroup 4				
15	‡	‡	‡	‡
16	* 31.2 (0.28)	17.8 (0.16)	34.9 (0.31)	113.0
17	* 15.6 (0.17)	13.8 (0.15)	21.2 (0.24)	90.0
18	* 26.9 (0.25)	15.3 (0.14)	66.0 (0.62)	106.0
Not classified in subgroups 1–4				
19	* 35.8 (0.28)	17.3 (0.14)	27.7 (0.22)	128.0
20	* 12.0 (0.16)	8.9 (0.12)	10.4 (0.14)	72.8
21	* 30.9 (0.24)	9.4 (0.07)	15.0 (0.12)	127.0
Reference ranges				
Enzyme activity§	12.0–40.0	6.0–25.0	90–281	45–105
Ratio¶	(0.17–0.56)	(0.08–0.45)	(0.90–4.70)	

*Isolated COX deficiency.

†COX activity in fibroblasts: activity is expressed as unit (μmol/min) and grams of total protein; range: 5.1–22.0 U/g total protein; coefficient of variation (cv) 17.3%; citrate synthase activity in fibroblasts: range: 8.5–44.0 U/g total protein; cv 23%.

‡For index patient 15, a combined RC defect of complex I (rest-activity 63%) and IV (rest-activity 29%) was detected according to the description of Krähenbühl *et al.*¹⁷ Owing to different application of methods, enzyme activities are not listed in table 1.

§RC activities in skeletal muscle: activity is expressed as unit (μmol/min) and grams of non-collagen protein (NCP). Complex I (NADH-ubiquinone oxidoreductase; cv 12.3%), complexes II+III (succinate cytochrome c oxidoreductase; cv 13.6%), complex IV (COX; cv 8.2%), citrate synthase (cv 6.19%). Activities differing from the reference range are marked in bold. Control values are given as 95% confidence interval of the mean.

¶An additional value in parentheses, reflecting the ratio between the activity of the respective RC complexes and the mitochondrial marker enzyme citrate synthase, is given to exclude artificial deficiencies resulting from a lower content of mitochondria in the samples. Ratio is expressed as unit/unit citrate synthase. Ratios differing from the reference range are marked in bold.

Measurements were done in duplicate; average values are given.

253, RV: 1240 - 1214, RV: 1431 - 1403, RV: 2173 - 2148. COX 6AM: FW: 33 - 54, RV: 369 - 346. COX 6b: FW: 21 - 42, RV: 338 - 315. COX 6c: FW: 26 - 48, RV: 348 - 322. COX 7AM: FW: 24 - 46, RV: 337 - 311. COX 7b: FW: 38 - 59, FW: 168 - 191, RV: 295 - 271, RV: 423 - 398. COX 7c: FW: 5 - 27, RV: 286 - 262. COX 8: FW: 21 - 43, RV: 297 - 319.

Cycle sequencing of nuclear gene fragments was done using the Dye Primer Cycle Sequencing Ready Reaction Kit (ABI). The sequences were determined with an Applied Biosystems 377 automated sequencer.

QUANTIFICATION OF HETEROPLASMIC mtDNA MUTATIONS

The mutations T7512C and 7472Cins were quantified as previously described.^{22, 23} The fragments were electrophoresed through a 3% agarose gel (NuSieve, FMC) and quantified after ethidium bromide staining using the Gel-Print2001 Station (MWG Biotech, Ebersberg, Germany) and ONE-DScan 1.0 (Scanalytics, CSPI).

Results

BIOCHEMICAL AND MORPHOLOGICAL STUDIES

Tables 1 and 2 list the clinical and biochemical features of the 21 unrelated index patients with COX deficiency selected for this study. Patient

5 had a marked reduction in COX activity in fibroblasts; however, no other enzyme was assayed. All other measurements were performed on skeletal muscle biopsies. Isolated COX deficiency was diagnosed in 14 patients, as defined by reduced activity of complex IV (0–73% of the lowest reference value) and normal activity of complexes I–III. Predominant reduction in COX activity with moderate deficiencies of complexes I–III was observed in six patients. COX activity was completely absent in one case (patient 2). No correlation of COX activity with clinical features, including age of onset, clinical course, and morphology, was observed.

