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Deoxythymidylate kinase, *DTYMK*, is a novel gene for mitochondrial DNA depletion syndrome

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ABSTRACT

Background: Mitochondrial DNA depletion syndrome is a group of heterogeneous diseases with non-specific presentation. The common feature is the quantitative depletion of mitochondrial DNA without qualitative defects. Diagnosis of these diseases poses a challenge and whole exome sequencing is often needed for their diagnoses.

Case: Two siblings of a quartet family, presenting with hypotonia, microcephaly and severe intellectual disability, have been diagnosed to harbor two heterozygous variants in *trans* in the *DTYMK* gene of the thymidine biosynthesis pathway. Mitochondrial DNA depletion has been demonstrated *in silico* in the more severe sibling.

Conclusions: We suggest the consideration of incorporating *DTYMK* as one of the associated genes of mitochondrial DNA depletion syndrome (MDDS). *DTYMK* may be the missing link in the mitochondrial nucleotide salvage pathway but further characterization and additional evidence would be needed.

1. Introduction

Mitochondrial DNA depletion syndrome (MDDS) has been described as the quantitative defects in the spectrum of mitochondrial DNA maintenance disorders [1]. This causes a significant drop in mitochondrial DNA in affected tissues, which is thought to account for the pathophysiology of MDDS [2], as most components of the respiratory chain are mitochondrial-encoded (except complex II, which is entirely encoded by nuclear DNA).

An array of presentation has been described [3–5]: muscle weakness, hepatic failure, renal tubulopathy during neonatal/infantile period to progressive encephalomyopathy, deafness, external ophthalmoplegia for those presenting during childhood/adolescence.

Diagnosis is challenging as the disease does not show any specific structural or biochemical abnormalities [6]. Presence of lactic acidemia and CSF lactate peak on MRI spectroscopy may suggest disorder of mitochondrial origin. In some patients, all of the investigations can be normal [7]. Diagnosis by mtDNA/nDNA ratio of 30% or enzymatic assay of respiratory chains in tissue biopsy [4,8] can be invasive and labor-intensive. As a consequence, many cases remain undiagnosed or misdiagnosed.

A number of genes have been associated with this heterogeneous condition over the years. Despite the array of genes involved, they converge onto genes regulating several common pathways: mtDNA synthesis/transcription (*POLG, POLG2, TWNK, TFAM, RNASEH1, MGME1* and *DNA2*), mitochondrial/cytosol nucleotide maintenance (*ABAT, AGK, DGUOK, MPV17, RRM2B, SLC25A4, SUCLA2, SUCLG1, TK2* and *TYMP*) and mitochondrial dynamics (*OPA1, MFN2* and *FBXL4*). Readers are referred to various reviews on this topic for further details [1,9].

With the advent of technology, many undiagnosed diseases enjoy the benefits with whole-exome sequencing (WES) [10,11]. Here we describe the clinical application of WES to two Chinese siblings where a novel gene potentially causing MDDS was identified: *DTYMK*. Further studies in their parents confirmed the two variants were in *trans* in both siblings. We have also attempted to demonstrate mitochondrial DNA (mtDNA) depletion *in silico* using data from whole genome sequencing (WGS).

DTYMK is a nuclear-encoded deoxythymidylate kinase, catalyzing the phosphorylation of deoxy-TMP to deoxy-TDP. This enzyme is ubiquitously expressed in all tissues and is the last unique enzyme in the pathway leading to dTTP production [12]. This has also been characterized as an essential gene in multiple studies and in the database of Online Gene Essentiality [13–15]; Stable supply of deoxyribonucleoside triphosphate is essential for DNA synthesis. A number of genes responsible for mitochondrial nucleotide maintenance have already been

