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Author

Rodriguez Almaraz, Jesus Eduardo

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Patterns of Genetic Alterations, Transposable Elements, and Immune Cells in
Supratentorial Glioma Patients: Clinical and Molecular Insights Towards Personalized
Treatment
by
Eduardo Rodriguez Almaraz

DISSERTATION

Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Epidemiology and Translational Science

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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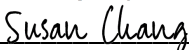


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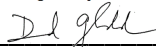
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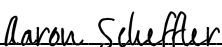
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Susan Chang



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David Glidden



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Aaron Scheffler

Committee Members

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By

Eduardo Rodriguez Almaraz

Dedication

To my parents, Maria Elena and Daniel, for showing me the power of perseverance. To Ariel, my wife, thank you for being my biggest cheerleader. And to Addison, my daughter, you are my greatest accomplishment.

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Dissertation committee:

Stephen Francis, Susan Chang, Aaron Scheffler, David Glidden

Other advisors:

David Solomon, Geno Guerra

Contributions

Chapter 2 has been published as a peer reviewed paper in Neuro-Oncology advances:

Eduardo Rodriguez Almaraz, Geno A Guerra, Nadeem N Al-Adli, Jacob S Young, Abraham Dada, Daniel Quintana, Jennie W Taylor, Nancy Ann Oberheim Bush, Jennifer L Clarke, Nicholas A Butowski, John de Groot, Melike Pekmezci, Arie Perry, Andrew W Bollen, Aaron W Scheffler, David V Glidden, Joanna J Phillips, Joseph F Costello, Edward F Chang, Shawn Hervey-Jumper, Mitchel S Berger, Stephen S Francis, Susan M Chang, David A Solomon, Longitudinal profiling of IDH-mutant astrocytomas reveals acquired RAS-MAPK pathway mutations associated with inferior survival, Neuro-Oncology Advances, Volume 7, Issue 1, January-December 2025, vdaf024, <https://doi.org/10.1093/noajnl/vdaf024>

Other contributors:

George Wendt, Helen Hansen, Mi Zhou, Lucie McCoy , Terri Rice, Paige Bracci, Annette Molinaro, John Wiencke, Margaret Wrensch

Patterns of Genetic Alterations, Transposable Elements, and Immune Cells in Supratentorial Glioma Patients: Clinical and Molecular Insights Towards Personalized Treatment

Eduardo Rodriguez Almaraz

Abstract

Gliomas are among the most common and aggressive primary brain tumors, with IDH-mutant and IDH-wildtype gliomas exhibiting distinct molecular and clinical characteristics. Despite advances in tumor classification and treatment strategies, prognostic uncertainty and therapeutic resistance remain major challenges in neuro-oncology. The present work investigates key genetic, molecular, and immune mechanisms that contribute to glioma progression and patient outcomes, with the goal of identifying novel biomarkers to improve risk stratification.

To address these challenges, three complementary projects were conducted using genomic, transcriptomic, and immune profiling information.

Transposable Elements in IDH-Wildtype Glioblastoma: Using RNA sequencing data from The Cancer Genome Atlas (TCGA), we analyzed the expression of transposable elements (TEs) in IDH-wildtype glioblastoma and their association with survival outcomes. Cox proportional hazards models and pathway enrichment analyses identified candidate TEs associated with reduced survival, proposing their role in genomic instability, and immune evasion.

Molecular Evolution of IDH-Mutant Astrocytomas: Longitudinal genomic profiling of 205 tumor specimens from patients with IDH-mutant astrocytomas to assess acquired mutations driving tumor progression and treatment resistance. UCSF500 next-generation sequencing panel was used to identify RAS-MAPK pathway alterations (in *KRAS*, *NRAS*, *BRAF*, *NF1*, and *LZTR1*) that were enriched in high-grade recurrent tumors and correlated with inferior survival, highlighting their potential as molecular targets for therapy.

Peripheral Immune Cell Dynamics in IDH-Mutant Gliomas After Surgical Resection: To investigate systemic immune changes in glioma patients, a longitudinal analysis of immune profiles of peripheral blood samples before and after surgery was conducted. Statistical modeling revealed shifts in circulating immune cell populations, particularly basophils and T-regulatory cells, that may reflect immune adaptations to tumor burden and surgical intervention.

Collectively, these studies provide insights into glioma biology, identifying novel genetic drivers, prognostic biomarkers, and immune alterations that could inform personalized treatment strategies. By integrating molecular and immune profiling, this work contributes to the advancement of precision oncology approaches for glioma diagnosis, risk stratification, and proposes potential therapeutic targets.

