Human Molecular Genetics, 2012, Vol. 21, No. 1 **66–75**
doi:10.1093/hmg/ddr438
Advance Access published on September 21, 2011

Thymidine kinase 2 mutations in autosomal recessive progressive external ophthalmoplegia with multiple mitochondrial DNA deletions

Henna Tyynismaa[1,*,†], Ren Sun[4,†], Sofia Ahola-Erkkilä[1], Henrikki Almusa[2], Rosanna Pöyhönen[1], Mari Korpela[5], Jari Honkaniemi[6], Pirjo Isohanni[1], Anders Paetau[3], Liya Wang[4], and Anu Suomalainen[1,2,5]

[1]Research Programs Unit, Molecular Neurology, Biomedicum Helsinki, Haartmaninkatu 8, [2]Institute for Molecular Medicine Finland (FIMM) and [3]Department of Pathology, University of Helsinki, Helsinki 00290, Finland, [4]Department of Anatomy, Physiology and Biochemistry, Section of Veterinary Medical Biochemistry, SLU, The Biomedical Centre, Uppsala SE-751 23, Sweden, [5]Department of Neurology, Helsinki University Central Hospital, Helsinki 00290, Finland and [6]Tampere University Hospital, Tampere 33520, Finland

Received August 4, 2011; Revised and Accepted September 19, 2011

Autosomal-inherited progressive external ophthalmoplegia (PEO) is an adult-onset disease characterized by the accumulation of multiple mitochondrial DNA (mtDNA) deletions in post-mitotic tissues. Mutations in six different genes have been described to cause the autosomal dominant form of the disease, but only mutations in the DNA polymerase gamma gene are known to cause autosomal recessive PEO (arPEO), leaving the genetic background of arPEO mostly unknown. Here we used whole-exome sequencing and identified compound heterozygous mutations, leading to two amino acid alterations R225W and a novel T230A in thymidine kinase 2 (*TK2*) in arPEO patients. TK2 is an enzyme of the mitochondrial nucleotide salvage pathway and its loss-of-function mutations have previously been shown to underlie the early-infantile myopathic form of mtDNA depletion syndrome (MDS). Our TK2 activity measurements of patient fibroblasts and mutant recombinant proteins show that the combination of the identified arPEO variants, R225W and T230A, leads to a significant reduction in TK2 activity, consistent with the late-onset phenotype, whereas homozygosity for R225W, previously associated with MDS, leads to near-total loss of activity. Our finding identifies a new genetic cause of arPEO with multiple mtDNA deletions. Furthermore, MDS and multiple mtDNA deletion disorders are manifestations of the same pathogenic pathways affecting mtDNA replication and repair, indicating that MDS-associated genes should be studied when searching for genetic background of PEO disorders.

INTRODUCTION

The circular 16.6 kb multi-copy genome of human mitochondria, the mitochondrial DNA (mtDNA), relies completely on proteins that are encoded by nuclear genes and imported into the mitochondria for its replication, copy number maintenance and repair. Therefore, mitochondrial diseases with mtDNA instability—mtDNA copy number depletion or multiple deletions—show autosomal inheritance (1,2). MtDNA depletion syndromes (MDS) manifest as severe, tissue-specific diseases of early childhood, whereas multiple mtDNA deletions accumulate during years and lead to adult-onset symptoms typically in the skeletal muscle and in the nervous system (3).

Adult-onset progressive external ophthalmoplegia (PEO) is a typical manifestation of multiple mtDNA deletions (4). The patients have mitochondrial myopathy with ptosis and weakness of the external eye muscles and in skeletal muscles and may have additional symptoms including sensory axonal neuropathy, optic atrophy, ataxia, hypogonadism and parkinsonism

*To whom correspondence should be addressed. Tel: +358 919125654; Fax: +358 919125610; Email: henna.tyynismaa@helsinki.fi
†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

(5–8). The molecular background of autosomal recessive PEO (arPEO) is not well known, as only mutations of mitochondrial replicative polymerase, *POLG* (MIM157640), have been described (9). Autosomal dominant PEO (adPEO) can be caused by mutations in genes that encode mtDNA replication proteins [*POLG* (9) and *POLG2* (MIM610131) (10) encoding the mitochondrial replicative DNA polymerase gamma and *C10Orf2* (MIM609286) (11) encoding for the replicative helicase Twinkle] or mitochondrial nucleotide pool maintenance proteins [*SLC25A4* (MIM609283) (12) encoding the adenine nucleotide translocator 1 (ANT1) and *RRM2B* (MIM613077) (13) encoding the ribonucleotide reductase small subunit p53R2], or *OPA1* [MIM605290] (14) encoding a protein involved in mitochondrial inner membrane fusion. Recessive defects in POLG, p53R2 and Twinkle also cause infantile mitochondrial encephalohepatopathies with mtDNA depletion (15). Tissue-specific MDS can be caused by mutations in the mitochondrial thymidine kinase 2 (*TK2*) [MIM609560] (16), the deoxyguanosine kinase *DGUOK* [MIM251880] (17) and *MPV17* [MIM256810] (18). Therefore, defects in proteins required for mtDNA replication or in those maintaining balanced nucleoside pools can result in either PEO or MDS, depending on the severity of the defect. MtDNA depletion and deletions may also co-exist in a patient, as occasionally seen in mitochondrial neurogastrointestinal encephalopathy (MNGIE), caused by mutations in the gene encoding thymidine phosphorylase (*TYMP*, MIM603041) (19), or in disorders caused by some *POLG* mutations (20).

Here we studied the molecular background of arPEO and multiple mtDNA deletions by whole-exome sequencing and describe that mutations in *TK2*, previously associated with MDS, can also underlie adult-onset mitochondrial myopathy.

RESULTS

Patients with arPEO

The two patients studied here (II:4 and II:5 in Fig. 1A) were daughters of healthy, non-consanguineous parents. The typical features of their disease included symmetrical blepharoptosis with age-of-onset at 40s, requiring blepharoplasty. Mildly restricted eye movements were noted, with slowly progressive ophthalmoplegia from late 40s to early 50s and slowly progressive proximal muscle weakness, preventing rising up from squatting position or walking the stairs, developed by the age of 50. Both developed muscle atrophy, one with scapular winging. One of the patients developed dysarthria because of facial muscle weakness and both reported dysphagia. Both patients were independently suspected for skin sarcoidosis. The patients died of non-neurological causes, i.e. metastatic breast cancer and acute pancreatitis.

Muscle biopsy analysis showed mitochondrial myopathy, with up to 10% COX-negative, succinate dehydrogenase (SDH)-positive ragged-red fibers and slight atrophic changes including fibrosis in late stage. One patient sample showed inflammation with T-cells, but no typical findings of inclusion body myositis. In a follow-up biopsy study, inflammation was not seen. Electroneuromyography showed myopathic findings, but no neuropathy. Serum lactate of patient II:4 and creatine kinase of patient II:5 were mildly elevated.

Multiple mtDNA deletions were detected in skeletal muscle by Southern blot analysis (Fig. 1C). MtDNA copy number, analyzed by real-time quantitative PCR utilizing the non-deleted gene region of 12S rRNA, was 60% of the controls' mean ($n = 18$) in the muscle of patient II:4 (Fig. 1B), which does not fill consensus criteria (<40%) of mtDNA depletion (21). The muscle DNA sample of patient II:5 was not available for mtDNA copy number analysis. Long-range PCR of mtDNA showed the presence of deleted mtDNA molecules in several regions of the brain but not in the cerebellum, heart or liver (Fig. 1D). Direct sequencing of the mitochondrial genome and the coding sequences of the previously identified PEO genes (*POLG*, *C10orf2*, *RRM2B*, *SLC25A4*, *POLG2*, *OPA1*) revealed no pathogenic mutations.

