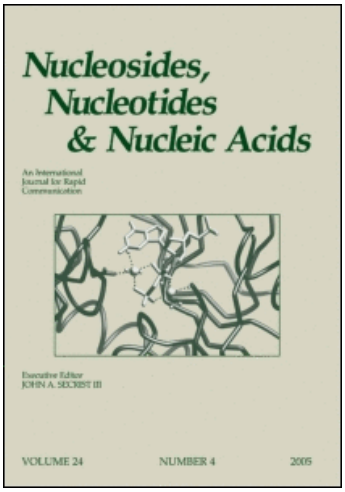

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Deoxyribonucleoside Kinases in Mitochondrial DNA Depletion

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ABSTRACT

Mitochondrial DNA (mtDNA) depletion syndromes (MDS) are a heterogeneous group of mitochondrial disorders, manifested by a decreased mtDNA copy number and respiratory chain dysfunction. Primary MDS are inherited autosomally and may affect a single organ or multiple tissues. Mutated mitochondrial deoxyribonucleoside kinases; deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK2), were associated with the hepatocerebral and myopathic forms of MDS respectively. dGK and TK2 are key enzymes in the mitochondrial nucleotide salvage pathway, providing the mitochondria with deoxyribonucleotides (dNP) essential for mtDNA synthesis. Although the mitochondrial dNP pool is physically separated from the cytosolic one, dNP's may still be imported through specific transport. Non -replicating tissues, where cytosolic dNP supply is down regulated, are thus particularly vulnerable to dGK and TK2 deficiency. The overlapping substrate specificity of deoxycytidine kinase (dCK) may explain the relative sparing of muscle in dGK deficiency, while low basal TK2 activity render this tissue susceptible toTK2 deficiency. The precise patho-physiological mechanisms of mtDNA depletion due to dGK and TK2 deficiencies

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remain to be determined, though recent findings confirm that it is attributed to imbalanced dNTP pools.

Key Words: Mitochondrial DNA depletion; Thymidine kinase 2; Deoxyguanosine kinase; Respiratory chain; Myopathy; Hepatocerebral; Deoxyribonucleoside; Deoxyribonucleotide.

INTRODUCTION

The mitochondria respiratory chain consists of 85 proteins assembled into five (I, II, III,IV,V) respiratory chain complexes. Only 13 out of the 85 respiratory chain subunits are encoded by mtDNA, while the remaining are nuclear encoded. Complex II is the only one that contains solely nuclear encoded proteins. The factors required for the replication and maintenance of the mitochondrial genome are nuclear encoded. Mitochondrial disorders may therefore be caused by defects in either genome and thus be transmitted either maternally or as Mendelian traits.[1]

Three molecular abnormalities are associated with the failure to maintain the integrity of mtDNA, with consequent decreased function of complexes I, III, IV and V. The first, are large scale-rearrangements (multiple mtDNA deletions) in patients with various forms of myopathy and progressive external ophtalmoplegia and were found to be associated with mutations in the mitochondrial DNA polymerase-γ (POLG), helicase (Twinkle) and adenosine nucleotide translocator (ANT1).[2] The second molecular abnormality is MDS, characterized by a quantitative rather than qualitative defect with severe reduction of mtDNA copy number. MDS are devastating tissue specific or multisystemic disorders usually presenting in infancy with hypotonia, and lactic acidosis.[3,4] Recently mutations in each of the two mitochondrial deoxynucleoside kinases were reported to be associated with mtDNA depletion; deoxyguanosine kinase (dGK) in hepatocerebral form and mitochondrial thymidine kinase (TK2) in myopathic form of MDS.[5–12] The third molecular phenomenon; mtDNA depletion with multiple deletions, occurs in patients with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), as a consequence thymidine phosphorylase (TP) deficiency.[13,14]

The association of mutations inTP, TK2 and dGK with MDS and the fact that antiviral nucleoside analogues may cause secondary mtDNA depletion,[15] indicate that mitochondrial deoxyribonucleotide metabolism play a pivotal in mtDNA synthesis and maintenance.