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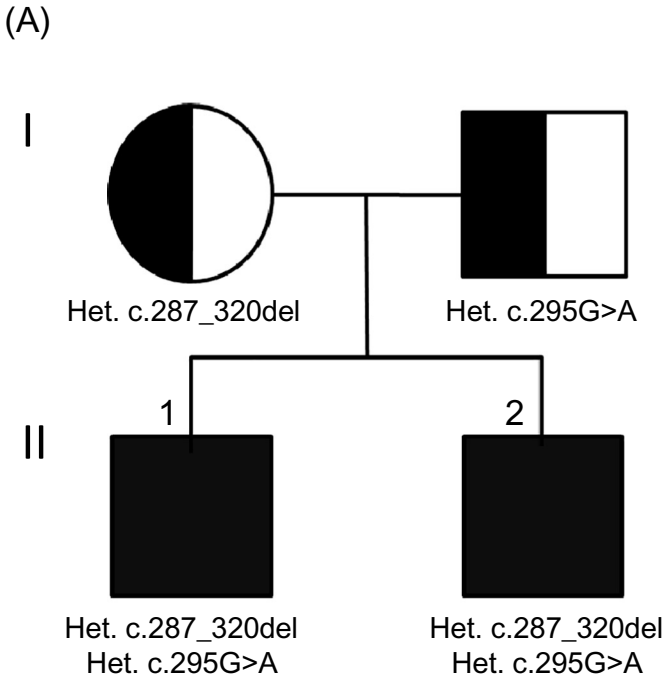

