Improving Precision Medicine for Pediatric Glioma: development of two novel clinical tests

The overall goal of this project is the development of two novel clinical tests to evaluate (1) the level of activation of targetable cellular growth pathways and (2) the degree of immune activation in the tumor microenvironment. These tests will use the NanoString nCounter platform, which uses barcoding technology to directly measure specific molecules in a complex mixture, including potentially DNA, RNA, and protein. These assays are cost-effective and have reliable results on clinical tissue samples up to 15-20 years old. The validation of both assays involves profiling several hundred tissue samples from the SickKids archive, and integrating this data with clinical annotations and additional testing modalities to provide biological insights into the mechanisms driving tumor growth and treatment resistance.

Activity 1: Cellular pathway activation panel.

Background:
Pediatric low-grade gliomas (LGG) are frequently driven by RAS-MAPK pathway alterations, many of which can be treated with targeted therapies. However, the clinical response to these therapies is variable, which we hypothesize is because the presence of a genomic alteration in the MAPK pathway does not necessarily imply that this pathway is the only one that is driving tumor growth. To investigate this, we designed a NanoString assay that integrates RNA and protein expression to quantify the activation of the MAPK, PI3K-AKT-mTOR, JAK-STAT, and NFkB pathways. We validated the assay on a range of cell lines that have been treated with perturbations in each pathway.

Progress/findings:
Over the past year I have developed the bioinformatic workflow for this assay and performed the analysis on samples we have run for test development and validation. We have run the assay on over 350 surgical samples, including 233 LGG, 112 high grade gliomas (HGG), 18 ependymoma, 10 medulloblastoma, and 11 non-tumor brain samples. LGGs with MAPK pathway genetic alterations have uniformly high MAPK pathway scores, but substantial variability in the other 3 pathways. In contrast, IDH-mutant LGGs have high PI3K pathway activation. HGGs generally have high PI3K pathway activation and variable MAPK pathway activation, which is higher in hemispheric HGG than in diffuse midline glioma. Public single cell RNA sequencing data from pilocytic astrocytomas (data from Reitman et al, Nat Comm 2019) demonstrates significant heterogeneity in pathway activation states within the tumor cells, as well as high pathway scores in some microglia.

Future work:
- Single cell RNA sequencing has been submitted for 8 PLGG samples with MEK-inhibitor response data to evaluate the role of intratumoral heterogeneity in treatment response and the influence of the tumor immune microenvironment.

- Expand cohort of MEK-inhibitor treated patients to identify predictors of treatment response.
- Finalize clinical validation and Ministry of Health approval.

Activity 2: Immuno-oncology panel and characterization of tumor-immune microenvironment.

Background: The tumor immune microenvironment (TIME) is a growing area of interest, however, the extent of immune activation in pediatric brain tumors is unknown. Although immunotherapy has not been successful in most CNS cancers, our group has previously described gliomas with germline mismatch repair deficiency (MMRD), which exhibit hypermutation and favorable responses to immune checkpoint inhibition (ICI). Therefore, detailed characterization of the CNS TIME is key for the development of novel immunotherapeutic strategies and application of existing ones in childhood brain tumors. To investigate this, we have developed a panel that includes markers reflecting cell types, therapeutic targets, and cellular pathways, as well as the 18-gene tumor inflammation signature (TIS), a biomarker for ICI response.

Progress/findings:
Over the past year I have developed the bioinformatic workflow for this assay and performed the analysis on samples we have run for test development and validation. We have tested over 500 brain tumors, including 266 low-grade gliomas (LGG), 170 high-grade gliomas (HGG), 91 MMRD tumors, 16 ependymomas, 46 medulloblastomas, and 36 non-tumor brain samples.

Overall, ependymomas and medulloblastomas have low levels of inflammation. IDH-mutant LGG are immunologically cold, while many gliomas with pediatric-LGG mutations have high levels of inflammation, including upregulation of immune checkpoints – indicating that ICI may be an effective strategy. Interestingly, in pediatric-LGG inflammation impacts outcome in tumors with the same genetic alterations. BRAF V600E-mutant LGG exhibiting high TIS have inferior prognosis, while no such relationship is observed in BRAF-fused tumors. Diffuse midline gliomas have higher inflammation than hemispheric HGG, indicating that these tumors are not immunologically cold, as has been previously reported. In MMRD tumors treated with ICI, high TIS correlates with improved survival and is independent from hypermutation and mutational burden. Furthermore, MMRD gliomas have high expression of several other immune checkpoints including LAG3, suggesting its value as an additional therapeutic target.