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Introduction

1.1 Background

Supratentorial gliomas, a diverse group of primary brain tumors, arise from the glial cells of the brain. These tumors represent a significant challenge in neuro-oncology due to their varied clinical presentations, heterogeneous molecular profiles, and often infiltrative growth patterns, which complicate treatment and contribute to variable prognoses.^{1, 2} Gliomas are classified according to the World Health Organization (WHO) grading system, which integrates histological features with molecular markers to categorize tumors from grade 2 (least aggressive) to grade 4 (most aggressive). Figure 1.1 depicts the diffuse glioma classification system.¹ This integrated approach is crucial for accurate diagnosis, prognostics, and treatment planning.

The cellular origin of supratentorial gliomas is thought to be from various glial cell types, including astrocytes, oligodendrocytes, and ependymal cells, or their progenitor cells.³ Astrocytomas, the most common type, exhibit a spectrum of malignancy, ranging from low-grade (diffuse astrocytoma, IDH-mutant) to high-grade (glioblastoma, IDH-wildtype). Oligodendrogliomas, characterized by their distinctive "fried egg" appearance histologically, often present mutations in the *IDH1/2* gene accompanied by whole arm co-deletion of chromosomes 1p and 19q.¹

Genetic and epigenetic alterations play a critical role in the development and progression of supratentorial gliomas. Key molecular oncogenic drivers include mutations in the *IDH1/2* genes, *TP53*, *EGFR*, and *TERT* promoter, among others.^{4, 5} These molecular alterations not only contribute to tumor growth and proliferation but also influence treatment response and patient survival.

For instance, *IDH1/2* mutations are associated with a better prognosis, while *EGFR* amplification is frequently observed in *IDH-1/2* wildtype astrocytomas grade 4.⁶

WHO 2021 diffuse glioma classification

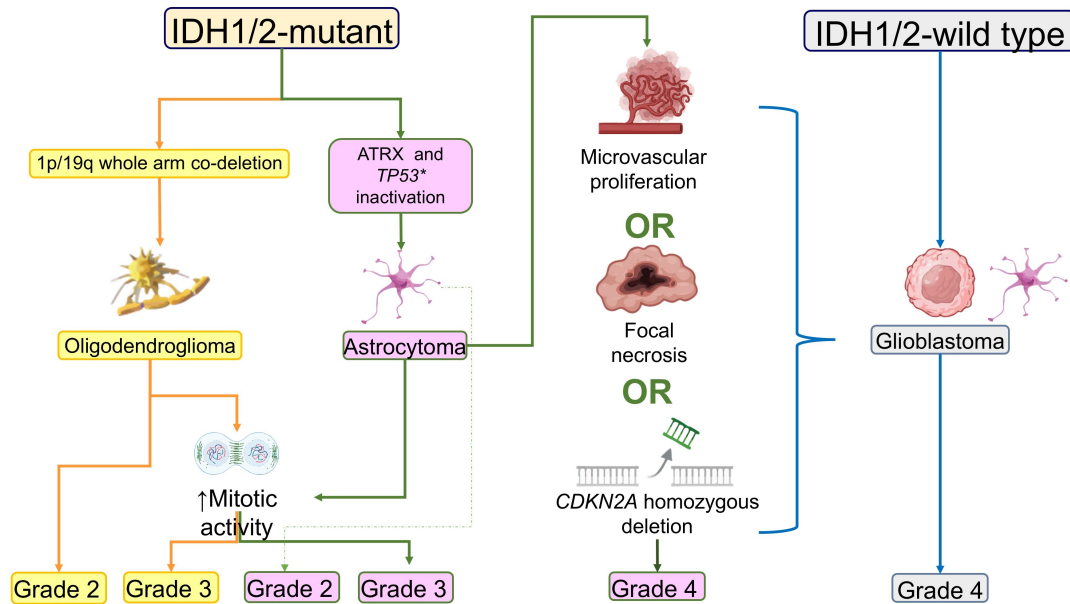


Figure 1.1 Algorithm for classification of diffuse gliomas according to CNS5 WHO 2021 guidelines