MITOCHONDRIAL DEOXYRIBONUCLEOTIDE METABOLISM

The dNP pool is separated from the cytosolic by the mitochondrial inner membrane (IM) which constitutes an impermeable barrier to charged molecules. dNP's may only be imported from the cytosol the to the mitochondria, through specialized transporters such as the deoxyribonucleotide carrier (DNC), or recycled from ribonucleosides through the mitochondrial salvage pathway.[16–18] De novo pathway enzymes in mitochondria are apparently lacking although there is one report of mitochondrial ribonucleotide reductase activity.[19]

The first phosphorylation step in the mitochondrial salvage pathway and is carried out by TK2 and dGK. TK2 is a pyrimidine kinase, phosphorylating thymidine (dThd), deoxycytidine (dCyt) and deoxyuridine (dUrd) while dGK is active towards the purine nucleosides, deoxyguanosine (dGuo) and deoxyadenosine (dAdo). TK2 and dGK show significant homology to the cytosolic deoxy cytidine kinase (dCK) and the insect multisubstrate deoxynucleoside kinases.[20–21] The mitochondrial kinases have overlapping substrate specificities with dCK which phosphorylates dThd, dCyt, dAdo and the cytosolic thymidine kinase 1 (TK1) specific for dThd and dUrd. TK1 is strictly S phase correlated and is expressed only in replicating cells.[18,20,21]

The subsequent phosphorylation steps of deoxyribonucleoside monophosphates and deoxyribonucleoside diphosphates are carried out in the cytosol, by nucleoside monophosphate kinases and the multifunctional nucleoside diphosphate kinase Mitochondrial mono- and diphosphate kinases are less studied. Adenylate kinases 3, 4 and the mitochondrial isoform of nucleoside diphosphate kinase are targeted to the mitochondrial matrix, and may also phosphorylate deoxyribonucleotides.[22,23] The existence of other mitochondrial NMPK's remains to be explored. The recent cloning of a mitochondrial UMP/CMP kinase from *Drosophila Melongaster* is an advance.[24]

The mitochondrial salvage pathway enzymes probably participate in the phosphorylation of nucleoside analogues and may cause secondary mtDNA depletion by interfering with pol-γ.[15] Nucleoside analogues may theoretically also act as mitochondrial deoxynucleoside kinase inhibitors and deprive mtDNA synthesis from natural substrates.

The activity of nucleoside kinases are opposed by the activities of mitochondrial 5′-nucleotidases. The only known enzyme in this group, mitochondrial 5′-deoxyribonucleotidase (dNT2), is suggested to play a role in mitochondrial thymidine metabolism.[25,26]

The regulation of dNP metabolism is of importance, as a balanced deoxyribonucleotide triphosphate (dNTP) pool is required for accurate DNA replication.[27] Not only shortage of dNP's but also a surplus may be harmful. The mtDNA aberrations and mtDNA point mutations found in patients with excessive thymidine and deoxyuridine due to TP deficiency underscores the importance of a proper regulation of dNP metabolism.[13,14]

The mitochondrial DNA synthesis occurs constitutively, throughout the whole cell cycle demanding a constant supply of dNTP's. The supply may be provided either by the mitochondrial salvage pathway or from the cytosol. However since cytosolic ribonucleotide reductase (RNR) and TK1 are downregulated in resting (G1) cells, the mitochondrial salvage pathway becomes the sole source of dNP's for mtDNA replication and cellular DNA repair.[18] Thus, non- replicative tissues would therefore be expected to be more vulnerable to alterations in the mitochondrial salvage pathway.

dGK DEFICIENCY WITH MITOCHONDRIAL DNA DEPLETION

Homozygocity mapping followed by candidate gene sequencing was used to identify the first reported mutation in the dGK gene (*DGUOK*) in 3 kindreds of Israeli–Druze origin with the hepatocerebral form of MDS. All patients were homozygous for

a single nucleotide deletion in the DGUOK gene, resulting in premature termination of translation. The pathogenicity was demonstrated by immunoblot analysis showing undetectable dGK protein in liver homogenates.[5] Consequently, *DGUOK* mutations were identified in 4 patients with liver failure due to MSD,from other ethnic backgrounds. Three homozygous to mutations resulting in a truncated dGK protein and one compound heterozygote for two missense mutations, predicted to affect critical residues in the dGK active site.[6,7]

