Development of functional screening for improved understanding of *TK2* genetic variants

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Introduction

- Thymidine kinase 2 deficiency (TK2d) (OMIM:609560) is an ultra-rare, life-threatening mitochondrial disease caused by autosomal recessive pathogenic variants in the thymidine kinase 2 gene (*TK2*)[1,2]
- Thymidine kinase 2 (TK2) is a mitochondrial enzyme involved in deoxynucleotide triphosphate (dNTP) metabolism for mitochondrial DNA (mtDNA) replication and repair[1]
- Impaired activity of this enzyme results in an imbalance in the dNTP pool, causing mtDNA depletion and/or multiple deletions in mtDNA,[1,2] leading to proximal, bulbar and axial muscle weakness[3]
- Pathogenic variants have been identified in all 10 exons of *TK2*; however, there is no clear genotype–phenotype correlation owing to the small number of affected individuals reported to date[3–5]
  - This variability presents a significant challenge in the classification of variants of uncertain significance (VUS), which can delay diagnosis[1,4]
- There is a need for improved understanding of TK2d pathobiology, to support more accurate resolution of *TK2* VUS

Objective

- To develop an *in vitro* functional assay that recapitulates disease biology to screen and evaluate the impact of *TK2* variants on mtDNA maintenance

Methods

Cell line generation and selection of *TK2* variants

- U2OS is a human osteosarcoma cell line that expresses *TK2* at the protein level[6]
- For this study, a U2OS *TK2* knockout pool line was generated externally using CRISPR, achieving approximately 70% CRISPR-edited cells
- Four pathogenic *TK2* variants previously described in the literature were selected for testing based on age of TK2d symptom onset in affected individuals and on the extent of mtDNA depletion and/or deletions in muscle biopsies from these individuals[4,5] (**Table 1**)

Table 1. Pathogenic *TK2* variants investigated in the present study[4,5]

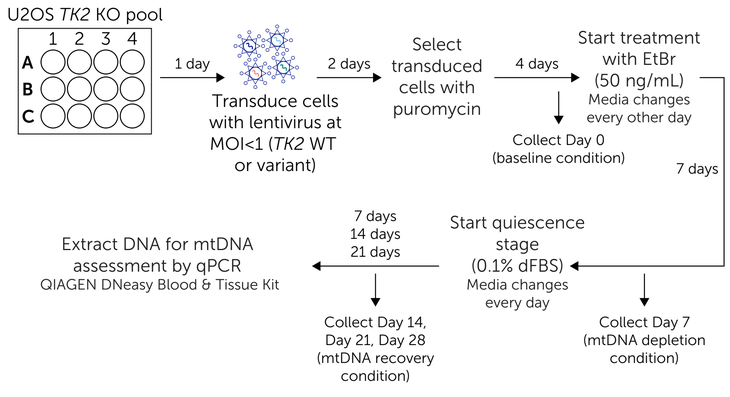
TK2 variant	Patients, n	mtDNA depletion, %, mean (SD)	Age of TK2d symptom onset, years, mean (SD)	Patients with deletion (if known), n	Patients without deletion (if known), n
NM_004614.5:c.323 C>T	18	33 (21)	11.4 (12.7)	8	2
NM_004614.5:c.388 C>T	5	20 (13)	0.3 (0.1)	NA	1
NM_004614.5:c.547 C>T	3	15 (NA)	1.1 (NA)	NA	NA
NM_004614.5:c.604-606del	11	55 (53)	39.3 (10.1)	4	NA

mtDNA, mitochondrial DNA; NA, not available; n, number; SD, standard deviation; *TK2*, thymidine kinase 2 gene; TK2d, thymidine kinase 2 deficiency.