Identification of TK2 mutations in arPEO by whole-exome sequencing

We sequenced the exon regions of the genome of patient II:4 by whole-exome next-generation sequencing technology. Exome targets of the patient's DNA were captured with the NimbleGen Sequence Capture 2.1M Human Exome v1.0 Array and sequenced with the use of the Illumina Genome Analyzer-IIx platform with 2 × 82 bp paired-end reads. The sequence reads covered 98.7% of targets (20-fold coverage 85.9%). The Pileup utility from the SAMTOOLS package was used for variant calling (22).

As a result of variant calling, 94 061 SNPs were identified. Those were then filtered for variants, which were not listed in dbSNP130 or in '1000 Genomes Project' and were within gene regions. Of the resulting 1775 variants, 149 were homozygous and 1626 were heterozygous (Fig. 2A). All homozygous variants, and of the heterozygous variants those of which two or more mapped to the same gene, were first subjected to a scan of known mitochondrial disease genes. Compound heterozygous missense mutations c.673C>T [p.R225W] and c.688A>G [p.T230A] (RefSeq accession no. NM_004614.3) in *TK2* were identified. Sanger sequencing confirmed the findings in both patients. DNA samples of the patients' parents or siblings were not available for the study and therefore we PCR-amplified, cloned and sequenced the corresponding genomic region in *TK2* of one of the patients and confirmed that the mutations were not allelic (Fig. 2B). The c.673C>T [p.R225W] mutation has previously been identified as homozygous or compound heterozygous (with R172W) in Finnish and Swedish pediatric patients to cause a severe myopathic form of infantile MDS (23,24). We did not find carriers of this mutation among 400 healthy Finnish control chromosomes. The c.688A>G [p.T230A] mutation T230A has not been previously described. Screening of 400 control chromosomes identified one heterozygous carrier of the novel mutation. We also screened *TK2* for mutations in samples from another recessive PEO family and from six sporadic PEO patients, but did not identify mutations.

Multiple sequence alignment of TK2 protein shows that the altered R225 and T230 residues are located within a highly conserved sequence block but are themselves not fully conserved even among vertebrates (Fig. 2C). The R225 is

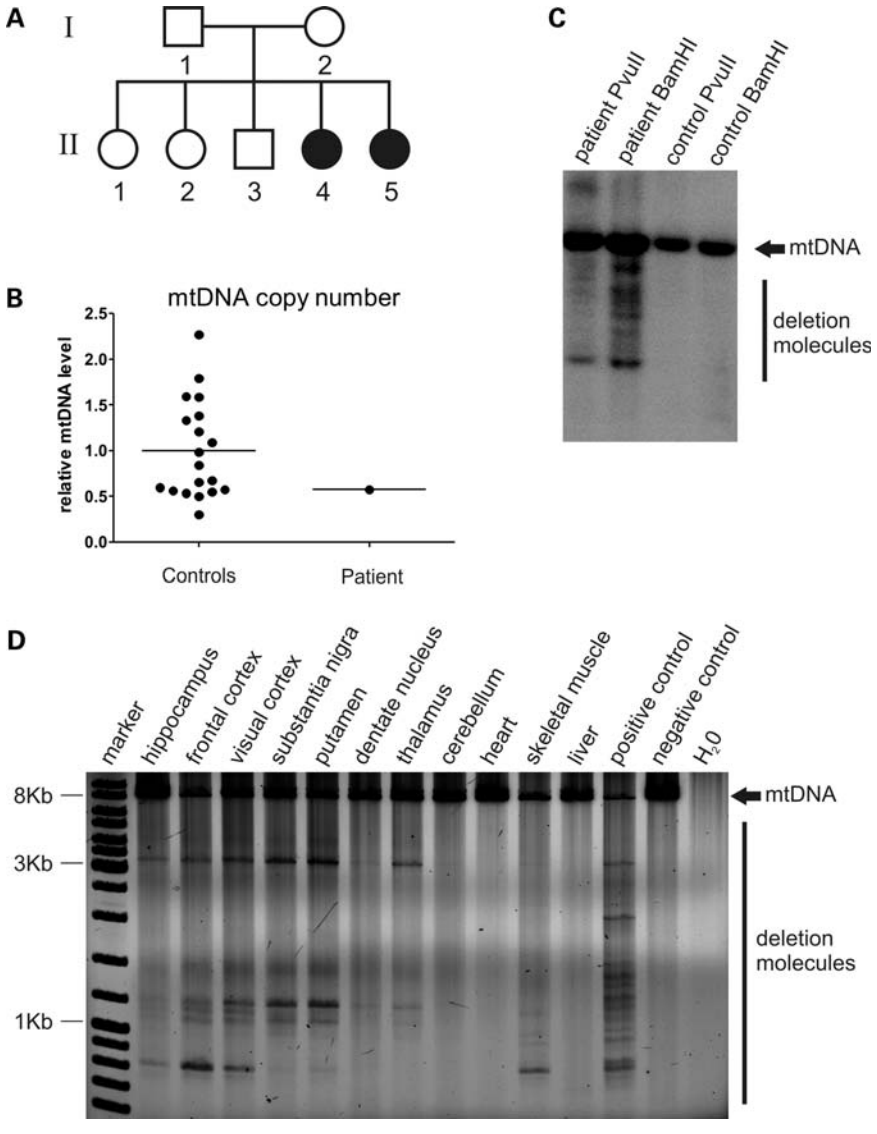

Figure 1. Mitochondrial DNA analysis. (**A**) Family pedigree. (**B**) The mtDNA copy number was determined by quantitative real-time PCR in skeletal muscle samples of 18 control individuals and patient II:4. Values are shown relative to the average of all control samples. (**C**) Southern analysis of mtDNA deletions shows multiple deletion molecules in the skeletal muscle sample of patient II:5, whereas only the full-length mtDNA is present in the control muscle sample. (**D**) Long-range PCR analysis of mtDNA deletions in multiple tissues of patient II:4. The heart, cerebellum and liver are devoid of deletion molecules, whereas some deletion molecules are visible in other brain regions and in the skeletal muscle. The positive control is a muscle sample from a patient with adPEO caused by a *RRM2B* mutation (13).

conserved from man to mouse but glutamine was found instead of arginine in that position in chicken, zebrafish and frog. T230 is conserved in many mammals and in zebrafish and frog but a valine or isoleucine is present in chicken and rodent TK2, respectively.

We carefully studied whether five previously identified heterozygous carriers of the c.673C>T [p.R225W] mutation (23) manifested any symptoms of mitochondrial disease. Family histories included hearing impairment, hypothyroidism, epilepsy, autism and mental retardation, but the carrier status did not cosegregate with any of the symptoms. Therefore, we concluded that c.673C>T [p.R225W] as a heterozygote did not cause mitochondrial disease in the studied families.

