

REVIEW ARTICLE

Defects in mitochondrial DNA replication and human disease

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Abstract

Mitochondrial DNA (mtDNA) is replicated by the DNA polymerase γ in concert with accessory proteins such as the mtDNA helicase, single stranded DNA binding protein, topoisomerase, and initiating factors. Nucleotide precursors for mtDNA replication arise from the mitochondrial salvage pathway originating from transport of nucleosides, or alternatively from cytoplasmic reduction of ribonucleotides. Defects in mtDNA replication or nucleotide metabolism can cause mitochondrial genetic diseases due to mtDNA deletions, point mutations, or depletion which ultimately cause loss of oxidative phosphorylation. These genetic diseases include mtDNA depletion syndromes such as Alpers or early infantile hepatocerebral syndromes, and mtDNA deletion disorders, such as progressive external ophthalmoplegia (PEO), ataxia-neuropathy, or mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). This review focuses on our current knowledge of genetic defects of mtDNA replication (POLG, POLG2, C10orf2) and nucleotide metabolism (TYMP, TK2, DGOUK, and RRM2B) that cause instability of mtDNA and mitochondrial disease.

Keywords: DNA polymerase γ , mitochondrial DNA replication, nucleotide pools, mitochondrial DNA depletion syndrome, progressive external ophthalmoplegia, ataxia-neuropathy

Introduction

The mitochondrial genome is a closed circular genome of 16,569 bp that codes for 37 genes, all of which are directly or indirectly involved in the production of ATP. Thirteen of these genes encode protein subunits involved in electron transport to carry out oxidative phosphorylation. The remaining 24 genes encode the transfer RNAs (22 genes) and ribosomal RNAs (2 genes) required for mitochondrial protein synthesis of the 13 polypeptides. Animal tissue cells can contain several thousand copies of mitochondrial DNA (mtDNA) spread out over hundreds of mitochondria (Miller et al., 2003). The mtDNA is located in discrete nucleoids in the inner mitochondrial matrix of the mitochondrion and contain between 1 and 2 copies of mtDNA (Kukat et al., 2011). MtDNA is replicated by the concerted action of DNA polymerase γ (pol γ) encoded by *POLG* or *POLG1* (alias), its accessory subunit, p55 encoded by *POLG2*, and replication factors such as the mitochondrial single-stranded DNA binding protein and the mtDNA helicase (*C10orf2* or Twinkle). Pol γ is the only known DNA polymerase to be found in mammalian

mitochondria and thus bears the burden of DNA replication and DNA repair functions (Graziewicz et al., 2006).

Mitochondrial diseases can be caused by genetic defects in mtDNA or in nuclear genes that encode proteins that function in the mitochondria (Wallace, 1999). The last 10 years has defined a list of genes linked to instability of mtDNA, either due to mtDNA depletion syndrome (MDS) or disorders characterized by multiple deletions, with or without point mutations. Table 1 list the current genes associated with MDS and deletion syndromes. Disorders associated with deletions and point mutations comprise commonly known disorders including progressive external ophthalmoplegia (PEO) and ataxia-neuropathy syndromes but also some very rare disorders of TCA cycle abnormalities (Copeland, 2008). MDS includes early childhood disorders such as Alpers syndrome, hepatocerebral syndromes, myocerebrohepatopathy spectrum disorders, and fatal myopathies (Wong et al., 2008; Saneto & Naviaux, 2010).

Defects in mtDNA and their associated disease can also arise in the production of mitochondrial nucleotide pools

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Table 1. Nuclear genes associated with instability of mitochondrial DNA in patients.

Gene	Clinical disorder	Consequence of mtDNA	Chromosome locus	Function
POLG(POLG1)	Ar Alpers or ataxia ArPEO/adPEO	Depletion Deletions	15q25	Mitochondrial DNA polymerase
POLG2	AdPEO		17q23-24	Pol γ accessory subunit
DNA helicase(C10orf2)	AdPEO Ar ataxia / encephalopathy	Deletions Depletion	10q24	Mitochondrial DNA helicase, Twinkle
TP(TYMP)	Ar MNGIE	Depletion Deletions	22q13.32	Thymidine phosphorylase
DGUOK	Ar encephalohepatopathy	Depletion	2p13	Mitochondrial deoxyguanosine kinase
TK2	Ar congenital myopathy	Depletion	16q22	Mitochondrial thymidine kinase
RRM2B	Ar encephalomyopathy Ad PEO	Depletion	8q23.1	p53-inducible small subunit of ribonucleotide reductase
ANT1(SLC25A4)	AdPEO	Deletions	4q34-35	Adenine nucleotide translocator
MPV17	Ar neurohepatopathy	Depletion	2p21-23	Mitochondrial inner membrane protein
SUCLA2	Ar encephalomyopathy	Depletion	13q12.2-q13.3	β -subunit, Succinate-CoA ligase
SUCLG1	Ar encephalomyopathy	Depletion	2p11.1	α -subunit, Succinate-CoA ligase
OPA1	Ad optic atrophy	Deletions	3q28-q29	Dynamin related GTPase