The clinical presentation of supratentorial gliomas is highly variable and depends on the tumor's location, size, and growth rate. Common symptoms include seizures, headaches, focal neurological deficits (e.g., hemiparesis, aphasia, visual field defects), and cognitive impairment.⁷ Diagnosis relies on a combination of neuroimaging techniques, primarily magnetic resonance imaging (MRI), which provides detailed information about tumor location, size, and characteristics, including contrast enhancement patterns and edema.⁸ Histopathological examination of tumor tissue obtained through biopsy or surgical resection remains the gold standard for definitive diagnosis and grading.¹

Treatment strategies for supratentorial gliomas are multimodal and tailored to the individual patient based on tumor type, grade, and molecular profile, as well as patient age and Karnofsky

performance status. Surgery, when feasible, is typically the first line of treatment, aiming for maximal safe resection.⁹ Radiotherapy and chemotherapy, particularly temozolomide, are often used as adjuvant therapies following surgery, particularly in high grade gliomas.¹⁰ Ongoing research is focused on developing novel diagnostic approaches that improve accuracy in diagnosis and allow for more precise treatment selection allowing improvement for outcomes for patients with these challenging tumors.^{11, 12}

This work in molecular and genetic epidemiology aims to contribute knowledge that improves diagnostics, thereby improving risk stratification and enabling clinicians to make more informed decisions in disease management. By integrating genetic and molecular insights, we aim to refine predictive models and propose pathways for future exploration of potential targeted therapeutic approaches. Furthermore, the findings of the present work generate hypotheses about the underlying biological mechanisms driving these observations, providing a foundation for future research and functional validation. Ultimately, this work not only advances scientific understanding but also has the potential to translate into meaningful clinical applications that improve patient outcomes.

Transposable element abundance as predictors of overall survival in IDH-wildtype glioblastoma: a TCGA analysis.

2.1 Introduction

Genomic instability is a hallmark of cancer initiation and progression.^{13–16} This instability can arise from various factors, including exposure to DNA-damaging agents, defects in DNA repair mechanisms (heritable or somatic), and the influence of ionizing radiation and alkylating agents.¹⁵ Genomic instability in human cancers is closely linked to the activity of mobile genetic elements, particularly transposable elements (TEs), which may both contribute to and be exacerbated by the mutagenic events commonly associated with tumorigenesis.¹⁶

TEs, which are repetitive sequences that include RNA transposons, DNA transposons, and endogenous retroviruses, constitute nearly half of the human genome.^{17–23} Retro elements (REs) are a class of TEs that propagate through an RNA intermediate, integrating back into the genome through reverse transcription.¹⁷ RE subtypes include short interspersed nuclear elements (SINE), long interspersed nuclear elements (LINE), and long terminal repeat (LTR) elements, the latter of which are derived from endogenous retroviruses (ERV). Together, LINEs and SINEs represent 33% of the human genome, while LTR retrotransposons, including ERVs account for approximately 8%.^{21, 24}

Historically, TEs were considered parasitic DNA elements, detrimental to genome integrity and host fitness due to their transpositional activity.¹⁷ However, advances in whole-genome sequencing and modern functional studies have revealed that, over millions of years of evolution, TEs have assumed crucial roles in various cellular and genetic functions.^{25, 26} These include the coordination of gene expression networks, pseudogene formation, somatic mosaicism, regulation of gene expression, evolution of regulatory networks, and epigenetic regulation of the genome.^{21, 23, 25, 27, 28} TEs have been implicated in normal brain development, where they play roles in the formation of neural networks critical for brain function^{17, 18, 21, 29} as well as in pathogenic processes.

DNA methylation is a crucial mechanism for silencing genomic TEs. In many forms of human malignancy, genome-wide hypomethylation compromises TE methylation, leading to increased retrotransposition and increased TE expression.^{30–32} Although few studies have focused on individual TEs, the most prominent family with active loci in human tumors is LINE-1.^{21, 28, 33} The existing research supports a model where LINE-1 promoter hypomethylation enables expression and retrotransposition.³²

LINE-1 promoter hypomethylation has been associated with various clinical measures in tumors, linking it to genomic instability and poor prognosis in various cancers such as non-small-cell lung cancer (NSCLC), colon cancer, and esophageal squamous cell carcinoma, as well as decreased overall survival and drug resistance in young breast cancer patients, recurrence of hepatocellular carcinoma post-resection, and more aggressive histology, shorter progression-free intervals, and poorer survival in ovarian cancer.^{30, 32, 34–37} Additionally, human endogenous retroviruses (HERVs) and their envelop splice products have been associated with tumorigenesis in a variety of cancers due to their activation of the c-MYC proto-oncogene³⁸ and with reduced survival in glioblastoma patients suggesting their role as oncogenic factors.³⁹