Structural modeling of the TK2 mutants

TK2 catalyzes the adenosine triphosphate (ATP)-dependent phosphorylation of deoxythymidine (dThd) and deoxycytidine (dCyt) in mitochondria. We built a TK2 structural model based on the structure of its homolog, the *Drosophila melanogaster* deoxynucleoside kinase (Dm-dNK), which has ~40% sequence identity to human TK2 (Fig. 3A). The overall structure of TK2 is similar to that of Dm-dNK with an α/β-architecture where a five-stranded parallel β-sheet together with α-helices forms the active site. The lid region and p-loop structure are important structural elements in accommodating the phosphate groups of ATP (25). As shown in the model structure, R225 is located on the tip of β4 and is

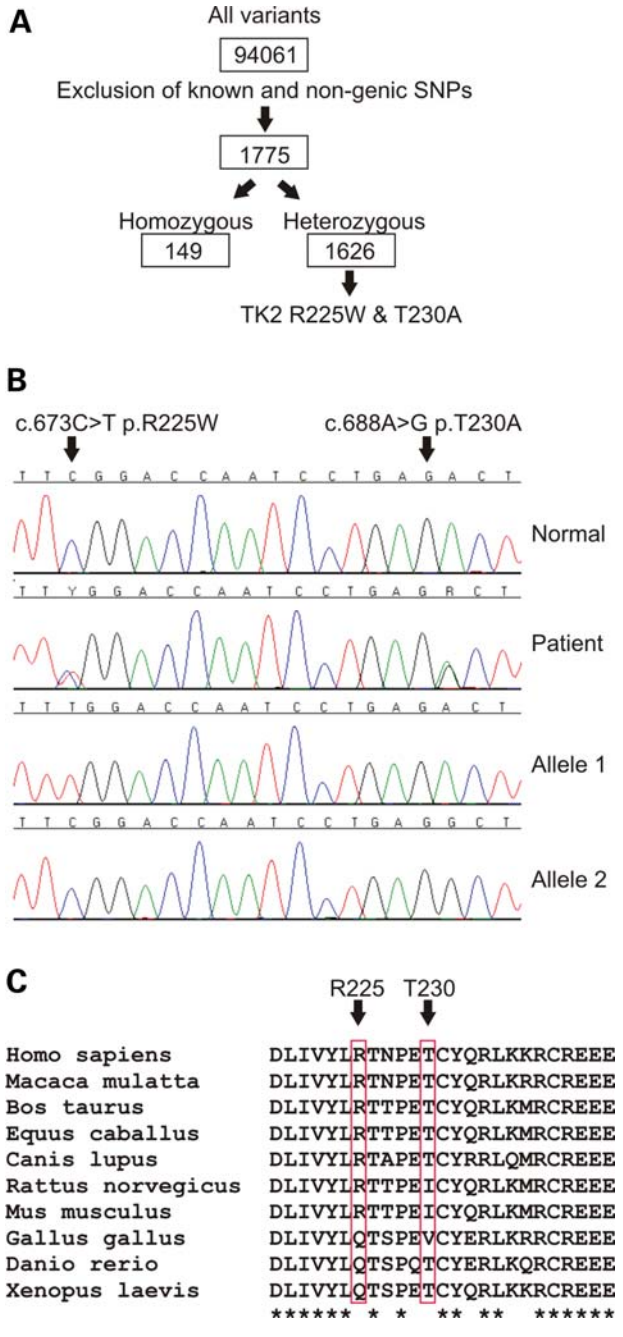

Figure 2. Exome sequencing in the identification of *TK2* mutations in arPEO. (**A**) Schematic representation of the exome data analysis and data filtering. The known SNP variants were excluded assuming the infrequency of the pathogenic variant. The homozygous and heterozygous variants were then screened for those affecting mitochondrial proteins. (**B**) DNA sequences of normal and patient's *TK2* gene. The result of the allelic cloning shows that the two mutations are compound heterozygous. (**C**) Cross-species protein conservation of *TK2*, flanking the altered amino acids p.R225W and p.T230A. The asterisks below the amino acids indicate fully conserved residues and show that the mutations are within a highly conserved sequence block but affect residues that are not fully conserved.

not directly involved in substrate binding. R225 is conserved in Dm-dNK and TK2, and the corresponding residue in human deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK) is asparagine (Fig. 3B). In the Dm-dNK structure with bound dCyd, ATP was modeled in the structure based on the structure homology to the herpes simplex virus TK, and according to this structure, R225 is located where the base moiety of ATP should bind (26). Substitution of R225 with a tryptophan is predicted to lead to the occupation of adenine-binding cleft with the bulkier side chain of tryptophan and loss of hydrogen bonds with surrounding amino acids. This is predicted to affect the binding affinity of ATP as well as the rate of catalysis.

The T230 residue is located in α8 which together with α9 forms the lid region (Fig. 3A), which is involved in the binding of phosphate groups of ATP and thus in catalysis. T230 is not conserved among the deoxynucleoside kinases (Fig. 3B), and the corresponding residue in human dCK is a threonine but in human dGK and Dm-dNK it is a valine. T230 is not directly involved in the binding of any substrate but indirectly through a hydrogen bond network to R234. This residue is conserved in all deoxynucleoside kinases and has been shown to interact with the phosphate donors for human dCK (27). Substitution of T230 with alanine is predicted to alter the hydrogen bonding pattern of R234, which could lead to lower catalytic rates.

TK2 activity of T230A and R225W mutants

Loss of TK2 activity is known to result in severe infantile MDS. Therefore, our finding of mutations in less conserved residues may be consistent with the late-onset mtDNA depletion phenotype. To test this, we expressed the identified mutants as recombinant proteins in *Escherichia coli* and measured their TK2 activity. Both R225W and T230A mutant proteins were purified and characterized with their natural substrates dThd, dCyd and ATP. R225W mutant had a higher K_m value for dThd and a similar K_m value for dCyd but the V_{max} values were very low when compared with the wt enzyme and the efficiency of R225W mutant was only ∼5% with dThd and ∼7% with dCyd when compared with the wt enzyme. Similarly, the T230A mutant had a higher K_m value for dThd but a similar K_m value for dCyd when compared with the wild-type enzyme. The V_{max} values decreased significantly with both substrates, resulting in low efficiency (V_{max}/K_m), ∼30% with dThd and 52% with dCyd when compared with the wt enzyme (Table 1).

When ATP was the variable substrate, both mutant enzymes exhibited higher K_m values and lower V_{max} values, which led to low catalytic efficiencies. T230A had an efficiency of 17 and 22%, whereas R225W had only 3 and 4% of activity when compared with the wt enzyme for the respective nucleosides (Table 1).

Recombinant human TK2 has been reported as a dimer or tetramer (28) and the native TK2 purified from human tissues as a monomer (29). Recombinant human TK2 proteins used in this study behaved like a monomer, and to evaluate the combined effect of the two mutants on total TK2 activity, we assayed both mutants simultaneously in an assay mixture containing different ratios of each mutant. As shown in Table 2, the total TK2 activity was decreased when the amount of R225W increased in the presence of standard amount of T230A, since R225W was less active. At 1:1 ratio, the TK2 specific activity was 22% of the wt TK2 activity. When only

