Determining the Effect of DLST on CD4 Cell Development in MYCN-driven Neuroblastoma using Transgenic Zebrafish

Yuyi Liu[1,2], Andrew Kwok-Ping Lam[2], Madison Rager[2], Hui Feng[2]

Winston Churchill High School, 11300 Gainsborough Rd, Potomac, MD 20854[1]; Boston University School of Medicine, Department of Pharmacology and Experimental Therapeutics, 71 East Concord Street, Boston, MA 02118[2]

Introduction

Zebrafish Cancer Model System

Zebrafish (*Danio Rerio*) are often utilized in cancer and stem cell research due to the following advantages:

- **% Similarity to Human Genome:**
  - 70% shared genes with human genome[1,2]
  - 82% of human disease genes are expressed in zebrafish[1]
- **Transducability of Human Genes:**
  - Target genes can be expressed by transduction to transgenic fish lines using CRISPR-Cas9
- **Fluorescence:**
  - Zebrafish are clear and can be imaged live without invasive surgery/dissection

MYCN-induced Neuroblastoma

Neuroblastoma is a high-risk childhood solid tumor that arises in the sympathetic nervous system.

- MYCN is a proto-oncoprotein expressed in ~20% of all neuroblastoma patients[3]
  - Attributed to larger tumor burden, higher metastasis, and poor survival outcomes[1,3]
  - Able to induce neuroblastoma *in vivo* in animal model systems modified with CRISPR-Cas9[1,3]
- eGFP (enhanced green fluorescent protein) expression driven under MYCN Dβh promoter to generate green tumors

DLST Pathway

Dihydrolipoamide s-succinyl transferase, or DLST, is a mitochondrial enzyme that is heavily involved in macromolecule synthesis and cellular energetics.

- Facilitates entrance of glutamine into Krebs cycle
- Catalyzes conversion of α-ketoglutarate (αKG) to succinyl CoA in during oxidative decarboxylation
- Reduces NAD+ to NADH for oxidative phosphorylation (OXPHOS)

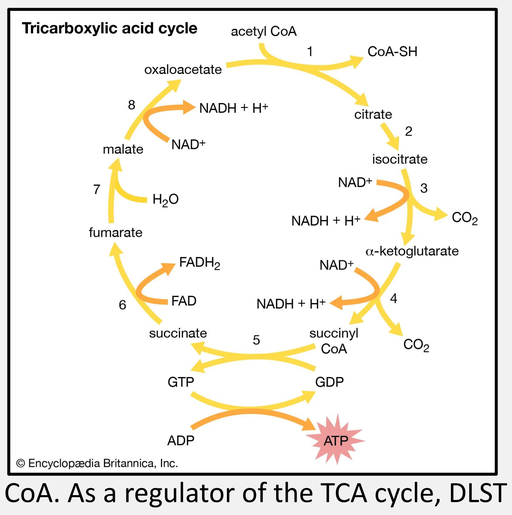

Figure 1. The tricarboxylic acid (TCA) cycle, more commonly referred to as the Krebs cycle, is a catabolic process necessary to supply the energy for the cell[4]. The enzyme DLST acts as a key checkpoint for the conversion of the α-ketoglutarate complex to succinyl CoA. As a regulator of the TCA cycle, DLST also has an important role in controlling the rate of OXPHOS and cancer metabolism.

T-cell and Thymus Visualization

- CD4 is a protein uniquely associated with immune cells such as T-cells and macrophages
- T-cells tagged red using RFP (red fluorescent protein) at zebrafish Cd4 promoter
- Thymus used for imaging because it is the primary site of T-cell development, high CD4 cell density

