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Respiratory chain activity in tissues from patients (MELAS) with a point mutation of the mitochondrial genome [$tRNA^{Leu(UUR)}$]

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A heteroplasmic point mutation (transition A to G at position 3243 in the mitochondrial $tRNA^{Leu(UUR)}$ gene is indicative for myo-encephalopathy with lactic acidosis and stroke-like episodes (MELAS). Decreased respiratory chain complex activities measured in different tissues from four patients with MELAS syndrome do not correlate with the proportion of mutated mitochondrial genome.

Myopathy; Encephalopathy; MELAS; Mitochondrial DNA mutation; Respiratory chain

1. INTRODUCTION

Myo-encephalopathy with lactic acidosis and stroke-like episodes (MELAS) is a rare mitochondrial (mt) disorder. Three independent groups [1–3] reported that in Japanese patients the disease is accompanied by a point mutation (transition A to G) of the mitochondrial genome within the $tRNA^{Leu(UUR)}$ at the Cambridge nucleotide number 3243. Recently we have confirmed these results in four Caucasian cases with MELAS [4]. It was speculated that this mutation might be the main cause of the disease. As respiratory chain enzymes are partially coded by the mt genome we asked the question of how activities of these enzymes are altered by the MELAS mutation. Here we describe the respiratory chain activities in muscle samples of four MELAS patients and in five different tissues from one particular MELAS case and compare the results with the proportions of wild-type mtDNA still present in these tissues.

2. PATIENTS AND METHODS

2.1. *Patients*

Case 1 is a 38-year-old man, who had the first symptoms of the disease at the age of 10. At the age of 30 he had several episodes of hemianopsia, hemiparesis, ataxia, frontal headache and generalized tonoclonic seizures. High blood lactate levels together with 'ragged red fibers' in skeletal muscle histology confirmed the diagnosis of MELAS. His mother and two brothers have sensoneural deafness and diabetes mellitus.

Case 2 is the elder brother of case 4. At the age of 14 years he presented with muscle weakness and a muscle biopsy was taken. Histologically 'ragged red fibers' and lipid accumulations were found. During the passed three years he had several stroke-like episodes and developed cortical blindness, hemiparesis, hearing loss, somnolence as well as intellectual retardation.

Case 3 is a 19-year-old girl with mental retardation and short stature. At the age of 16 she had a first attack of headache with vomiting. High blood lactate levels and histologically finding of 'ragged red fibers' with lipid accumulation in skeletal muscle confirmed the MELAS syndrome.

Case 4 was the elder sister of case 2. She showed retarded development and presented with muscle weakness at the age of 7 years. At that time a muscle biopsy was taken and histologically lipid accumulation and an increased number of mitochondria were found. During the last two years of her life she became more and more dement, had several stroke-like episodes and developed facial palsy, convulsions, hemiparesis, cortical blindness, sensoneural hearing loss. She died at the age of 12 years.

2.2. *Methods*

DNA extraction and activity measurements of the respiratory chain complexes were performed in muscle biopsy samples (cases 1, 2 and 3) or in post-mortem autopsy tissues (case 4). All biopsies were taken for diagnostic reasons in vivo after obtaining informed consent of the patients.

Extraction and labelling of DNA. Total DNA preparation was performed by standard proteinase K procedure as described [5]. MtDNA as probe for hybridization was purified from human placenta according to Drouin [6] and labeled with the ECL (enhanced chemiluminescence) gene detection system as recommended by the supplier (Amersham, Germany).

Analysis of mtDNA. Total patient DNA (2–3 μg) was digested overnight with 10 U restriction endonuclease *Apa*I (Boehringer Mannheim, Germany) according to the manufacturers instructions,

Abbreviations: bp, base pairs; CPEO, chronic progressive external ophthalmoplegia; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibers; mt, mitochondrial

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subjected to electrophoresis on 1.0% agarose gels, transferred to nylon membranes (Hybond-N, Amersham, Germany) and further processed as recommended by the manufacturer for the ECL system. MtDNA/1-kb ladder (BRL, Germany) was used as marker DNA. Exposure times between 10 s and 10 min were sufficient to get scannable signals (X-Omat RP, Kodak, Germany). Densitometry was performed using the Elscript 400 UVR Scanner (Hirschmann, Munich). Measurements of the respiratory chain complexes (complex I: NADH-ubiquinone oxidoreductase; complex II+III: succinate-cytochrome *c* oxidoreductase; complex IV: cytochrome *c* oxidoreductase; complex I–IV: NADH-O_2 oxidoreductase) were performed as described elsewhere [5,7].

3. RESULTS

Digestion with the endonuclease *Apa*I usually cuts the mtDNA at positions 16459, 9269, 8253, 4431 and 1466 and produces five fragments of 7190 bp, 3822 bp, 2965 bp, 1576 bp and 1016 bp in length (Fig. 1, lane C). The transition A to G at position 3243 creates a new restriction site for the enzyme leading to a cleavage of the 2965 bp fragment into two smaller fragments with molecular weights of 1780 bp and 1185 bp. The resulting seven band pattern was found in skeletal muscle samples from all of our four MELAS patients as well as in five different tissues from case 4 [4]. As in all MELAS tissues, wild-type and mutated mtDNA coexisted together and the genomes were heteroplasmic. *Apa*I digestion of mtDNA from skeletal muscle of five controls yielded the expected normal five band pattern. Fig. 1 shows the results for cardiac muscle (CM_4) and liver (L_4) of case 4, for skeletal muscle of case 3 (SM_3), and for skeletal muscle of one representative control (C).