Of note, the majority of retroelement insertions identified in cancers are found in intronic and intergenic regions.⁴⁰ The impact of these insertions remains a central question in the field. Like other mutations in non-coding DNA, mobile DNA insertions can disrupt protein binding sites and regulatory sequences.⁴¹ However, TEs are unique in that they can introduce complex sequence modules to a locus. Full-length LINE-1 transpositions, for instance, may position their promoter to produce new RNA isoforms, potentially disrupting genes.⁴² Alu repeats, though understudied in cancer, have internal sequences that are prone to 'exonization'.⁴³ Additionally, gene rearrangements associated with retrotransposition or post-insertion recombinations that delete intervening sequences may have significant implications.⁴⁴

The selective expression of repetitive sequences in cancers may provide opportunities for cancer detection, prognosis, or targeted therapy, and their RNAs and proteins may play roles in oncogenic transformation. Moreover, acquired insertions have the potential to act as driver mutations, disrupting functions or creating new ones at loci where they insert.^{23, 45} The current paradigm of molecular genetics underlying cancer development and therapeutic strategies largely focus on protein-coding DNA sequences in the human genome and the interactions of their protein products. However, TEs emerge as potential therapeutic targets due to their dual role: host genomes domesticate and utilize these repetitive elements to regulate host gene transcription and genome organization, while cancers exploit them to deregulate transcriptional networks and promote genome instability, suggesting that repression of these elements may contribute to therapy resistance.⁴⁶ This duality suggests that TEs could serve as both biomarkers and therapeutic targets in cancer treatment. Several therapeutic strategies including the repression of TE activity through gene therapy have been proposed.^{44, 47–49}

Altogether, the growing evidence of TEs and their roles in normal human tissue development

and disease highlights their potential as promising therapeutic targets in cancer. Herein, we aim to investigate the expression patterns of TEs and their association with survival outcomes in IDH-wildtype glioblastoma. Furthermore, the goal of the present work is to explore the potential of TEs as biomarkers and therapeutic targets, which could lead to improved treatment strategies for this aggressive tumor type.

2.2 Methods

2.2.1 Sample Selection

We utilized RNA sequencing (RNA-seq) data of tumor samples obtained from The Cancer Genome Atlas (TCGA) for glioblastoma cases.⁵⁰ To meet the latest diagnostic guidelines WHO 2021 guidelines¹, cases with IDH1/2 mutations were excluded. Only samples submitted to the TCGA glioblastoma project (TCGA-GBM) with IDH-wildtype status were included. The final cohort of available IDH-wildtype glioblastoma tumors consisted of 151 samples.

2.2.2 Expression Quantification

Transposable element abundance levels were quantified using the RDiscoverTE pipeline version 1.7.0.³⁵ Briefly, RDiscoverTE utilizes annotations from Gencode and RepeatMasker to identify and count TE expression within the human genome. TEs were classified into 1052 distinct subfamilies across five classes: LINE, SINE, LTR, SVA, and DNA transposons.³⁵ Expression/abundance was quantified across the whole genome, within introns (intron), within exons (exon), and within intergenic regions (intergenic). Effective library sizes were calculated using the relative log expression (RLE) method⁵¹, and read counts were standardized to counts per million (CPM) prior to analysis. TEs with a median expression level of 0 CPM across the 151 samples were excluded

from all subsequent analyses.

2.2.3 Individual TE expression and overall GBM survival

Cox proportional hazard models were utilized to assess the association of individual TE expression levels with overall survival, adjusting for age at initial diagnosis and sex. Hazard ratios (HR) with associated p-values adjusted using the false discovery rate (FDR) method less than 0.05 were deemed statistically significant (FDR $q < 0.05$). Sensitivity analyses further adjusting for known prognostically relevant somatic markers CDKN2A and MGMT promoter methylation were conducted on the sample subset with available information. To identify subjects with high TE expression, unadjusted percent survival distributions based on binned TE expression levels were estimated using Kaplan-Meier curves. For each nominated TE, samples were split into those with high expression levels (>80th percentile) and normal expression levels ($\leq$ 80th percentile). Figure 2.1 shows the survival curves for each quintile.

2.2.4 TE Dysregulation Score Analysis

To evaluate the effect of broad overexpression/dysregulation of TEs on survival, we calculated a TE overexpression score (TEOS). For each individual, TEOS represents the number of TEs which are overexpressed within that tumor sample. Here, overexpression of a specific TE was defined to be >80th percentile of values from all included GBM samples. Standardized TEOS were evaluated for survival associations using Cox proportional hazards models adjusting for age and sex. Significant association was determined at a $p < 0.05$ threshold.