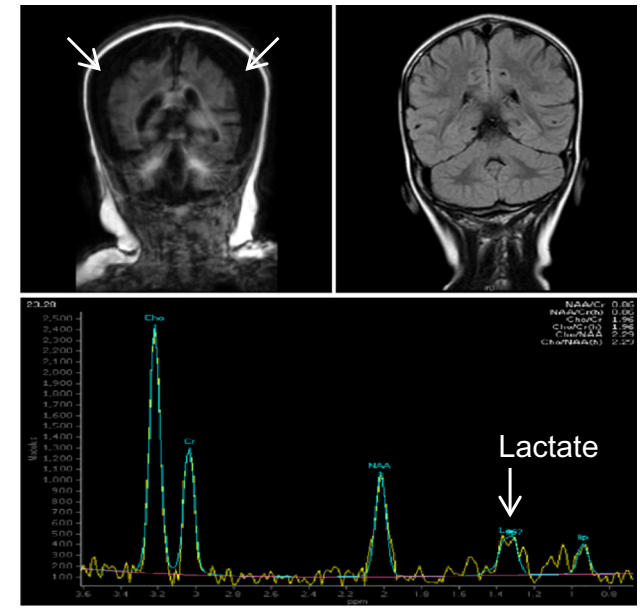

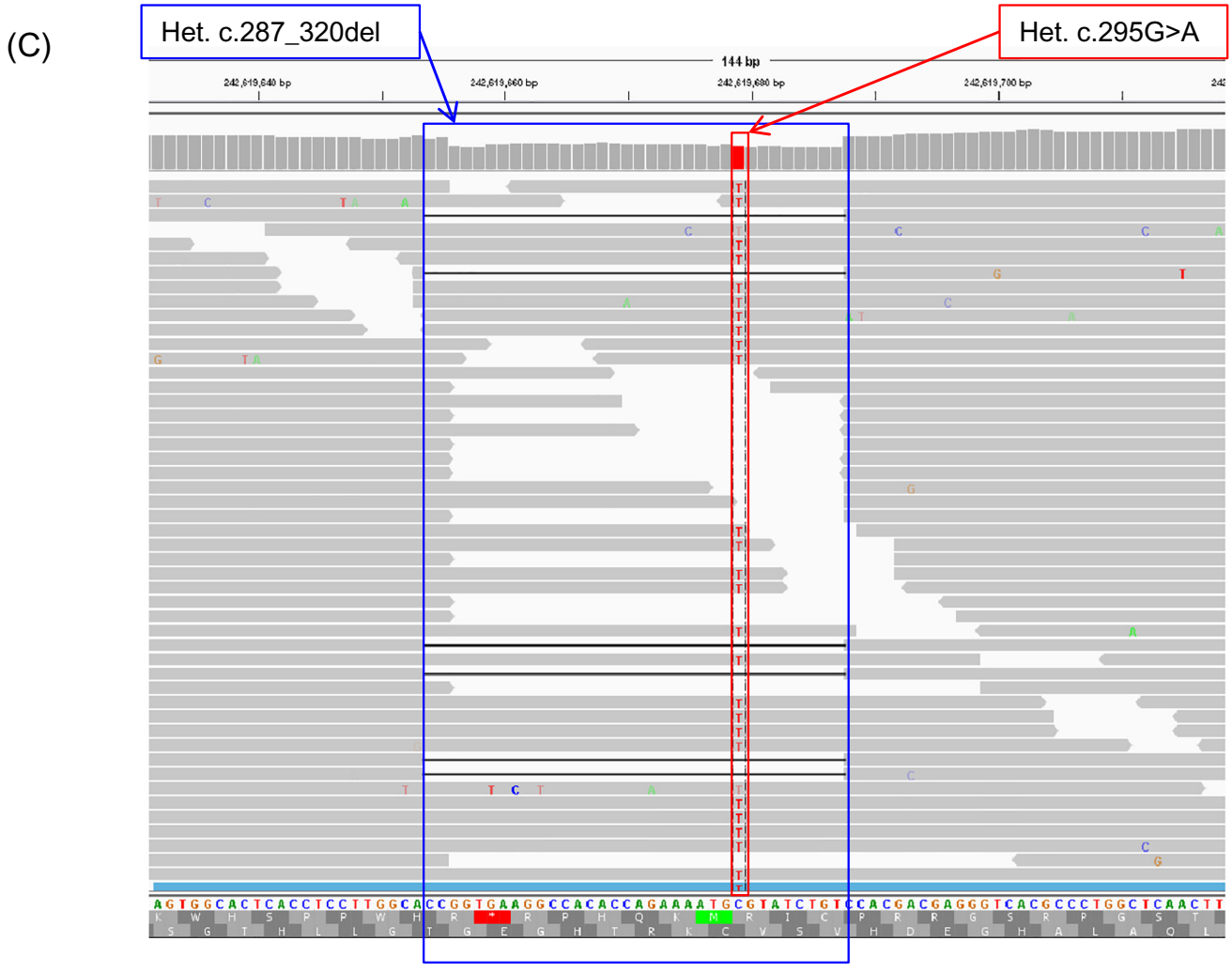

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Fig. 1. WES results of the family and imaging findings of the two siblings. (A) Pedigree of the quartet family. (B) Magnetic resonance imaging and spectroscopy of the siblings. Upper panel shows coronal plane with T1 imaging of the more severe younger sibling (top left) and the older sibling (top right). More profound cerebral atrophy noted in the more severe younger sibling with doublet lactate peak detected at around 1.3 ppm (bottom). (C) Compound heterozygous of NM_012145.3:c.287_320del; p.Asp96Valfs*8 and NM_012145.3:c.295G > A;p.Ala99Thr in the younger sibling. Note the two mutations are mutually exclusive and this figure alone already supports the fact that these mutations are in *trans*.

associated with MDDS [3]. To our understanding, these siblings could represent the first family with MDDS due to *DTYMK* mutations.

2. Cases

We described 2 brothers, aged 1 and 6 years, with microcephaly, hypotonia and severe intellectual disability and they shared several biochemical abnormalities including raised serum lactate, pyruvate and alanine (Fig. 1A).