Current data suggest marked phenotype/genotype correlation; in all patients with deleterious mutations of the *DGUOK* gene, brain and liver were clinically involved and mitochondrial respiratory chain was also decreased in heart and variably decreased in muscle. In the only patient who harbored missense mutations associated with significant residual dGK activity, the liver was exclusively involved. Four years following liver transplantation, the patient is healthy, exhibiting mild intermittent tubular dysfunction.[5–7]

TK2 DEFICIENCY WITH MITOCHONDRIAL DNA DEPLETION

Initially 4 patients with skeletal myopathy and MDS, missense mutations in the TK2 gene were described.[8] Post publication, one of the original patient, surviving longer than the others, developed encephalopathy (H. Mandel, personal communication). The pathogenicity of the mutations was confirmed by enzymatic analysis in the patients' muscle and by in characterization of recombinant mutant TK2.[28]

Since than, ten additional patients harboring six new mutations have been reported.[9–12] All patients expressed skeletal muscle involvement . Why some patients also have CNS involvement could not readily be explained by the genotype (Table 1). The different phenotypes may be related to different expression of other genes regulating mitochondrial dNP metabolism such as dNT2.[25,26] Contrary to dGK mutations, none of the patients was homozygous for deleterious mutations, suggesting that a total knock out of TK2 may severely impede fetal development.

Regardless of the specific gene mutation, the diagnosis of mtDNA depletion is problematic. It is accomplished through Southern blot, comparing mtDNA to nuclear DNA ratio relatively to age matched controls. The method is cumbersome and cut off points vary between different laboratories. Based upon the finding of TK2 mutations in a patient with residual mtDNA in muscle reaching 60% of the control, it became clear that cut off points should be re-defined, in order to avoid false negative diagnoses (Table 1). With the growing number of case reports, the place of biochemical and histochemical findings that were previously considered obligatory for diagnosis is also questioned. For example, muscle fibers which do not stain for cytochrome −C oxidase (COX), are present in most patients but were absent in one patient and were only detected in repeated biopsies in another one.[11,24] Similarly, a decrease in respiratory chain activities relative to complex II, was detected in the first muscle biopsy in all but one patient; in this patient, a repeated biopsy at a later age revealed respiratory chain deficiency suggesting that mtDNA depletion may precede a functional defect in respiratory chain.[11] In still another TK2 deficient patient, muscle biopsy suggested isolated COX deficiency with normal activity of all other complexes and only moderately decreased mtDNA content.[12]

Table 1. Characteristics of mtDNA depletion due to TK2 mutations.

Genotype/ phenotype	TK2 I22M/ I22M I153M/ I153M*	TK2 H90N/ H90N H121N/ H121N*	TK2 H90N/ T77M H121N/ T108M*	TK2 T77M/ T77M T108M/ T108M*	TK2 142insG*/	TK2 I121N/I121N I212N/I212N*	TK2 R152G/ K171del R183G/ K202del*	TK2 R183G/ R254X*
Age of onset	24m,15m	24m	12m,16m	12m,14m,16m	5m	8m,12m,8m	3y	2m
Affected tissues	Skeletal muscle +/− lower motor neurons	Skeletal muscle	Skeletal muscle	Skeletal muscle	Skeletal muscle	Skeletal muscle +/− CNS	Skeletal muscle	Skeletal muscle CNS Kidney
Lactic acidosis	+	+	(+)	−	−	+	nd	+
Elevated CK	+	+	(+)	+	nd	+	nd	nd
COX negative muscle fibers	+	−	+	+	+	+	+	+
Muscle mtDNA**	6% − 14%	21%	6%	10%–20%	37%	16%–22%	10%	60%
Fibroblasts mtDNA**	nd	115%	nd	nd	nd	92%,nd,nd	nd	106%
Muscle RC defect	nd	I,III,IV,V	I,III,IV	I,III,IV	I,III,IV	I,III,IV,V	I,III,IV	IV
Fibroblast RC defect	nd	_	nd	nd	nd	_	nd	nd
Muscle TK2 activity**	28% dThd	45% dThd 32% dCyt	36% dThd	nd	nd	14%,23% dThd 13% dCyt	nd	nd
Fibroblast TK2 activity**	nd	47% dThd 31% dCyt	nd	nd	nd	52% dThd 5% dCyt	9% dThd	nd
Reference	[9]	[8,29,33]	[9]	[12]	[10]	[8,29,33]	[11]	[10]