TE-subfamily scores were also computed to evaluate if dysregulations within certain subfamilies (e.g. ERV1 subfamily) were associated. Significant subfamily associations were determined at a

threshold of $p < 0.001$ based on the number of independent tests.

All statistical analyses were conducted using R (version 4.3.2)⁵²; data visualization was carried out using ggplot2 (version 3.5.1) for generating various plots including heatmaps, expression plots and survival curves.⁵³

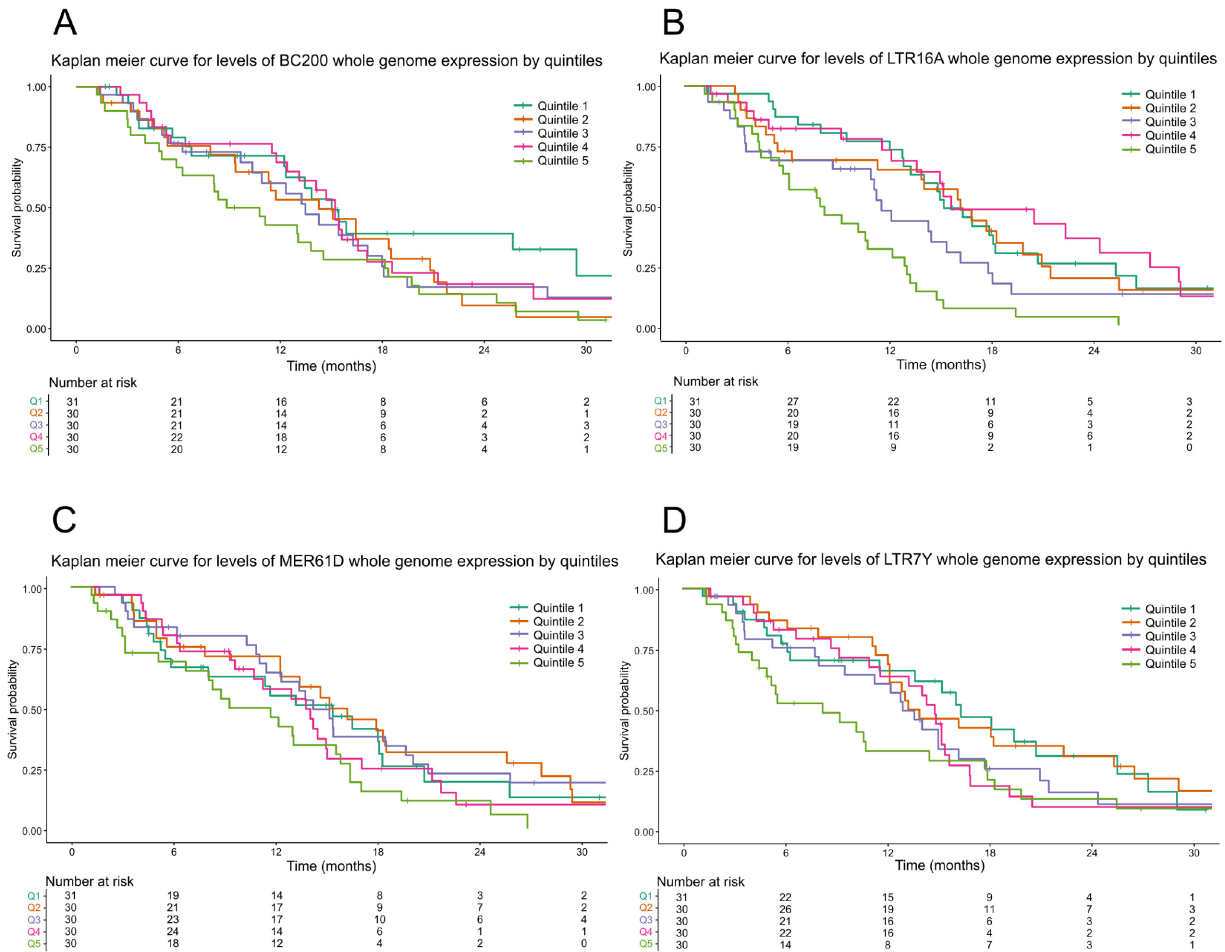


Figure 2.1 Kaplan Meier plots of transposable elements nominated via whole genome expression levels stratified by quintiles. (A) BC200, (B) LTR16A, (C) MER61D, (D) LTR7Y

2.2.5 Association of TEs and relevant genes

To establish links between expression of particular TEs and the possible genes they regulate, Spearman correlation coefficients were estimated for each TE/gene pair within tumor samples,

with corresponding p-values. TE-gene pairs were determined to be significantly correlated using an FDR adjusted q-value threshold of <0.05 .