Using densitometric scanning we found that the proportion of preserved wild-type mtDNA in skeletal muscle ranged from 7% in case 1 to 68% in case 4 (Fig. 2A). A similar range of variation was seen in the five different tissues from case 4, where the wild-type mtDNA varied from 19% in brain up to 68% in skeletal muscle (Fig. 3A).

Fig. 2 compares the proportion of non-mutated mtDNA with activity values of the respiratory chain complexes I, II + III and IV in skeletal muscle from the four MELAS patients. Complex I deficiency and to a lesser extent also decreased complex IV activity was found in patients 1 and 4, while in all cases no reduced complex II + III activities were found. Cases 1–3 showed a very low proportion of normal mtDNA between 7% and 16% while in case 4 we found 68%. There was no clear-cut correlation between the proportion of the mutated mtDNA and reduced enzyme activities. In particular cases 2 and 3 had nearly the same high amount of mutated mtDNA in muscle. In contrast to case 2 who had a severe complex I defect, case 3 had biochemically normal values. Comparison of the DNA data with the biochemical activity values measured in five different tissues from case 4 yielded similar results (Fig. 3). Although the proportion of mutated mtDNA was lower

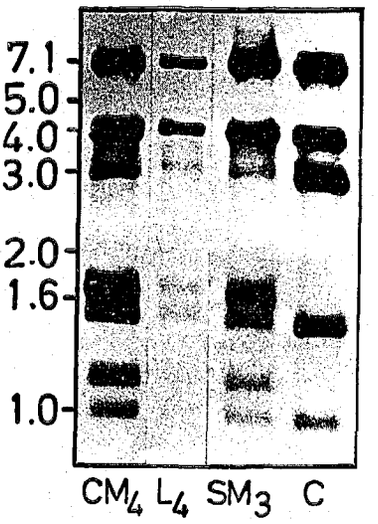
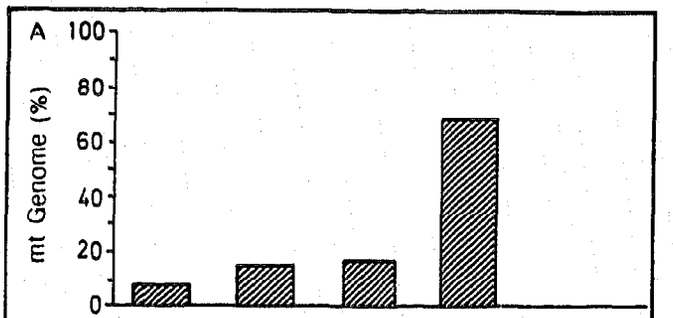


Fig. 1. Southern blot after digestion by *Apa*I of mtDNA from cardiac muscle (CM_4) and liver (L_4) of case 4, and from skeletal muscle (SM_3) of case 3 and a healthy control (C). The wild-type pattern is shown in lane C, where 5 different bands can be seen (M_r: 7190 bp, 3822 bp, 2965 bp, 1576 bp, and 1016 bp). Mutated MELAS mtDNA always shows two additional bands of approximately 1.8 kb and 1.2 kb, which result from a fifth recognition site in the 2965 bp fragment. As a result of heteroplasmy, this latter fragment is still present in the MELAS tissues.

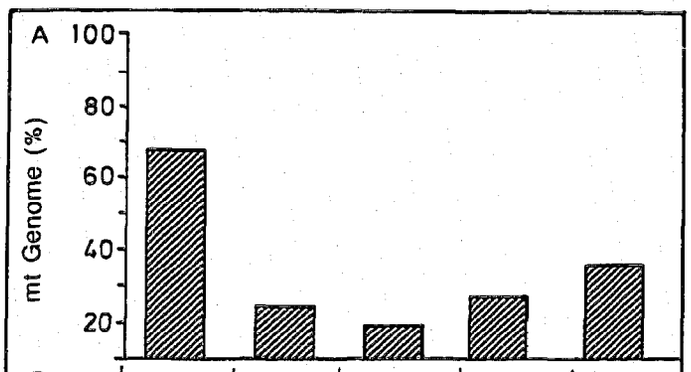
in skeletal muscle than in diaphragm, liver and heart all respiratory chain activities were measured within the reference ranges in these three latter tissues. This was not the case in skeletal muscle. In brain a complex I deficiency was detectable.