The younger brother was 12 months old, who was a full term baby born by cesarean section for breech presentation with birth weight of 2.455 kg (around 50th percentile). The baby was noted to have congenital microcephaly (40.7 cm at 6-month-old, slightly below 3rd percentile), hypotonia with global delay at 6 months old. He suffered from status epilepticus associated with febrile illness at nearly 7 months old and subsequently had developmental regression, hypertonia, cortical blindness and dysphagia. CT brain performed on the same day of status epilepticus showed no focal lesion or edema, while MRI brain done 1 week later revealed bilateral symmetric restricted diffusion at caudate heads, basal ganglia, thalami, corona radiate, cerebellar dentate nuclei and crus cerebri. Cerebrospinal fluid (CSF) screened for infection and anti-NMDA receptor anti body were negative. Repeat MRI brain at 10 month old showed marked cerebral atrophy with bilateral subdural haemorrhagic effusion, and lactate peak in the MR spectroscopy (Fig. 1B). Serum lactate (1.7 to 4.5 mmol/L; Ref: 0.7–2.1) and alanine level (541 to 584 umol/L; Ref: 143–439) were raised while the CSF lactate was normal. Other metabolic screen including blood glucose, ammonia, carnitine, homocysteine, biotinidase, creatine metabolism and urine for organic acid, amino acids and reducing substance were normal. Muscle biopsy was not available for investigation. His target genetic panel for epilepsy was unremarkable. At the time of writing at the age of 25 months old, patient had epilepsy on multiple anti-convulsants, mixed spastic quadriplegia with dystonic cerebral palsy, undescended testes, laryngomalacia, oromotor dysphagia requiring gastrostomy feeding, sensorineural hearing loss and cortical blindness.

His 6-year-old elder brother also had mild congenital microcephaly (head circumference of 31 cm, below 3rd percentile) and low birth weight of 2.1 kg, born at full term. He had hypotonia, global developmental delay and severe intellectual disability. He could walk independently and speak few single words. His vision and hearing were normal and he had no seizure attack. His serum lactate fluctuated from 2 to 3.5 mmol/L (Ref. 0.7–2.1) and alanine had been slightly raised and then normalized while the MRI brain was normal (Fig. 1B). Muscle biopsy was also not available for investigation. His head circumference remained static in the past 3 years and is now 5 cm below 3rd percentile. At the age of 7 years old, patient had severe intellectual disability (about 19-month-old mental age) and lower limb dystonia. He also developed an episode of sudden onset coma with respiratory failure requiring intubation. Glasgow coma score (GCS) was 5 on admission with bilateral 4 mm sluggish pupils. Cause of sudden collapse could not be identified after extensive microbiological and biochemical investigations. Electroencephalogram (EEG) only showed encephalopathic background with occasional sharps/spikes and slow wave discharges over frontal region and was not in status epilepticus. GCS gradually recovered with conscious level normalizing 2 days after admission.

Both parents were asymptomatic.

3. Materials and methods

3.1. Whole exome sequencing (WES)

Blood samples were collected from the siblings and the parents. Informed consent had been obtained from the family. Methods for DNA extraction and WES analysis had been previously described [10,11]. Target enrichment was performed using SureSelect Target Enrichment System Human All Exon V4 target kit (Agilent Technologies). Sequencing reaction was performed on Illumina HiSeq 2000 sequencer with 100 bp paired-end. Analysis of sequencing data was done with VariantStudio (Version 2.2.1, Illumina) and in-house established bioinformatics pipeline.

3.2. In silico analysis of mitochondrial DNA depletion

Whole genome sequencing (WGS) was performed with Illumina whole genome sequencing kit analyzed on NextSeq 500. Data analysis was performed with VariantStudio (Version 2.2.1, Illumina) and in-house established bioinformatics pipeline, establishing the median read depth in WGS. Mitochondrial genome was analyzed with mtDNA Variant Processor and mtDNA Variant Analyzer from Illumina BaseSpace. mtDNA:nDNA ratio was calculated by dividing mtDNA average read depth with median read depth obtained from WGS.

4. Results

4.1. Identification of compound heterozygous mutations in the two siblings

Two mutations were identified in the *DTYMK* gene from the siblings. The first one is a frameshift mutation NM_012145.3:c.287_320del; p.Asp96Valfs*8 and the second one is a missense mutation NM_012145.3:c.295G > A;p.Ala99Thr (Fig. 1C). The siblings share the same mutations in *DTYMK* gene (Supplementary Fig. 1). The first mutation was inherited from the mother and the second mutation was inherited from the father (Supplementary Fig. 1). Both of the brothers were compound heterozygous for the 2 mutations. No other pathogenic genes could be detected in the symptomatic siblings.

