Thymidine kinase 2 defects can cause multi-tissue mtDNA depletion syndrome

Alexandra Götz,1,∗ Pirjo Isohanni,1,2,∗ Helena Pihko,2 Anders Paetau,3 Riitta Herva,4 Outi Saarenpää-Heikkilä,5 Leena Valanne,6 Sanna Marjavaara1 and Anu Suomalainen1,7

1Research Programme of Molecular Neurology, Biomedicum-Helsinki, University of Helsinki, Helsinki, 2Department of Paediatric Neurology, Hospital for Children and Adolescents, Helsinki University Central Hospital, Helsinki, 3Department of Pathology, University of Helsinki and Helsinki University Central Hospital, Helsinki, 4Department of Pathology, Oulu University Hospital, Oulu, 5Paediatric Clinics, Tampere University Hospital, Tampere, 6Helsinki Medical Imaging Center, University of Helsinki and 7Department of Neurology, Helsinki University Central Hospital, Helsinki, Finland

∗These authors contributed equally to this work.

Correspondence to: Prof. Anu Suomalainen, Research Programme of Molecular Neurology, Biomedicum-Helsinki, room c523a, Haartmaninkatu 8, University of Helsinki, 00290 Helsinki, Finland
E-mail: anu.wartiovaara@helsinki.fi

Mitochondrial DNA depletion syndrome (MDS) is a severe recessively inherited disease of childhood. It manifests most often in infancy, is rapidly progressive and leads to early death. MDS is caused by an increasing number of nuclear genes leading to multisystemic or tissue-specific decrease in mitochondrial DNA (mtDNA) copy number. Thymidine kinase 2 (TK2) has been reported to cause a myopathic form of MDS. We report here the clinical, autopsy and molecular genetic findings of rapidly progressive fatal infantile mitochondrial syndrome. All of our seven patients had rapidly progressive myopathy/encephalomyopathy, leading to respiratory failure within the first 3 years of life, with high creatine kinase values and dystrophic changes in the muscle with cytochrome c oxidase-negative fibres. In addition, two patients also had terminal-phase seizures, one had epilepsia partialis continua and one had cortical laminar necrosis. We identified two different homozygous or compound heterozygous mutations in the TK2 gene in all the patients: c.739 C → T and c.898 C → T, leading to p.R172W and p.R225W changes at conserved protein sites. R172W mutation led to myopathy or encephalomyopathy with the onset during the first months of life, and was associated with severe mtDNA depletion in the muscle, brain and liver. Homozygosity for R225W mutation manifested during the second year of life as a myopathy, and showed muscle-specific mtDNA depletion. Both mutations originated from single ancient founders, with Finnish origin and enrichment for the new R172W mutation, and possibly Scandinavian ancestral origin for the R225W. We conclude that TK2 mutations may manifest as infantile-onset fatal myopathy with dystrophic features, but should be considered also in infantile progressive encephalomyopathy with wide-spread mtDNA depletion.

Keywords: mitochondrial DNA depletion syndrome; thymidine kinase 2; myopathy; encephalomyopathy; mtDNA

Abbreviations: APP = amyloid beta precursor protein; CK = creatine kinase; COX = cytochrome c oxidase; CytB = cytochrome B; dNTP = deoxy-nucleotidetriphosphate; EEG = electroencephalography; ENMG = electroneuromyography; GT = glutamyl transeptidease; Mb = mega-basepair; MDS = mitochondrial DNA depletion syndrome; mtDNA = mitochondrial DNA; SDH = succinate dehydrogenase; SMA I = spinal muscular atrophy type I; TK2 = thymidine kinase 2.