B

	SM_1	SM_2	SM_3	SM_4	controls
Complex I	1.8	0.4	12.8	5.8	12 - 26.4
Complex II+III	39.5	11.0	13.8	8.7	6 - 25
Complex IV	59.5	81.0	141.0	50.0	90 - 281
Complex I-IV	n.d.	0.3	10.3	n.d.	9 - 37

Fig. 2. (A) Proportion of wild-type mtDNA from skeletal muscle (SM_i) of four patients with MELAS as percent of total mtDNA; index i denotes the case number. Depicted values are means of 1–3 densitometric scannings. (B) Biochemical measurements in muscle homogenates of complex I (NADH-ubiquinone oxidoreductase), complex II + III (succinate-Cyt *c* oxidoreductase) and complex IV (cytochrome *c* oxidase), complex I–IV (NADH-O_2 oxidoreductase). Activity is expressed as μmol/min and grams of non-collagen protein. Measurements were done at least in triplicates; average values are given.



B	SM_4	CM_4	B_4	L_4	D_4
Complex I	5.8 (12.0-26.4)	9.6 (2.7-31.6)	2.9 (18.2-64.0)	9.2 (2.2-22.5)	6.6 (3.9-30.3)
Complex II +III	8.7 (6.0-25.0)	13.2 (9.0-40.2)	26.4 (21.6-41.5)	16.7 (3.9-14.9)	9.6 (15.3-27.6)
Complex IV	50.0 (90-281)	168 (88.2-311)	50.2 (60.5-339)	99.5 (13.7-48.7)	69.9 (51-238)

Fig. 3. (A) Proportion of wild-type mtDNA extracted from skeletal muscle (SM_4), cardiac muscle (CM_4), brain (B_4), liver (L_4) and diaphragm (D_4) of case 4; index denotes the case number. Depicted values are means of 1–3 densitometric scannings. (B) Biochemical measurements of respiratory chain complex activities in the different tissues. Activity is expressed as described in Fig. 2. Measurements were done at least in triplicates per tissue, average values are given. Control ranges are given in parentheses.

4. DISCUSSION

13 polypeptides (subunits of the respiratory chain complexes), 2 ribosomal RNAs and 22 tRNAs are encoded by the mitochondrial genome. Among the heterogenous group of mitochondrial encephalomyopathies deletions of the mtDNA were found in patients with chronic progressive external ophthalmoplegia (CPEO) and Kearns-Sayre syndrome [5,8–13], while defined point mutations were observed in patients with Leber's opticus neuropathy [14] and in patients with myoclonus epilepsy with ragged red fibers (MERRF) [15,16]. Sequencing of the mtDNA from patients with MELAS yielded a transition A to G at position 3243 of the mt genome within the $tRNA^{Leu(UUR)}$ gene. This mutation was reported in 36/41 Japanese patients with MELAS by three independent groups [1–3], but was not found in 83 controls and in 5 cases with MERRF [1]. One patient with CPEO, who exhibited no larger mtDNA deletion, also had the mutation at position 3243 [1]. We could confirm these results in four Caucasian patients with MELAS [4]. Thus the fact that the A to G transition within the $tRNA^{Leu}$ seems to be specific, but not exclusive is not restricted to one ethnic group.

The adenine at position 3243 within the DHU loop of the mitochondrial $tRNA^{Leu(UUR)}$ is functionally conserved during evolution [1,3]. A second function of the gene which encodes for the $tRNA^{Leu(UUR)}$ is to play a role in the partial termination of the heavy strand transcription causing a more frequent ribosomal RNA synthesis [17]. The high proportion of mutated mtDNA found in some of our patients (>90%) indicates that most of the cells should be affected. Assuming a direct correlation between the molecular and biochemical level this should be reflected by lowered or missing activities of the respiratory chain complexes, which are partially encoded by the mtDNA. As we show here, this is not the case. There is no clear-cut correlation between the proportion of mutated mtDNA and reduced complex I and/or IV activities in skeletal muscle or other tissues. Furthermore only focal complex IV deficiencies are usually observed histochemically in muscle samples from MELAS patients. Using specific antibodies in immunoblot technique, Jinnai et al. [18] demonstrated that beside the mitochondrially encoded subunit 2 also the nuclear encoded subunits 6 and 7 of complex IV were decreased in skeletal muscle of a MELAS patient. Focal complex IV defects were also obtained in skeletal muscle of case 3 (unpublished results) and in other tissues from case 4 by one of us [19]. We cannot exclude that the activity of mitochondrial Mg-ATPase, which has two mitochondrial encoded subunits, but cannot be measured in muscle homogenates, is more related to the degree of mutated mt genome populations. Nevertheless comparison of biochemical and molecular biological results for SM_3 and SM_4 as well as diaphragm, cardiac muscle and skeletal muscle in case 4 points towards additional factors (additional mutations or nuclear influences) involved in the pathogenesis of this form of mitochondrial encephalomyopathies. Therefore the point mutation at position 3243 of the $tRNA^{Leu(UUR)}$ gene might be indicative, but is probably not the only cause for MELAS. A similar conclusion might be drawn from the fact that the clinical picture of this disease is dominated by cardiac and cerebral symptoms although other tissues exhibit the mt mutation. Further studies have to be done to answer the question which at present unknown additional factors can explain how genotype and phenotype are connected in this disease.

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