SUPPLEMENTAL DATA

METHODS

Patients

Five patients suffering from mitochondrial myopathy associated with multiple mtDNA deletions were included in the study. Mutations in TK2 (RefSeq NP_004605.4) were identified for all 5 patients. Patient 1 (P1) was compound heterozygote for the mutations p.K202del and p.T108M; patient 2, 3 and 4 (P2, P3 and P4) were homozygous for the p.K202del mutation; patient 5 (P5) and patient 6 (P6) were homozygous for the p.T108M mutation. Patient 7 (P7) was compound heterozygote for the mutations p.R192K and p.T108M. Besides the adult patients, we also studied skin fibroblasts from 3 healthy controls (C) and four infantile patients suffering from severe mtDNA depletion: patient 8 (P8, described in (1)) was compound heterozygote for the mutations p.K202del and p.S51Ifs*99; patient 9 (P9) was compound heterozygote for the mutations p.R130W and p.Y208C; patient 10 (P10) was compound heterozygote for the mutations p.R192K and p.H121N; and patient 11 (P11, described in (2)) was compound heterozygote for the mutations p.K202del and p.R183G. All mutations but p.Y208C in P9 had been previously described (3, 4). This novel mutation affects a highly conserved residue and scores as probably damaging on functionality prediction softwares (PolyPhen-2; score:1). Pathogenicity of the p.Y208C mutation was finally proved after observing a severe reduction of TK2 activity (16% residual activity) in fibroblasts derived from P9. All subjects gave informed consent in accordance with our Institutional Review Boards and the Declaration of Helsinki.
Biochemical and Histological analysis and Mitochondrial DNA investigations

Biochemical, histological and molecular investigations were performed on skeletal muscle biopsies. Measurement of respiratory chain complexes activity, biochemical determinations and histology analysis were performed as previously described (5, 6). mtDNA deletions were investigated by long-range PCR and/or Southern blot and mtDNA copy number was assessed by quantitative PCR as previously described (7, 8).

Genetic analysis

For patients 1, 2, 4 and 5, the coding region of 13 genes that had been related with mtDNA maintenance (DGUOK, MFN2, MPV17, OPA1, POLG, POLG2, RRM2B, SLC25A4, SUCLA2, SUCLG1, TK2, PEO1 and TYMP) was PCR enriched by an IonAmpliSeq™ Custom panel (Life Technologies). “In silico” panel coverage (exons and exon-intron boundaries) was 97.39%.

The pool of PCR products was followed by DNA libraries preparation using the Ion AmpliSeq Library kit 2.0 and massive parallel sequencing by Ion PGM instrument (Ion Torrent, Life technologies). Sequence alignment, variant calling and annotation were performed by Ion Torrent Suite Software™ and Annovar software (9). Whole-exome sequences from patient 3 were captured by SureSelect XT HumanAllExon 50Mb (Agilent) and later sequenced with Illumina HiSeq2000 (Illumina). Variants have been selected by the following criteria: i) 1000 genomes MAF lower than 0.0050, ii) deleterious effects (nonsynonymous SNV, frameshift mutation, stoploss SNV, etc) and iii) presence in “Mitocarta” gene list (10).
TK2 activity determination

Primary cultured fibroblasts were obtained from skin biopsies of patients 3 and 4. Cells were seeded in 3 plates of 100 mm diameter in DMEM with 4.5 g/L glucose, supplemented with 2 mM L-glutamine, 100 U/mL penicillin and streptomycin, and 10% dialyzed FBS (Invitrogen) in a humidified incubator at 37°C and 5% CO₂. After tight confluence was reached, FBS was reduced to 0.1% to induce quiescence. Twelve days later cells were washed with ice-cold PBS and collected using a cell scraper. Cells were pelleted and stored at -80°C until further use. We measured TK2 activity following a published method (11). 250 µl of extraction buffer (0.5% Triton X, 2 mM EDTA, 1 mM DTT, 10 mM Tris HCl pH 7.5 supplemented with protease inhibitors Complete Mini, Roche) were added to the cell pellets, plus a further addition of NaCl up to 0.2 M. After vortex homogenization, extracts were centrifuged at 19,000 g and 4°C for 20 min. Protein concentration in the supernatants was determined by a BCA Protein Assay (Bio-Rad). Seven µg of extracted protein in a final volume of 10 µl were mixed with 10 µl of 2x dilution buffer (1 mg/ml BSA, 10 mM Tris HCl pH 7.5, 2 mM DTT, 5 mM ATP) and 20 µl of 2x reaction mix (100 mM Tris HCl pH 7.5, 10 mM ATP, 10 mM MgCl₂, 1 mg/ml BSA, 10 mM NaF, 4 mM DTT, 1 mM 5-Bromouracil (TP inhibitor) and 5 µM of 1,000 cpm/pmol substrate, [5′-³H]5-(2-bromovinyl)-2’-deoxyuridine (Moravek biochemicals). After 60 min at 37°C, 30 µl of reaction was spotted on Whatman DE81 filters and air dried. Subsequently, filters were washed three times for 5 min in 5 mM ammonium formate and placed in vials to elute with 2 ml of 0.1 M HCl, 0.2 M NaCl for 30 min at room temperature. Radioactivity in the eluate was counted after adding 7 ml of BCS Biodegradable Counting Scintillant Liquid (Amersham Biosciences) in a LS
6000 scintillation counter (Beckman). Enzyme activity was calculated as pmol product/min/mg protein.