Introduction

Mitochondrial DNA depletion syndrome (MDS) is a group of severe autosomal recessive disorders affecting the skeletal muscle, liver, brain or several tissues (Moraes et al., 1991). MDS manifests often in infancy, with average fatality by the age of 3 years, irrespective of the presentation (McFarland et al., 2002). However, some children manifest later with a slowly progressive phenotype and survive into adulthood (Vu et al., 1998). MDS is characterized by severe reduction of mitochondrial DNA (mtDNA) copy number in the affected
Patients, Materials and Methods

Patients

Seven infants of Finnish origin presented rapidly progressive muscle weakness leading to death of six of them and permanent ventilator support in one. Their parents were not known to be consanguineous, but three families originated from the same rural area. Information of ancestors’ birthplaces was obtained by parent interviews. Patients 5, 6 and 7 were previously described in a series of congenital muscular atrophy type I (SMA I) in clinical severity. Two index patients were found to have severe muscle-specific mtDNA depletion and TK2 mutations. Search for TK2 mutations in similar phenotypes led to identification of five more patients, including encephalomyopathies with multi-tissue mtDNA depletion.

Patient 1

Patient 1 was the second child of the family, born after in vitro fertilization at term. Her mother has hypothyroidism and has had cardiac arrhythmias, whereas father and elder brother are healthy. The patient had normal early development and could walk without support at 12 months of age. Progressive muscle weakness was noticed at 18 months: she could not crawl up the stairs and had to push herself with hands up from the floor. On examination at 1 year 9 months of age, she had severe generalized muscle weakness and absent deep tendon reflexes, and her cognitive development was normal. Her disease progressed rapidly during the following months, within 6 months she lost all antigravity movements and after a year she needed gastrostomy. She died of respiratory failure 18 months after the disease onset.

Patient 2

Patient 2 was the first child of healthy parents. The pregnancy, her birth and early developmental milestones were normal. At 7 months of age, she could raise herself up to standing position, but thereafter her motor development slowed down. At the age of 1 year 4 months, upon a respiratory tract infection, her condition deteriorated during a couple of weeks: she stopped crawling, could not rise up and held her head with difficulty. Her disease progressed rapidly. At the age of two she went into respiratory failure and needed permanent ventilator support. Her cognitive skills could not be formally assessed, but appeared normal. She has had epileptic seizures since the age of 2 years and the seizures evolved to epilepsy partialis continua. Repeated EEGs showed slow background activity and focal spikes, multi-spikes and spike-slow wave discharges. Brain MRI was normal at 16 months of age, but at 5 years showed cortical and central atrophy. Presently, she is 5 years of age, hospitalized and ventilator-dependent, with extreme muscle weakness and atrophy, cardiomyopathy and has gastrostomy. She has had fractures of large bones, e.g. femoral bone, during daily routine care, possibly caused by inactivity. She has retained minimal movement in her eyelids and finger tips, and she communicates by opening and closing her eyes.

Patient 3

Patient 3 was the third child of healthy parents with two healthy siblings. The pregnancy and delivery were normal. She was referred for investigations because of poor head control since the age of 5 months. On examination at 6 months of age she was an alert baby, with poor muscle tone and severe proximal muscle weakness and absent reflexes. The muscle weakness progressed rapidly and she died of respiratory failure at the age of 12 months.

Patient 4

Patient 4 was born as the first child of healthy parents after normal pregnancy and delivery. On examination at 3 months of age she was alert, but showed severe proximal muscle weakness, hypotonia and absent reflexes. Her muscle weakness progressed rapidly. She had short seizures with focal epileptic discharges in EEG from the age of 6 months, and she died of respiratory failure at the age of 6 months.

Patient 5

Patient 5 was the second child of healthy parents, born after normal pregnancy and delivery. Her brother was healthy. Her weight gain was poor, and her motor development was slow from 3 months of age. On examination at 5 months she was alert, but had severe muscle weakness and hypotonia, with absent reflexes. Her muscle weakness progressed rapidly, and she developed short focal seizures. She died of pneumonia and respiratory failure at the age of 7 months.

Patient 6

Patient 6 was the second child of a healthy father and a mother with aortic stenosis with two healthy daughters. The pregnancy
and delivery were normal. Severe progressive muscle weakness was noticed at 3 months of age, and he died of pneumonia at the age of 6 months. The diagnosis of congenital dystrophy was made based on the high creatine kinase (CK)-value and dystrophic changes in the muscle.