Future work:
- Validate findings using multiplex immunohistochemistry on selected tissue samples.
- Single cell RNA sequencing has been submitted for selected LGG and HGG samples to better isolate and characterize immune cell populations.
- Expand cohort of MMRD patients with ICI treatment response.
- Finalize clinical validation and Ministry of Health approval.

Presentations:

- Immunological characterization of pediatric brain tumors has clinical implications for patient management and prognosis. *Society for Neuro-Oncology.* Tampa, FL. Nov 17-20, 2022. Abstract Submitted.
- Comprehensive immunological gene expression profiling of pediatric brain tumors. *International Society of Pediatric Neuro-oncology*. Hamburg, Germany. June 12-15, 2022. Poster presentation (presented by co-author Liana Nobre in person).
- Implementation of a clinical assay for targetable cellular pathway activations. *American Association of Neuropathology*. Bonita Springs, FL. June 10, 2022. Podium presentation (see attached slides).
    - Recipient of Richard Davis travel award for best clinically oriented abstract by a trainee with MD.
- Immunologic characterization of pediatric brain tumors. *University of British Columbia Clinician Investigator Program Research Day.* June 3, 2022, Vancouver BC. Poster presentation (see attached poster).

Publications:

- Briggs M, Das A, Firth H, Levine AB, et al. Recurrent posterior fossa group A ependymoma in a young child with constitutional mismatch repair deficiency. Submitted to *Neuropathology and Applied Neurobiology* (impact factor=8.09), under review.
- Haizel-Cobbina J, Thakkar R, Richard K, Du L, Levine AB, Bennett J, Hawkins C, Tabori U, Dewan MC. Clinical and molecular features of disseminated pediatric low-grade glioma and glioneuronal tumors: A systematic review and survival analysis. Submitted to *Neuro-oncology Advances*, under review.

Implementation of a clinical assay for targetable cellular pathway activations

Adrian Levine, MD, FRCPC
Neuropathology Research Fellow, Hospital for Sick Children, Toronto, ON
Clinician Investigator Program, University of British Columbia

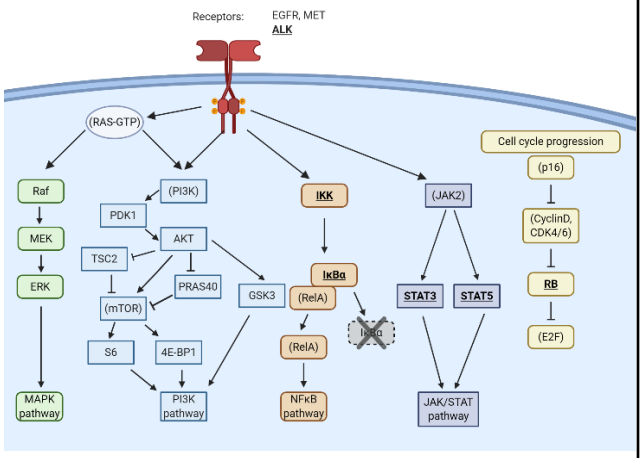
American Association of Neuropathology, 98th annual meeting
Bonita Springs, FL
June 8-12, 2022

1

Introduction

Cellular pathways and targeted therapies

- Many pediatric brain tumors are candidates for targeted therapies
- Issues with treatment stratification based on DNA alterations:
  - Mutation ≠ pathway activation
  - Additional pathways may be upregulated
- **Hypothesis: integration of gene and protein expression will better predict response to targeted therapy than DNA mutations alone**
- 4 key actionable pathways:
  - **MAPK, PI3K, NF-kB, JAK/STAT**