Results

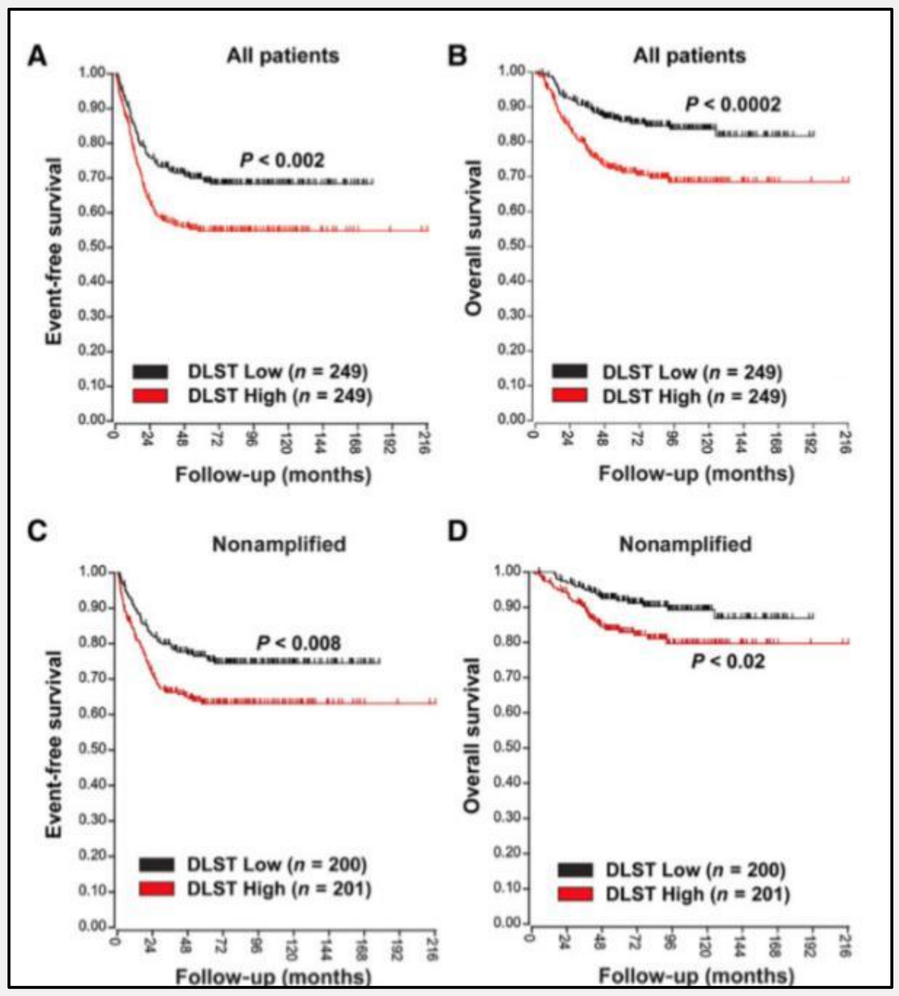

Figure 2. Kaplan-Meier survival curves for variable DLST expression in neuroblastoma[5]. Charts demonstrate that high DLST expression indicates poor prognosis in all forms of neuroblastoma. All data was compiled from the R2 public genomic database.

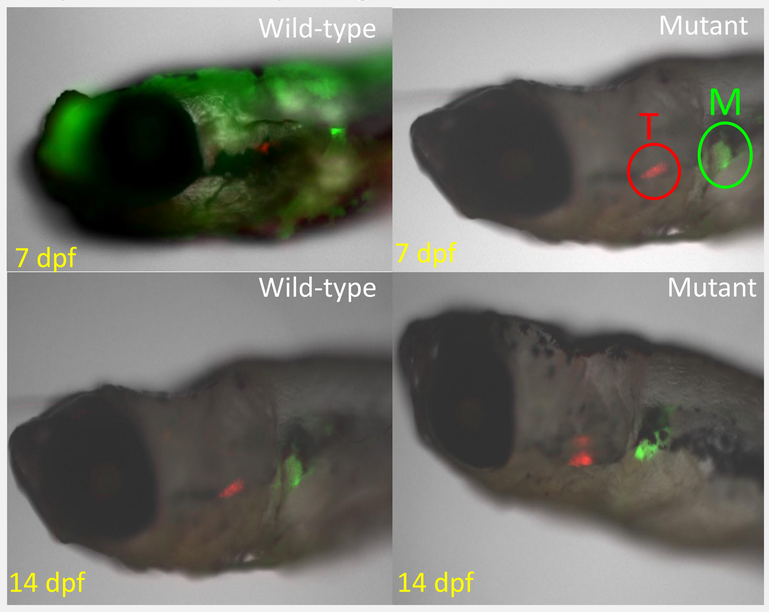

Figure 3. Overlaid images showing RFP-tagged thymus (T) and eGFP-tagged neuroblastoma (M). Observational evidence from images taken at 7 and 14 dpf show that the thymus is brighter in loss of function mutant fish. Fish were imaged at (min:0, max:140) brightness, 150ms (7dpf) and 50ms (14dpf) exposure.

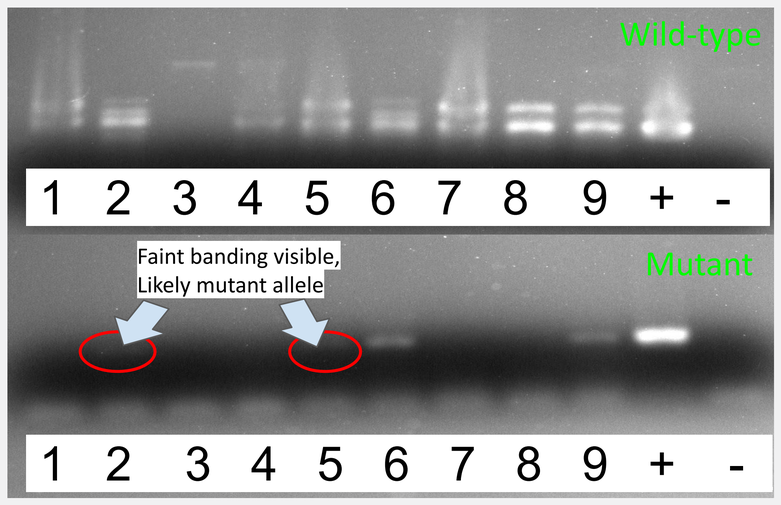

Figure 4. Results from wild-type (top) and mutant (bottom) *dlst* allele genotyping. Banding for samples 2, 5, 6, and 9 indicate presence of *dlst* mutant gene. Due to poor sampling outcomes and fragility of embryonic samples, not all pictured samples were included in final data.