**Patient 7**

Patient 7 was the brother of Patient 6. He was born at term after normal pregnancy. His hypotonia raised concern at 3 months of age. He had frequent respiratory infections. On examination at 5 months of age he was alert but had muscle weakness and hypotonia, with no head control. He had high CK and dystrophic changes in the muscle. His muscle weakness progressed, and he died at the age of 8 months of pneumonia, with the diagnosis of congenital dystrophy.

All studied patients (P1–5) had liver transaminases 2- to 4-fold the normal range while CK was elevated 5- to 10-fold. Transaminase increase is generally associated with high CK, and therefore this finding cannot be interpreted as direct evidence for liver dysfunction. However, Patient 2 has had consistently increased gamma glutamyl transpeptidase (GT) values (71–893 U/l; normal <50 U/l) from the age of 3 years. As her gamma GT values were over 300 U/l already when antiepileptic medication included clobazam, oxcarbazepine or gabapentine that often do not cause liver dysfunction, the increase of gamma-GT may be a sign of disease-associated liver dysfunction.

**Control subjects**

As ‘muscle controls’ we used biopsy samples of vastus lateralis from age-matched subjects, who were deemed not to have a muscle disease. As ‘brain and liver controls’ we used paraffin-embedded or frozen post-mortem samples from age-matched subjects, with no mitochondrial disease. In addition to age-matching, each paraffin-embedded patient sample was matched with controls with similar post-mortem (<24 h) and formalin-fixation times, and frozen samples were matched with frozen control samples. The matched control groups were the following: C1, frozen muscle biopsy specimens of children of 3, 10 and 43 months of age; C2, cerebral cortex, basal ganglia and cerebellum autopsy samples from children of 2, 3 and 7 months of age, and C3, liver autopsy sample of a patient of <12 months of age, all matched for similar post-mortem and formalin-fixation times; C4, frozen autopsy samples of cerebral cortex and cerebellum of children of 8 and 9 months of age, and C5, frozen liver biopsy specimens of children of 3, 9, 11, 30, 38 and 41 months of age.

**Morphologic analysis**

Open surgical biopsies from vastus lateralis muscle were taken under generalized anaesthesia. The samples were snap-frozen in isopentane and processed for histochemical analyses for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) activities, as well as for the following stainings: haematoxylin–eosin, Van Gieson, modified Gomori trichrome, periodic acid–Schiff, oil red-O, NADH-tetrazolium reductase and adenosine triphosphatase at pH 10.4, 4.6 and 4.3 preincubations. Samples for ultrastructural studies were fixed by glutaraldehyde and examined with a Jeol JEM-1200EX electron microscope. Autopsy information with neuropathological examination on formalin-fixed brain was available of Patients 1, 3, 4 and 5. Autopsy samples of Patients 1, 4 and 5 were available to us.

**Biochemical analysis of respiratory chain enzyme activities**

The mitochondria were isolated from a fresh muscle biopsy specimen, and the respiratory chain enzyme activities, rotenone-sensitive NADH: cytochrome c oxidoreductase (CI + III), antimycin-sensitive succinate: cytochrome c oxidoreductase (CII + III), SDH (CII), COX (CIV) and citrate synthase, were analysed as previously described (Majander et al., 1995).

**DNA analyses**

The Homo sapiens chromosome 16 reference assembly sequence, which contains the human TK2 genomic DNA sequence (NC_000016) and TK2 mRNA (NM_004614), and newly revised TK2 protein sequence (NP_004605.3) were used as reference sequences. Of note, the recent revision of TK2 sequence described additional N-terminal amino acids, changing the amino acid numbering compared with that used in previous publications, utilizing old reference sequence XM_007855 (Galbiati et al., 2006).

Samples for DNA-analysis were snap-frozen in liquid nitrogen and stored at −80°C. Total DNA from frozen muscle, brain and liver samples (either autopsy or muscle biopsy-derived), as well as from paraffin-embedded formalin-fixed autopsy samples was extracted by standard methods (Shibata et al., 1988).