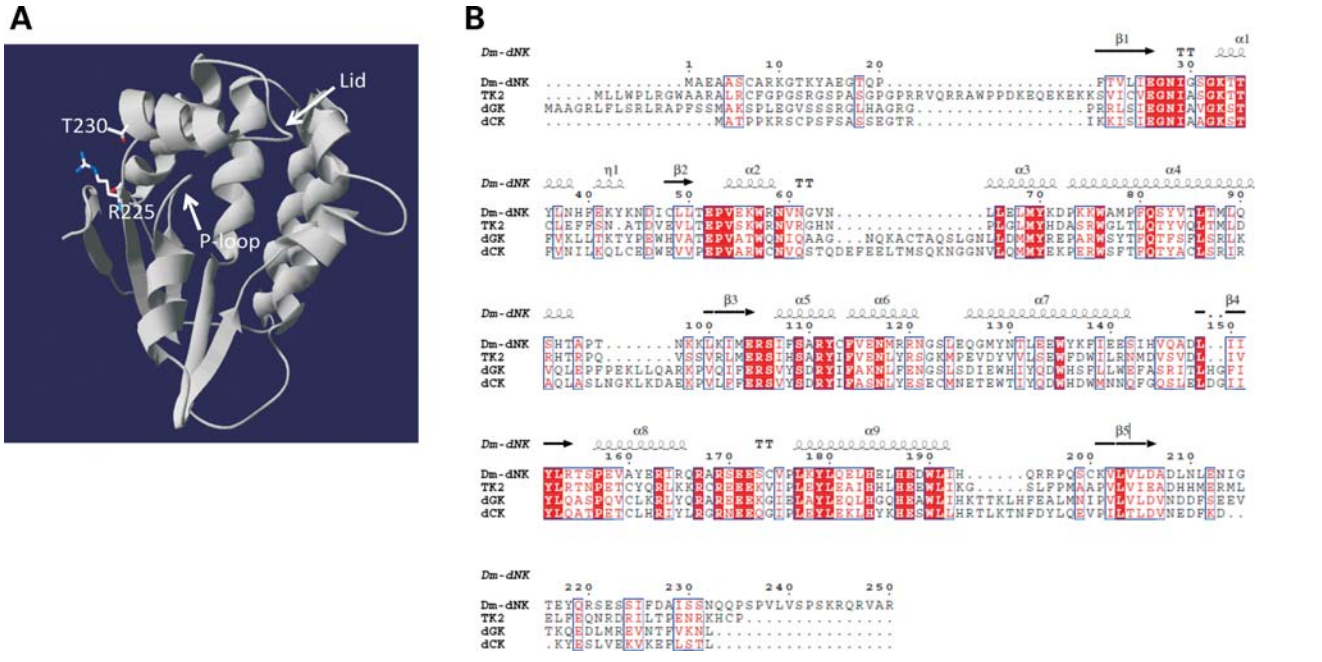

Figure 3. Modeled human TK2. (**A**) The TK2 model structure based on the structure of Dm-dNK. Residues R225 and T230 are indicated. (**B**) Amino acid sequence alignment of Dm-dNK (accession no. AAF55615) with human TK2 (accession no. NP_004605), dGK (accession no. NP_550438) and dCK (accession no. CAG38764). The secondary structure of Dm-dNK is shown on the top of the sequence.

Table 1. Steady-state kinetic parameters of recombinant wild-type and mutant TK2

	Variable dThd			Variable dCyd		
	K_m (μm)	V_{max} (nmol/min/mg)	V_{max}/K_m (%)	K_m (μm)	V_{max} (nmol/min/mg)	V_{max}/K_m (%)
Wild-type	2.9 ± 0.2	888.6 ± 34.8	306.4 (100)	11.7 ± 1.0	839.8 ± 14	71.8 (100)
T230A	6.0 ± 0.4	564.7 ± 28.1	94.1 (30.7)	13.8 ± 2.4	522.8 ± 40	37.9 (52.8)
R225W	5.1 ± 0.5	89.5 ± 40.9	17.5 (5.7)	12.7 ± 0.4	71.3 ± 29	5.6 (7.8)
	Variable ATP/fixed dThd			Variable ATP/fixed dCyd		
Wild-type	1.8 ± 0.4	969.8 ± 66	539 (100)	2.7 ± 0.1	582.9 ± 64.6	215.9 (100)
T230A	5.2 ± 1.7	485.1 ± 2.4	93 (17.3)	7.7 ± 2.5	370.3 ± 21.5	48.1 (22.3)
R225W	4.8 ± 0.4	85.0 ± 37	18 (3.3)	6.0 ± 1.9	57.4 ± 21.1	9.6 (4.4)

Data were from assays of two batches of enzyme preparations and given as means ± SD. Data in parentheses are the relative efficiency (V_{max}/K_m) of the mutant when compared with that of the wild-type set to 100. The concentrations of dThd and dCyd were varied from 0.5 to 41 μm in the presence of 2 mM ATP. With ATP as the variable substrate (10–2000 μm), the concentration of dThd or dCyd was kept at 50 μm.

T230A was present, the TK2 activity was 38% of the wt TK2 activity. When R225W was kept constant with increasing amount of T230A, the total activity reached the maximum of 33% of the wt TK2 activity (Table 2).

TK2 activity levels in mitochondria isolated from fibroblasts derived from the patients

Mitochondrial TK2-specific activity was determined using tritium-labeled dThd and dCyd at saturating conditions. The TK2-specific activity with dThd and dCyd was 22 and 28%, respectively, of the control values for patient II:4, whereas they were 42 and 54%, respectively, of the control values for patient II:5. A notable change was the ratio of the specific activities with dThd and dCyd, which was higher in the two patients when compared with controls (Table 3).

DISCUSSION

Here we report TK2 mutations in adult-onset arPEO with mtDNA instability. By whole-exome sequencing, we identified compound heterozygous mutations, leading to amino acid changes R225W and T230A, in *TK2*. Both the *in vitro* activity of recombinant mutant proteins and the TK2 activity in patients' cells indicated a functional defect caused by the two mutations, confirming that *TK2* is a novel gene for arPEO.

TK2 is a key enzyme in the mitochondrial nucleotide pool maintenance, and therefore essential for mtDNA synthesis. For mammalian mitochondria, dNTPs are generated via two

Table 2. Specific activity (nmol/min/mg) of recombinant T230A and R225W mutant proteins mixed at different ratios

Ratio	T230A:R225W (%)	R225W:T230A (%)
1:0	338.0 ± 11 (38)	18.0 ± 1.6 (2.0)
1:1	198.3 ± 31 (22)	198.3 ± 31 (22)
1:2	138.9 ± 12 (16)	243.2 ± 26 (27)
1:5	78.1 ± 1.9 (8.8)	289.0 ± 27 (33)
1:10	56.9 ± 3.7 (6.4)	265.1 ± 11 (30)

Assays were performed with 10 μm tritium-labeled dThd as the substrate. Data are given as means ± SD. Data in parentheses are percent activity when compared with the wild-type TK2 with a specific activity of 888.6 nmol/min/mg for dThd.

Table 3. TK2 activity (pmol/min/mg) in mitochondrial extracts from control and patient fibroblasts (pmol/min/mg)

	dThd (%)	dCyd (%)	Ratio dCyd/dThd
Control	59.6 ± 5.1 (100)	40.3 ± 4.3 (100)	0.67
Patient II:4	13.3 ± 1.5 (22)	11.3 ± 0.5 (28)	0.85
Patient II:5	25.0 ± 0.1 (42)	21.9 ± 0.2 (54)	0.88

Assays were performed with 10 μm tritium-labeled dThd or dCyd as the substrate in the presence of 2 mM ATP as described in Materials and Methods and data are given as means ± SD.

complementary pathways: the cytoplasmic *de novo* and the mitochondrial salvage pathway. TK2 participates in the pyrimidine deoxynucleoside salvage for mtDNA maintenance in post-mitotic tissues by phosphorylating dThd, dCyd and deoxyuridine as a first step of generating dNTP substrates for mtDNA replication. Recessive loss-of-function mutations in *TK2* cause the myopathic form of MDS (16,23). The disease manifests during the first and second year of life, with a rapidly progressive, early fatal myopathy that sometimes mimics spinal muscular atrophy (16,23,30). Disorders with mtDNA depletion or multiple deletions have been suggested to form a continuum, the mtDNA manifestation and age-of-onset depending on the severity of the functional defect in a given protein (3). Indeed, *RRM2B* mutations may manifest as recessive infantile lethal multisystem disorders with MDS (31), as recessive juvenile encephalopathies with mtDNA depletion or deletions (32) or as dominant adult-onset PEO with multiple mtDNA deletions (13). Similar patterns of manifestations occur with defects of mtDNA replication polymerase gamma or helicase Twinkle (9,11,33,34), and typically mtDNA depletion associates with early-onset disorders with fatal disease course, whereas adult-onset disorders show multiple mtDNA deletions. The previous examples and the current *TK2* mutations in both MDS and arPEO indicate that all MDS genes should be considered as candidates for autosomal PEO, and vice versa.

