Frequency of mitochondrial transfer RNA mutations and deletions in 225 patients presenting with respiratory chain deficiencies

Michaela Jaksch, Stephanie Kleinle, Curt Scharfe, Thomas Klopstock, Dieter Pongratz, Josef Müller-Höcker, Klaus-D Gerbitz, Sabina Liechti-Gallati, Hanns Lochmuller, Rita Horvath

Metabolic Disease Centre Munich-Schwabing and Institute of Clinical Chemistry, Diagnostic Molecular Biology, and Mitochondrial Genetics, Munich, Germany
M Jaksch
R Horvath
K D Gerbitz

Human Molecular Genetics, Department of Paediatrics and Clinical Research, University of Berne, Switzerland
S Kleinle
S Liechti-Gallati

Genome Technology Centre, Stanford University, Palo Alto, CA, USA
C Scharfe

Department of Neurology, Grosshadern, LMU Munich, Germany
T Klopstock

Friedrich-Baur-Institut, LMU Munich, Germany
D Pongratz
H Lochmuller

Institute of Pathology, LMU Munich, Germany
J Müller-Höcker

Correspondence to: Dr Jaksch, Academic Hospital Schwabing, Koelner Platz 1, 80804 Muenchen, Germany, Michaela.Jaksch@lrz.uni-muenchen.de

Revised version received 12 July 2001
Accepted for publication 18 July 2001

Abstract

***Objective*—To evaluate the frequency of pathogenic mtDNA transfer RNA mutations and deletions in biochemically demonstrable respiratory chain (RC) deficiencies in paediatric and adult patients.**

***Methods*—We screened for deletions and sequenced mitochondrial transfer RNA genes in skeletal muscle DNA from 225 index patients with clinical symptoms suggestive of a mitochondrial disorder and with biochemically demonstrable RC deficiency in skeletal muscle.**

***Results*—We found pathogenic mitochondrial DNA mutations in 29% of the patients. The detection rate was significantly higher in adults (48%) than in the paediatric group (18%). Only one pathogenic mutation was detected in the neonatal group. In addition, we describe seven novel transfer RNA sequence variations with unknown pathogenic relevance (six homoplasmic and one heteroplasmic) and 13 homoplasmic polymorphisms. One heteroplasmic transfer RNA$^{Leu(UUR)}$ A>G mutation at position 3274 is associated with a distinct neurological syndrome.**

***Conclusions*—We provide an estimation of the frequency of mitochondrial transfer RNA mutations and deletions in paediatric and adult patients with respiratory chain deficiencies.**

(*J Med Genet* 2001;**38**:665–673)

Keywords: mtDNA; tRNA mutations; respiratory chain deficiency

Our present knowledge of inherited mitochondrial disorders is largely restricted to the classical, genetically heterogeneous respiratory chain (RC) deficiencies, which may present as highly variable neuromuscular diseases with onset at any age. The term "mitochondrial disorders" is often restricted to mtDNA encoded syndromes with biochemically decreased activities of one or more respiratory chain (RC) complexes.[1 2] The respiratory chain is embedded in the inner mitochondrial membrane and it constitutes the major cellular ATP providing system. Complexes I, III, IV, and V are encoded by both the mitochondrial and the nuclear genome, while complex II is exclusively nuclear encoded. Complex IV is the terminal enzyme of the proton pumping respiratory chain and complex V (ATPase) is driven by this protonic force to synthesise ATP from ADP+P. Several non-structural, nuclear encoded proteins are additionally involved in transcription, translation, transport, and assembly of RC complex subunits and these genes are thought to play a major role in autosomal recessive RC deficiency disorders. Little is known about these additional, non-structural genes in isolated RC deficiencies with a few exceptions, *SURF1, SCO2, SCO1*, and *COX10*, which all cause isolated cytochrome c oxidase (COX, complex IV) deficiency.[3–9] So far, no common mutations have been identified in the 10 nuclear encoded structural subunits of the COX complex.[10 11] In contrast, four pathogenic mutations have been reported in mtDNA encoded structural subunits of COX.[12–15] This differs from the recessive isolated complex I deficiencies in which a number of causal mutations have been attributed to nuclear complex I subunits.[16] To date, however, most known mutations associated with RC deficiencies are the maternally inherited mitochondrial transfer (t)RNA mutations and sporadic deletions, which are found in different complex neurological disorders. RC deficiencies, independent of whether they are of nuclear or mitochondrial origin, present as highly variable neuromuscular diseases with onset at any age.[17 18] RC deficiencies in neonates and infants are usually more severe and lethal events are more frequent. In contrast, RC deficiencies in older children and adults are typically less progressive and present more often as a syndromic encephalomyopathy.[18]

There are numerous questions on the genetic causes of RC deficiency and their relevance in mitochondrial disorders still remains elusive. Thus, we focused on the overall pathogenetic relevance of mt tRNA mutations and deletions in RC deficiencies using a retrospective and follow up approach (1992-2000). We report here on the frequencies of these mutations in a very large collection of 225 paediatric and adult patients.

Patients and methods

PATIENTS

Patients presenting with clinical features suggestive of a mitochondrial disorder and with a biochemically demonstrable RC defect in skeletal muscle were included in this study. Patients with age at onset between birth and 15 years were classified as paediatric cases. All

Table 1 Clinical features of the 225 patients presenting with RC deficiencies

Clinical presentation	No	Paediatric	Adult	Known pathogenic mtDNA mutation	
				Total	Paed/adult
KSS	10	3	7	9	3/6
CPEO	21	4	17	16	3/13
Pearson	1	1	0	1	1/0
MM	53	25	28	9	2/7
MNGIE*	1	0	1	1	0/1
MELAS	21	6	15	15	6/9
Diab/Df	3	0	3	3	0/3
Diab/MM	1	0	1	1	0/1
PME/Df	5	5	0	5	5/0
MERRF	4	4	0	3	3/0
MEM	25	18	7	2	2/0
Cong LA	42	42	0	0	0/0
Hepatopathy†	3	3	0	0	0/0
Leigh syndrome‡	23	21	2	0	0/0
CMP§	12	9	3	0	0/0
Total	225	141	84	65	25/40