We amplified and sequenced the TK2 exons and exon–intron boundaries as in (Saada et al., 2001), using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Warrington, Cheshire, UK) on an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence processing was done with Sequencher 4.5 software (GeneCodes). For single-nucleotide detection of mutant nucleotides we utilized solid-phase minisequencing, according to (Suomalainen and Syvanen, 2000). Specific primers were designed for both the mutations:

- **Exon6_F**: Biotin 5′-TCTCTTTTCCCCCTGAAGTGATGATTCTCTT-3′
- **Exon6_R**: 5′-CCTCTCATACAGGTTTTCTACAAAAATGTAT CTTGGCG-3′
- **Exon6_R Detection**: 5′-AAATGCAGCTCTCCTCAACGC-3′
- **Exon8_F**: Biotin 5′-GAGCGAGCTCTGAGACGACAC-3′
- **Exon8_R**: 5′-CCTCTATCCGACATCTTCTTTAATCC-3′
- **Exon8_R Detection**: 5′-ACAAGTCTAGAGTGATGCTC-3′

For mtDNA quantification, the mitochondrial cytochrome b (Cytb) in mtDNA and the nuclear amyloid beta precursor protein (APP) genes were simultaneously amplified by quantitative TaqMan real-time PCR assay in the ABI Prism 7000 Detection System Cycler, using the following primers and probes:

- **hCytb_F**: 5′-GCGGCTCTGTATGATACAAAT-3′
- **hCytb_R**: 5′-AAGTACCGCTGATGTACACC-3′
- **hAPP_F1**: 5′-TGTGTCCTCCAGTGCTA-3′
- **hAPP_R1**: 5′-CAGTCTGCTGATGTCAGTG-3′
- **h_cytB-FAM**: 6-FAM 5′-CACCAGAGCCTCAACCCGCGC-3′
- **h_APP_VIC**: VIC 5′-CCCTGAACCTGACATCACAATGTG GTAG-3′
- **h_APP_VIC**: VIC 5′-CCCTGAACCTGACATCACAATGTG GTAG-3′

As amplification standards we used human APP or Cytb cDNAs cloned in PCR-TOPO vector (Invitrogen, Carlsbad, California, USA), amplified parallel to the samples and used at concentration dilutions of 10⁵–10⁶ copies. Of the genomic DNA, 25 ng was amplified using TaqMan Universal PCR Master Mix.
(Applied Biosystems, Roche, Branchburg, New Jersey, USA), 900 nM of each primer and 250 nM of each probe in 30 μl volume in following conditions: an enzyme activation step for 2 min at 95°C, followed by denaturing for 10 min at 95°C, and 40 amplification cycles of 15 s at 95°C, and 1 min at 60°C. Fluorescence acquisition was done at the end of each annealing step, and data were analysed using the ABI Prism 7000 SDS software (Applied Biosystems, FosterCity, CA, USA). The ratio of mtDNA to nuclear DNA obtained by comparison to the standard curve, created using known copy numbers of standard plasmids with Cy5B or APP genes as amplification templates, was used as a measure of mtDNA content (Livak and Schmittgen, 2001), with typically 2–5 replicate analyses per one sample. The mtDNA sequence was determined as in (Pietilainen et al., 2008), and multiple mtDNA deletion analysis was done as in (Van Goethem et al., 2004).

**Haplotype analysis**
We analysed five polymorphic dinucleotide markers flanking the TK2 gene (D16S3021, D16S3019, D16S397, D16S421 and D16S3025). PCR was performed in 0.2 mM of dNTPs, 0.4 μM of PCR primers, 0.8 μCi of [33P]dATP and 0.6 U of thermostable DNA polymerase (Dynazyme II, Finnzymes, Espoo, Finland), in 30 μl of its buffer, with an initial cycle at 95°C for 3 min, followed by 30–35 cycles at 94°C for 30 s, 55–56°C (optimized for each primer pair separately) for 30 s, and 72°C for 1 min and a final elongation at 72°C for 10 min. The radioactive PCR products were electrophoresed through non-denaturing 8% polyacrylamide gels, and autoradiography was done on Biomax MR films (Kodak, Rochester, NY, USA) for 1–2 days.

**Bioinformatics**
Human TK2 wild-type protein sequence (NP_004605) and the sequence introducing the TK2 mutations were analysed for post-translational modifications and protein structure using the PredictProtein analysing tool (www.predictprotein.org).