Cell transduction

- The *TK2* full-length coding sequence (NM_004614.5) was cloned downstream of a ubiquitin C promoter into a lentiviral expression plasmid backbone containing EGFP:T2A-puro under the control of a cytomegalovirus promoter
  - The full-length wild type (WT) *TK2* was replaced by the coding sequence containing the variants described in **Table 1**
  - Lentivirus was produced by co-transfecting HEK23T cells with the *TK2* expression plasmid and with the plasmids pMD2.G and psPAX2
- U2OS *TK2* knockout pool cells were cultured in McCoy's 5A medium supplemented with 10% dialyzed fetal bovine serum (dFBS) and 1% penicillin-streptomycin
- Cells were seeded at 100 000 cells per well in 12-well plates and incubated overnight at 37°C, 5% CO_2
- After this incubation, cells were transduced at a multiplicity of infection of 0.3 with WT *TK2* or *TK2* variants
- Duplicate wells were transduced with each lentiviral construct
  - Transduction was performed in the presence of Polybrene (8 µg/mL), which was subsequently removed 24 hours after transduction
- Two days after transduction, cells were selected with puromycin for 4 days; following this, cells at the Day 0 time point were collected (baseline condition; **Figure 1**)

Figure 1. Overview of cell transduction and schedule of cell collection



dFBS, dialyzed fetal bovine serum; EtBr, ethidium bromide; KO, knockout; MOI, multiplicity of infection; mtDNA, mitochondrial DNA; qPCR, quantitative polymerase chain reaction; *TK2*, thymidine kinase 2 gene; WT, wild type.

Ethidium bromide-induced mtDNA depletion and post-treatment recovery

- Pathogenic variants may not consistently show mtDNA depletion *in vitro*
- Ethidium bromide (EtBr) is commonly used to intercalate into and deplete mtDNA by interfering with transcription and replication, allowing assessment of variant-specific recovery dynamics over time[7–9]
- Therefore, transduced cells were incubated with a low concentration of EtBr (50 ng/mL) for 7 days to drive mtDNA depletion without affecting nuclear DNA (nDNA)[9]
  - At the Day 7 time point, EtBr-treated cells were collected (mtDNA depletion condition; **Figure 1**)
- EtBr was withdrawn from the remaining cells, which were subsequently grown under quiescent conditions for up to 21 days using 0.1% dFBS to drive *TK2* over thymidine kinase 1 gene (*TK1*) expression[10]
  - Thymidine kinase 1 (TK1) is a cytosolic enzyme of the nDNA salvage pathway that recycles thymidine for nucleotide synthesis[8]
  - In contrast to *TK2*, expression of *TK1* is cell-cycle dependent, with peak levels during S-phase and with degradation after division[8]
- Cells were collected during the quiescence stage at the Day 14, Day 21 and Day 28 time points (mtDNA recovery condition; **Figure 1**)

DNA extraction and mtDNA copy number quantification

- Total DNA was extracted from cell pellets using the DNeasy Blood & Tissue Kit (QIAGEN) and quantified using a NanoDrop spectrophotometer
- Quantification of mtDNA and nDNA was performed via quantitative polymerase chain reaction (qPCR) in the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems), using standard fast cycle conditions for TaqMan Fast Advanced Master Mix (Applied Biosystems) in 384-well plates with 25 ng of total DNA per well in quadruplicate for each sample
  - mtDNA copy number was quantified in relation to nDNA[7,10]
  - A custom-designed NADH-ubiquinone oxidoreductase chain 1 (*ND1*) TaqMan assay was used to quantify relative mtDNA, and the Copy Number Reference TaqMan assay for ribonuclease P (*RNase P*; Applied Biosystems) was used for nDNA quantification
  - The mtDNA copy number was calculated using the formula $2^{\Delta(Ct)}$, in which Ct is the difference of average cycle threshold values between *RNase P* (nuclear gene) and *ND1* (mitochondrial gene)[7]
- A one-way ANOVA with Dunnett's multiple comparisons test was used to test for statistical differences in mtDNA copy number between the *TK2* variants and WT *TK2* at baseline (Day 0)
- A two-way ANOVA with Dunnett's multiple comparisons test was used to test for statistical differences in mtDNA copy number between the *TK2* variants and WT *TK2* at baseline (Day 0) and all subsequent time points (Day 7, Day 14, Day 21 and Day 28)