Cong LA, congenital lactic acidosis; CMP, cardiomyopathy; CPEO, chronic progressive external ophthalmoplegia; Df, deafness; Diab, diabetes; KSS, Kearns-Sayre syndrome; MELAS, mt myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; MEM, mt encephalomyopathy; MERRF, myoclonus epilepsy with ragged red fibres; MH, muscular hypotonia; MM, mt myopathy; MNGIE, mt neurogastrointestinal encephalomyopathy; PME, progressive myoclonus epilepsy.
**TP* mutations were excluded.
†mtDNA deletion was excluded.
‡*SURF1* mutations were excluded.
§*SCO2* mutations were excluded.

other patients were included in the adult group. Skeletal muscle DNA from 225 unrelated paediatric (n=141) and adult (n=84) index patients presenting with different mitochondrial disorders and with combined or isolated RC deficiencies of complexes I, III, or IV were examined (table 1). Patients with deficiencies of the pyruvate dehydrogenase complex, complex II, V, and secondary RC deficiencies, for example Menkes disease, were excluded. To focus on RC deficiencies as the ultimate selection criteria, we also excluded patients with typical clinical and morphological findings of a mitochondrial disorder, such as chronic progressive external ophthalmoplegia (CPEO) and Kearns-Sayre syndrome (KSS), without a biochemically detectable respiratory chain deficiency but positive for a mitochondrial deletion or pathogenic point mutation.

In the paediatric group, severe congenital lactic acidosis was the most prominent finding in 42 cases. The clinical course was progressive with death within the first two years in 35 cases. Twenty-three paediatric patients had a classical mitochondrial disorder, such as KSS, CPEO, Pearson syndrome, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), progressive myoclonus epilepsy and deafness (PME/Df), or myoclonus epilepsy with ragged red fibres (MERRF). In 25 patients, mitochondrial myopathy (MM) was the leading symptom, and 18 patients presented with both encephalopathy and myopathy (MEM). Three patients suffered from severe hepatopathy. Twenty-one patients had features compatible with LS based on the criteria proposed by Rahman *et al*.[19] Nineteen patients with LS died within the first four years of life, whereas two patients are still alive aged 4½ and 15 years. Nine patients suffered from hypertrophic or dilated cardiomyopathy (CMP).

In the adult group, the clinical course was generally less progressive. The clinical presentation was that of a classical mitochondrial disorder in 44 cases, such as KSS, CPEO, MELAS, diabetes (Diab)/Df, Diab/MM, or mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). Age at onset in this group ranged from 15 to 68 years. Two adult patients presented with LS. Single and multiple symptoms suggestive of mitochondrial disorders were present in the remaining 40 cases (MEM, MM, CMP). The relative proportion of phenotypes in the paediatric and the adult group is summarised in table 1.

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 in supernatants of homogenised fresh frozen muscle biopsy specimens according to the methods of Fischer *et al*.[20] To exclude isolated complex II deficiencies, we additionally measured complex II activity using succinate as substrate alone with phenacinemethosulphate as electron acceptor. To exclude artificial deficiencies resulting from a lower content of mitochondria in muscle, the following inclusion criteria were used: a residual, normalised (U/U citrate synthase) activity in one of the complexes I, III, and IV (or combined) <90%, when compared to the lowest reference values.

DNA ANALYSIS

Total genomic DNA was extracted from skeletal muscle, leucocytes, and fibroblasts according to standard purification protocols (Qiagen). MtDNA was screened for rearrangements by Southern blotting as described previously.[21] In 44 cases, a "prescreening" using long PCR was performed.[22] Single strand conformation polymorphism (SSCP) analysis of all 22 mitochondrial tRNA genes was performed in all 225 patients. Electrophoresis was done as described previously.[23 24] Mitochondrial tRNA genes were amplified using the primer pairs according to Jaksch *et al*[11] and Kleinle *et al*.[25] Direct sequencing of all PCR products with deviating SSCP patterns was performed on an ABI 377 DNA Sequencer (Applied Biosystems, PE) using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit. A complete sequence analysis of the 22 mitochondrial tRNA genes was performed in 25 patients without electrophoretic deviating patterns but with clinical features highly suggestive of a classical mitochondrial disorder.

MtDNA point mutations were quantified by restriction fragment length polymorphism (RFLP) analysis based on fluorescence detection on an ABI 377 DNA Sequencer (ABI, PE) using primers labelled with 6FAM. Pathogenic mutations (T618C, A3243G, T3271C, G5703A, T5814C, 7472insC, T7512C, G7497A, A8344G, T8993G/C) were additionally analysed by standard RFLP analysis. The

A3274G mutation was amplified using a forward mismatch primer to create a *Pst*I restriction site in the case of mutation (5'-GCCCGGTA ATCGCATAAAACTTAAAACTCT-3'). The A4435G mutation was analysed by *Nla*III digestion. Serial dilutions were resolved on a 4% denaturing polyacrylamide gel and analysed with the GeneScan computer program (ABI, PE). Base exchanges were classified as homoplasmic in the absence of additional single and heteroduplex strands on SSCP gels as well as absence of additional fluorescence in the sequencing results. Heteroplasmy levels of pathogenic mtDNA mutations in skeletal muscle were expected to be higher than the detection limit of direct sequencing of about 20%. The G15930A variant was estimated after sequence analysis by scanning the fluorescence proportions of nucleotides G and A and by scanning the heteroduplex formation on the silver stained PAA gel after SSCP analysis.

CRITERIA OF PATHOGENICITY IN tRNA SEQUENCE CHANGES

MtDNA specific criteria of pathogenicity were (ordered according to relevance): (1) cosegregation of mutations with the clinical phenotype; (2) heteroplasmy; (3) absence of mutations in >100 healthy controls; (4) functional impairment in one or more enzyme complexes of the RC; (5) haplogroup divergency of identical mutations in different index patients; and (6) phylogenetic conservation of the affected nucleotide.[26] In the case of homoplasmy, pathogenicity was mainly ruled out by the presence of the identical homoplasmic tRNA mutation in healthy maternal relatives.

Results

MtDNA from muscle biopsy specimens was analysed for mt tRNA mutations and deletions in 141 paediatric and 84 adult patients indicative of a mitochondrial disorder and with biochemically demonstrable RC deficiency in skeletal muscle. Clinical phenotype, sex, age at onset, family history, and biochemical results are summarised for all patients with mtDNA mutations in tables 1 and 2.

KNOWN PATHOGENIC mtDNA DELETIONS AND tRNA MUTATIONS

We found deletions of the mtDNA in 12.4% of patients and known pathogenic mutations in the mitochondrial tRNA genes Leu(UUR), Lys, Ser(UCN), Phe, Asp, Thr, Cys, and Leu (CUN) in 16.4%.