**Results**

**Clinical features**
Table 1 summarizes the symptoms and signs of the patients. No patient showed pseudohypertrophy of muscles, and muscle atrophy came late in the disease course. The disease-onset was acute or subacute in all patients and led to loss of all motor skills rapidly from one to few months from disease-onset, as in SMA I. Cognitive state was consistent to the patients’ ages. In electroneuromyography (ENMG), all studied patients had polyphasic motor units as a sign of myopathic changes. Nerve conduction velocities were normal. Patient 1 had also spontaneous electrical activity, fibrillations, as a sign of lower motor neuron involvement. EEGs were performed on patients 2, 4 and 5, and it showed normal or slow background activity with localized or secondarily generalized spike and slow-wave activity.

**Muscle morphology and histochemistry**
Table 1 summarizes the light microscopic findings in the patients’ muscle biopsy samples. Two representative biopsy findings are described in detail:

**Patient 2**
At 21 months of age, the muscle showed marked fibre size variation and atrophy, slight fibrosis, increased neutral lipids and up to 60–70% COX-negative fibres. Scattered necrotic fibres were seen. Electron microscopy demonstrated increased lipid vacuoles, but mitochondria appeared normal (not shown).

**Patient 4**
At 5 months of age, the muscle showed severe dystrophic changes with marked fibre size variation and fibrosis rendering a preliminary diagnosis of congenital muscle dystrophy (Fig. 1A). A few centronuclear, possibly regenerating fibres were seen, as well as occasional necrotic fibres. Most muscle fibres were of ragged-red type and >70% COX-negative fibres were detected (Fig. 1B). In electron microscopy, mitochondria were increased in number especially subsarcolemmally and often abnormal in shape with concentric cristae (not shown).

**Autopsy findings**
The macroscopic and light microscopic findings of thoroughly sampled brains of Patients 1 and 3 were within normal range, except for slight terminal capillary congestion and marginal oedema. Patient 4 displayed a focal mid-layer laminar necrosis of the visual cortex with neuronal loss and gliosis (Fig. 1C), consistent with ischaemic injury. Similar mild changes were also seen in the dentate gyrus of hippocampus. Purkinje cell damage was detected in the cerebellar cortex (Fig. 1D and E). Patients 4 and 5 had mild to moderate, mainly macrovesicular steatosis peripherally in the liver (Fig. 1F). The spinal cords of Patients 1, 3 and 4 showed no changes in histological analysis.

**TK2 sequence analysis**
Of a total of 41 screened patients with infantile muscle hypotonia we could diagnose seven with mutations in the TK2. We found two different missense mutations in TK2 (Fig. 2). In exon 8, C > T transversion at nucleotide 898 (Genbank acc.no: NM_004614), causing an amino acid change from arginine to tryptophane, and in exon 6 at nucleotide 739 C > T, which also changed an arginine to tryptophane. Both amino acids are highly conserved in species (Fig. 2). Table 1 indicates the mutations found in each patient.
mtDNA analysis
Quantification of mtDNA by real-time PCR revealed severe mtDNA depletion in the skeletal muscle (Fig. 3A) in all studied patients. In addition, severe mtDNA depletion in the brain and liver of Patients 4 and 5 were detected, whereas the brain of Patient 1 showed normal mtDNA amount and the liver of Patient 1 showed only slightly reduced mtDNA amount compared with the liver and brain samples of the age-matched controls (Fig. 3B and C). This result was verified by Southern blotting (data not shown).
Complete mtDNA sequence of Patients 1 and 2 was analysed from muscle DNA, and no pathogenic mutations were found. Number of polymorphic variants was not increased. In PCR analysis, no large-scale mtDNA deletions were found.

Biochemical analysis
Table 2 shows the respiratory chain enzyme activities in fresh muscle homogenates of Patients 1 and 2. The CI + III dependent activities were reduced to 20% or 25% of controls per citrate synthase, in P1 and P2, respectively, and CII + CIII activities were reduced to 30% and 60% of controls per citrate synthase. COX was most severely affected of the enzyme complexes, with 17% and 19%, respectively, remaining activities compared with the controls. Also a moderate reduction of CII, which is not dependent on mtDNA-encoded subunits, was seen in both patients. In Patient 2, citrate synthase activity was increased, as a sign of mitochondrial proliferation.