The TK2-arPEO is among the latest onset of PEO disorders with multiple mtDNA deletions. In clinical manifestation, it resembles closely adPEO caused by defects in another protein of nucleotide synthesis, p53R2. Both disorders manifest as late-onset, slowly progressive myopathies, with no indication of peripheral nervous system involvement. However, mild late-onset CNS involvement cannot be excluded, as our patient showed dysphagia and dysarthria, combined with partial Purkinje-cell loss. *TK2* mutations should be suspected in sporadic patients with late-onset PEO with multiple mtDNA deletions.

We evaluated the functional effects of the two mutations by characterization of purified recombinant mutant enzymes and by structural analysis. The mutations were found to reduce TK2 activity and alter the specificities of dThd and dCyd phosphorylation. Kinetic studies of the purified mutant enzymes showed that mutations affected the binding affinities of dThd and ATP but not dCyd to serve as substrates, suggesting that amino acid residues involved in substrate binding are different for dThd and dCyd. The major impact of these two point mutations on TK2 activity was decreases in V_{max} values with both substrates.

The TK2 structural model predicted that the substitution of R225 with a tryptophan may lead to the occupation of adenine-binding cleft by the bulky side chain of tryptophan and loss of hydrogen bonds with the surrounding amino acids. As demonstrated with enzyme kinetic studies of the recombinant mutant enzyme, R225W mutation resulted in lower binding affinity, ~2-fold changes of the K_m value with ATP. The catalytic rate of R225W was only ~3–4% when compared with the wild-type enzyme. Substitution of T230 with an alanine probably altered the hydrogen bonding pattern of R234, an important residue in ATP binding and catalysis, which may lead to lower catalytic rates. The T230A mutant had higher K_m values for ATP and dThd and lower V_{max} values for all substrates. These findings support the pathogenic nature of the two identified TK2 mutations.

The R225W has been previously identified in Finnish pediatric MDS patients as a homozygous mutation (23). We found the TK2 activity of the recombinant R225W mutant protein to be very low, consistent with the severe mtDNA depletion when present as a homozygote. The longest surviving R225W homozygote patient had 95% depletion at 21 months of age (23). The R225W mutant is undoubtedly a near-total loss-of-function allele. However, we found no indication that heterozygous carriers would have disease symptoms.

The T230A mutation has not been previously described. We found it as a heterozygous variant in one of our Finnish control chromosomes, suggesting enrichment within the genetically isolated country. The T230 residue was conserved among species but not fully, which was also true for the R225. Both residues were located within a highly conserved sequence block but the lesser degree of conservation of the mutated residues themselves suggested that some residual TK2 activity may be retained in the mutant proteins. We found the TK2 activity of the recombinant T230A protein to be reduced compared with that of the wild-type but exceeding that of the R225W mutant. In a ratio of 1:1, the R225W and T230A combination showed 22% specific activity in comparison with the wild-type. The decreased TK2 activities measured in the mitochondria of patient fibroblasts supported the recombinant protein findings. These results are consistent with the compound heterozygous mutations as the cause of adult-onset PEO in our patients.

Two major genetic causes are known to lead to the accumulation of multiple mtDNA deletions in post-mitotic tissues.

First, dominant mutations in genes encoding for mtDNA replication proteins (POLG, POLG2, Twinkle) have been suggested to lead to increased replication stalling and subsequently to double-stranded DNA breaks that predispose to incorrectly repaired mtDNA molecules (35,36). The second group of defects (p53R2, ANT1) affects the balance of nucleoside/nucleotide supply for mtDNA replication (12,37). These could also induce replicase stalling and double-strand breaks, because of the low availability of DNA building blocks and thereby mutagenesis. In MNGIE, intramitochondrial dTTP levels are elevated because the defective thymidine phosphorylase is unable to catabolize thymidine (38), which according to a recent *in vitro* model leads to mtDNA instability because an excess of dTTP limits dCTP availability (39). Our study has identified TK2 mutations as a new cause for altered nucleotide pools underlying mtDNA deletion disease. Compromised phosphorylation of deoxypyrimidines in mitochondria is expected to lead to unbalanced dTTP and dCTP pools, which could stall mtDNA replication. Based on our findings of *RRM2B* (13) and *TK2* mutations in adult-onset mtDNA instability disorders, both the cytoplasmic *de novo* synthesis and the mitochondrial nucleoside salvage pathway are essential also in adult skeletal muscle to maintain balanced nucleotide pools for mtDNA replication.

In summary, we have identified TK2 mutations as a new cause of arPEO with multiple mtDNA deletions. Whole-exome sequencing combined with the knowledge of mitochondrial proteome is a powerful combination to identify genetic causes of mitochondrial disease.

MATERIALS AND METHODS

Subjects

All samples were taken according to the Declaration of Helsinki, with informed consent. The study was performed with the research permit from the Department of Neurology, Helsinki University Central Hospital. Patient II:4 was a woman, who developed symmetrical progressive ptosis from the age of 47 years, treated with blepharoplasty at the age of 52. She had proximal leg muscle weakness with mild muscle atrophy (difficulty in rising up from squatting position and in heel walking) from the age of 49 years and PEO from the age of 52 years. Muscle weakness symptoms in the legs were slowly progressive, but she could walk unaided at the age of 64. She developed mild dysarthria and dysphagia. She had skin sarcoidosis since the age of 49, and later during her 60s, she needed medication for high blood pressure and hypercholesterolemia. At the age of 64, she suffered from pancreatitis and deceased because of associated pneumonia and respiratory insufficiency.

Muscle MRI at the age of 55 years showed reduced mass of both quadriceps muscles and focal muscle atrophy in the distal end of left musculus vastus intermedius. EMG was myopathic with signs of polymyositis and spontaneous activity in the proximal musculature. Single-fiber EMG was normal. Serum lactate was marginally increased (2.8, normal value $<$2.4 mmol/l), but creatine kinase was normal. In the spiroergometric examination, the patient had reduced aerobic performance with the total residual performance of 45% of age- and sex-matched controls.

Muscle biopsy from tibialis anterior muscle showed signs of mild myositis with T-cell infiltration, but with no typical findings of inclusion body myositis. In histochemical analysis, up to 10% of fibers were cytochrome *c* oxidase (COX)-negative, SDH-positive and up to 3–4% were ragged red fibers. Second biopsy sample from vastus lateralis a couple of years later was myopathic with mild endomysial fibrosis without any signs of clear inflammation. Neuropathologic examination showed findings consistent with mitochondrial myopathy, with no indication of neuropathy. Hippocampus and thalamus showed anoxic-ischemic lesions and the cerebellum mild dropout of Purkinje cells, which were considered to be a likely consequence of terminal ischemia.