Nine out of 10 KSS patients had deletions of variable size (2-7 kb in length and 40 to 70% of mutant DNA). In all KSS patients, RRF, COX negative fibres, and moderate complex I and IV deficiencies were found (90% residual activity related to the lower reference range limit).

Twenty-one patients presented with the clinical picture of CPEO. MtDNA deletions were detected in 15 CPEO patients, one of them showing multiple deletions. One CPEO patient was positive for the pathogenic 3243 $tRNA^{Leu(UUR)}$ mutation. Muscle histology showed RRF in all CPEO patients. Biochemically, CPEO patients presented with mild deficiencies of RC complexes I (n=5) or IV (n=4) or both (n=7). There were no striking differences in the clinical, histological, or biochemical presentation of CPEO patients with deletions, tRNA mutations, or without detected mtDNA mutation.

Of 21 MELAS patients, 14 had the common A3243G $tRNA^{Leu(UUR)}$ mutation and one had the T3271C $tRNA^{Leu(UUR)}$ mutation. Seven of the 3243 positive patients showed RRF. Biochemically, the 3243 positive patients showed a combined complex I and IV deficiency and in one case an isolated complex I defect was present.

The severity of the enzymatic defects was more pronounced in MELAS patients (about 50% residual activity in complexes I and IV) when compared to mtDNA deletion syndromes with similar rates of heteroplasmic mutant DNA (80%). Three out of four patients with myoclonus epilepsy carried the typical $tRNA^{Lys}$ mutation at np 8344. All had RRF on muscle morphology, as well as a combined complex I and IV deficiency (about 45% residual activity). Progressive myoclonus epilepsy was diagnosed in five patients with apparently homoplasmic tRNA $Ser^{(UCN)}$ mutations.[11 27] In these cases, an isolated COX deficiency was found in four of the five index patients (about 40% residual activity).

In 53 patients, myopathy was the leading symptom on clinical examination. In 15 patients, a mitochondrial origin was suggested by the presence of RRF on muscle biopsy and they were therefore classified as MM. In the remaining 38 patients both light and electron microscopy pointed to MM because of the presence of abnormal mitochondria. In two of the 15 patients with RRF, mtDNA deletions were detected. Pathogenic mitochondrial tRNA mutations were found in seven MM patients (A3243G, n=3; G7497A, n=2[27]; T618C, n=1[25]). One infant had a combination of two homoplasmic mutations (G5703A and A15923G) with marginal reduction in complex IV activity (90% residual activity of the lowest reference value, when related to the mitochondrial marker enzyme CS).

In the remaining 44 patients with muscular symptoms no mtDNA mutations were detectable, although six patients presented with RRF and remarkable RC defects. Most of the MM patients presented either with isolated complex IV deficiency (n=22) or with combined RC deficiency (n=22). Twenty-one patients from the MM group had an early onset disease (<2 years) with pronounced RC deficiencies and, in general, no RRF were seen (complex I deficiency, n=1; complex IV, n=10; combined complex I and IV, n=10).

One infant presenting with a severe progressive encephalomyopathy which was later diagnosed to be the result of subacute sclerosing panencephalitis carried a homoplasmic T5814C mutation.[28] The patient's mother (homoplasmic for the mutation in white blood cells) had mild, non-specific neurological symptoms. A second infant with a progressive

Table 2 Genotype-phenotype correlations

Patient No	Onset (mean or range)	Sex		Clinical phenotype	Mutation	% mutant (mean)	Conservation		Morphology		RC deficiency			Family history	Haplogroup affiliation according to ref 21
		F	M				nt	W-C bp	RRF	CCO^{-}	CI	CIII	CIV		
(A) Genotype-phenotype correlation in 65 patients with known pathogenic mtDNA mutations															
Deletions (n=28)															57%: D^{C}; 21%: 4216; 11%: 12308; 7%: 12705; 4%: 15904
1–9	15 y	8	1	KSS	del	60	~	~	+	+	+	+	+	s	
10–23	23 y	7	7	CPEO	del	60	~	~	+	+	+	+	+	s	
24	25 y	1	0	CPEO	dels	20	~	~	+	+	+	+	+	f	
25	2 d	0	1	Pearson	del	60	~	~	+	+	++	++	++	s	
26	40 y	0	1	MM	del	50	~	~	+	+	+	+	++	s	
27	42 y	0	1	MM	dels	40	~	~	+	+	++	+	++	s	
28	20 y	1	0	MNGIE	dels	30	~	~	−	−	+	−	+	f	
Known pathogenic tRNA mutations															
					$tRNA^{Leu(UUR)}$ (n=23)										*For 3243 (n=22)*: 50%: 4216; 27%: D^{C}: 27%; 14%: 12308; 9%: 12705
29–42	12 y	4	10	MELAS	A3243G	62	+	~	+	+	++	−	+	1s 13f	
43	27 y	0	1	CPEO	A3243G	50			+		++	−	−	f	
44–46	29 y	1	2	Diab/Df	A3243G	28			−	−	+	−	−	f	
47	40 y	0	1	Diab/MM	A3243G	65			+	+	++	−	−	s	
48–50	28 y	1	2	MM	A3243G	80			+	+	++	−	−	?	
51	?	0	1	MELAS	T3271C *$tRNA^{Ser(UCN)}$*	45	−	+	+	+	+	−	−	?	−
52–54	2 y–8 y	2	1	PME/Df	7472insC	100	+	+	−	−	−	−	++	1s 2f	Variable[27]
55–56	5 mth–14 y	1	1	PME/Df	T7512C	100	+	+	−	−	+	−	++	1s 1f	Variable[27]
57–58	3 y–30 y	1	1	MM	G7497A *$tRNA^{Lys}$*	100	−	+	+	−	++	−	++	1s 1f	Variable[27]
59–61	11 y	1	2	MERRF	A8344G *$tRNA^{Phe}$*	60	+	~	+	+	++	−	++	f	D^{C}: n=2; 4216: n=1
62	26 y	0	1	MM	T618C $tRNA^{Asp/Thr}$	95	−	+	+	+	++	+	−	f	−
63	1 y	0	1	MM, CMP	G5703A	100	−	+	+	+	−	−	+	s	−
					A15923G *$tRNA^{Cys}$*	100	+	~							
64	*11 y*	*0*	*1*	*MEM/SSPEE*	*T5814C $tRNA^{Leu(CUN)}$*	*100*	+	+	−	−	−	−	+	*f*	−
65	*8 m*	*0*	*1*	*MEM/GA I*	*T12297C*	*100*	+	~	−	−	+	−	−	*s*	−