Morphological studies on skeletal muscle probes were available for 18 patients (table 1). In most of the patients, normal morphology of mitochondria was found by light and electron microscopy (60%), while four cases (22%) showed mitochondrial proliferation, two (11%) had ragged red fibres, and in one case (5%) lipid accumulation was seen.

CLINICAL CLASSIFICATION OF PATIENTS AND FAMILY HISTORY

The patients were classified clinically into five subgroups (table 1). Patients 1–3 (subgroup 1) had rapidly progressive hypertrophic cardiomyopathy with death by 5 months of age. Leigh's disease was diagnosed in patients 4–6 of subgroup 2, based on bilateral basal ganglia lesions on MRI. Patients 7 and 8 (subgroup 3a) had fatal infantile myopathy with hypotonia and death from respiratory failure by the age of 4 months. Patients 9–14 (subgroup 3b) manifested slowly progressive hypotonia with other features, including psychomotor retardation, extrinsic asthma, and congenital lactic acidosis. Benign reversible infantile myopathy could not be excluded in this group. Patients 15–18 had a combination of sensorineural hearing loss, ataxia, myoclonic epilepsy, and mental retardation (subgroup 4). Patients 19–21 had other clinical features which did not fit into the above categories (table 1). Four of the patients, including two of the four in subgroup 4, had affected first degree relatives. Consanguinity (first cousin parents) was reported in three cases (patients 1, 7, and 8). The mothers of patients 7 and 8 both reported miscarriages; patient 7 has two asymptomatic sibs, while patient 1 was an only child. While consanguinity suggests possible autosomal recessive inheritance, maternal inheritance could not be excluded in any of the 21 index patients.

SCREENING OF NUCLEAR COX SUBUNIT GENES COX 4–COX 8 FROM SKELETAL MUSCLE CDNA

To confirm that the correct COX cDNAs were amplified (corresponding to the published cDNA sequences), PCR fragments amplified from control muscle cDNA were sequenced. All patients were directly sequenced for mutations in the COX 4 gene and, as with the SSCP results, no variants were detected. Furthermore, all nuclear COX genes were sequenced completely from patient 1, who presented with a severe and isolated COX defect. (Parental

Table 3 Evolutionary conservation of nucleotide position 6480 (COX I)

Human mtDNA position Inserted amino acid (patient 18) Inserted nucleotide (patient 18) Conserved amino acids	nt 6480									
	I					I				
	a					a				
	V	L	I	T	A	V	L	L	L	L
Human	gtc	cta	atc	aca	gca	gtc	cta	ctt	ctc	ctc
	V	L	I	T	A	V	L	L	L	L
Cow	gta	ata	att	acc	gcc	gta	cta	cta	cta	cta
	V	M	I	T	A	V	L	L	L	L
Opossum	gta	ata	atc	aca	gca	gta	tta	ctc	ctt	ctt
	V	M	I	T	A	V	L	L	L	L
Rattus	gta	cta	att	aca	gcc	gtc	cta	cta	ctt	ctt
	V	L	I	T	A	V	L	L	L	L
Mus	gta	ctt	att	aca	gcc	gta	ctg	ctc	cta	cta
	V	L	I	T	A	V	L	L	L	L
Phoca	gta	tta	atc	aca	gcg	gtg	ctc	cta	cta	cta
	V	L	I	T	A	V	L	L	L	L
Baleo	gtc	cta	gtc	aca	gca	gta	cta	ctc	cta	cta
	V	L	V	T	A	V	L	L	L	L
X laevis	gta	tta	atc	aca	gct	gta	ctt	tta	ctt	ctt
	V	L	I	T	A	V	L	L	L	L

consanguinity in this patient would suggest autosomal recessive inheritance.)

For the COX 5b gene, four genomic PCR fragments including all exons were screened by SSCP. Sequencing of control fragments and of the patient's fragments only showed four base alterations in intronic regions: 1102 insG, G1118T, A1201T, and A1202T. These base changes do not represent pathogenic mutations, since they were present in all of the sequenced probes (n=4) as well as in the control muscle DNA.

Thus, no disease associated mutations were identified in any of the nuclear encoded COX genes.