Patient II:5 was a woman, who was healthy until the age of 30/40 years, when she developed bilateral ptosis, operated three times at the age of 41, 46 and 48. In her early 40s, she developed both proximal arm and leg weakness. This weakness gradually increased but she did not seek for medical advice until at the age of 54 years. At his time, she had mild ophthalmoplegia and difficulties in walking because of the proximal weakness of leg musculature. At the age of 59, the patient showed only minor ptosis with normal eye movements. There was no weakness in facial musculature, also speech, articulation as well as swallowing were normal, although she reported mild dysphagia. Scapular winging was noted on both sides. She used the help of her arms to rise up from the squatting position. Also, muscle weakness in the trunk was noticed. Serum creatine kinase value was mildly over normal limits, 183 U/l (normal values 0–150), and serum lactate was normal, 0.70 mmol/l (normal values 0.70–1.80). In spiroergometric examination (simultaneous testing of ergometric performance, oxygen utilization and carbon dioxide production, with serum lactate monitoring), she showed reduced performance of 70% compared with age- and sex-matched controls.

Muscle biopsy from vastus lateralis muscle showed typical signs of mitochondrial myopathy with ~10% of negatively staining fibers in COX histochemistry and around 5% of ragged-red fibers. There were no signs of fibrosis, fat accumulation or inflammation.

Other medical conditions included erythema nodosum and sarcoidosis of the skin and the lungs which was diagnosed at the age of 41 and spontaneously cured in 3 years. Breast cancer with lymph node metastasis was diagnosed at the age of 60. The patient died due to metastatic breast cancer at the age of 70. No autopsy was performed.

MtDNA deletion analysis

Total DNA extracted from patient and control tissues was used for both the Southern and long-range PCR analysis of mtDNA deletions. For the Southern blot, DNA was restriction digested with *Bam*HI and *Pvu*II restriction enzymes (Fermentas). Both linearize mtDNA but from separate sites. The samples were electrophoresed through a 0.8% agarose gel. The DNA was transferred on a Hybond-N+ (GE Healthcare) membrane, which was then hybridized with a ^{32}P-labeled probe, cloned

fragment of mtDNA (nt 1–740). The radioactive signal was detected on an autoradiography film.

Long-range PCR analysis was performed using Phusion polymerase (New England BioLabs) with GC buffer, and 15 ng of genomic DNA was used as the template. The PCR amplifications were done according to the manufacturer's instruction with an annealing temperature of 63°C and an extension time of 12 min. Primers used for this assay were located at nt 8238–8262 (forward) and 16 496–16 465 (reverse), amplifying a 8.2 kb product from the normal mtDNA molecule and often harboring mtDNA deletions in patients.

MtDNA depletion analysis

MtDNA was quantified using real-time quantitative PCR. A portion of the 12S ribosomal RNA gene was used as the mitochondrial gene target and a portion of a single copy gene amyloid precursor protein (APP) as the nuclear gene target. The QPCR reaction was performed using DyNAmo^{TM} Flash SYBR Green QPCR Kit (Finnzymes) with primers 5′-AAACTGCTCGCCAGAACACT-3′ and 5′-TAGGCTGA GCAAGA GGTGGT-3′ for 12S rRNA and 5′-TGTGT GCTCTCCCAGGTCTA-3′ and 5′-CAGTTCTGGATGGTC ACTGG-3′ for APP. The PCR reactions were performed in triplicate and the mean values of the measurements were used for the analyses.

MtDNA sequencing

MtDNA sequencing was performed by PCR amplification of the genome in two fragments, followed by sequencing with several internal primers as recently described in Gotz *et al.* (40).

Exome sequencing

The patient's genomic DNA was isolated from cultured fibroblasts and the exome targets were captured with the NimbleGen Sequence Capture 2.1M Human Exome v1.0 Array (Roche NimbleGen) and sequenced with the use of the Illumina Genome Analyzer-IIx platform (Illumina). The 2 × 82 bp paired-end sequence reads were first trimmed from any possible B quality block from the end of reads, removing any pair which had a read smaller than 36 bp. Then the quality scores were converted to the Sanger phred score using Emboss (version 6.3.1) (41) and aligned to the hg19 reference genome with the Burrows–Wheeler Alignment (version 0.5.8C) tool (42). Any potential PCR duplicates were removed using Picard MarkDuplicates (version 1.32). The Pileup utility from the SAMTOOLS package was used for variant calling (22). The resulting SNPs were then recalculated using quality values. A ratio between the sum of quality values of reference (R) and variant (V) calls was calculated: $R/(R + V)$, discarding any where the ratio was above 0.8. If there were two variant calls and no reference, the call with a higher quality value was used in the place of a reference. Any call with a ratio smaller than 0.2 was assumed to be homozygous and the rest heterozygous. The identified SNPs were then filtered for variants, which were not listed in dbSNP130 or in 1000 Genomes Project and were in gene regions. The human MitoCarta was used to extract the variants that were in genes encoding for mitochondrial proteins (43).

Allelic cloning

The genomic region of TK2 harboring the identified mutations was PCR-amplified from patient's DNA, using primers 5′-CAAGATCCTTTTGGGGGAAT-3′ and 5′-CTCTCTCTG CAAACAAGGGC-3′. The PCR product was cloned into pCR 2.1-TOPO (Invitrogen) and several clones were sequenced.

Determination of the carrier frequency

DNA samples of 200 healthy Finnish controls were used to determine the carrier frequencies of the identified mutations. For the R225W mutation, minisequencing method was used with conditions described in Gotz *et al.* (23). For the T230A mutation, DNA samples were subjected to PCR amplification with the primers that were also used for allelic cloning (see above). The PCR products were then digested with the *Bsu*36I restriction enzyme (Thermo Scientific). The product from the normal allele is cut into two fragments, whereas the T230A mutant product is cut into three fragments. Among the 200 controls, one sample was identified to carry a heterozygous T230A mutation by the restriction digestion analysis, and the finding was subsequently confirmed by Sanger sequencing.

Cloning, expression and purification of R225W and T230A mutants

To obtain the R225W and T230A mutant cDNAs, total RNA was first extracted from patient fibroblasts using the Trizol reagent (Invitrogen). The RNA was used to generate cDNA with random primers and M-MLV Reverse Transcriptase (Promega) followed by the amplification of TK2 cDNA with primers 5′-ATGCGACCGGGTCTCTTTAAG-3′ and 5′-CA GCAGGCATTTTTCAGA CA-3′ and Phusion polymerase in HF buffer (New England BioLabs). The products were extracted from agarose gel, cloned into TOPO Blunt (Invitrogen) and sequenced.

For the production of the recombinant proteins, the mutant cDNAs containing either R225W or T230A were PCR-amplified using the primers 5′-CACCACATG CCATCAGT GATCTGTGTCGA-3′ and 5′-CACGGATCCTATGGGC AATGCTTCCGA-3′ and subcloned into the pEXP5-NT/TOPO® vector (Invitrogen) following the manufacturer's instructions and verified by sequencing. Recombinant T230A and R225W proteins, containing an N-terminal fusion 6×His tag, were expressed and purified as previously described for the wild-type human TK2 (44).

Mitochondrial isolation from fibroblasts

The human fibroblast cells were cultured in DMEM medium supplied with 2 mM glutamine and antibiotics. The cells were harvested and washed twice with PBS and mitochondria were isolated essentially as previously described (45). The final mitochondrial pellets were resuspended in buffer containing 5 mM Tris–HCl, pH 7.4, 320 mM sucrose, 2 mM EGTA and

0.5% NP-40. Mitochondrial proteins were extracted by freezing and thawing three times and sonication for 5 min in an ice-water bath. Protein concentrations were determined by the Bio-Rad protein assay using BSA as the standard.

TK2 enzyme assays

Of the radiolabeled substances, [*methyl*-^{3}H]-thymidine (20.0 Ci/mmol) was obtained from PerkinElmer and [5-^{3}H(N)]-2′-deoxycytidine (26.8 Ci/mmol) from Moravek Biochemicals Inc. Non-radioactive nucleosides and ATP were purchased from Sigma.