Table 2 continued

Patient No	Onset (mean or range)	Sex F	Sex M	Clinical phenotype	Mutation	% mutant	Conservation nt	Conservation W-C bp	Morphology RRF	Morphology CCO^-	RC deficiency CI	RC deficiency CIII	RC deficiency CIV	Family history	Healthy carriers	Haplogroups according to ref 21
(B) Genotype-phenotype correlations in 26 patients with novel mtDNA mutations																
					$tRNA^{Phe}$											D^C + 12308
66–67	20 y	1	1	MM	T629C	100	−	~	+	+	−	−	+	s	NA	
14	23 y	1	0	CPEO	A644G	100	+	+						s	+	−
					$tRNA^{Leu(UUR)}$											
68	11 y	0	1	ATX, SHL, MM, RP	A3274G	40	−	−	+	+	++	−	−	s	−	−
					$tRNA^{Met}$											
69–70	20 y	2	0	MM	T4418C	100	−	~	+	+	+	−	−	s	NA	D^C + 4216 12308var
71	45 y	1	0	CPEO	A4435G	13	+	~	−	−	−	−	+	s	+	−
72	8 y	0	1	CPEO	T4452C	100	+	~	−	−	−	−	+	s	NA	−
73	8 y	0	1	MM	T4454C	100	−	~	−	−	−	−	+	s	NA	−
					$tRNA^{Trp}$											
72					T5553C	100	−	−								
					$tRNA^{Asn}$											
74	10 y	1	0	MM	A5715G	100	−	~	−	−	+	−	−	s	NA	−
					$tRNA^{Cys}$											
75	6 y	1	0	ATX, SHL, RP, Df	G5773A	100	−	~	−	−	+	−	−	s	+	D^C
76	34 y	0	1	MM	G5773A	100			−	+	++	−	++	s	NA	D^C
					$tRNA^{Tyr}$											
77	Birth	0	1	Cong LA	C5839T	100	−	~	−	−	+	−	+	s	+	−
78	Birth	1	0	Cong LA	A5843G	100	−	~	−	−	−	−	+	s	−	−
					$tRNA^{Ser(UCN)}$											
79	Birth	1	0	MEM	A7500G	100	+	~	−	−	−	(+)	+	s	NA	−
					$tRNA^{Lys}$											
80–81	Birth	1	2	MEM, MH	A8343G	100	−	~	−	+	++	++	++	s	+	D^C
					$tRNA^{His}$											
27					C12153T	100	−	~							NA	−
82	34 y	1	0	MM	A12172G	100	−	~	−	+	−	−	+	s	NA	−
					$tRNA^{Thr}$											
83	Birth	0	1	Hepat	G15894A	100	−	+	−	+	−	−	+	s	NA	−
84	5 y	1	0	MEM	G15897A	100	−	−	−	+	−	−	++	s	+	D^C
85	34 y	1	0	MM	G15897A	100			−	+	+	−	+	s	NA	−
86	6 y	0	1	MEM	G15930A	100	−	~	−	−	+	−	−	s	+	D^C
87	Birth	1	0	MM	G15930A	100			−	−	+	−	−	f	+	−
88	Birth	1	0	Hepat	G15930A	30			−	−	−	(+)	+	s	+	−
89–90	4–7 y	1	1	MEM	T15941C	100	−	~	−	−	−	(+)	+	1s 1f	+	D^C + 12308
					$tRNA^{Pro}$											
91	39 y	1	0	CPEO	T15968C	100	−	+	−	+	−	−	+	s	NA	−

F, female; M, male; %, % mutant mtDNA in skeletal muscle; conservation nt, W-C bp, conservation of the nucleotide and Watson-Crick base pair; RRF, ragged red fibres; CCO^-, cytochrome c oxidase negative fibres; C, complex; haplogroup, haplogroup affiliation; ATX, ataxia; Hepat, hepatopathy; RP, retinopathy; SHL, sensorineural hearing loss; y, year; d, day; mth, months; del, single deletion; dels, multiple deletions; ~, no relevance; +, high residual activity of 85–95% of the lowest reference range; ++, residual activity of 50–85% of the lowest reference range; +++, severe deficiency with residual activities of <50% when compared to the lowest reference range; s, single; f, familial; var, variable; id, identical; NA, not analysed. Patients 64 and 65 are typed in italics as our data did not confirm primary pathogenic influence of the tRNA mutations.

encephalomyopathy which was later diagnosed as glutaraciduria type I had a homoplasmic T12297C mutation.[29] In one patient with congenital Pearson syndrome, a single mtDNA deletion was found. This was the only patient in the neonatal group with known and confirmed pathogenic mtDNA mutations. Pathogenic tRNA mutations in infants with an onset between 0.5 and 5 years were found in seven cases (5% out of 141 patients from the paediatric group). The positive detection rate of both mt tRNA mutations and deletions then increased constantly with age in the group between 5 and 15 years.

NOVEL PATHOGENIC mt tRNA MUTATION

One patient presenting with a syndrome of progressive MM, cataract, sensorineural hearing loss, cerebellar ataxia, psychomotor retardation, retinopathy, and psychosis had a novel heteroplasmic G3274A mutation in $tRNA^{Leu\ (UUR)}$ in skeletal muscle. This mutation was absent in white blood cells of the patient and of three maternal family members as well as in 400 controls. Biochemical analysis of skeletal muscle showed an isolated, significant complex I deficiency.

NOVEL mt tRNA SEQUENCE VARIATIONS WITH UNKNOWN PATHOGENIC RELEVANCE

Seven novel sequence variations with unknown pathogenic relevance were found in five different mitochondrial tRNA genes. First, a homoplasmic T629C ($tRNA^{Phe}$) change was detected in two female adult patients belonging to different haplogroups and both presenting with MM and RRF. Another homoplasmic T4418C ($tRNA^{Met}$) sequence variation was present in two adult female patients with MM, and in one of each RRF were detected. Both patients also belonged to different haplogroups. Third, a female patient presenting with late onset CPEO carried a A4435G mutation in $tRNA^{Met}$ with 13% mutant DNA in skeletal muscle, 10% in buccal cells, and 8% in leucocytes. Three so far unaffected sons carried this mutation in a heteroplasmic state (30%, 4%, 75% of mutant DNA in white blood cells). The remaining homoplasmic sequence variations, T4452C ($tRNA^{Met}$), A7500G ($tRNA^{Ser(UCN)}$), G15894A ($tRNA^{Thr}$), and T15968C ($tRNA^{Pro}$), affect conserved nucleotides or Watson-Crick base pairs.[26] None of these novel base changes was detectable in 400 controls.