Haplotype analysis and ancestral origins
Figure 4 summarizes the haplotype analysis. The patients and the heterozygote carriers of R172W shared a common core haplotype of three polymorphic dinucleotide markers, extending over a chromosomal region of 1.1 Mb. However, in some patients with R172W the shared haplotype extended over 3.3 Mb. The R225W patients shared a common haplotype of two polymorphic dinucleotide markers extending over a chromosomal region of ~700 kb. Figure 5 shows the birthplaces of the grandparents and great-grandparents of the patients.

Bioinformatic analysis of the mutant TK2
Secondary structure analysis showed that the missense change R225W is located at the first position of a putative tyrosine kinase phosphorylation site (residue 225–232, -RTNPETCY-)abolishing it. The R172W missense change at the third position of a putative protein kinase c phosphorylation site (residue 170–172, -SVR-) abolishes the site.
We describe here mitochondrial TK2 defects in seven Finnish infants, and the first autopsy studies of such patients. All our patients showed severe dystrophic myopathy with mtDNA depletion, and two were also verified to have multi-tissue manifestation with mtDNA depletion in the skeletal muscle, brain and the liver. Four patients were homozygous for a novel TK2 mutation leading to amino acid change R172W, one patient was homozygous for R225W change, previously described in Swedish patients (note: R225W has been previously reported as R183W) (Tulinius et al., 2005), and two patients were compound heterozygotes for both. Since no other TK2 mutations were found in our representative material of 41 childhood myopathies, these two mutations are likely to explain the molecular background of myopathic MDS in Finland. No previous patients homozygous for either mutation have been reported, allowing us a unique opportunity for genotype–phenotype correlation. The phenotype of R172W was among the most severe reported in TK2 deficiency, with disease-onset in the first months of life, rapid loss of all motor skills and death within few months. Compound heterozygosity for R172W/R225W resulted in almost equal disease severity. The R225W homozygosity mimicked the disease course often reported with TK2–MDS (Oskoui et al., 2006), with normal motor development up to 1.5 years, rapid progressive myopathy, leading to loss of all antigravity muscle power and respiratory failure at 3 years of age. Interestingly, the R225W homozygosity lead to muscle-specific manifestation, whereas R172W lead to multi-tissue mtDNA depletion involving the muscle, brain and the liver.

![Histological findings in TK2 deficiency](image)

**Fig. 1** Histological findings in TK2 deficiency. Muscle biopsy sample from Patient 4 shows (A) marked fibre variation, atrophic fibres, endomysial fibrosis and fatty degeneration (frozen section, haematoxylin–eosin ×400, original magnification) and (B) high proportion of dark blue cytochrome c oxidase-negative and SDH-positive fibres (frozen section, COX-SDH ×200; original magnification). (C) Visual cortex from Patient 4. Between arrows: a spongiotic zone of laminar necrosis with neuronal loss and gliosis. (Paraffin section, HE ×40 original magnification). (D) Neocortical mid-laminar pyramidal layers of Patient 5 harboured many pyknotic red neurons consistent with ischaemic injury (arrow). (Paraffin section, HE ×400 original magnification). (E) In the cerebellar cortex of Patient 5 pyknotic red Purkinje cells (arrow) could easily be found. (Paraffin section, HE ×200 original magnification). (F) In the liver parenchyma of Patient 5 periportal, mainly macrovesicular steatotic vacuolization (arrows) could be seen. (Paraffin section, HE ×200 original magnification).
All our patients had severe muscle weakness resembling SMA I. Previously, two patients with TK2 mutations have been described as 'SMA-like', with loss of motor units and fibrillations (Mancuso et al., 2002) or with ‘neuropathic’ findings (Oskoui et al., 2006) in ENMG. Our patients had myopathic ENMG except for Patient 1, who also had fibrillations as a sign of lower motor neuron involvement. However, none of our TK2 patients had giant motor units, reflecting denervation–reinnervation and typical for SMA. In our patients, the disease was sometimes accompanied by encephalopathy with partial seizures, but none of our patients showed progressive cognitive impairment or ocular myopathy, as reported in (Galbiati et al., 2006). Three patients had seizures as a late manifestation, and one of them showed laminar cortical necrosis in the occipital region. Lack of molecular diagnosis and rapidly developing respiratory insufficiency led one patient to ventilator treatment (compound heterozygous for R172W/R225W), which has kept her alive to the present age of 5 years. She has minimal muscle power, epilepsia partialis continua—a typical seizure type in mitochondrial diseases—and severe cortical atrophy. The R172W patients showed clear clinical signs consistent with CNS