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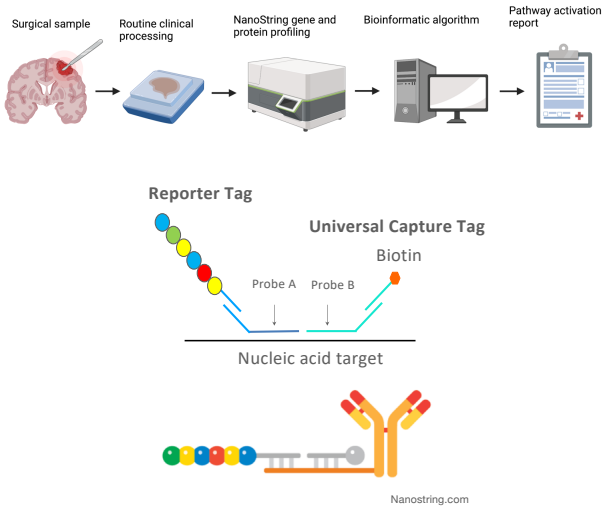
Methods

NanoString assay for pathway signatures and protein expression

- Multiplexed measurement of RNA and protein
- Robust results on FFPE tissue and time/cost effective in clinical workflow
- RNA pathway signatures using **PROGENy** Bioconductor package
- Selected phosphorylated proteins in each pathway



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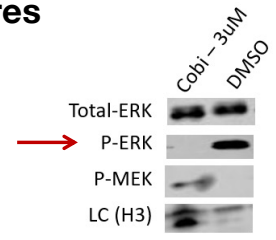
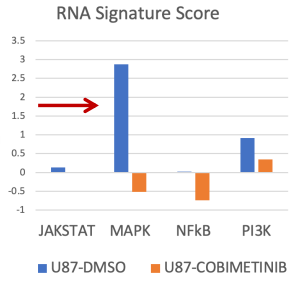
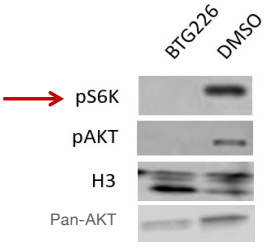
Methods

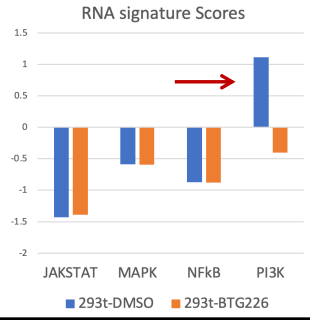
Cell line validation of NanoString scores

- Validated on cell lines with perturbations
  - Activation: BRAF V600E, PTEN knockout
  - Inhibitors: Cobimetinib (MEK), BTG226 (PI3K)
- Pathway scores and protein levels decrease with inhibitors
- Inhibitors have greater effect on cells with high baseline pathway activation









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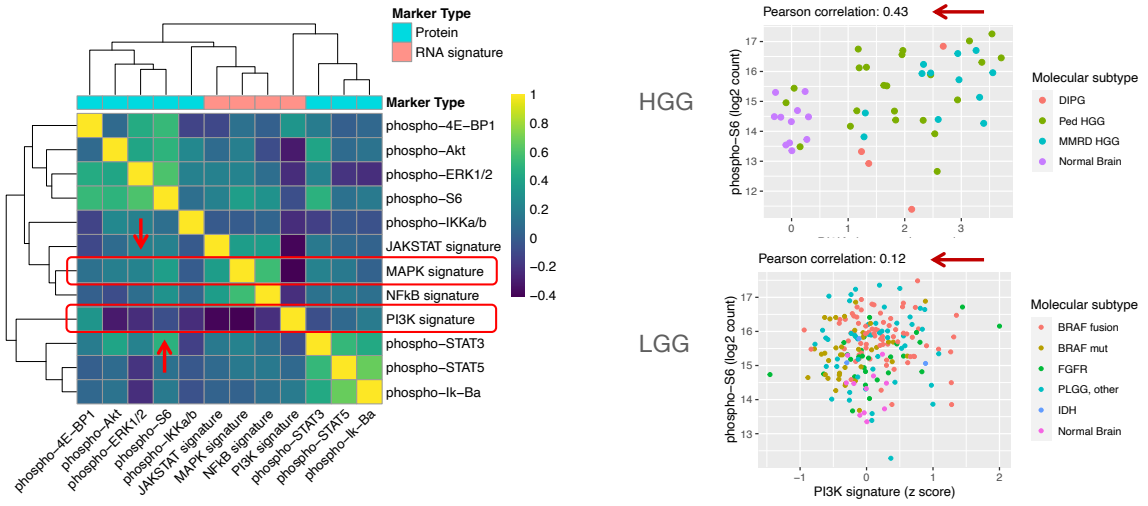
Results