NOVEL mt tRNA POLYMORPHISMS

Thirteen novel homoplasmic sequence changes were classified as polymorphisms using the criteria for pathogenicity mentioned in the methods section: A644G ($tRNA^{Phe}$), T4454C ($tRNA^{Met}$), T5553C ($tRNA^{Trp}$), A5715G ($tRNA^{Asn}$), G5773A ($tRNA^{Cys}$), C5839T ($tRNA^{Tyr}$), A5843G ($tRNA^{Tyr}$), A8343G ($tRNA^{Lys}$), C12153T, A12172G ($tRNA^{His}$), G15897A, G15930A, T15941C ($tRNA^{Thr}$).

ADDITIONAL ANALYSES

Twenty-five patients with findings highly suggestive of an underlying defect in the mtDNA (RRF and a syndromic neurological disorder) were directly sequenced for mutations in all 22 tRNA genes and, as with the SSCP results, no variants or mutations were detected.

Discussion

RC complex deficiencies are in part caused by molecular defects of the mtDNA. The clinical phenotypes are heterogeneous and range from mild forms of glucose intolerance in adults to fatal infantile encephalomyopathies. Besides large scale rearrangements, pathogenic mtDNA mutations impairing RC function are frequently represented by point mutations in mitochondrial tRNA genes. So far, more than 60 different pathogenic mutations and more than 50 polymorphic sites have been described in these tRNA genes (see also http://www.gen.emory.edu/mitomap.html). The difficulties in deciding to what extent mtDNA (and nuclear DNA) analyses should be performed in patients with RC deficiencies emphasises the importance of investigating a large collection of patients for the presence of pathogenic mitochondrial mutations. To evaluate the proportion of mitochondrial tRNA mutations and deletions in patients with biochemically demonstrable functional impairment of the RC, we have performed a comprehensive study in 225 paediatric and adult patients suffering from different types of mitochondrial disorder.

KNOWN PATHOGENIC mt tRNA MUTATIONS AND DELETIONS

Of all 38 reported pathogenic tRNA mutations found, 87% were in the three tRNA genes Leu(UUR) (23/38), Ser(UCN) (7/38), and Lys (3/38). Interestingly, both tRNAs Ser (UCN) and Leu(UUR) are redundant genes, thereby possibly pointing to an important compensating mechanism in evolution. The most frequent tRNA mutation was A3243G $tRNA^{Leu(UUR)}$, which occurred in 22 cases. Thus, these three tRNAs represent the most important "mutational hot spots" in mitochondrial disease.[30 31]

In this study, 12.4% of the patients presented with deletions (11% with single deletions, 1% with multiple deletions) underlining the frequency of sporadic, large mtDNA rearrangements. As for tRNA mutations, deletions were found predominantly in older children and in adult patients (tables 1 and 2A). Although high loads of deleted mtDNA molecules (>70%) are likely to impair RC function to a significant extent, activities repeatedly showed a moderate decline when compared to mt tRNA mutations with similar or even less mutated molecules. Our findings are consistent with studies from other groups when relating the rate of mtDNA deletions to the clinical, histochemical, and biochemical profiles.[32 33] These observations may in part reflect methodical problems, which could be solved by using more sensitive tests in the future.[34] Conversely, it is known that normal RC activities do not exclude the presence of mtDNA deletions or point mutations. A

considerable number of our patients with deletions or low mutant loads of pathogenic tRNA point mutations (for example, the 3243 mutation in $tRNA^{Leu(UUR)}$) did not show RC deficiencies, but had characteristic clinical and morphological findings suggestive of a mitochondrial disorder (not included in this study).

An unexpected finding was the presence of an apparently homoplasmic T>C mutation at position 12297 in one of our patients with a (later) clear diagnosis of glutaraciduria type I (GA I) and without cardiac problems. The mutation has been described in association with familial dilated cardiomyopathy.[29] Similarly, the T5814C mutation[28] was found homoplasmic in skeletal muscle of the severely affected index patient and in white blood cells of his mother suffering from non-specific, mild neurological symptoms. The index patient was later diagnosed as suffering from a subacute sclerosing panencephalitis. Further studies are needed to prove a clear pathogenic role for these mutations.

NOVEL PATHOGENIC mt tRNA MUTATION

We have detected one novel heteroplasmic $tRNA^{Leu(UUR)}$ G3274A mutation in skeletal muscle (25% mutant load) of a patient with progressive myopathy, deafness, ataxia, psychomotor retardation, retinopathy, and psychosis. Muscle morphology showed numerous ragged red as well as COX negative fibres. The mutation affects a non-conserved Watson-Crick base pair in the anticodon stem of $tRNA^{Leu(UUR)}$; however, it was not detectable in the patient's blood or in blood samples of three maternal relatives indicating its sporadic or somatic origin. On the other hand, detection of a very low amount of mutant DNA, <3%, is difficult and heteroduplex formation may have influenced the accuracy of the scanning results. Similar to most $tRNA^{Leu(UUR)}$ mutations described so far, this novel mutation is also associated with an isolated complex I deficiency.

NOVEL mt tRNA SEQUENCE VARIATIONS WITH UNKNOWN PATHOGENIC RELEVANCE

Seven novel homoplasmic and one novel heteroplasmic tRNA sequence variations are classified as "of unknown pathogenic relevance" for the following reasons. (1) The affected np is phylogenetically conserved and no additional relatives are available to confirm or exclude cosegregation with the disease. (2) Haplogroup diversity points to a possible pathogenic influence usually confirmed for pathogenic tRNA mutations.[21] (3) The base exchanges are found in patients suffering from a similar disorder. (4) The absence in 400 controls suggests that they do not belong to common European polymorphisms.[21 35]

Population studies, single fibre analyses, and functional studies are needed to classify these mutations better as pathogenic.

NOVEL mt tRNA POLYMORPHISMS

We describe 13 novel nucleotide changes without obvious primary pathogenic contribution; however, an additive or synergistic deleterious effect cannot be ruled out in any of these cases. In one patient, the G15930A base exchange was found in a heteroplasmic state, possibly indicating a recent mutational event, but the variant is also present homoplasmically in healthy maternal relatives of two other patients, thereby making a primary pathogenetic influence unlikely.