**Table 2** Mitochondrial respiratory chain activities in muscle (nmol/min/mg) (per citrate synthase)

<table>
<thead>
<tr>
<th>Activity</th>
<th>P1 (22 months)</th>
<th>P2 (21 months)</th>
<th>Controls (0–17 years)</th>
</tr>
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<tbody>
<tr>
<td>CI + III (NADH:cyt c reductase)</td>
<td>100 (0.04)</td>
<td>93 (0.05)</td>
<td>273 ±134 (0.2)</td>
</tr>
<tr>
<td>CII + III (succinate: cyt c reductase)</td>
<td>93 (0.03)</td>
<td>120 (0.06)</td>
<td>131 ± 72 (0.1)</td>
</tr>
<tr>
<td>CII (SDH)</td>
<td>101 (0.04)</td>
<td>72 (0.04)</td>
<td>150 ± 50 (0.11)</td>
</tr>
<tr>
<td>CIV (COX)</td>
<td>722 (0.26)</td>
<td>540 (0.29)</td>
<td>2068 ± 888 (1.50)</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>2807</td>
<td>1849</td>
<td>1375 ± 494</td>
</tr>
</tbody>
</table>

NADH = reduced nicotinamide adenine dinucleotide; Cyt c = cytochrome c.

All our patients had severe muscle weakness resembling SMA I. Previously, two patients with TK2 mutations have been described as ‘SMA-like’, with loss of motor units and fibrillations (Mancuso et al., 2002) or with ‘neuropathic’ findings (Oskoui et al., 2006) in ENMG. Our patients had myopathic ENMG except for Patient 1, who also had fibrillations as a sign of lower motor neuron involvement. However, none of our TK2 patients had giant motor units, reflecting denervation–reinnervation and typical for SMA. In our patients, the disease was sometimes accompanied by encephalopathy with partial seizures, but none of our patients showed progressive cognitive impairment or ocular myopathy, as reported in (Galbiati et al., 2006). Three patients had seizures as a late manifestation, and one of them showed laminar cortical necrosis in the occipital region. Lack of molecular diagnosis and rapidly developing respiratory insufficiency led one patient to ventilator treatment (compound heterozygous for R172W/R225W), which has kept her alive to the present age of 5 years. She has minimal muscle power, epilepsy partialis continua—a typical seizure type in mitochondrial diseases—and severe cortical atrophy. The R172W patients showed clear clinical signs consistent with CNS
manifestation of the disease. Our findings also show that the aggressive skeletal muscle involvement of R172W patients would likely be followed by manifestations in other post-mitotic tissues, if the patients did not die of respiratory muscle weakness.

Recently, prominent vacuolar changes in spinal cord neurons were found in TK2 knock-in mice (Akman et al., 2008), but the spinal cords of three of our patients were investigated and found to be normal. As the TK2 mice did not have prominent muscle involvement, the differences between the TK2 disease in humans and mice may reflect different tissue-specific dNTP pool regulatory pathways.

Demonstration of mtDNA depletion in the brain in two children with R172W change strongly suggested that the CNS symptoms and signs are MDS associated. R172W also