Wild-type and mutant TK2 activities were determined by the radiochemical method using the DE-81 filter paper (44). The standard reaction mixture (50 μl) contained 50 mM Tris/HCl, pH 7.6, 0.5 mg/ml BSA, 5 mM $MgCl_2$, 2 mM ATP, 5 mM dithiothreitol and [^{3}H]-dThd or dCyd. To determine the kinetic parameters, the concentrations of dThd or dCyd were varied from 0.5 to 41 μm with a fixed concentration of ATP (2 mM) or variable ATP concentration (10–2000 μm) at fixed dThd or dCyd concentration (50 μm). The amount of enzyme used in the reactions was 1 ng (wt), 10 ng (T230A) and 50 ng (R225W). The TK2 activity was given as nanomoles of dTMP/dCMP formed per milligram of protein per minute. The data were analyzed by Sigma Plot Enzyme Kinetic Module version 1.1 (SPSS Inc.) and the K_m and V_{max} values were calculated using the Michaelis–Menten equation.

Mitochondrial TK2 activity was determined using 10 μm [^{3}H]-dThd or [^{3}H]-dCyd as described above and 15 mM NaF was included in the reaction mixture. About 4–10 μg of protein (3 μl) was used in each assay. Data were from two to four independent assays and given as mean ± SEM.

Structural modeling

A human TK2 3D model structure was built by comparative protein modeling using the SWISS-MODEL computer algorithm (46) and the Dm-dNK structure as the template (PDB entry 1OT3). Swiss-PdbViewer was used in the structural and mutational analysis.

ACKNOWLEDGEMENTS

We would like to thank Professor Staffan Eriksson for valuable discussions. We would like to acknowledge the contribution of late Dr Hannu Somer in diagnosing the patients.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Academy of Finland (to H.T. and A.S.), Helsinki Biomedical Graduate School (to S.A.-E.), Sigrid Juselius Foundation and University of Helsinki (to A.S.), the Swedish Research Council and partly by a grant from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (to L.W.).

REFERENCES

1. Moraes, C.T., Shanske, S., Tritschler, H.J., Aprille, J.R., Andreetta, F., Bonilla, E., Schon, E.A. and DiMauro, S. (1991) mtDNA depletion with variable tissue expression: a novel genetic abnormality in mitochondrial diseases. *Am. J. Hum. Genet.*, **48**, 492–501.
2. Zeviani, M., Moraes, C.T., DiMauro, S., Nakase, H., Bonilla, E., Schon, E.A. and Rowland, L.P. (1988) Deletions of mitochondrial DNA in Kearns–Sayre syndrome. *Neurology*, **38**, 1339–1346.
3. Suomalainen, A. and Isohanni, P. (2010) Mitochondrial DNA depletion syndromes—many genes, common mechanisms. *Neuromuscul. Disord.*, **20**, 429–437.
4. Zeviani, M., Servidei, S., Gellera, C., Bertini, E., DiMauro, S. and DiDonato, S. (1989) An autosomal dominant disorder with multiple deletions of mitochondrial DNA starting at the D-loop region. *Nature*, **339**, 309–311.
5. Luoma, P., Melberg, A., Rinne, J.O., Kaukonen, J.A., Nupponen, N.N., Chalmers, R.M., Oldfors, A., Rautakorpi, I., Peltonen, L., Majamaa, K. *et al.* (2004) Parkinsonism, premature menopause, and mitochondrial DNA polymerase gamma mutations: clinical and molecular genetic study. *Lancet*, **364**, 875–882.
6. Melberg, A., Lundberg, P.O., Henriksson, K.G., Olsson, Y. and Stalberg, E. (1996) Muscle-nerve involvement in autosomal dominant progressive external ophthalmoplegia with hypogonadism. *Muscle Nerve*, **19**, 751–757.
7. Servidei, S., Zeviani, M., Manfredi, G., Ricci, E., Silvestri, G., Bertini, E., Gellera, C., Di Mauro, S., Di Donato, S. and Tonali, P. (1991) Dominantly inherited mitochondrial myopathy with multiple deletions of mitochondrial DNA: clinical, morphologic, and biochemical studies. *Neurology*, **41**, 1053–1059.
8. Suomalainen, A., Majander, A., Haltia, M., Somer, H., Lonnqvist, J., Savontaus, M.L. and Peltonen, L. (1992) Multiple deletions of mitochondrial DNA in several tissues of a patient with severe retarded depression and familial progressive external ophthalmoplegia. *J. Clin. Invest.*, **90**, 61–66.
9. Van Goethem, G., Dermaut, B., Lofgren, A., Martin, J.J. and Van Broeckhoven, C. (2001) Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions. *Nat. Genet.*, **28**, 211–212.
10. Longley, M.J., Clark, S., Yu Wai Man, C., Hudson, G., Durham, S.E., Taylor, R.W., Nightingale, S., Turnbull, D.M., Copeland, W.C. and Chinnery, P.F. (2006) Mutant POLG2 disrupts DNA polymerase gamma subunits and causes progressive external ophthalmoplegia. *Am. J. Hum. Genet.*, **78**, 1026–1034.
11. Spelbrink, J.N., Li, F.Y., Tiranti, V., Nikali, K., Yuan, Q.P., Tariq, M., Wanrooij, S., Garrido, N., Comi, G., Morandi, L. *et al.* (2001) Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat. Genet.*, **28**, 223–231.
12. Kaukonen, J., Juselius, J.K., Tiranti, V., Kyttala, A., Zeviani, M., Comi, G.P., Keranen, S., Peltonen, L. and Suomalainen, A. (2000) Role of adenine nucleotide translocator 1 in mtDNA maintenance. *Science*, **289**, 782–785.
13. Tyynismaa, H., Ylikallio, E., Patel, M., Molnar, M.J., Haller, R.G. and Suomalainen, A. (2009) A heterozygous truncating mutation in RRM2B causes autosomal-dominant progressive external ophthalmoplegia with multiple mtDNA deletions. *Am. J. Hum. Genet.*, **85**, 290–295.
14. Hudson, G., Amati-Bonneau, P., Blakely, E.L., Stewart, J.D., He, L., Schaefer, A.M., Griffiths, P.G., Ahlqvist, K., Suomalainen, A., Reynier, P. *et al.* (2008) Mutation of OPA1 causes dominant optic atrophy with external ophthalmoplegia, ataxia, deafness and multiple mitochondrial DNA deletions: a novel disorder of mtDNA maintenance. *Brain*, **131**, 329–337.
15. Isohanni, P., Hakonen, A.H., Euro, L., Paetau, I., Linnankivi, T., Liukkonen, E., Wallden, T., Luostarinen, L., Valanne, L., Paetau, A. *et al.* (2010) POLG1 manifestations in childhood. *Neurology*, **76**, 811–815.
16. Saada, A., Shaag, A., Mandel, H., Nevo, Y., Eriksson, S. and Elpeleg, O. (2001) Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. *Nat. Genet.*, **29**, 342–344.
17. Mandel, H., Szargel, R., Labay, V., Elpeleg, O., Saada, A., Shalata, A., Anbinder, Y., Berkowitz, D., Hartman, C., Barak, M. *et al.* (2001) The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. *Nat. Genet.*, **29**, 337–341.