HAPLOGROUP TESTING: A HELPFUL TOOL TO DISCRIMINATE PATHOGENIC MUTATIONS FROM POLYMORPHIC SITES?

It has been repeatedly shown[36 37 21] that deleterious mtDNA point mutations and deletions are not associated with specific mtDNA haplogroups indicating sporadic and recent events, whereas normal variants or less pathogenic mutations are mostly restricted to single maternal lineages.[21] Some base exchanges in this study do not fit into this model, for example, variants T7547C, T15941C, A15924G, and G15897A. They are detectable in different lineages, but they are also homoplasmic in healthy maternal relatives tested. Three hypotheses can be proposed to explain this. (1) Several mutational hot spots are located within tRNA genes without functional effects or (2) the base exchanges have only minor pathogenetic power compensated by a nuclear background in the relatives or (3) the variants occurred before the apparent haplogroup defining polymorphism (that is, an inaccuracy in the phylogenetic analysis). An interesting finding is the high proportion of haplogroup 4216 in certain mitochondrial diseases, as already discussed in a recent report.[21] In the present study, the proportion of the 4216 haplogroup was 50% in patients carrying the 3243 mutation (11 out of 22). Compared to controls (19%),[21] this sequence variation should therefore be considered as a susceptibility locus.

MUTATIONAL HOT SPOTS

It is of interest that some mitochondrial tRNA genes present with a highly variable number of nucleotide changes indicating different susceptibility to mutate. Both, the $tRNA^{Leu(UUR)}$ and $tRNA^{Thr}$ show most of the detected nucleotide changes when compared to the wild type sequence.[38] However, influence on mitochondrial tRNA function by nucleotide changes seems to vary widely. While the $tRNA^{Leu(UUR)}$ presents as a "mutational hot spot" for pathogenic mtDNA mutations, most nucleotide changes of $tRNA^{Thr}$ have a polymorphic character.

CORRELATION BETWEEN LOCATION OF tRNA MUTATION AND CLINICAL PHENOTYPE

Regarding the localisation of all pathogenic tRNA mutations found in this study and those previously described (http://www.gen.emory.edu/mitomap.html), they are (1) rarely located in variable loops of the tRNA genes, except as reported by Chalmers *et al*,[39] (2) next to pathogenic mutations causing severe neuromuscular disorders (for example, G8342A and A8344G[40 41]), homoplasmic polymorphisms (for example, A8343G as found several times in this study) do not cosegregate

with the disease, and (3) pathogenic mutations are randomly distributed (except variable loop and anticodon) and therefore do not concentrate in specific regions. It has been proposed that post-transcriptional modifications of single base pairs are strongly required for correct folding and aminoacylation of human tRNA genes.[42] This would largely explain the positional selection of some mutated bases with no functional effect of next located homoplasmic base exchanges.

MUTATIONS IN OTHER CANDIDATE GENES

This study has focused on optimised strategies to detect mt tRNA mutations and deletions in patients with RC deficiency disorders. The extent of mutations in coding regions of the mtDNA as well as in nuclear genes is unknown. Recently, mutations in the mitochondrial structural genes have been described in patients with predominant complex III deficiency or isolated complex IV deficiency.[12–15 43] Therefore, mutations of mtDNA protein coding genes should still be considered in our patients without detectable tRNA mutation or deletion.

DETECTION RATE OF mtDNA tRNA MUTATIONS AND DELETIONS IN ADULTS AND PAEDIATRIC PATIENTS

The detection rate of mtDNA defects is significantly higher in adults (40 out of 84 patients, 48%) than in children (25 out of 141 patients, 18%). These data correspond to the findings of Shoffner[44] and clearly point to a major contribution of nuclear gene mutations in RC disorders of infantile or early onset. We found no mtDNA defect in patients with a disease onset between birth and 6 months, except in one neonate presenting with Pearson syndrome, thus indicating an almost exclusive nuclear mode of inheritance in this age group. The detection rate of mitochondrial tRNA mutations and deletions increased constantly with the patients' age showing a peak between 16 and 27 years (fig 1). A cumulative or threshold effect of mtDNA mutations could explain a late onset of the disease in most patients.

The total detection rate for pathogenic mtDNA mutations and deletions in this study was 29%. In a comparable study, 35 adult patients with clinical and morphological data suggestive of a mitochondrial disorder were screened for tRNA mutations using denaturing gradient gel electrophoresis (DGGE).[45] In this study, Sternberg *et al*[45] found 13 patients (37%) positive for heteroplasmic tRNA mutations. Ten out of 35 patients carried the 3243 mutation (29%), one patient had the 8344 mutation (2.9%), and two patients carried novel heteroplasmic mutations. Interestingly, a similar detection rate is obtained in our study when relating pathogenic tRNA mutations to the adult group (19 out of 84 patients, 23%). These findings indicate a comparable detection rate for tRNA mutations in clinically/morphologically or clinically/biochemically preselected adult patients. In contrast, $tRNA^{Ser\ (UCN)}$ mutations would have been missed using clinical and morphological preselection criteria alone. This is owing to two different facts. First, the 7472 and 7512 mutations are not associated with characteristic morphological findings like RRF. Second, immunohistochemistry would fail to show COX negative fibres in the case of a homoplasmic distribution of the mutations.[27] This situation is also observed in infantile RC deficiencies. Characteristic histological findings indicative of a mitochondrial disorder are rarely observed in these cases especially in the relatively common isolated complex I deficiencies, which are not visible on histochemical testing.

GENETIC TESTING BASED ON CLINICAL CRITERIA ALONE

By comparison, the detection rate in unselected patients (more than 3000 blood/tissue samples were analysed over the past 10 years for mtDNA mutations based on clinical criteria alone; LHON cases are not included) was very low (1%). These data again clearly emphasise that mtDNA analysis should be done after morphological and biochemical precharacterisation.

mtDNA MUTATIONS ARE A FREQUENT CAUSE OF RESPIRATORY CHAIN DEFICIENCIES

In conclusion, mitochondrial tRNA mutations and deletions contribute to almost one third of RC deficiency disorders of both adult and paediatric patients >5 years, thus being the first candidates for a fast screening approach. In contrast, nuclear gene mutations are the main cause of RC deficiencies in neonates and infants. Identification of such nuclear genes will provide further insights into the heterogeneous field of mitochondrial RC deficiency disorders.

We greatly appreciate the excellent technical assistance of A Zimmermann, K Schulte, and J Drechsel. We thank the families whose collaboration made this study possible. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ja 802/1-2) (MJ), the Friedrich-Baur-Stiftung (MJ), and the Ernst und Berta Grimmke Stiftung (MJ, RH).