Ad, autosomal dominant; Ar, autosomal recessive.

needed for DNA replication. Mitochondria depend heavily on either mitochondrial transport proteins or salvage pathway enzymes to supply deoxynucleotide triphosphates required for mtDNA replication (Figure 1). This review focuses on the genes and gene products identified as disease alleles and that function either directly at the replication fork or in metabolism of deoxynucleotide triphosphate pools used as the precursors for DNA replication. Other genes, such as *OPA1*, *MPV17*, *ANT1*, or *SUCLA2*, *SUCLG1* that cause mitochondrial depletion or deletion syndromes but not directly involved in these two processes have been reviewed elsewhere (Copeland, 2008).

Defects in proteins that act at the fork

MtDNA is replicated and repaired by DNA polymerase γ (pol γ) the only known DNA polymerase to be found in animal cell mitochondria. The holoenzyme of pol γ in humans consists of a catalytic subunit (encoded by *POLG* at chromosomal locus 15q25) and a dimeric form of its accessory subunit (encoded by *POLG2* at chromosomal locus 17q24.1). The catalytic subunit is a 140 kDa enzyme (p140) that has DNA polymerase, 3'-5' exonuclease and 5' dRP lyase activities (Kaguni, 2004; Graziewicz et al., 2006). The accessory subunit is a 55 kDa protein (p55) required for tight DNA binding and processive DNA synthesis (Lim et al., 1999). The pol γ holoenzyme functions in conjunction with the mtDNA helicase, Twinkle or *C10orf2*, and the mtSSB to form the minimal replication apparatus (Korhonen et al., 2004; Falkenberg et al., 2007). Gene mutations in *POLG*, *POLG2* and *C10orf2* have been implicated in mitochondrial disease and are discussed below.

DNA polymerase γ catalytic subunit, *POLG*

To date, nearly 200 mutations in *POLG* have been reported in association with mitochondrial diseases (<http://tools.niehs.nih.gov/polg/>) (Longley et al., 2005; Copeland, 2008; Longley et al., 2010; Walter et al., 2010; Tang,

2011)). *POLG*-related disorders are currently defined by at least six major phenotypes of neurodegenerative disease that include: Alpers-Huttenlocher syndrome, childhood myocerebrohepatopathy spectrum, myoclonic epilepsy myopathy sensory ataxia, the ataxia neuropathy spectrum, autosomal recessive progressive external ophthalmoplegia (arPEO), and autosomal dominant progressive external ophthalmoplegia (adPEO) (Wong et al., 2008; Cohen et al., 2010; Saneto and Naviaux, 2010). Other signs and symptoms have been associated with *POLG* mutations such as male infertility, testicular cancer, and Parkinsonism and are reviewed in (Saneto and Naviaux, 2010).

POLG and PEO phenotypes

Mutations in *POLG* were first identified in 2001 by Van Goethem and colleagues as a locus for PEO (Van Goethem et al., 2001). PEO is a mitochondrial disorder associated with mtDNA depletion and/or accumulation of mtDNA point mutations and deletions (Zeviani et al., 1989; Hirano et al., 2001; Van Goethem et al., 2001). PEO is characterized by late onset (between 18 and 40 years of age) bilateral ptosis and progressive weakening of the external eye muscle, resulting in blepharoptosis and ophthalmoparesis, proximal muscle weakness, and wasting as well as exercise intolerance. Skeletal muscles of PEO patients present red ragged fibers and lowered activity of respiratory chain enzymes. Multiple large-scale deletions of mtDNA isolated from muscle biopsies were first demonstrated in Italian families with heritable autosomal dominant PEO (adPEO) by Zeviani and colleagues (Zeviani et al., 1989).

To date, with the exception of one mutation, all autosomal dominant *POLG* mutations responsible for developing PEO are mapped to the polymerase domain of pol γ . Two of the substitutions, R943H and Y955C, change side chains that interact directly with the incoming dNTP (Graziewicz et al., 2004). These enzymes retain less than