Complex RNA to protein correlations



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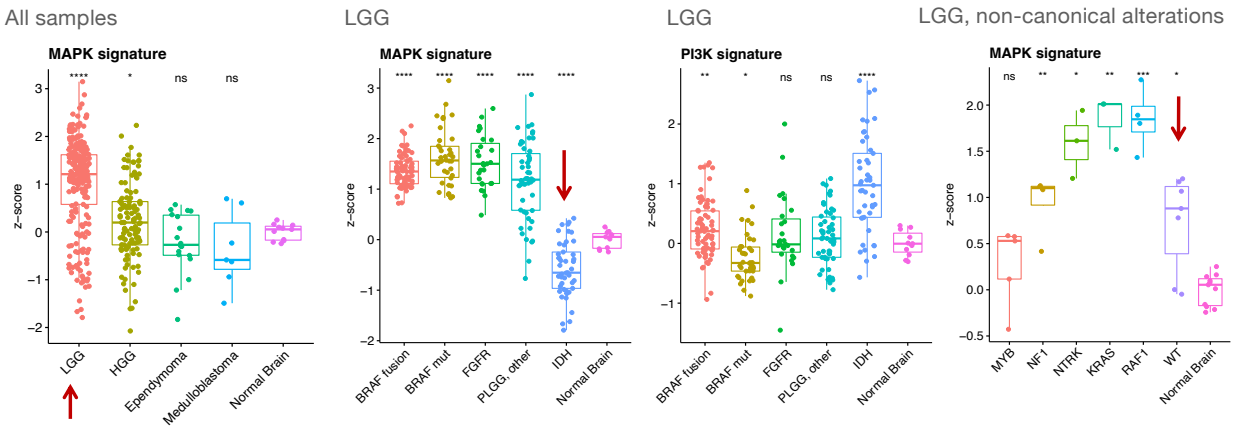
Results

Low-grade glioma: high MAPK pathway activation



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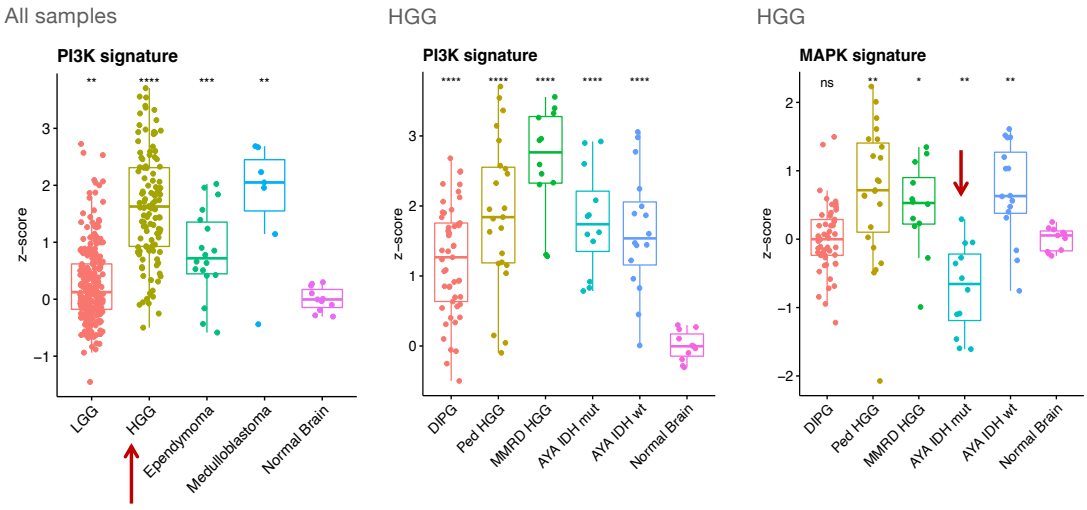
Results