1 Zeviani M, Tiranti V, Piantadosi C. Mitochondrial disorders. *Medicine* 1998;**77**:59-72.
2 Hanna MG, Nelson IP. Genetics and molecular pathogenesis of mitochondrial respiratory chain diseases. *Cell Mol Life Sci* 1999;**55**:691-706.
3 Zhu Z, Yao J, Johns T, Fu K, De Bie I, Macmillan C, Cuthbert AP, Newbold RF, Wang J, Chevrette M, Brown GK, Brown RM, Shoubridge EA. SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. *Nat Genet* 1998;**20**:337-43.
4 Tiranti V, Hoertnagel K, Carozzo R, Galimberti C, Munaro M, Granatiero M, Zelante L, Gasparini P, Marzella R, Rocchi M, Bayona-Bafaluy MP, Enriquez JA, Uziel G, Bertini E, Dionisi-Vici C, Franco B, Meitinger T, Zeviani M. Mutations of SURF-1 in Leigh disease associated with cytochrome c oxidase deficiency. *Am J Hum Genet* 1998;**63**:1609-21.
5 Tiranti V, Jaksch M, Hofmann S, Galimberti C, Hoertnagel K, Lulli L, Freisinger P, Bindoff L, Gerbitz D, Comi GP, Uziel G, Zeviani M, Meitinger T. Loss-of-function mutations of SURF-1 are specifically associated with Leigh syndrome with cytochrome c oxidase deficiency. *Ann Neurol* 1999;**46**:161-6.
6 Papadopoulou LC, Sue CM, Davidson M, Tanji K, Nishino I, Sadlock JE, Krishna S, Walker W, Selby J, Glerum DM, Van Coster R, Lyon G, Scalais E, Lebel R, Kaplan P, Shanske S, De Vivo DC, Bonilla E, Hirano M, DiMauro S, Schon EA. Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in SCO2, a COX assembly gene. *Nat Genet* 1999;**23**:333-7.
7 Jaksch M, Ogilvie I, Yao J, Kortenhaus G, Bresser H-G, Gerbitz K-D, Shoubridge EA. Mutations in SCO2 are associated with a distinct form of hypertrophic cardiomyopathy and cytochrome c oxidase deficiency. *Hum Mol Genet* 2000;**9**:795-801.