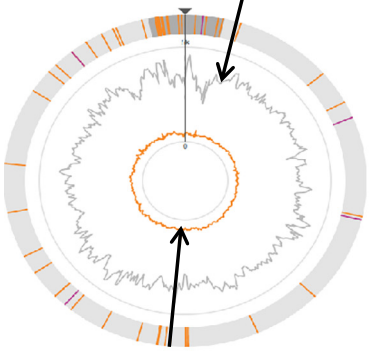
4.2. mtDNA depletion in the more severe sibling

Mitochondrial DNA associated Leigh syndrome due to m.8993 T > G variant (which is not known to cause mtDNA depletion) was used as the negative control. *POLG*-related MDDS was used as the positive control with mtDNA depletion demonstrated with mtDNA:nDNA of 11.6 which is 15.6% of the negative control (Fig. 2A–D). The percentage of control was lower at 65.5 in the more severe younger sibling than the older, less severe sibling (Fig. 2D). Of note, mtDNA analysis did not detect any differences in sequences across the two siblings (Data not shown).

5. Discussion

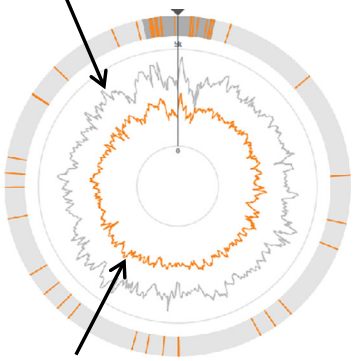
Hypotonia, microcephaly and intellectual disability are non-specific presentations. Elevated lactate and presence of lactate peak in spectroscopy, together with these non-specific symptoms, hint towards disorders related to mitochondrial dysfunction in the younger sibling. Diagnosis was only made after WES of the quartet family, supporting

(A) Read depth of mt genome with m.8993T>G mutation



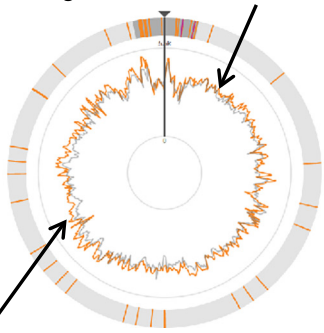
Read depth of mt genome in *POLG*-related mtDNA depletion

(B) Read depth of mt genome with m.8993T>G mutation



Read depth of mt genome in the more severe younger sibling with *DTYMK* mutation

(C) Read depth of mt genome with m.8993T>G mutation (Grey)



Read depth of mt genome in the less severe older sibling with *DTYMK* mutation (Orange)

(D)

	Nuclear DNA	mtDNA	mt:nDNA	% of control (Blood)
m.8993T>G	47	3500	74.5	100.0
POLG	43	500	11.6	15.6
DTYMK (severe)	41	2000	48.8	65.5
DTYMK (milder)	40	3500	85.4	114.6

(E)