High-grade glioma: high PI3K pathway activation



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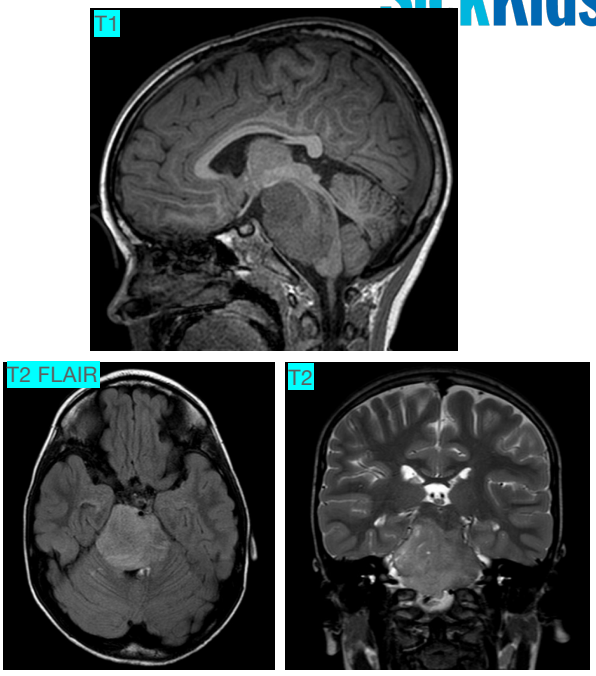
Case presentation

Clinical presentation

- 6 year old boy, presented with 10 days ataxia following fever
- On exam: headache, cerebellar ataxia, left eye diplopia
- Imaging: expansile pontine mass, strongly suspicious for diffuse midline glioma
- Underwent stereotactic biopsy to gain further information as to biology of tumor



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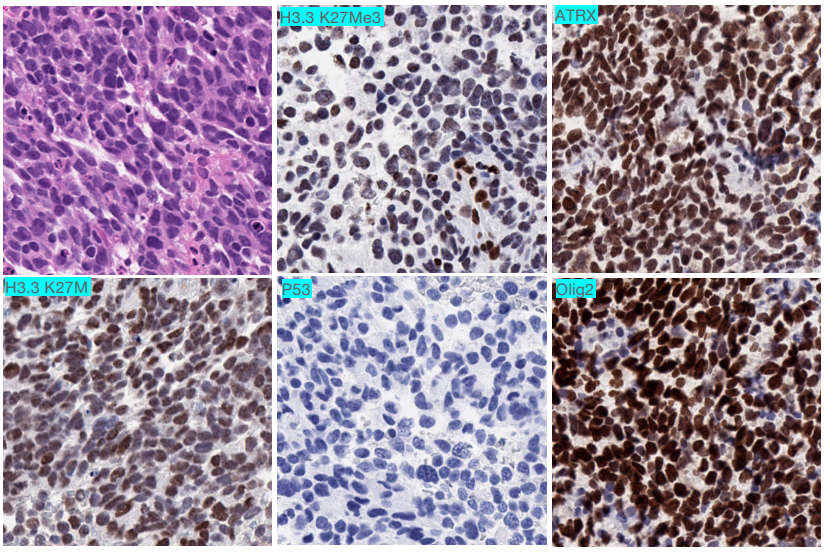
Case presentation

Pathology

- Diffuse midline glioma, H3-K27 altered (WHO grade 4)
  - H3.3 K27M
  - P53 p.D186Mfs*61 (frameshift deletion)
  - **PTEN p.F341V**
    - Loss of function
    - Subclonal (VAF 0.14)