8 Valnot I, von Kleist-Retzow JC, Barrientos A, Gorbatyuk M, Taanman JW, Mehaye B, Rustin P, Tzagoloff A, Munnich A, Rotig A. A mutation in the human heme A:farnesyltransferase gene (COX10) causes cytochrome c oxidase deficiency. *Hum Mol Genet* 2000;**9**:1245-9.
9 Valnot I, Osmond S, Gigarel N, Mehaye B, Amiel J, Cormier-Daire V, Munnich A, Bonnefont JP, Rustin P, Rotig A. Mutations of the SCO1 gene in mitochondrial cytochrome c oxidase (COX) deficiency with neonatal-onset hepatic failure and encephalopathy. *Am J Hum Genet* 2000;**67**:1104-9.
10 Adams PL, Lightowlers RN, Turnbull DM. Molecular analysis of cytochrome c oxidase deficiency in Leigh's syndrome. *Ann Neurol* 1997;**41**:268-70.
11 Jaksch M, Hofmann S, Kleinle S, Liechti-Gallati S, Pongratz D, Müller-Höcker J, Baldwin Jedele K, Meitinger T, Gerbitz KD. A systematic mutation screen of 10 nuclear and 25 mitochondrial candidate genes in 21 patients with cytochrome c oxidase (COX) deficiency reveals tRNASer(UCN) mutations in a subgroup with syndromal encephalopathy. *J Med Genet* 1998;**35**:895-900.
12 Keightley JA, Hoffbuhr KC, Burton MD, Salas VM, Johnston WS, Penn AM, Buist NR, Kennaway NG. A microdeletion in cytochrome c oxidase (COX) subunit III associated with COX deficiency and recurrent myoglobinuria. *Nat Genet* 1996;**12**:410-16.
13 Manfredi G, Schon EA, Moraes CT, Bonilla E, Berry GT, Sladky JT, DiMauro S. A new mutation associated with MELAS is located in a mitochondrial DNA polypeptide coding gene. *Neuromusc Disord* 1995;**5**:391-8.
14 Comi GP, Bordoni A, Salani S, Franceschina L, Sciacco M, Prelle A, Fortunato F, Zeviani M, Napoli L, Bresolin N, Moggio M, Ausenda CD, Taanman JW, Scarlato G. Cytochrome c oxidase subunit I microdeletion in a patient with motor neuron disease. *Ann Neurol* 1998;**43**:110-16.
15 Tiranti V, Corona P, Greco M, Taanman JW, Carrara F, Lamantea E, Nijtmans L, Uziel G, Zeviani M. A novel frameshift mutation of the mtDNA COIII gene leads to impaired assembly of cytochrome c oxidase in a patient affected by Leigh-like syndrome. *Hum Mol Genet* 2000;**9**: 2733-42.
16 Smeitink J, van den Heuvel L. Human mitochondrial complex I in health and disease. *Am J Hum Genet* 1999;**64**: 1505-10.
17 Chinnery PF, Turnbull DM. Mitochondrial medicine. Review. *Q J Med* 1997;**90**:657-67.
18 Munnich A, Rötig A, Chretien D, Saudubray JM, Cormier V, Rustin P. Clinical presentations and laboratory investigations in respiratory chain deficiency. *Eur J Pediatr* 1996;**155**:262-74.
19 Rahman S, Blok RB, Dahl HHM, Danks DM, Kirby DM, Chow CW, Christodoulou J, Thornburn DR. Leigh syndrome: clinical features and biochemical and DNA abnormalities. *Ann Neurol* 1996;**39**:343-51.
20 Fischer JC, Ruitenbeek W, Gabreels FJ, Janssen AJM, Renier RO, Sengers RCA, Stadhouders AM, ter Laak HJ, Trijbels JM, Veerkamp JH. A mitochondrial encephalomyopathy: the first case with an established defect at the level of coenzyme Q. *Eur J Pediatr* 1986;**144**:441-4.
21 Hofmann S, Jaksch M, Bezold R, Mertens S, Aholt S, Paprotta A, Gerbitz KD. Population genetics and disease susceptibility: characterization of central European haplogroups by mtDNA gene mutations, correlation with D-loop variants and association with disease. *Hum Mol Genet* 1997;**6**:1835-46.
22 Kleinle S, Wiesmann U, Superti-Furga A, Krähenbühl S, Boltshauser E, Reichen J, Liechti-Gallati S. Detection and characterization of mitochondrial DNA rearrangements in Pearson and Kearns-Sayre syndromes by long PCR. *Hum Genet* 1997;**100**:643-50.
23 Jaksch M, Gerbitz KD, Kilger C. Screening for mitochondrial DNA (mtDNA) point mutations using nonradioactive single strand conformation polymorphism (SSCP) analysis. *Clin Biochem* 1995;**28**:503-9.
24 Liechti-Gallati S, Schneider V, Neeser D, Kraemer R. Two buffer PAGE system-based SSCP/HD analysis: a general protocol for rapid and sensitive mutation screening in cystic fibrosis and any other human genetic disease. *Eur J Hum Genet* 1999;**7**:590-8.
25 Kleinle S, Schneider V, Moosmann P, Brandner S, Krähenbühl S, Liechti-Gallati S. A novel mitochondrial tRNA-(Phe) mutation inhibiting anticodon stem formation associated with a muscle disease. *Biochem Biophys Res Commun* 1998;**247**:112-15.
26 Sprinzl M, Horn C, Brown M, Ioudovitch A, Steinberg S. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res* 1998;**26**:148-53.
27 Jaksch M, Klopstock T, Kurlemann G, Dorner M, Hofmann S, Kleinle S, Hegemann S, Weissert M, Muller-Hocker J, Pongratz D, Gerbitz KD. Progressive myoclonus epilepsy and mitochondrial myopathy associated with mutations in the tRNA(Ser(UCN)) gene. *Ann Neurol* 1998;**44**:635-40.
28 Manfredi G, Schon EA, Bonilla E, Moraes CT, Shanske S, DiMauro S. Identification of a mutation in the mitochondrial tRNA (Cys) gene associated with mitochondrial encephalopathy. *Hum Mutat* 1996;**7**:158-63.
29 Tessa A, Vilarinho L, Casali C, Santorelli FM. MtDNA-related idiopathic dilated cardiomyopathy. *Eur J Hum Genet* 1999;**7**:847-8.
30 Moraes CT, Ciacci F, Bonilla E, Ionasescu V, Schon EA, DiMauro S. Two novel pathogenic mitochondrial DNA mutations affecting organelle number and protein synthesis. Is the tRNALeu(UUR) gene an etiologic hot spot? *J Clin Invest* 1993;**92**:2906-15.
31 Sue CM, Tanji K, Hadjigeorgiou G, Andreu AL, Nishino I, Krishna S, Bruno C, Hirano M, Shanske S, Bonilla E, Fischel-Ghodsian N, DiMauro S, Friedman R. Maternally inherited hearing loss in a large kindred with a novel T7511C mutation in the mitochondrial DNA tRNA(Ser(UCN)) gene. *Neurology* 1999;**52**:1905-8.
32 Goto YI, Koga Y, Horai S, Nonaka I. Chronic progressive external ophthalmoplegia: a correlative study of mitochondrial DNA deletions and their phenotype expression in muscle biopsies. *J Neurol Sci* 1990;**100**:63-9.
33 Fassati A, Bordoni A, Amboni P, Fortunato F, Fagiolari G, Bresolin N, Prelle A, Comi G, Scarlato G. Chronic progressive external ophtalmoplegia: a correlative study of quantitative molecular data and histochemical and biochemical profile. *J Neurol Sci* 1994;**123**:140-6.
34 Wiedemann FR, Vielhaber S, Schröder R, Elger CE, Kunz WS. Evaluation of methods for the determination of mitochondrial respiratory chain enzyme activities in human skeletal muscle samples. *Anal Biochem* 2000;**279**:55-60.
35 Torroni A, Huoponen K, Francalacci P, Petrozzi M, Morelli L, Scozzari R, Obinu D, Savontaus ML, Wallace DC. Classification of European mtDNAs from an analysis of three European populations. *Genetics* 1996;**144**:1835-50.
36 Howell N, McCullough DA, Kubacka I, Halvorson S, Mackey D. The sequence of human mtDNA: the question of errors versus polymorphisms. *Am J Hum Genet* 1992;**50**: 1333-40.
37 Lehtonen MS, Meinila M, Hassinen IE, Majamaa K. Haplotype-matched controls as a tool to discriminate polymorphisms from pathogenic mutations in mtDNA. *Hum Genet* 1999;**105**:513-14.
38 Anderson A, Bankier AT, Barrel BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. *Nature* 1981;**290**:457-65.
39 Chalmers RM, Lamont PJ, Nelson I, Ellison DW, Thomas NH, Harding AE, Hammans SR. A mitochondrial DNA tRNA(Val) point mutation associated with adult-onset Leigh syndrome. *Neurology* 1997;**49**:589-92.
40 Tiranti V, Carrara F, Confalonieri P, Mora M, Maffei RM, Lamantea E, Zeviani M. A novel mutation (8342G→A) in the mitochondrial tRNA(Lys) gene associated with progressive external ophthalmoplegia and myoclonus. *Neuromusc Disord* 1999;**9**:66-71.
41 Shoffner JM, Lott MT, Lezza AM, Seibel P, Ballinger SW, Wallace DC. Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. *Cell* 1990;**61**:931-7.
42 Helm M, Florentz C, Chomyn A, Attardi G. Search for differences in post-transcriptional modification patterns of mitochondrial DNA-encoded wild-type and mutant human tRNALys and tRNALeu(UUR). *Nucleic Acids Res* 1999;**27**:756-63.
43 Andreu A, Hanna MG, Reichmann H, Bruno C, Penn AS, Tanji K, Pallotti F, Iwata S, Bonilla E, Lach B, Morgan-Hugh J, DiMauro S. Exercise intolerance due to mutations in the cytochrome b gene of mitochondrial DNA. *N Engl J Med* 1999;**341**:1037-44.
44 Shoffner JM. Maternal inheritance and the evaluation of oxidative phosphorylation diseases. *Lancet* 1996;**348**:1283-8.
45 Sternberg D, Danan C, Lombes A, Laforet P, Girodon E, Goossens M, Amselem S. Exhaustive scanning approach to screen all the mitochondrial tRNA genes for mutations and its application to the investigation of 35 independent patients with mitochondrial disorders. *Hum Mol Genet* 1998;7:33-42.