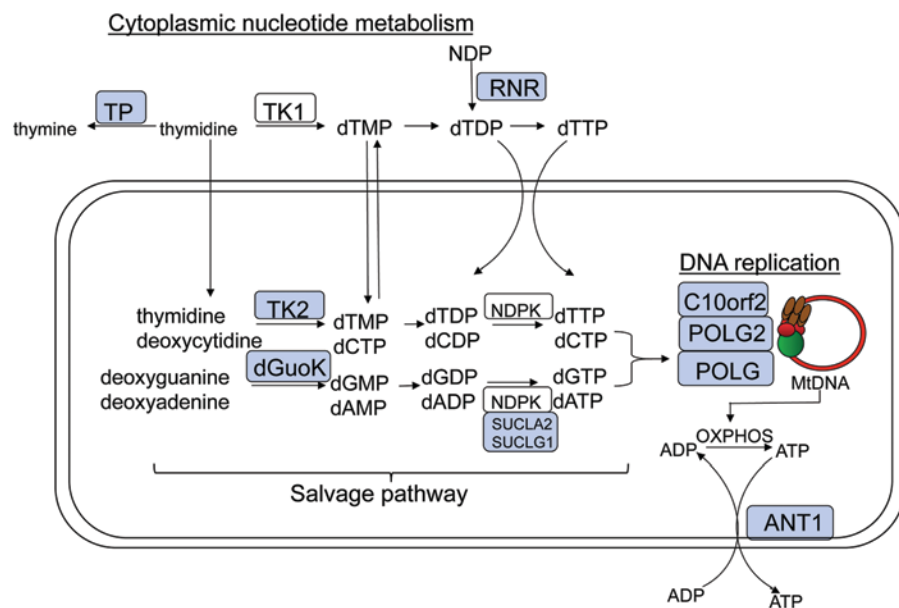


Figure 1. Schematic diagram of the mitochondria representing enzyme pathways that cause mtDNA mutations or depletion when disrupted. Gene products associated with MDS or mtDNA mutations are labeled as light blue boxes.

1% of the wild-type polymerase activity and display a severe decrease in processivity. The significant stalling of DNA synthesis and extremely low catalytic activities are the two most likely causes of the severe clinical presentation in R943H and Y955C heterozygotes (Graziewicz et al., 2004). The Y955C substitution also increases nucleotide misinsertion errors 10–100 fold in the absence of exonucleolytic proofreading (Ponamarev et al., 2002). In a yeast model developed to evaluate the homologous mutation in the yeast *Mip1* gene we found that Y757C mutant (Y955C in humans) demonstrated enhanced mtDNA damage indicative of oxidative damage and very high petite frequency (Stuart et al., 2006). In a related study, Baruffini et al. showed that this high petite frequency could be rescued by treatment with antioxidants or up regulation of ribonucleotide reductase (Baruffini et al., 2006). In a mouse transgenic model in which the Y955C *POLG* was targeted to the heart, the mouse developed cardiomyopathy, loss of mtDNA, enlarged heart, and increased levels of 8-oxo-dG in their mtDNA (Lewis et al., 2007). Collectively, these phenotypes suggest that patients harboring the Y955C mutation may have elevated oxidative damage and may benefit from antioxidant therapy.

Significant cosegregation of parkinsonism with mutations in *POLG* gene was described in two individuals with adPEO (Luoma et al., 2004). Parkinsonism associated with *POLG* mutations manifested several years after initial disease symptoms. In addition many women with PEO from *POLG* mutations go through early menopause and suffer from high gonadotropin and low estrogen concentrations, indicative of premature ovarian failure (Luoma et al., 2004; Pagnamenta et al., 2006).

Most of the mutations found in *POLG* are associated with autosomal recessive PEO (arPEO) and patients with

PEO are often compound heterozygotes with two arPEO alleles. For example, the A467T mutation has been found in *trans* with other *POLG* missense mutations in PEO, ataxia-neuropathy and Alpers syndrome (Van Goethem et al., 2003; Naviaux and Nguyen, 2004). The A467T mutation was found in two pedigrees as a homozygous mutation and associated with severe ataxia in mid-life (Van Goethem et al., 2004).

***POLG* and Alpers syndrome and myocerebrohepatopathy spectrum disorders**

Alpers syndrome is a rare but severe, heritable, autosomal recessive MDS disease that afflicts young children. Within the first few years of life, patients exhibit progressive spastic quadriplegia, progressive cerebral degeneration leading to mental deterioration and seizures, cortical blindness, deafness, liver failure, and eventual death. Naviaux et al reported an Alpers patient with reduced electron transport chain function, dicarboxylic aciduria, fulminant hepatic failure, refractory epilepsy, and lactic acidosis which resulted in death at 42 months (Naviaux et al., 1999). Analysis of mtDNA and pol γ activity from skeletal muscle biopsy indicated a reduction of mtDNA content to 30% of wildtype levels and no detectable pol γ activity (Naviaux et al., 1999). Sequencing of the *POLG* gene in two unrelated pedigrees revealed a heterozygous G to T nonsense mutation in *POLG* that converts Glu873 (GAG) to a stop codon and the heterozygous A467T substitution just after the exonuclease domain in the linker region (Naviaux & Nguyen, 2004). Pol γ mRNAs with the E873stop mutation are degraded from the pool of mRNAs by nonsense mediated decay resulting in mono-allelic expression of *POLG* containing only the A467T mutation (Chan et al.,