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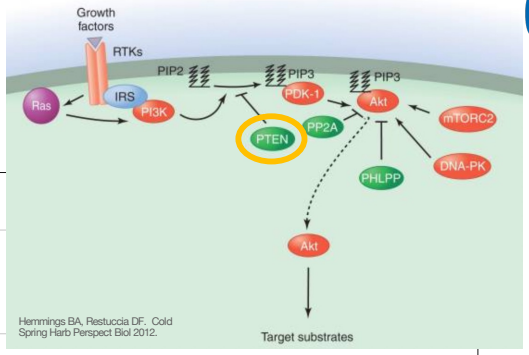
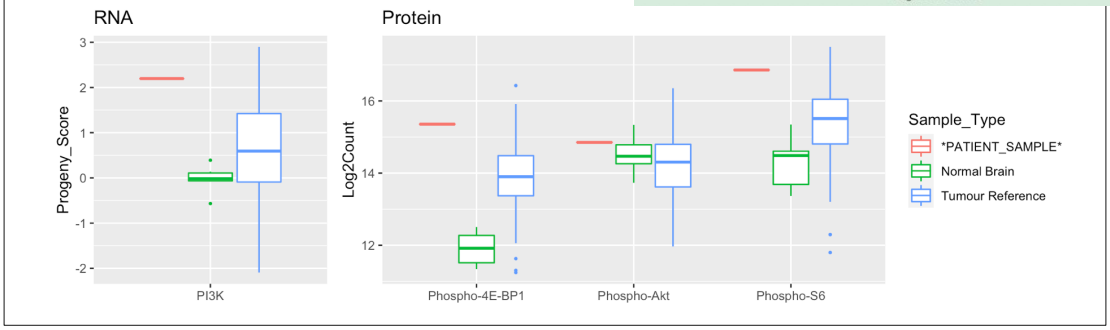
Case presentation

Pathways assay results



PI3K pathway

```
RNA signature score = 2.2  (percentile 91.3)
Phospho-S6: count = 118866  (percentile 95.6)
Phospho-4E-BP1: count = 41961  (percentile 96.4)
Phospho-Akt: count = 29582  (percentile 77.2)
```



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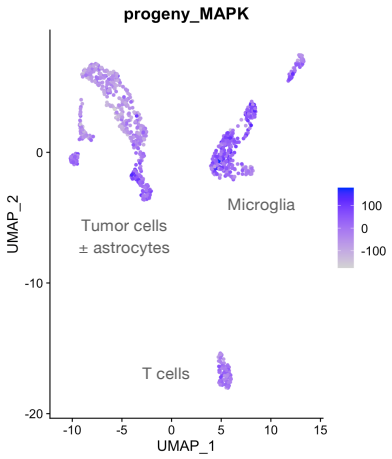
Conclusions and future work

- Developed clinical assay to identify pathway activations using RNA and protein expression
- High variability in samples with same genetic driver
- Pediatric LGG: high MAPK pathway activation
- HGG and IDH-mutant LGG: high PI3K pathway
- **Future:** investigate intratumoral heterogeneity as mechanism of treatment failure using single cell sequencing



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Thank you and questions!

- **Dr. Cynthia Hawkins**
- **Hawkins lab members**
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  - Scott Milos, MSc
  - Rob Siddaway, PhD
  - Scott Ryall, PhD
  - Ben Laxer
  - Monique Johnson, MSc
  - Michelle Ku, MSc
  - Rodney Lyn, PhD
- **Dr. Uri Tabori**
- **Dr. Gino Somers and SickKids Department of Pediatric Laboratory Medicine**

Laboratory Medicine & Pathobiology
UNIVERSITY OF TORONTO

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Immunologic characterization of pediatric brain tumors

Adrian Levine, MD, FRCPC; Liana Nobre, MD; Scott Milos, MSc; Anirban Das, MD; Rob Siddaway, PhD; Uri Tabori, MD; Cynthia Hawkins, MD, PhD, FRCPC

INTRODUCTION

Brain tumors are the most common solid tumor in children and the leading cause of cancer mortality.