SCREENING OF MITOCHONDRIAL COX GENES COX I-COX 3 FROM SKELETAL MUSCLE DNA

Fourteen PCR amplified DNA fragments (average size 290 bp) encompassing genes for COX I-III subunits were screened by SSCP. The reproducibility of results was confirmed using control fragments harbouring defined mutations (T9698C, T6776C). A total of seven mutated sites were detected. Of these, six variants represented silent mutations: C6557T, T6671C, and T6776C (COX I) and T9698C, T9716C, and T9899C (COX III). Nucleotide variants in flanking regions were not included in the results (n=3). In index patient 18 and in her less severely affected mother and sister, a homoplasmic G6480A mutation in the COX I gene changing isoleucine to valine at position 193 was detected (in addition to a heteroplasmic pathogenic tRNA mutation as discussed below). The novel G6480A mutation is highly conserved (table 3) and was absent in 300 normal controls.

Table 4 MtDNA haplogroup affiliation in patients 15-18

Nt	Locus	Base exchange	Amino acid exchange	Cons	Index patients			
					16 (7472Cins)	17 (7472Cins)	15 (C7512T)	18 (C7512T)
7028	COXI	C/T	Syn	Low	-	+	+	+
4216	ND1	T/C	Y/H	Low	-	-	-	+
12308	tRNA ^{Leu(CUN)}	A/G	-	High	-	-	-	-
12705	ND5	C/T	Syn	Low	-	-	+	-
15904	tRNA ^{Thr}	C/T	-	Low	-	+	-	-
10463	tRNA ^{Arg}	T/C	-	High	-	-	-	+
15928	tRNA ^{Thr}	G/A	-	Medium	-	-	-	+
Haplogroup association according to the classification of ref 24					D ^C	D ¹⁵⁹⁰⁴	D ¹²⁷⁰⁵	D ⁴²¹⁶

SCREENING OF ALL MITOCHONDRIAL tRNA GENES FROM SKELETAL MUSCLE DNA

In total, 13 PCR fragments (average size 310 bp) encompassing mitochondrial tRNA genes were investigated using SSCP and direct sequencing. For each fragment, control PCR fragments harbouring distinct and known mutations were run together with the patients' probes to test the reproducibility of SSCP conditions. Four tRNA variants were found: G7521A in tRNA^{Asp}, T7547C in tRNA^{Asp}, T10463C in tRNA^{Arg}, and G15928A in tRNA^{Thr}, the two latter known haplogroup specific polymorphisms. Nucleotide variants in flanking regions (n=>10) were not included in the results.

In the four patients of clinical subgroup 4, two separate previously reported pathogenic tRNA^{Ser(UCN)} mutations were identified. An insertion of one cytosine at nt 7472 of the tRNA^{Ser(UCN)} was found in skeletal muscle DNA from patients 16 and 17 but not in over 300 normal controls. Both patients had 100% mutant DNA, as estimated by additional PCR based RFLP quantification (detection limit 5% heteroplasmy). Heteroplasmy of the mutation was found in the matrilineage of both families (mother and brother of patient 16 were both clinically affected and had 65% and 75% mutant mtDNA in skeletal muscle, respectively; the unaffected mother of patient 17 had 5% mutant mtDNA in leucocytes).

A tRNA^{Ser(UCN)} mutation at nt 7512 was detected in patients 15 and 18. In patient 15, with infantile onset deafness, myoclonic epilepsy, ataxia, and mental retardation, this mutation was found in homoplasmic form in muscle and blood. Heteroplasmy of the mutation was found in the matrilineage of the family (an asymptomatic maternal aunt had 75% and the unaffected brother had 30% mutant mtDNA in leucocytes, respectively). In patient 18, 95% mutated mtDNA was detected in skeletal muscle; this patient died of multisystemic failure at 36 years of age. His mildly affected mother and sister had a lower degree of mutated mtDNA in leucocytes (40% and 45% respectively).

OTHER mtDNA MUTATIONS

Frequent pathogenic mtDNA mutations associated with MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) and MERRF (myoclonus epilepsy and ragged red fibres) syndromes in tRNA^{Leu(UUR)} and tRNA^{Lys} (nt 3243, 3250, 3271, 8344, 8356), as well as the Leigh's disease associated mutation at nt 8993, were excluded by RFLP analysis, and large scale rearrangements were excluded using Southern blotting.