2005b). To date the number of reported Alpers and other early childhood hepatocerebral syndromes associated *POLG* mutations has risen to nearly 100 different types of mutations (Ferrari et al., 2005; Nguyen et al., 2006; Horvath et al., 2006; Wong et al., 2008; Tang et al., 2011) (see Human DNA Polymerase γ Mutation Database: <http://tools.niehs.nih.gov/polg/>). In all cases, the *POLG* mutations in Alpers patients are recessive. Many of these mutations also occur in PEO patients as autosomal recessive mutations. The A467T mutation is the most frequently found pathogenic mutation in *POLG*. The frequency of the A467T mutation has been found to exist in approximately 0.6% in the Belgian normal population (Van Goethem et al., 2001), 0.69% in the British population (Horvath et al., 2006), 1% in the Norwegian populations (Winterthun et al., 2005), and less than 0.2% in 380 Finnish controls (Luoma et al., 2005). However, the A467T allele has been estimated to occur in 36% of all alleles associated with *POLG* disease, as shown by five large mitochondrial disease population studies identifying *POLG* mutations (Ferrari et al., 2005; De Vries et al., 2007; Horvath et al., 2006; Nguyen et al., 2006; Wong et al., 2008). Biochemical analysis has demonstrated that the recombinant A467T pol γ is unstable and retains only 4% activity compared to WT enzyme as a result of >5-fold decrease in k_{cat} and a 4.7-fold increase in $K_{\text{m(dNTP)}}$ (Chan et al., 2005a). Additionally, the A467T pol γ is compromised for its interaction with the accessory subunit.

Analysis of a cluster of Alper's mutations in the thumb-palm domain has shown a striking correlation with the severity of the defect and the degree of conservation (Kasiviswanathan et al., 2009). Mutations in the most conserved sites represented by G848S, T851A, R852C and R853Q exhibited less than 1% WT enzyme activity (Kasiviswanathan et al., 2009). Mutations in codons for less conserved amino acids (Gln879 and Thr885) resulted in only moderate reduction in activity (Kasiviswanathan et al., 2009).

Yeast has been a useful model system to study mutations in residues that are conserved between yeast (in the *Mip1* gene) and man (Stuart et al., 2006; Baruffini et al., 2007; Baruffini et al., 2011; Baruffini et al., 2006; Stumpf et al., 2010). In a study of 31 mutations in the conserved regions in *Mip1*, 20 mutations were shown to disrupt mtDNA replication (Stumpf et al., 2010). The sporadic mutations, Q308H, R807C, G1076V, R1096H and S1104C, caused decreased polymerase activity leading to mtDNA depletion and mitochondrial dysfunction. Interestingly, point mutagenesis in mtDNA as a consequence of these *POLG* mutations, especially in the exonuclease region, were insufficient to explain pathogenesis (Stumpf et al., 2010). Instead, mutations in the exonuclease domain cause pathogenesis by disrupting polymerase activity and not by decreasing fidelity (Stumpf et al., 2010; Szczepanowska & Foury, 2010). For the majority of the disease substitutions that have been studied *in vitro*, the biochemical defects correlate with the severity and age of onset found in patients (Chan & Copeland, 2009).

***POLG* and ataxia neuropathy spectrum and myoclonic epilepsy myopathy sensory ataxia phenotypes**

Mutations in *POLG* can also cause ataxia-neuropathy syndrome with onset in the early teens to late thirties. This ataxia, also termed mitochondrial associated ataxia syndrome (MIRAS) (Hakonen et al., 2005), spino-cerebellar ataxia-epilepsy syndrome, or sensory ataxic neuropathy dysarthria and ophthalmoparesis (SANDO) (Van Goethem et al., 2004) is caused by autosomal recessive mutations in *POLG* producing multiple mtDNA deletions in the affected individuals. Symptoms of ataxia involving mutations in *POLG* include peripheral neuropathy, dysarthria, mild cognitive impairment, involuntary movements, psychiatric symptoms, myoclonus, and epileptic seizures. Ataxic patients who are homozygous for the A467T mutation present with symptoms in their early to late teens (Van Goethem et al., 2004; Winterthun et al., 2005; Tzoulis et al., 2006). SANDO patients have also been found to have compound heterozygous mutations with the A467T mutation in one *POLG* allele and either R3P, L304R, or R627W in the other (Van Goethem et al., 2003). One patient with ataxia-myopathy syndrome was shown to have A467T in one *POLG* allele and R627Q mutation in cis with the Q1236H single nucleotide polymorphism (SNP) in the other *POLG* allele (Luoma et al., 2005). Other patients with ataxia were found to be heterozygous with the A467T mutation on one allele and W748S in cis with the E1143G mutation in the other allele. The latter mutation, E1143G, was originally identified as a SNP in 4% of the general population (Longley et al., 2005). However, the accumulated reports of this mutation with other *POLG* mutations in mitochondrial disease suggest that E1143G may augment the disease process (Hudson & Chinnery, 2006; Hudson et al., 2006). The W748S mutation in combination with E1143G has been found as a frequent cause of ataxia (Winterthun et al., 2005; Hakonen et al., 2005). Haplotype analysis of the Finnish population demonstrates a carrier frequency of 1:125 for the W748S mutation with a common ancestor origin of this diseased allele in the ancient European population (Hakonen et al., 2005). We have found that the W748S mutation alone causes the polymerase to have a low catalytic activity and a severe DNA-binding defect (Chan et al., 2006). The E1143G substitution partially rescues the deleterious effects of the W748S mutation and appears to modulate the effects of disease mutations in *POLG*.