Immune checkpoint inhibition (ICI) has been incredibly successful in some cancers but not in brain tumors, outside of rare mismatch repair deficient (MMRD) cases

- Low tumor mutation burden
- Lack of biomarkers and challenge with repeat sampling
- Intra-tumoral heterogeneity

Immunosuppressive tumor immune microenvironment :

- Low tumor infiltrating lymphocytes
- Tumor associated macrophages with “M2" anti-inflammatory phenotype:
- Arginase, TGFβ, IL10, IDO
- Systemic corticosteroids (for cerebral edema)

AIMS

1. **Develop and clinically validate an immuno-oncology assay**
2. **Characterize the immune landscape across pediatric brain tumors**
3. **In depth understanding of immune cell types in pediatric gliomas**

METHODS

Clinical 103 probe immuno-oncology gene expression panel

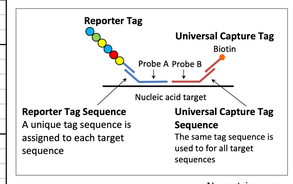
NanoString nCounter

- Simultaneous, multiplexed direct measurement of specific molecules in a complex mixture
- Robust results on FFPE tissue and extensively used at SickKids for clinical panels
- Hybridization of Reporter and Universal Capture Tags with unlabeled, end-user developed, probes

Tumor inflammation signature

- 18-gene signature to evaluate inflammation of tumor microenvironment
- Validated using retrospective analysis of KEYNOTE basket trials as predictive of response to ICI

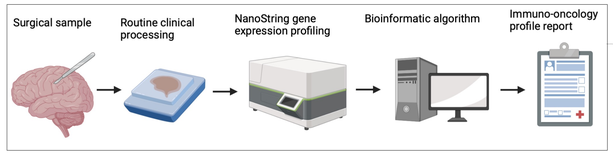
Biologic Process	Gene	Function
APC abundance	PSB10	Immunoproteosome
	HLA-DQA1	MHC class II
	HLA-DRB1	MHC class II
	CMKLR1	Chemokine receptor
T/NK cell abundance	HLA-E	MHC Class I
	NKG7	Cytolytic granule protein
	CD8A	MHC I coreceptor
Interferon activity	CCL5	Chemoattractant
	CXCL9	Chemoattractant
	CD27	T cell activation
	CXCR6	T cell activation
	IDO1	T cell inhibitor
	STAT1	Mediates IFN response
T cell exhaustion	TIGIT	T cell inhibitor
	LAG3	T cell inhibitor
	PD-L1 (CD274)	T cell inhibitor
	PD-L2	T cell inhibitor
	CD276 (B7-H3)	T cell inhibitor



Nanostring.com

RESULTS

1. Overview of immune activation in pediatric brain tumors and incorporation into clinical workflow



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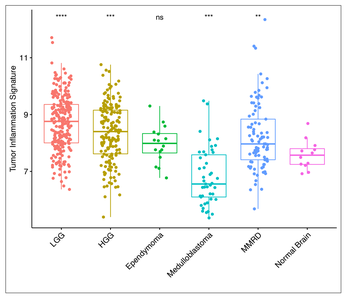
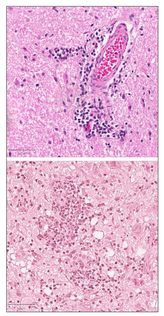
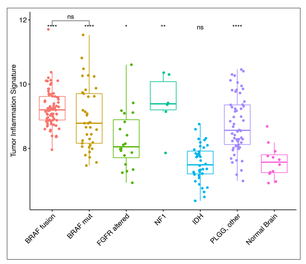
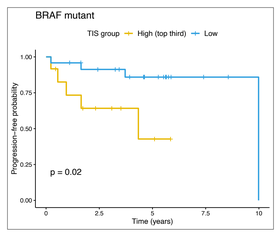
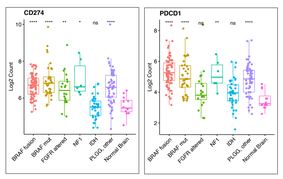


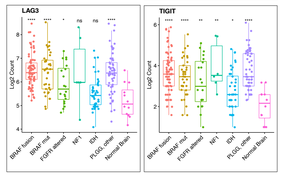


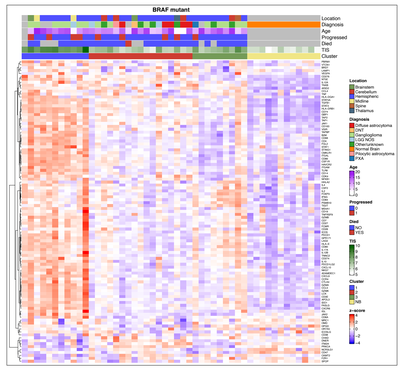
2. Low grade gliomas: distinct profiles between pediatric and adult tumors and within genetically defined subtypes