Results

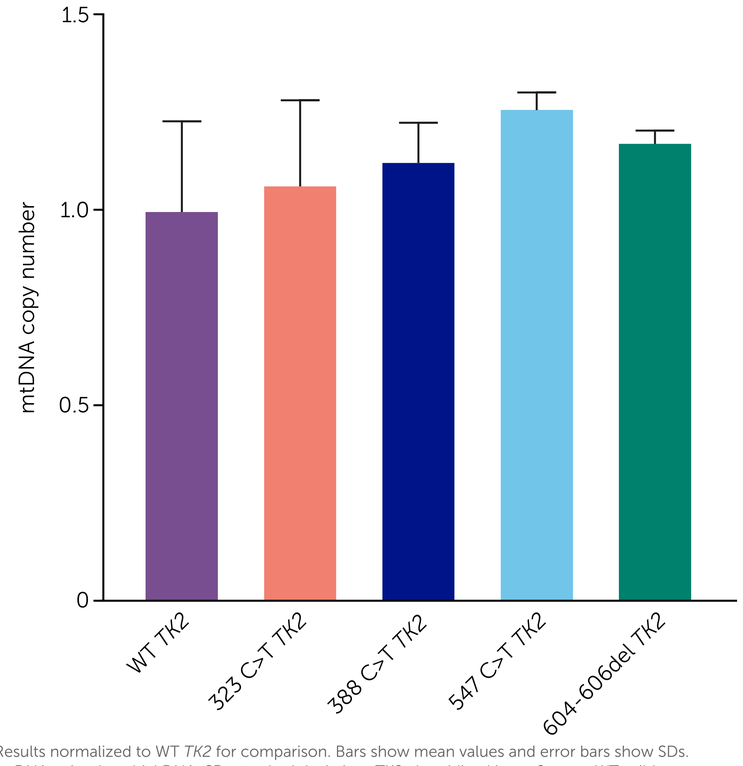
Baseline mtDNA copy number in *TK2* variants

- The baseline differences observed in mtDNA copy number between the *TK2* variants and WT *TK2* were not significant (**Figure 2**), highlighting the need to use EtBr to induce mtDNA depletion for the measurement of mtDNA recovery

mtDNA copy number in *TK2* variants after mtDNA depletion and post-treatment recovery

- As expected, substantial mtDNA depletion occurred after EtBr treatment (Day 7) for all *TK2* variants and WT *TK2* (**Figure 3**)
- When mtDNA recovery of all the *TK2* variants was tracked and compared with that of WT *TK2*, a slight delay was found in mtDNA recovery for the variant c.604-606del on Day 14 and Day 21, but was not significantly different to WT *TK2*
  - This variant shows the highest levels of mtDNA depletion in patient-derived samples[4,5] (**Table 1**)
- However, mtDNA levels for all four of the *TK2* variants were comparable to those for WT *TK2* after 21 days of EtBr treatment (Day 28)