***POLG2*, the accessory subunit of pol γ**

Mutation of the *POLG2* gene is rarely described as compared to mutations in the gene for the catalytic subunit. The first mutation described (c.1352G>A; G451E) was in a late onset adPEO patient with multiple mtDNA deletions in muscle and ptosis (Longley et al., 2006). The G451E substitution was in a region of the p55 protein that interacts with the p140 subunit, and caused a

decreased in processivity of the enzyme complex due to a compromised p55-p140 subunit interaction (Longley et al., 2006). The second case also involved a late onset adPEO patient with mtDNA deletions and harbored a c.1207-1208ins24 mutation, causing mis-splicing and skipping of exon 7, thus impairing the C-terminal domain required for enzyme processivity (Walter et al., 2010). More recently, in a cohort of 112 patients with mitochondrial disease and suspected of *POLG* involvement but absent of *POLG* mutations, 8 heterozygous mutations were identified where 7 mutations were novel (Young et al., 2011). Biochemical analysis of these 7 variant p55 proteins (G103S, L153V, P205R, R369G, D386E, S423Y, and L475DfsX2) revealed that G103S, L153V, D386E and S423Y were similar to wild-type, and the P205R and R369G p55 variants had reduced stimulation of processivity and decreased affinity for the catalytic subunit (Young et al., 2011). The L475DfsX2 protein was unable to bind the p140 catalytic subunit, unable to bind dsDNA and was generally unstable. The failure to enhance processivity in the catalytic subunit by these mutant variants would cause the complex to stall during DNA replication and is consistent with the accumulation of mtDNA deletions detected in PEO.

The mtDNA helicase, the *TWINKLE* or *C10orf21* gene

The mtDNA helicase encoded by the *PEO1* or *TWINKLE* gene is related to the phage T7 gp4 helicase-primase. This gene was first identified as a locus for PEO on chromosome 10, *C10orf2* (Spelbrink et al., 2001). The derived amino acid sequence showed significant sequence homology to the C-terminal end of T7 gp4 helicase but lacked critical primase-associated sequences found in T7 gp4 (Spelbrink et al., 2001; Garrido et al., 2003). Screening of the *C10orf2* gene in individuals with adPEO, associated with multiple mtDNA deletions, identified 11 different mutations that co-segregated with the disorder in 12 affected families (Spelbrink et al., 2001). At least 23 additional missense mutations in *C10orf2* associated have been reported in adPEO (Van Hove et al., 2009; Fratter et al., 2010). Although mutations in *C10orf2* are mainly associated with adPEO, several reports have described recessive mutations as a cause of either epileptic encephalopathy with mtDNA depletion or infantile-onset spinocerebellar ataxia (Hudson et al., 2005; Hakonen et al., 2007; Lonnqvist et al., 2009).

Mouse transgenic models that overexpressed several of the PEO mutations in *C10orf2* recapitulated many of the symptoms observed in human PEO including multiple mtDNA deletions, progressive respiratory dysfunction, and cytochrome c oxidase deficiency (Tynismaa et al., 2005; Tynismaa & Suomalainen, 2009). Overexpression of dominant disease variants of the mtDNA helicase in cultured human or Schneider cells results in stalled mtDNA replication or depletion of mtDNA (Wanrooij et al., 2007;

Matsushima & Kaguni, 2007; Goffart et al., 2009), which emulates the disease state. Two of five adPEO mutants exhibited a dominant negative phenotype with mtDNA depletion in Schneider cells (Matsushima & Kaguni, 2007). Disease mutations in the *C10orf2* gene have been evaluated by study of the recombinant helicase harboring these mutations as a homogeneous population. Analysis of the recombinant helicase purified from baculoviral infected insect cells has demonstrated defects in the helicase due to disease mutations. Disease mutations in the linker region were shown to disrupt protein hexamerization and abolish DNA helicase activity (Korhonen et al., 2008). Four mutations in the N-terminal domain demonstrated a dramatic decrease in ATPase activity (Holmlund et al., 2009). A comprehensive study of recombinant disease variants overproduced and purified from *E. coli* has revealed that all of the disease variants display some level of activity where mild to moderate defects were seen variably over 20 different variants with defects in helicase activity, ATP hydrolysis and stability (Longley et al., 2010). All of the variants displayed efficient DNA binding. This study emphasises the need to optimize *in vitro* conditions for biochemical analysis of disease variants (Longley et al., 2010). The moderate defects demonstrated *in vitro* is consistent with the delayed onset the adPEO associated with mutation of *C10orf2*.