Fig. 3 The mtDNA copy number analysis by quantitative real-time PCR (Q-PCR) in patients with TK2 mutations. (A) Skeletal muscle mtDNA copy number is dramatically decreased in all TK2-patients. Frozen muscle biopsy samples of patients P1 (21 months), P2 (21 months), P4 (5 months) and pooled controls C1 (3, 10 and 43 months; as specified in methods); autopsy-derived formalin-fixed muscle of patient P5 (7 months). (B) The brain (cortex, cerebellum, basal ganglia) and liver of TK2-patients with the R172W mutation show severe mtDNA depletion. Brain and cerebellum samples of patient P4 (7 months), the pooled controls C2 (2, 3 and 7 months), the liver sample of P4 and the control C3 (<12 months) were autopsy-derived, formalin-fixed paraffin samples, matched with similar post-mortem and formalin-fixation times. (C) Patient P1 homozygous for the R225W TK2 mutation has high amounts of mtDNA in the brain and slightly reduced in the liver, at 3 years of age. Pooled controls C4 (8 and 9 months; frozen brain samples) and C5 (3, 9, 11, 30, 38 and 41 months of age; frozen liver biopsy specimens). The mtDNA copy number of one of the controls in each pool (10-month old in pool C1, 7-month old in C2 and 9-month old in C4) was set as 100% reference point. The results are average values of two to five Q-PCR experiments, each with triplicate samples.

Fig. 4 Haplotypes of the Finnish TK2 patients. The DNA marker names are shown without ‘D16S’. P1–4 are TK2 patients and C1–2 are heterozygote carriers of either TK2 mutation. The haplotype associated with the R172W change is shown in gray, and the haplotype associated with the R225W change is shown in black.
led to partial mtDNA depletion in the liver, with macrovesicular steatosis, but to no clinical signs. This is the first report of TK2-associated mtDNA depletion in non-muscle tissues and clearly confirms the clinical suspicion of multi-organ involvement. Our single homozygous patient for R225W, with severe muscle-restricted mtDNA depletion, manifested later and lived longer than those homozygous or compound heterozygous for R172W change. We demonstrate here unique mutation-specific tissue manifestations of TK2-MDS. As both R225W and R172W affect potential phosphorylation sites, our findings suggest that single residues can differentially modulate tissue-specific functions of mitochondrial TK2, possibly through modification of phosphorylation status. Mechanisms involved may give important insights into tissue specificity of MDS.

The haplotype analysis showed that the R172W and R225W changes were associated with single specific haplotypes, indicating that they originated from single ancestral founders. The grandparents and great-grandparents of our patients with R172W mutation (left), of patients with R225W mutation (right).

Both TK2 mutations described here changed a highly conserved hydrophilic arginine to hydrophobic tryptophan in the TK2 polypeptide. R172W change is novel, although the same amino acid changed to glutamine, combined with Y154N change, was mentioned in a recent survey of myopathic MDS, without further clinical details of this specific patient (Sarzi et al., 2007a). R225W and R225G have been described as compound heterozygous in pure childhood myopathies (Carrozzo et al., 2003; Vila et al., 2003; Tulinius et al., 2005), and R225G, combined with a truncating mutation, in infantile-onset encephalomyopathy (Carrozzo et al., 2003). The causative role of both mutations is further supported by the ancestral mutation-specific haplotypes and their full co-segregation with the disease in multiple families with similar disease. Further, TK2 mutations were not found in 68 chromosomes of Finnish patients with other types of childhood myopathies.

All our patients studied showed just 5–10% residual mtDNA amount in muscle compared with age-matched controls, at the time of their muscle biopsy and diagnosis. The longest surviving R225W homozygote patient showed 95% depletion at 21 months of age, whereas the patient with the most severe phenotype, R172W homozygote, had 90% depletion already at 5 months of age. This shows that at the time of diagnosis, the muscle has already lost the majority of its mtDNA. Quite surprisingly, the low mtDNA amount was still capable to maintain 20–30% of residual activities of the respiratory chain enzyme complexes I, III and IV, containing mtDNA-encoded subunits, which suggests compensatory mechanisms, possibly involving increased mitochondrial transcript or protein half-lives (Vila et al., 2008). We detected also downregulation of complex II, with no mtDNA-encoded subunits, probably reflecting a secondary effect due to the severe dystrophy of the muscle.

In conclusion, our study shows that TK2 mutations should be considered when searching for the molecular background of progressive muscle disease with SMA I-like clinical severity. Furthermore, we show that TK2 gene can also underlie MDS with brain and liver involvement and that the tissue specificity of the manifestation may be modified by single missense mutations.

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