*= numbering according to Wang et al.[28]; **= residual; nd = not determined or not reported; + = present; − = absent; (+) = mildly elevated; RC = respiratory chain; CK = serum creatine phosphokinase; CNS = central nervous system.

Determination of TK2 activity in muscle, though most diagnostic, is problematic owing to the low basal TK2 activity in muscle, and the possibility of contamination by cytosolic TK1 activity. Measuring both dThd and dCyt phosphorylation in isolated mitochondria may be the most sensitive option.[8] Muscle is by far superior to fibroblasts throughout the diagnostic evaluation of TK2 deficiency. The near normal mtDNA content in patient fibroblasts, could be attributed to the import of dNT's from the cytosol in these replicating cells.[17] Upregulation of transcription and/or translation may further compensate for mutated TK2 activity in fibroblasts (Table 1). Hence, mutation analysis is the least invasive and most accurate for the diagnosis of TK2 deficiency in MDS patients.

Since the first descriptions of mutations in the DGUOK and TK2 genes in MDS patients, several studies of the frequency of mutations in these genes among MDS patients have been reported. Current data suggest that TK2 mutations are harbored by only ~10% of the patients with the myopathic form of MDS and dGK deficiency occurs in some 15% of the patients with the hepathocerebral form of MDS.[6,9,10] Many cases remain unaccounted for and the search for candidate genes, likely those involved in mitochondrial deoxynucleotide metabolisms and mtDNA replication, is ongoing.[3,4,10,25]

HYPOTHESIS OF PATHO-PHYSIOLOGICAL MECHANISM

Tissue Specificity

Although mtDNA depletion may affect a number of tissues, liver failure and skeletal muscle involvement is typical in dGK and TK2 deficiency, respectively.

The sparing of replicating tissues, such as cells of the hematopoetic system, may be explained by the import of dNT's supplied from the cytosolic de novo and salvage pathways. Cytosolic import may also provide dNT's during fetal development, as fetal tissues may be regarded as replicative. This most likely explains the normal in-utero development in most patients with mtDNA depletion.[17,30]

The mechanism of sparing non- replicating tissues, such as muscle in dGK deficiency and liver in TK2 deficiency is less clear. DGK deficient tissues may be rescued by dCK activity, which has substrate specificity overlapping that of dGK. However, in brain and liver, dCK activity is low, which explain the vunerability of these tissues to dGK deficiency.[5,31]

In TK2 deficiency, it is not comprehensible that the liver is spared since both de novo pathway, as well as TK1 and dCK are down regulated in this tissue. The most plausible explanation may be, that the residual mutant TK2 activity in liver is still sufficient to meet the demand for mitochondrial dNTP production. This possibility is supported by the finding, that basal TK2 activity relatively to mtDNA content in liver mitochondria is relatively high (Table 2).[30]

Up to date, no patient with homozygous truncation of the TK2 protein and total abolishment of TK2 activity has been reported. Theoretically, such a patient would have a multi-system disorder including liver failure.

The vulnerability of skeletal muscle to TK2 mutations is probably due to low basal muscleTK2 activity, combined with high demand for mitochondrially encoded

Table 2. TK2 and dGK activities relative to COX activity and mtDNA content.