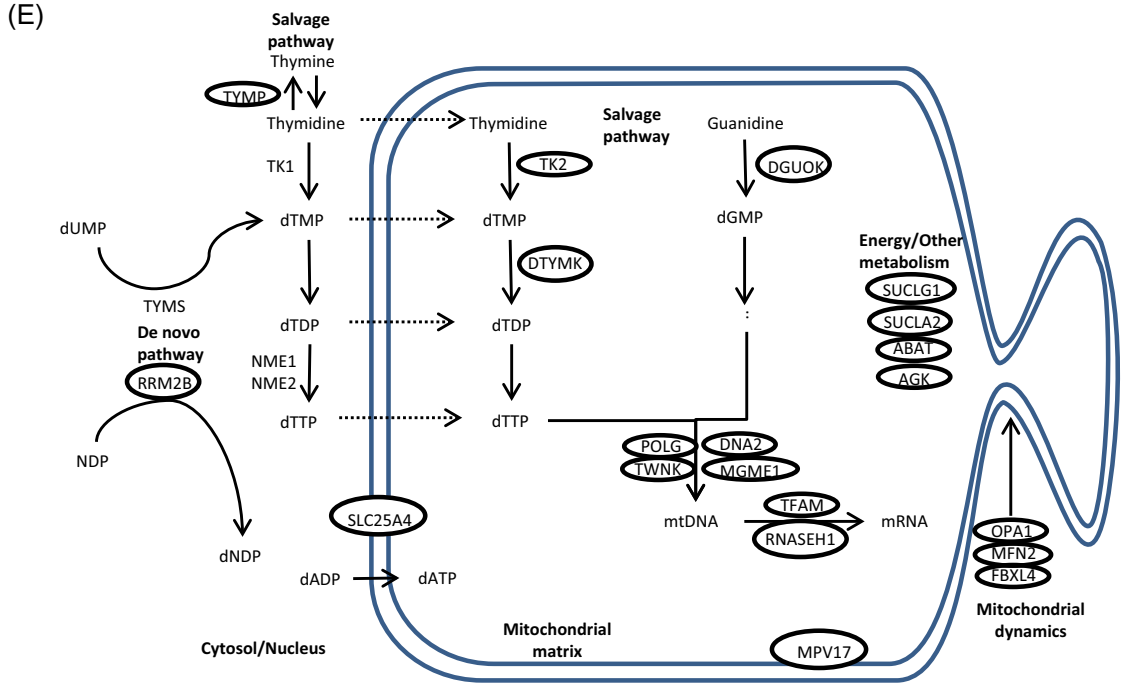


Fig. 2. *In silico* demonstration of mtDNA depletion with whole genome sequencing data. (A)–(C) Comparison of read depth of negative control (a non-MDDS mitochondrial cytopathy m.8993T > G), positive control (*POLG*-related MDDS) and that of the two siblings in mitochondrial genome. (D) Table showing the mtDNA:nDNA ratio of the above cases. (E) Schematic representation of genes associated with mitochondrial DNA depletion syndrome, in the context of mitochondrial nucleotide metabolism. Genes that have been associated with MDDS were encircled. Nucleotides within mitochondria can be produced from the salvage pathway by recycling nucleotides within mitochondria or transported from cytosol, of which the later is limited in quiescent cells. Thus, in non-replicating cells, mtDNA replication is expected to be highly dependent on the salvage pathway of mitochondria. Due to spatial constraint, cellular localization for ABAT, AGK, SUCLA2, SUCLG1, OPA1, MFN2 and FBXL4 may not be accurately represented on this diagram.

the importance of WES for the diagnosis in these cases.

NM_012145.3:c.287_320del is a known mutation documented on Database for Single Nucleotide Polymorphisms (dbSNP: rs1005167119) without information on its clinical significance. This variant is present only in East Asian population at a low frequency of 0.01087% according to Genome Aggregation Database. It is predicted to cause frameshift mutation beginning at the 96th residue out of 212 in the encoded DTYMK. Virtually all MDDS are inherited as autosomal recessive and most mutations causing autosomal diseases are loss-of-function in nature [16]. Furthermore, frameshift and nonsense mutations in the two genes upstream of *DTYMK*, *TK2 and TYMP*, can also cause autosomal recessive MDDS [17]. Hence, as an enzyme-encoding gene, it was reasonable to assume loss-of-function as a disease causing mechanism of this gene. As such, based on published guidelines from the American College of Medical Genetics and Genomics (ACMG), [18], this variant may be classified as pathogenic.

The other variant is a single nucleotide change at c.295G > A (dbSNP: rs887888951). This variant is detected in East Asian population and non-Finnish European at a low frequency of 0.01002% and 0.001548% respectively. It is predicted to be disease causing and damaging by MutationTaster and SIFT respectively. Combined Annotation-Dependent Depletion (CADD) score for this variant was 24.5 [19]. To better understand the effect of this missense variant, a program called HOPE was used to provide structural information [20]. The resulted missense variant, p.Ala99Thr, introduced a hydrophilic residue into the protein core and may cause loss of hydrophobic interactions within the core. The missense change is also predicted to break the alpha-helix in which p.Ala99 resides. Further characterization would be needed to fully understand the pathogenicity of this variant.