Defects in mitochondrial nucleotide metabolism

Mitochondrial dNTP pools arise either through active transport of cytosolic dNTPs or through the purine and pyrimidine salvage pathways by action of two mitochondrial deoxyribonucleoside kinases, thymidine kinase 2 (*TK2*) and deoxyguanosine kinase (*DGUOK*) (Figure 1). In non-dividing cells cytosolic *TK1* and dNTP synthesis is down-regulated forcing the burden of mitochondrial dNTP pool synthesis on the two mitochondrial deoxyribonucleoside kinases. Mutations in the genes for enzymes involved in this salvage pathway cause several forms of MDS. Computational models suggest that this salvage pathway is insufficient to support rapid mtDNA replication and the mitochondrial salvage pathway serves more as a backup role for the supply of nucleotide precursors for mtDNA replication (Gandhi & Samuels, 2011). This model helps to explain why defects in *TK2* are restrictive to muscle and present as myopathic MDSs (Mancuso et al., 2002). An alternative pathway for mitochondrial pools of nucleotides involves transport from cytoplasmic pools, arising from reduction of ribonucleotides by ribonucleotide reductase (RNR). Nucleotide pools arising from RNR would then be imported into the mitochondria as dNTPs to mix with dNTPs derived from *TK2/DGUOK* pathways (Figure 1).

Thymidine phosphorylase, TP or *TYMP*

Thymidine phosphorylase (TP) is part of the pyrimidine salvage pathway required for the reversible reaction

catalyzing thymidine and phosphate to thymine and deoxyribose-1-phosphate (Figure 1). A defect in this gene (*TYMP*) causes mitochondrial neurogastrointestinal encephalopathy (MNGIE) due to the accumulation of thymidine and uracil in the blood. Because mitochondria rely heavily on salvage pathways for generating intramitochondrial dNTP pools, the mitochondria will uptake the excess thymidine which stimulates the synthesis of unbalanced and elevated dTTP levels by thymidine kinase 2 in the mitochondria. The unbalanced mitochondrial deoxynucleotide pools result in mtDNA depletion, multiple deletions, and point mutations. MNGIE is an autosomal recessive disorder caused by mutations in the gene for thymidine phosphorylase (Nishino et al., 1999; Hirano et al., 2005). There are over 30 mutations in TP associated with MNGIE (Hirano et al., 2005). Similar to PEO, the disease is associated with multiple deletions and depletion of mtDNA in affected tissues (Hirano et al., 2001). The onset usually takes place between the second and fifth decade of life and typical clinical features include ptosis, progressive external ophthalmoparesis, gastrointestinal dysmotility, cachexia, peripheral neuropathy, myopathy, and leukoencephalopathy (Hirano et al., 2004; Nishino et al., 2000). TP deficiency leads to increased concentrations of circulating deoxythymidine (Spinazzola et al., 2001) and deoxyuridine (Marti et al., 2003), which generates imbalanced mitochondrial deoxyribonucleoside triphosphate pools, the effect of which can be responsible for increased mtDNA mutagenesis (Song et al., 2003). Analysis of thymidine and deoxyuridine levels in post-mortem samples of MNGIE patients have revealed that the absence of TP activity causes an accumulation of thymidine and nucleosides, higher than observed in the plasma of these patients (Valentino et al., 2007).

The majority (>80%) of mtDNA mutations found in tissues from MNGIE patients are T to C transitions preceded by a short run of As (Nishigaki et al., 2003). This signature mutation suggests a “next-nucleotide effect” caused by the more common T:dGMP misinsertion (Longley et al., 2001) which is quickly extended by the elevated dTTP concentration resulting from TP deficiency in the mitochondria of MNGIE cells (Nishigaki et al., 2003). Additionally, elevated concentrations of dTMP derived from the increased thymidine can inhibit the exonuclease activity of pol γ (Lim & Copeland, 2001). HeLa cells grown in the media supplemented with 50 μ M thymidine causes mtDNA deletions and elevated mitochondrial pools of dTTP and dGTP, a result that recapitulated many of the genetic effects seen in MNGIE (Song et al., 2003). These results support a mutagenic mechanism involving competition between dGTP and dATP for incorporation opposite to template T reported by Nishigaki and colleagues (Nishigaki et al., 2003).