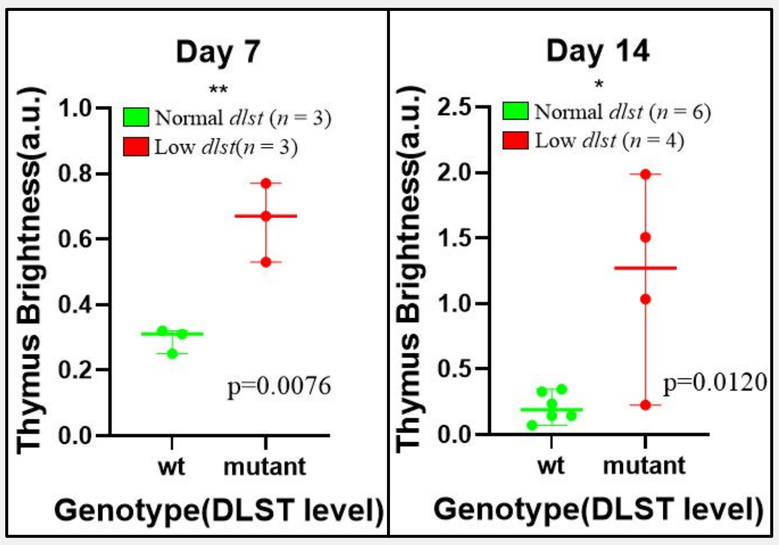

Figure 5. Median normalized thymus brightness for mutant and wild-type fish. P-values were generated using unpaired two-tailed t-tests and demonstrate significance at both time points. Significance intervals determined by the following: *, p ≤ 0.05;**, p ≤ 0.01.

Discussion

- DLST enzyme could have direct impact on T-cell activation
- DLST may affect the premalignant tumor and indirectly lead to proliferation of T-cells
- More exploration is needed to isolate the role of DLST on immune cell development

Next Steps:

- Analyze existing images for noticeable differences in tumor size
- Extend study to 6 weeks to analyze the entire CD4 development process
  - Fish develop functional T cells after 3-6 weeks[3]
- Repeat experiment with non-tumorigenic fish to determine if CD4 cell proliferation is tumor-specific

Conclusions

- *dlst* mutant zebrafish have improved prognosis compared to wild-type
- Mutant *dlst* fish with had brighter thymuses
  - Significance at 7 (p=0.0076) and 14 (p=0.012)dpf
- Demonstrated link between *dlst* reduction and heightened CD4 cell development

Methods

- **Selective Breeding:** Transgenic fish lines were generated by crossing parents expressing MYCN;eGFP and CD4;mCherry and sorted to RFP+ GFP+ fish. Original parents were all modified using CRISPR-Cas9.
- **Fish Genotyping:** Zebrafish embryos were cut in half at the tail region and placed into 25µL of lysis buffer with 2.5µL of proteinase K (PK) enzyme overnight to extract DNA. Afterwards, PCR test was performed for both wild-type and mutant variants of the allele using 1903-c forward primers with HCP (wild-type) and IPL3 (mutant) reverse primers. Samples were run through 2% agarose gel at 110V for ~25 minutes and imaged under UV light to detect banding.
- **Imaging:** Zebrafish were fixed in paraformaldehyde (PFA) at both 7 and 14 days post fertilization (d.p.f.). After soaking overnight, samples were washed using PBS (phosphorus buffered saline) and placed in 30% sucrose solution. Fish were then imaged under brightfield, UV red, and UV green filters to generate images showing fish body, thymus, and tumor.
- **Image Processing:** ImageJ was used to highlight glowing areas corresponding to the thymus in the fish to determine total thymus brightness intensity. The same area region was then captured from the image background and used to eliminate background fluorescence. Finally, a measurement of the eye was taken and used to normalize data by size.

References

1. Patton, E. E.; Zon, L. I.; Langenau, D. M. Zebrafish Disease Models in Drug Discovery: From Preclinical Modelling to Clinical Trials. Nature Reviews Drug Discovery 2021, 20 (8), 611–628.
2. Miao, K. Z.; et al. Tipping the Scales with Zebrafish to Understand Adaptive Tumor Immunity. Frontiers in Cell and Developmental Biology 2021, 9.
3. Zhu, S.; et al. Activated ALK Collaborates with MYCN in Neuroblastoma Pathogenesis. Cancer Cell 2012, 21 (3), 362–373.
4. The Editors of Encyclopaedia Britannica. tricarboxylic acid cycle; Encyclopaedia Britannica, 2013.
5. Anderson, N. M.; et al. Metabolic Enzyme DLST Promotes Tumor Aggression and Reveals a Vulnerability to OXPHOS Inhibition in High-Risk Neuroblastoma. Cancer Research 2021, 81 (17), 4417–4430.

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