Given the allele frequency of the two rare variants in East Asian population, the chance of a compound heterozygous individual of this genotype would be 1.089×10^{-8} (or 1 in ~91,810,000).

5.1. Homeostasis of mitochondrial nucleotide pool

Before explaining the potential association of *DTYMK* with MDDS, the mitochondrial pyrimidine metabolism must first be reviewed. Deoxynucleoside triphosphates (dNTPs) are essential units for DNA replication both within nucleus and mitochondria. Two pathways have been described for the synthesis of dNTP: *de novo* and salvage pathway [21]. *De novo* pathway is part of the folate-mediated one-carbon biometabolic network for the synthesis of purines, thymidylate and remethylation of homocysteine to methionine [22]. In the context of pyrimidine nucleotide synthesis, *de novo* pathway reduces ribonucleotides to produce deoxyribonucleotides. The *de novo* pathway is considered to operate in cytosol only [23]. Salvage pathway phosphorylates and recycles existing deoxynucleosides to supply dNTPs for replication and it operates both in cytosol and mitochondria (Fig. 2C).

The timing of replication adds another dimension to the homeostasis of dNTPs within mitochondria. DNA replication occurs separately in both nucleus and mitochondria; Replication in nucleus only occurs during S-phase while that in mitochondria occurs independently round-the-clock. Hence in quiescent cells, dNTP synthetic pathways in cytosol do not contribute significantly to mitochondrial dNTPs [24] and mitochondrial DNA replication in quiescent cells rely mostly on salvage pathway within mitochondria. For instance, dTTP production in rat cardiomyocyte mitochondria is catalyzed solely by *TK2* of the salvage pathway in mitochondria [25].

Spatially, as a compartmentalized organelle, import of dNTPs into mitochondria requires dedicated transporters. Solute carrier family 25 (SLC25) is probably the most well-characterized family of mitochondrial transporters [26]. Within the 24 characterized subfamilies (stratified based on their substrate specificity), 10 of them act as nucleotides or dinucleotides transporters [27]. *SLC25A4* encodes the heart-/muscle-specific ADP/ATP carrier, also known as the *ANT1* gene [28] and is one of the genes associated with MDDS. *SLC25A33* and *SLC25A36* have been identified to be the mitochondrial pyrimidine nucleotide transporters [29]. They have yet to be associated with any human diseases.

5.2. DTYMK as a candidate gene for mitochondrial TMPK

Focusing on thymidylate biosynthesis, thymidine is phosphorylated by either of the salvage pathway TK1 in cytosol or TK2 in mitochondria to dTMP. Thymidine may also be converted back to thymine in cytosol by the TYMP; the deficiency of which is known to cause mitochondrial neurogastrointestinal encephalopathy syndrome (MNGIE) which is another MDDS [30]. Mutation in genes of the cytosolic thymidine salvage pathway (*TK1*, *NME1* and *NME2*) has not been associated with MDDS while deficiency of TK2 deficiency of the mitochondrial salvage pathway does [31].

Thymidine monophosphate kinase activity has been detected [32] but the enzyme itself has yet to be characterized. Reduced level of DTYMK in cancer cell lines has been shown to cause reduced dTDP level [33], coinciding with the reduced dTTP level in TK2 deficient patient cell lines [34]. In addition, DTYMK is the last unique enzyme in the dTTP biosynthetic pathway with the conversion of dTDP to dTTP catalyzed by a non-specific nucleoside diphosphate kinase (NME1 and NME2).

Though so far lacking support from functional study, DTYMK can be a candidate for mitochondrial thymidine monophosphate kinase. Different isoforms and transcript of DTYMK has been annotated electronically to be present in cytosol and mitochondria [35]. Localization study and further characterization of DTYMK should shed light on its role in mitochondrial thymidine metabolism. *DTYMK* may also be considered to be included as part of the gene panel of MDDS.

Furthermore, supplementation of deoxypyrimidine monophosphate for TK2 deficiency has been shown to rescue the phenotype to some extent *in vitro* [36,37] and in mouse models [38]. Whether a similar principle works for DTYMK deficiency, *i.e.* dTDP supplementation, awaits future studies.