**FIGURE LEGENDS**

**Figure e-1: Histochemical findings in muscle biopsies**  Histochemical analysis of muscle biopsies evidenced the existence of COX negative that were SDH-hyper-reactive fibers in all patients. Ragged-red fibers were also present in most of the patients. Atrophy or necrosis affecting isolated fibers were less frequently observed (i.e. HE stain for P1). Some scattered regenerative fibers were also identified. HE: hematoxylin-eosin stain; COX(SDH): Cytochrome oxidase stain alone or in combination with Succinate-dehydrogenase stain; GT: Gomori trichrome stain. (*) helps localizing those COX-negative fibers that are otherwise hardly noticeable.
Figure e-1
Figure e-2: mtDNA characterization and TK2 activity determination

Multiple deletions were observed in mtDNA of all patients by long range PCR (A) which were positive also by Southern blot analysis for all patients except P4 (B). TK2 activity in skin fibroblasts from patients and healthy controls. TK2 activity was measured in quiescent fibroblasts obtained from 2 adult (P3 and P4) patients, 3 age-matched healthy controls and 4 paediatric patients (P8-P11). All results correspond to the average value obtained in 2-3 independent determinations. The error bar indicates standard deviation of the values obtained for the three controls (C).
**Figure e-2**

**A**

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<th>P1</th>
<th>C</th>
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**B**

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**C**

![Graph showing TK2 activity](image)

**TK2 activity (pmol of product/min/mg)**

- P8
- P9
- P10
- P11
- P3
- P4
SUPPLEMENTAL TABLE

Table e-1: Genetic and phenotypic description of previously reported TK2-deficient patients with mild forms of the disease

<p>| Patient identification (Id) in the original publication (Pa-Pc appear as P1-P3 (12)) | Mutation according to RefSeq NP_004605.4 | Residual levels of mtDNA in muscle biopsies as quantified by real-time PCR. Results are shown as percentage of control samples. Histochemical findings (d) and Respiratory chain complexes activity (e) on muscle biopsies are indicated when available. f) Onset of the clinical manifestations is indicated in years (y) or months (m) when available. g) TK2 activity expressed as percentage of control samples CS: Citrate Synthase. NA: Information not available. |</p>
<table>
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<tr>
<th>REF</th>
<th>Patient Id</th>
<th>Mutation</th>
<th>mtDNA Levels</th>
<th>Main Clinical Features</th>
<th>Histochemical Findings</th>
<th>Respiratory Chain Activities</th>
<th>Onset Progression</th>
<th>TK2 Activity</th>
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<td>6</td>
<td>--</td>
<td>p.T108M</td>
<td>45%</td>
<td>Muscle weakness; Respiratory insufficiency; Dysarthria; Dysphagia</td>
<td>50% COX-</td>
<td>CI deficiency; Increased CS</td>
<td>Infancy (24m); Slow</td>
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<td>Pa</td>
<td>p.T108M</td>
<td>30%</td>
<td></td>
<td>Muscle weakness; Restrictive respiratory failure</td>
<td>7% COX- and RRF; Type 1 are predominant; Nuclear internalization</td>
<td>Combined CI, CIII and CIV deficiency</td>
<td>Infancy; Slow</td>
<td>40%</td>
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<td>Pb</td>
<td>p.R90C</td>
<td>30%</td>
<td></td>
<td>Ptosis; Dysarthria; muscle weakness; Mild restrictive respiratory failure</td>
<td>4% COX- and RRF; Type 1 are predominant; Nuclear internalization</td>
<td>Combined CI, CIII and CIV deficiency</td>
<td>Adolescence; Slow</td>
<td>4%</td>
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<td>Pb</td>
<td>p.W4VfsX40</td>
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<td>Pc</td>
<td>p.T108M</td>
<td>30%</td>
<td></td>
<td>Progressive muscle weakness; Moderate restrictive respiratory failure</td>
<td>20% COX- and RRF; Type 1 are predominant; Nuclear internalization</td>
<td>Combined CI, CIII and CIV deficiency</td>
<td>Infancy; Slow</td>
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<td>II4</td>
<td>p.R183W</td>
<td>60%</td>
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<td>Ptosis; PEO; Proximal muscle weakness and atrophy; mild dysphagia</td>
<td>10% COX- and RRF; Fibrosis in late stage</td>
<td>NA</td>
<td>Adulthood (40y)</td>
<td>25%</td>
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<td>II5</td>
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<td>Ptosis; PEO; Proximal muscle weakness and atrophy; Mild dysphagia; Mild dysarthria</td>
<td>10% COX-; RRF; Fibrosis in late stage</td>
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<td>Adulthood (40y)</td>
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</table>
| 4 | -- | p.Q35*  
  p.K194N | 185% | PEO; Limb-girdle dystrophy-like; Progressive muscle weakness; Rapidly progressive respiratory failure | 20% COX-; RRF | NA | Late adulthood (74y) | NA |
REFERENCES TO SUPPLEMENTAL DATA