18. Spinazzola, A., Viscomi, C., Fernandez-Vizarra, E., Carrara, F., D'Adamo, P., Calvo, S., Marsano, R.M., Donnini, C., Weiher, H., Strisciuglio, P. *et al.* (2006) MPV17 encodes an inner mitochondrial membrane protein and is mutated in infantile hepatic mitochondrial DNA depletion. *Nat. Genet.*, **38**, 570–575.
19. Nishino, I., Spinazzola, A. and Hirano, M. (1999) Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. *Science*, **283**, 689–692.
20. Tzoulis, C., Papingji, M., Fiskestrand, T., Roste, L.S. and Bindoff, L.A. (2009) Mitochondrial DNA depletion in progressive external ophthalmoplegia caused by POLG1 mutations. *Acta Neurol. Scand. Suppl.*, **189**, 38–41.
21. Poulton, J. and Holt, I.J. (2009) 163rd ENMC International Workshop: nucleoid and nucleotide biology in syndromes of mitochondrial DNA depletion myopathy 12–14 December 2008, Naarden, The Netherlands. *Neuromuscul. Disord.*, **19**, 439–443.
22. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G. and Durbin, R. (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, **25**, 2078–2079.
23. Gotz, A., Isohanni, P., Pihko, H., Paetau, A., Herva, R., Saarenpaa-Heikkila, O., Valanne, L., Marjavaara, S. and Suomalainen, A. (2008) Thymidine kinase 2 defects can cause multi-tissue mtDNA depletion syndrome. *Brain*, **131**, 2841–2850.
24. Tulinius, M., Moslemi, A.R., Darin, N., Holme, E. and Oldfors, A. (2005) Novel mutations in the thymidine kinase 2 gene (TK2) associated with fatal mitochondrial myopathy and mitochondrial DNA depletion. *Neuromuscul. Disord.*, **15**, 412–415.
25. Johansson, K., Ramaswamy, S., Ljungcrantz, C., Knecht, W., Piskur, J., Munch-Petersen, B., Eriksson, S. and Eklund, H. (2001) Structural basis for substrate specificities of cellular deoxyribonucleoside kinases. *Nat. Struct. Biol.*, **8**, 616–620.
26. Knecht, W., Sandrini, M.P., Johansson, K., Eklund, H., Munch-Petersen, B. and Piskur, J. (2002) A few amino acid substitutions can convert deoxyribonucleoside kinase specificity from pyrimidines to purines. *EMBO J.*, **21**, 1873–1880.
27. Hazra, S., Konrad, M. and Lavie, A. (2010) The sugar ring of the nucleoside is required for productive substrate positioning in the active site of human deoxycytidine kinase (dCK): implications for the development of dCK-activated acyclic guanine analogues. *J. Med. Chem.*, **53**, 5792–5800.
28. Barroso, J.F., Carvalho, R.N. and Flatmark, T. (2005) Kinetic analysis and ligand-induced conformational changes in dimeric and tetrameric forms of human thymidine kinase 2. *Biochemistry*, **44**, 4886–4896.
29. Munch-Petersen, B., Cloos, L., Tyrsted, G. and Eriksson, S. (1991) Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. *J. Biol. Chem.*, **266**, 9032–9038.
30. Oskoui, M., Davidzon, G., Pascual, J., Erazo, R., Gurgel-Giannetti, J., Krishna, S., Bonilla, E., De Vivo, D.C., Shanske, S. and DiMauro, S. (2006) Clinical spectrum of mitochondrial DNA depletion due to mutations in the thymidine kinase 2 gene. *Arch. Neurol.*, **63**, 1122–1126.
31. Bourdon, A., Minai, L., Serre, V., Jais, J.P., Sarzi, E., Aubert, S., Chretien, D., de Lonlay, P., Paquis-Flucklinger, V., Arakawa, H. *et al.* (2007) Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. *Nat. Genet.*, **39**, 776–780.
32. Shaibani, A., Shchelochkov, O.A., Zhang, S., Katsonis, P., Lichtarge, O., Wong, L.J. and Shinawi, M. (2009) Mitochondrial neurogastrointestinal encephalopathy due to mutations in RRM2B. *Arch. Neurol.*, **66**, 1028–1032.
33. Hakonen, A.H., Isohanni, P., Paetau, A., Herva, R., Suomalainen, A. and Lonnqvist, T. (2007) Recessive Twinkle mutations in early onset encephalopathy with mtDNA depletion. *Brain*, **130**, 3032–3040.
34. Naviaux, R.K. and Nguyen, K.V. (2004) POLG mutations associated with Alpers' syndrome and mitochondrial DNA depletion. *Ann. Neurol.*, **55**, 706–712.
35. Goffart, S., Cooper, H.M., Tyynismaa, H., Wanrooij, S., Suomalainen, A. and Spelbrink, J.N. (2009) Twinkle mutations associated with autosomal dominant progressive external ophthalmoplegia lead to impaired helicase function and in vivo mtDNA replication stalling. *Hum. Mol. Genet.*, **18**, 328–340.
36. Krishnan, K.J., Reeve, A.K., Samuels, D.C., Chinnery, P.F., Blackwood, J.K., Taylor, R.W., Wanrooij, S., Spelbrink, J.N., Lightowlers, R.N. and Turnbull, D.M. (2008) What causes mitochondrial DNA deletions in human cells? *Nat. Genet.*, **40**, 275–279.
37. Kawamata, H., Tiranti, V., Magrane, J., Chinopoulos, C. and Manfredi, G. (2011) adPEO mutations in ANT1 impair ADP-ATP translocation in muscle mitochondria. *Hum. Mol. Genet.*, **20**, 2964–2974.
38. Lopez, L.C., Akman, H.O., Garcia-Cazorla, A., Dorado, B., Marti, R., Nishino, I., Tadesse, S., Pizzorno, G., Shungu, D., Bonilla, E. *et al.* (2009) Unbalanced deoxynucleotide pools cause mitochondrial DNA instability in thymidine phosphorylase-deficient mice. *Hum. Mol. Genet.*, **18**, 714–722.
39. Gonzalez-Vioque, E., Torres-Torronteras, J., Andreu, A.L. and Marti, R. (2011) Limited dCTP availability accounts for mitochondrial DNA depletion in mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). *PLoS Genet.*, **7**, e1002035.
40. Gotz, A., Tyynismaa, H., Euro, L., Ellonen, P., Hyotylainen, T., Ojala, T., Hamalainen, R.H., Tommiska, J., Raivio, T., Oresic, M. *et al.* (2011) Exome sequencing identifies mitochondrial alanyl-tRNA synthetase mutations in infantile mitochondrial cardiomyopathy. *Am. J. Hum. Genet.*, **88**, 635–642.
41. Rice, P., Longden, I. and Bleasby, A. (2000) EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet.*, **16**, 276–277.
42. Li, H. and Durbin, R. (2009) Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics*, **25**, 1754–1760.
43. Pagliarini, D.J., Calvo, S.E., Chang, B., Sheth, S.A., Vafai, S.B., Ong, S.E., Walford, G.A., Sugiana, C., Boneh, A., Chen, W.K. *et al.* (2008) A mitochondrial protein compendium elucidates complex I disease biology. *Cell*, **134**, 112–123.
44. Wang, L., Saada, A. and Eriksson, S. (2003) Kinetic properties of mutant human thymidine kinase 2 suggest a mechanism for mitochondrial DNA depletion myopathy. *J. Biol. Chem.*, **278**, 6963–6968.
45. Palacino, J.J., Sagi, D., Goldberg, M.S., Krauss, S., Motz, C., Wacker, M., Klose, J. and Shen, J. (2004) Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. *J. Biol. Chem.*, **279**, 18614–18622.
46. Peitsch, M.C. (1996) ProMod and Swiss-Model: Internet-based tools for automated comparative protein modelling. *Biochem. Soc. Trans.*, **24**, 274–279.