5.3. In silico demonstration of mtDNA depletion

Diagnosis of MDDS is often done by demonstration of two deleterious mutations *in trans* in the relevant genes. Doubtful genetic findings may be supplemented by demonstration of mtDNA depletion. The historical standard to demonstrate mtDNA depletion was the reduced mtDNA to nuclear DNA ratio by Southern blot analysis. This method is labor-intensive with radioactive substance involved. Biochemical proof of reduced activities in components of electron transport chain would be equally tedious and is subject to sample integrity and assay availability. Real-time quantitative PCR (qPCR) has been described [39,40] but requires amplification of fixed, limited positions in the nuclear and mitochondrial genome. Here, whole genome sequencing data (without the bias from PCR and/or enrichment in WES) was used to demonstrate mtDNA depletion *in silico*.

mtDNA depletion has been demonstrated in the blood sample of the more severe sibling at 65.5% of control. Although it may not be directly comparable, referencing the data from qPCR of Dimmock et al. [39], mtDNA depletion cutoff of 70% has a specificity of 80% while cutoff at 60% has a specificity of 91% in blood. Sensitivity of qPCR in blood for MDDS is, on the other hand, relatively low with 51% when using a cutoff of 70%. Demonstration of mtDNA depletion in blood tends to be more specific than being sensitive, especially when a lower cut-off was used [39]. Highest sensitivity may be seen when using samples from liver where mtDNA depletion of 50% can still achieve a sensitivity of 100%.

As the degree of mtDNA depletion in blood is highly variable in MDDS while mtDNA depletion is more consistently observed in muscle or liver tissues [39], it would be interesting to see if similar pattern can be seen in the whole genome sequencing data obtained from liver and muscles.

5.4. Intra-familial variability of presentation

The two siblings described in the current study harbor the same set of mutations in *DTYMK* but with significantly different manifestation. In fact, intrafamilial and interfamilial variability have been frequently described across MDDS in *MFN2* [41], *TYMP* [42,43], *RRM2B* [44,45], *TK2* [7,46], and others. Taking TK2 deficiency for illustration, Paradas et al. [46] described the proband born of consanguineous parents with homozygous c.323C > T in *TK2* gene presenting with indolent myopathy at the age of 22 years old (symptom onset since childhood). His other siblings had more aggressive disease courses with death at the age of 3 and another with stillbirth at 34 weeks' gestation. Two more infantile deaths were documented from previous generations in the same consanguineous family.

In the current study, both siblings harbor the same mutations with common symptoms of microcephaly, hypotonia and severe intellectual disability. Their symptom onset ages were similar around at age of 6 months. However, the younger siblings had a more severe presentations evidenced by status epileptics with cortical blindness and developmental regression at 7 months old. The elder brother, on the other hand, developed sudden onset coma with respiratory failure at the age of 7. The evidence had been summarized in Supplementary Fig. 1D.

6. Conclusion

We have described the identification of compound heterozygous mutations in *DTYMK* from this quartet family with the use of WES, highlighting the importance of WES for the diagnosis of cases with non-specific presentations. DTYMK was poorly characterized though it is known to phosphorylate dTMP to dTDP. Cancer cell line studies showed depletion of dTDP with loss of DTYMK, suggesting DTYMK accounts for a major source of dTDP. *TK2* and *TYMP,* probably upstream of *DTYMK*, are well-known genes associated with MDDS.

Based on the facts (1) that *DTYMK* is an essential gene, (2) that mtDNA depletion has been demonstrated and (3) the potential role of DTYMK in thymidylate biosynthesis supported by biochemical studies such as the reduced dTDP level in cancer cell lines reduced in DTYMK level, *DTYMK* may be considered as a new member of gene under MDDS. Further characterization of DTYMK and its identified mutations would be needed to further substantiate its role in mitochondrial nucleotide salvage pathway. We would also await additional evidence from more clinical cases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.06.028.

Declaration of Competing Interest

None.

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