HAPLOGROUP AFFILIATION OF INDEX PATIENTS FROM SUBGROUP 4

Haplogroup associations of patients 15-18 with tRNA^{Ser(UCN)} mutations were analysed by testing for branching positions at nt 7028, 4216, 12308, 12705, and 15904, as recently described by our group.¹⁹⁻²⁴ Patients 16 and 17 carrying the 7472 mutation, and patients 15 and 18 carrying the 7512 mutation, belonged to different European mtDNA haplogroups (table 4).

Discussion

Most cases of COX deficiency are sporadic and remain unexplained at the molecular genetic level. We therefore first initiated a systematic screening of nuclear genes for structural COX subunits as most obvious nuclear candidate genes for COX deficiency. Twenty-one patients with biochemically defined isolated or predominant COX deficiency were selected for this purpose. We included mitochondrial COX genes as well as mitochondrial tRNA genes in our screening study since family history could not exclude maternal inheritance of the disease in any of our patients.

A genotype-phenotype correlation was provided by the finding of tRNA mutations in each of the four patients in the subgroup with sensorineural hearing loss, ataxia, myoclonic epilepsy, and mental retardation (subgroup 4). This was the only group of patients with any demonstrable pathogenic mutation. Each patient in subgroup 4 had a mutation which affected tRNA^{Ser(UCN)}, patients 16 and 17 with an apparently homoplasmic cytosine insertion at nt 7472, and patients 15 and 18 with a mutation at nt 7512 (heteroplasmic in patient 18 and apparently homoplasmic in patient 15).

The pathogenicity of these mutations is supported in several ways. First, the mutations were heteroplasmic in the matrilineage of both families. A second indicator of the pathogenicity of these mutations is their different mitochondrial haplogroup affiliations, showing the independent origin of these mutations (table 4). Pathogenic mtDNA mutations arise independently in multiple families, consistent with their occurrence in different haplogroups.²⁴⁻²⁵ In contrast, most polymorphisms are found in strict association with a single mtDNA haplogroup.

The 7472 disease mutation, which presumably changes the secondary structure of the T ψ C loop region, has been described in a heteroplasmic form in a family with myoclonus, sensorineural hearing loss, and ataxia.²² Cybrid studies indicated a decrease in oxidative phosphorylation.

The second tRNA^{Ser(UCN)} mutation at nt 7512 was detected in a further two index patients of subgroup 4 (patients 15 and 18). Nakamura *et al.*²³ described a heteroplasmic T7512C mutation in a family with a MELAS/MERRF overlap syndrome, which disrupts a highly conserved base pair in the acceptor stem in the tRNA^{Ser(UCN)}.

An unexpected finding was a second mutation, G6480A, in patient 18 and in her mother and sister. This mutation is also mitochondrial in origin but, unlike 7472 and 7512, occurs in a COX encoding gene. This mutation is located within an evolutionarily conserved part of the COX I gene; however, the presence of both mutations in all affected family members makes it difficult to estimate the relative pathogenicity of each mutation separately.

The lack of mutations in nuclear COX subunit genes in this group is probably significant. There are several possible reasons for this. The sensitivity of our SSCP protocol, although

estimated to be >90%,²⁰ might allow some mutations to be missed.

A common genetic defect in one of the structural COX genes seems less likely since complete sequencing of DNA from a patient with a severe isolated COX deficiency and consanguineous parents, and the patient of Adams *et al.*²⁶ with Leigh's disease and COX deficiency, was normal. Similarly, no mutation was found in the mitochondrially encoded COX subunits I-III in a series of 18 patients with isolated COX deficiency.²⁷

Since no promoter or intron regions were investigated, the screening was incomplete. Future analysis of the genomic intron/exon structure of the nuclear COX genes is important for a complete screening and other candidate genes need to be identified and investigated. Yeast studies show that a substantial number of other nuclear genes are required for mitochondrial COX gene transcription and translation, mitochondrial import, and folding of nuclear encoded COX proteins, subunit assembly, and turnover.²⁸⁻²⁹ To date, most of the human genes corresponding to these proteins are unknown, but rapid and powerful strategies for identifying human mitochondrial genes have recently been reported.³⁰

In conclusion, our data indicate that mutations in tRNA^{Ser(UCN)} are a cause of mitochondrial syndromic encephalopathy with COX deficiency. Further research will be necessary to identify the other gene defects responsible for this complex group of disorders.

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