Several lines of therapy or treatment of MNGIE are underway. Hemodialysis has been shown to transiently reduce thymidine levels in blood (Spinazzola et al., 2002). Thymidine levels in blood can be reduced in MNGIE

patients by repeated platelet infusions (Lara et al., 2006). Allogeneic stem cell transplantation has been shown to have some success in restoring TP activity and lowering plasma thymidine levels (Hirano et al., 2006) however this approach shows toxicity of myeloablative conditional and graft rejections. More recently, hematopoietic gene therapy by the introduction of TP with lentiviral vectors into TP-deficient B-lymphoblastoid cells and TP/Upp1 knockout mice was shown to restore TP activity with the concomitant reduction of nucleosides in peripheral blood (Torres-Torronteras et al., 2011).

Mitochondrial thymidine kinase, *TK2*

In a study of four unrelated patients with fatal myopathy during infancy and MDS, Saada et al found reduced mitochondrial respiratory chain function and reduced amounts of mtDNA in muscle (Saada et al., 2001). Sequence analysis of the thymidine kinase 2 (*TK2*) gene identified either the homozygous H90R mutation or the homozygous I181D mutation in these patients. Activity analysis in mitochondrial lysates from these patients revealed reduced TK2 activity (Saada et al., 2001). DNA sequence by other groups have identified over 20 missense mutations found either as recessive homozygous or as compound heterozygous mutation and one stop codon from a total (Mancuso et al., 2002; Mancuso et al., 2003; Carrozzo et al., 2003; Wang et al., 2005; Galbiati et al., 2006; Alberio et al., 2007; Gotz et al., 2008; Blakely et al., 2008; Collins et al., 2009; Lesko et al., 2010). TK2 defects due to two compound heterozygous mutations, R172W and R225W, were also found to be responsible for multi-tissue MDS in five unrelated patients and two brothers (Gotz et al., 2008). A review of these mutations in the structure of the human *TK2* shows a random distribution of these mutations over the *TK2* gene (Eriksson and Wang, 2008). *TK2* gene mutations primarily affect muscle tissue with no effect on liver, brain, heart, or skin. Quantitation of TK2 activity in various tissues relative to mtDNA or cytochrome c oxidase activity helps to explain the tissue specificity of this disorder (Saada et al., 2003).

Kinetic analysis of two *TK2* mutants, I212N and H121N demonstrated that the I212N mutant enzyme has less than 1% activity while the H121N mutant enzyme has a 2–3 fold lower V_{\max} as compared to the wild type *TK2* enzyme (Wang et al., 2003). Biochemical analysis of two other *TK2* mutations, T77M and R161K, found as compound heterozygous mutation in one patient revealed that the recombinant enzyme only retained 2–4% activity (Wang et al., 2005). Introduction of the H126N mutation in mouse knockin mice caused rapid progressive weakness 10 days after birth followed by death between 2 and 3 weeks (Akman et al., 2008). These mice recapitulated many of the symptoms of MDS and showed *TK2* deficiency, unbalanced dNTP pools and mtDNA depletion, along with electron chain respiratory defects (Akman et al., 2008).

Mitochondrial deoxyguanosine kinase, *DGUOK*

Deoxyguanosine kinase is the other mitochondrial deoxyribonucleoside kinase and phosphorylates the purine nucleosides into nucleotide monophosphates (Figure 1). Homozygosity mapping in three consanguineous kindreds with hepatocerebral MDS identified a region on chromosome 2p13 that included the deoxyguanosine kinase gene (Mandel et al., 2001). Sequence analysis of this gene identified a nucleotide deletion (204delA) that segregated with the disease (Mandel et al., 2001). In a screen of 21 patients with MDS, Salviati et al. identified 3 patients (14%) with *DGUOK* gene mutations (Salviati et al., 2002). Phenotypes of these three patients were highly variable, including one patient who developed liver failure but responded well to liver transplant (Salviati et al., 2002). To confirm that the *DGUOK* mutations did indeed disrupt enzymatic function, Wang et al. characterized recombinant *DGUOK* proteins with these substitutions (Wang & Eriksson, 2003). They found that the R142K variant had very low activity with dG and no activity with dA. The E227K protein had normal affinity for dG and dA but low catalytic efficiency indicating that this mutation disrupts catalysis without affecting substrate binding. The C-terminal truncated variants were inactive. Further analysis later by Wang et al. identified one patient with MDS, muscular weakness, and exercise intolerance due to a severe mitochondrial myopathy that harbored the L250S mutation in *DGUOK* (Wang et al., 2005). Examination of the recombinant L250S-*DGUOK* protein revealed <1% activity as compared to the wild type enzyme with differential competition between dC and dT as substrates (Wang et al., 2005). De Camaret et al. identified two missense mutations, N46S and L266R in a 10-year-old child with hepatocerebral form of MDS (Mousson De Camaret et al., 2007). Kinetic analysis mitochondrial fractions from skin fibroblasts derived from this patient as compared to normal cells showed 10 and 14% kinase activity for dG and dA substrates, respectively (Mousson De Camaret et al., 2007). Freisinger also identified five new mutations and two previously described mutations in six children with infantile hepatoencephalopathies and MDS (Freisinger et al., 2006). There has been nearly 40 different mutations described in the *DGUOK* gene, mostly presented as homozygous mutations from over 25 different probands (Freisinger et al., 2006; Alberio et al., 2007; Dimmock et al., 2008). In a screen of nine kindreds, Dimmock et al. noted that mutations that result in truncated proteins (nonsense, splice site or frameshift mutations) predominate over missense mutations (Dimmock et al., 2008). Patients with null mutations usually present with early onset liver failure with significant neurological symptoms with death before 2 years of age (Dimmock et al., 2008). Interestingly, five patients were identified in newborn screening with elevated tyrosine or phenylalanine levels (Dimmock et al., 2008).