Tissue	TK2 activity	dGK activity	COX activity	TK2/ COX ratio	dGK/ COX ratio	Tk2/ mtDNA ratio	dGK/ mtDNA ratio
Liver	100%	93%	32%	94%	83%	100%	100%
Skeletal muscle	10%	45%	100%	3%	17%	5%	26%
Heart	72%	100%	77%	28%	38%	38%	56%
Fibroblast	84%	71%	25%	100%	100%	63%	57%
100% value	0.077*	0.015*	1509*	17×10^{-5}	3×10^{-5}	13×10^{-5}**	2×10^{-5}**

Mitochondria isolated from various human tissues were analyzed for TK2 (dThd), dGK (dGuo), complex IV activity and mtDNA content.[31] The percentages are presented relatively to the tissue expressing the highest value. The last row presents the absolute values corresponding to 100%.

*nmol/min/mg protein.

**nmol/min(activity)/ng mtDNA.

respiratory chain proteins and mtDNA (Table 2).[31] The balance between TK2 activity and demand for its products in skeletal muscle, is likely fine-tuned and near a threshold level. This may be exemplified by the fact that homozygocity for the ''relatively mild'' TK2 mutation H90N is still associated with devastating consequences.[8] Kinetic studies of the cloned mutant enzyme demonstrated markedly increased Km values for ATP(the main phosphate donor for TK2 activity). The mutant TK2, may have initiated a vicious cycle with decreased dTMP and dCMP production with consequent mtDNA depletion and respiratory chain dysfunction. Decreased ATP production, would than in turn inhibit TK2 activity.[28]

Mitochondrial Deoxyribonucleotide Pool Imbalance

The exact events leading to mtDNA depletion are yet to be determined. Most probably mutated dGK and TK2, induce imbalances in the mitochondrial dNP pools. The nature of dNT pool aberrations in dGK and TK2 deficiency is the focus of two recent studies.

The finding that mitochondrial DNA depletion can be prevented by dGMP and dAMP supplementation in serum depleted dGK deficient fibroblasts, provides evidence that deoxynucleoside monophosphate limitation may trigger mtDNA depletion.[32]

Additionally, we recently, measured all four mitochondrial dNTP pools, in resting fibroblast from patients with TK2 deficiency. Imbalanced dNTP pools, with specifically decreased dTTP content and lowered dTTP/dCTP and deoxypyrimidine triphosphate/ deoxypurine triphosphate ratios were observed. The patients fibroblasts did not express mtDNA depletion. Still, our findings illustrate the tendency of dNTP pool disturbances in TK2 deficiency.[33] It is anticipated that dNTP pool aberrations would be more pronounced in non- replicating tissues such as muscle. The lack of available tissues expressing mtDNA depletion from TK2 deficient patients hampers the proceeding of additional studies.

The consequences of imbalanced dNTP pools would be expected include mtDNA mutations as well as depletion, due to decreased fidelity of polymerase-γ.[28] This is probably the cause with TP deficiency, as MNGIE patients accumulate qualitative as well as quantitative mtDNA defects.[14,34] In contrast, no mutations were detected in the mitochondrial t-RNA genes in one of the first reported TK2 patients. Additionally, dGK and TK2 deficiency was not accompanied by deletions or other qualitative mtDNA aberrations.[5,8] Possibly decreased dNTP abundance is less mutagenic than the corresponding excess, as DNA polymerase is forced to stall. Consequently, with decreased DNA synthesis but with a more efficient proofreading.[27]

CONCLUSIONS

Mitochondrial DNA depletion due to dGK and TK2 deficiently are included in an emerging group of disorders with impaired mtDNA maintenance. The diagnosis and characterization of these disorders require a multi disciplinary approach including clinical, biochemical and molecular characterization. dGK and TK2 genes should be sequenced but mutations are found only in a small fraction of patients with mtDNA depletion syndrome, and the search for additional candidate genes should continue. The exact patho-physiological mechanisms of TK2 and dGK deficiencies remain to be determined but recent findings show that they are most likely attributed differential expression of deoxyribonucleoside kinases and to imbalanced dNTP pools.

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