Figure 2. mtDNA copy number in *TK2* variants at Day 0

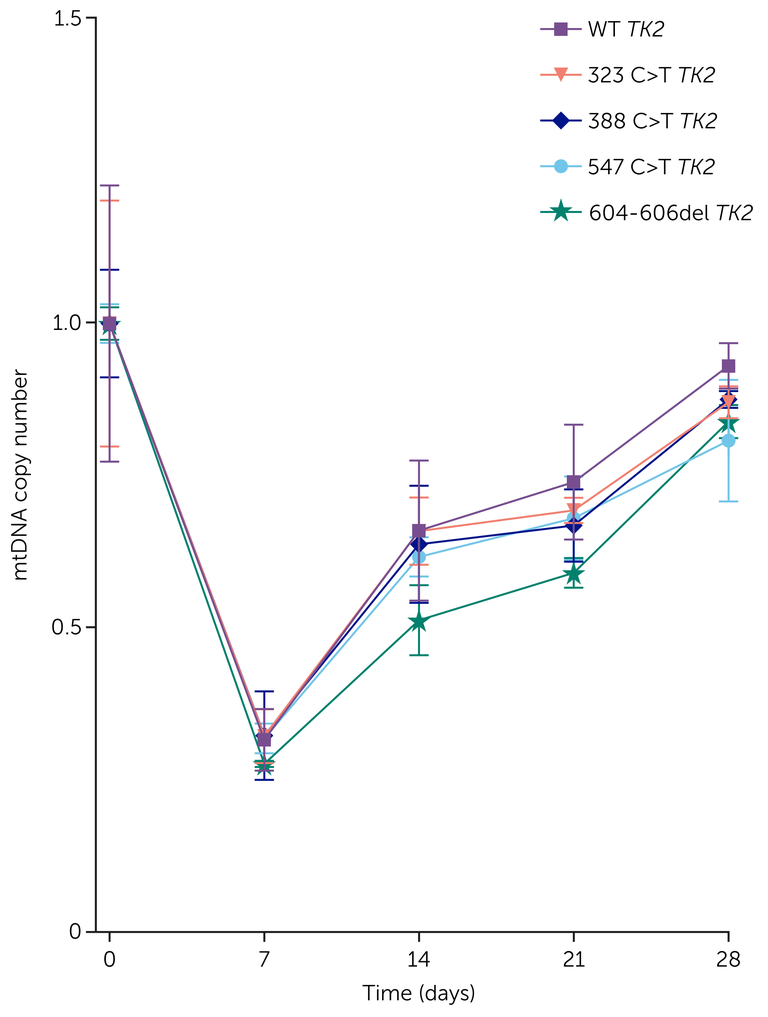


Results normalized to WT *TK2* for comparison. Bars show mean values and error bars show SDs. mtDNA, mitochondrial DNA; SD, standard deviation; *TK2*, thymidine kinase 2 gene; WT, wild type.

Discussion, conclusions and outlook

- Compared with WT *TK2*, the *TK2* variants studied here did not have a marked effect on mtDNA copy number depletion
  - Given the known role of TK1 in cytosolic deoxythymidine triphosphate synthesis and the high levels of *TK1* expression in cancer cells,[8] it is hypothesized that TK1 may partially compensate for TK2d in immortalized cell lines, although its contribution to mtDNA copy number in this assay remains unclear
- Therefore, this *in vitro* model using the U2OS *TK2* knockout pool line with EtBr treatment did not accurately recapitulate TK2d disease biology because the compensatory effects of TK1 could not be evaluated
  - Potential compensation by TK1 in models of TK2d may affect the ability to detect the effects of individual *TK2* variants *in vitro*; however, this hypothesis requires further research
- In this assay, mtDNA depletion phenotypes were mostly undetectable for the pathogenic variants tested, suggesting that patient-derived cells are the most appropriate source of information for investigating the effects of individual *TK2* variants on mtDNA

Figure 3. mtDNA copy number in *TK2* variant cell lines at baseline (Day 0), after mtDNA depletion with EtBr treatment (Day 7) and during post-treatment recovery (Days 14–21)



mtDNA copy number levels for each *TK2* variant have been normalized to their respective levels at Day 0. Markers show mean values and error bars show SDs.
EtBr, ethidium bromide; mtDNA, mitochondrial DNA; SD, standard deviation; *TK2*, thymidine kinase 2 gene; WT, wild type.

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Disclosures: Maria Esteban Lopez is an employee of UCB. Sarah Chang, Kaja Zarakowska and Ashley Jermusyk are employees of and stockholders in UCB.

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