Currently, two forms of deoxyguanosine kinase deficiency have been described, the hepatocerebral MDS which presents as a multisystem disorder and an isolated hepatic disease later in infancy or childhood (Scaglia et al., 2009). The majority of affected individuals have the multisystem illness with hepatic disease (cholestasis) and neurologic dysfunction evident within weeks of birth (reviewed in (Scaglia et al., 2009)).

RRM2B, the p53 inducible small subunit of ribonucleotide reductase

In contrast to the mitochondrial salvage pathway, nucleotide precursors required for DNA replication can be directly obtained by reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates by ribonucleotide reductase. Ribonucleotide reductase is made up of two subunits, a large catalytic subunit, R1, and the smaller R2 subunit. Cells have two forms of the R2 subunit, a cell cycle regulated form that is maximally expressed in S-phase, and a p53-inducible form known as p53R2. The p53R2 form is required for a basal level of DNA repair and mtDNA synthesis in non-proliferating cells. In 2007 Bourdon et al. (Bourdon et al., 2007) identified the *RRM2B* gene encoding p532B as a candidate disease gene from a genome-wide linkage analysis in a family with severe muscle mtDNA depletion. Sequence analysis of *RRM2B* in this family and three other affected families identified nonsense, missense and splice-site mutations and in-frame deletions within the *RRM2B* gene that were not found in control chromosomes (Bourdon et al., 2007). Severe mtDNA depletion from *RRM2B* disruption was also confirmed in *Rrm2b*^{-/-} mice demonstrating the essential role of this gene in mtDNA nucleotide metabolism and mitochondrial disease (Bourdon et al., 2007).

Similar to *POLG* related disorders the symptoms and signs associated with *RRM2B* mutations cover a wide spectrum by age and disease. A 42-year-old woman with mitochondrial neurogastrointestinal encephalopathy and mtDNA depletion in muscle was found to have two pathogenic mutations, R110H and R121H in the *RRM2B* gene (Shaibani et al., 2009). These two mutations are believed to affect the dimerization of the RNR small subunit. *RRM2B* mutations have also been described in PEO patients. A heterozygous stop codon mutation was described in two families with autosomal dominant PEO (Tyyntismaa et al., 2009). The stop codon mutation resided in exon 9 and escaped nonsense-mediated decay resulting in a dominant negative protein truncated by 26 amino acids and. This C-terminal region is believed to be essential for interaction with the R1 large subunit of RNR. To determine the frequency of *RRM2B* mutations in patients with PEO, Fratter et al. studied 75 probands with mtDNA deletions and PEO symptoms (Fratter et al., 2011). They found that 16% of the subjects contained mutations in the *RRM2B* gene for a total of 10 different *RRM2B* variants in 12 subjects. Seven patients had

a single heterozygous missense mutations resulting in truncated proteins and two patients harbored compound heterozygous mutation.

Conclusion

The role of nuclear genes and the disease mutations in these genes that function in the maintenance of our mtDNA has gained increased appreciation over the last 10 years. Disease of mtDNA stability are found in core proteins of mtDNA replication or in genes involved in supplying the mitochondrial nucleotide precursors needed for DNA replication (Figure 1). With our awareness of disease mutations in these genes the frequency of mitochondrial patients due to mutation in these genes will continue to increase. As an example, the number of individual harboring a recessive pathogenic mutation in *POLG* has been estimated to approach 2% of the population (Cohen & Naviaux, 2010). However, the varied polymorphic nature of these diseases as well as the age of presentation due to these gene mutations stumps our understanding and challenges clinician and researchers. While *in vitro* biochemistry and yeast models have proven very effective in understanding the biochemical and pathological defects, animal models are becoming an essential tool for predicting the *in vivo* consequences of heritable mutations with these genes. The next 10 years of research should be illuminating in this field.

Declaration of interest

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