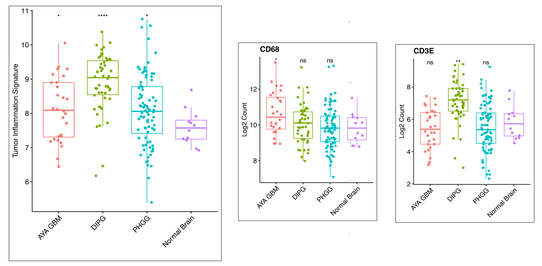


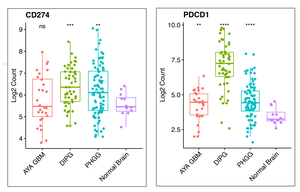


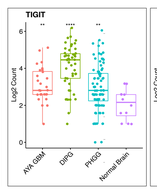


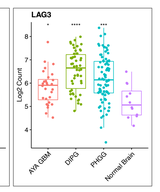


3. High grade gliomas: high levels of immune activation in some tumors contradict previous reports characterizing as “immunologically cold”

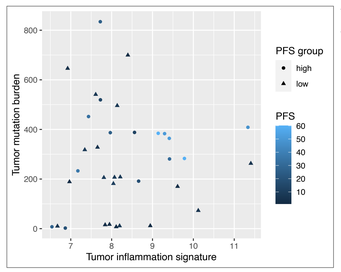


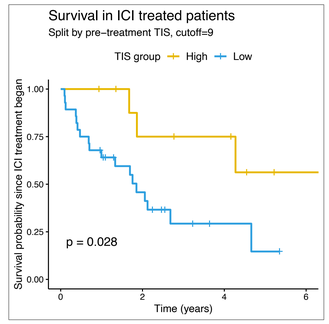


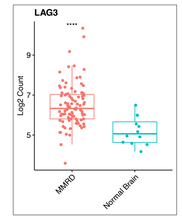


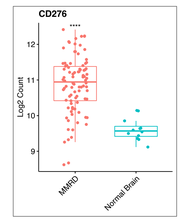


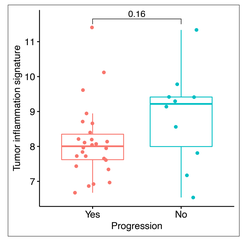
4. Mismatch repair deficient tumors: clinical implications of immune profiling



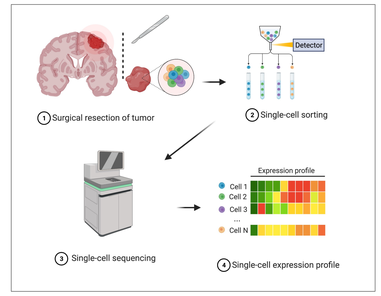








5. Single cell RNA sequencing in diffuse midline glioma identifies predominantly macrophage with immunosuppressive phenotype



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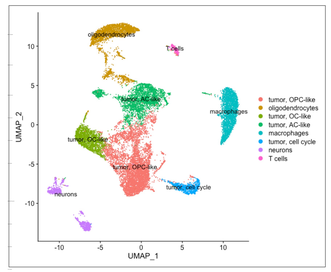

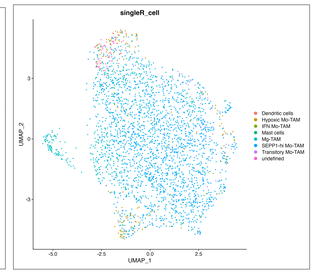

CONCLUSIONS

- Pediatric brain tumors have wide variability in immune activation levels, including within genetically defined subtypes
- Tumor inflammation signature has prognostic and predictive utility in some tumor types
- Increased expression of immune checkpoints and other therapeutic targets may indicate potential treatment strategies

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3. Pombo Antunes AR, Scheyltjens I, Lodi F, et al. Single-cell profiling of myeloid cells in glioblastoma across species and disease stage reveals macrophage competition and specialization. Nat Neurosci 2021;24(4):595–610.

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Laboratory Medicine and Pathobiology, University of Toronto
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