For each TE, the list of significantly correlated genes were then analyzed for pathway enrichment using the clusterProfileR package (bioconductor library version 3.19).⁵⁴ The top 15 pathways for each TE were reported.

Enrichment analysis was subcategorized by disease pathway enrichment and gene ontology (GO) pathway enrichment. Disease pathway enrichment focused on identifying enriched pathways or biological processes that were directly associated with specific diseases. In contrast, GO enrichment analysis identified which biological processes, molecular functions, or cellular components were statistically overrepresented on a given set of genes.

2.3 Results

This analysis included 151 IDH-wildtype GBM tumor samples, each from a distinct subject, in accordance with the WHO 2021 classification guidelines of central nervous system tumors.¹ Descriptive statistics for subject demographics and molecular characteristics are summarized in Table 2.1. The median age of cases was 62 years, with an interquartile range (IQR) of 53-70.5 years. The cohort included 53 females and 98 males. The median survival time from surgery confirming diagnosis was 11.7 months with an IQR of 5.3 - 17.1 months.

2.3.1 Individual Transposable Elements Associated with Glioblastoma Survival

An initial set of 4634 transposable elements was considered as estimated by REdiscoverTE, with their abundance measured across the whole genome, within exons, introns, and in intergenic

Table 2.1. Cohort Characteristics

Variable	Overall (N=151)
Age, mean (SD)	61.85 (11.98)
Sex, n (%)	
Male	98 (64.9)
Female	53 (35.1)
Self-reported race, n (%)	
White	124 (82.11)
Black or African American	23 (15.23)
Asian	3 (2)
Not reported	1 (0.66)
Last known vital Status, n (%)	
Alive	31 (20.5)
Deceased	120 (79.5)
Overall survival (months), median (SD)	11.5 (10.56)
IDH1/2 status, n (%)	
Wildtype	151 (100)
Mutant	0 (0)
CDKN2A homozygous status, n (%)	
Deleted	108 (71.5)
Non-deleted	34 (22.5)
Unavailable	9 (6)
MGMT promoter status, n (%)	
Methylated	53 (35.1)
Unmethylated	69 (45.7)
Unavailable	29 (19.2)

regions. After filtering for TEs with median expression greater than zero across all included samples, 1093 TEs with non-zero expression in whole genome were retained. Specifically, 585 TEs were found within exons, 939 within introns, and 1062 in intergenic regions. These counts represent distinct TEs in each genomic region, meaning a TE located in one region is not considered in another.

We observed significant survival associations with expression of 4 TEs across whole genome (BC200, LTR7Y, LTR16A, and MER61D), exonic expression of 5 TEs (BC200, LTR16A, L1ME3B, LTR79, and HAL1M8), and 2 TEs (LTR7Y, FLAM-C) in intronic expression. Of note, in all cases, only over expression of TEs was associated with significantly shorter survival times (FDR-adjusted $q < 0.05$). No association between TEs expressed within intergenic regions and survival differences in IDH WT GBMs. Additionally, no under expressed TEs were associated with survival. Full results are available in appendix tables 6.1 through 6.4. Figure 2.3 shows a correlation matrix for statistically significant correlations between the nominated TEs by genomic region.

Elevated expression of BC200 was associated with decreasing survival times (Hazard Ratio, $HR=1.75$, 95% CI: 1.28-2.22, FDR- $q=0.0042$), where those with expression >80th quantile had a median survival of only 9.81 months compared to 14.70 months for those <80th quantile of expression (figure 2.2(A)). Similarly, increased LTR7Y abundance was associated with worse prognosis ($HR=2.24$, 95% CI: 1.55-3.25, FDR- $q=0.011$), with high expressors having a median survival of 8.35 months compared to 14.53 months for low/medium expressors (figure 2.2(E)). Lastly, abundance of MER61D was associated with inferior survival ($HR=1.48$, 95% CI: 1.20-1.82, FDR- $q=0.055$), with a 9.34- and 14.27-month survival, for high and low/medium MER61D expressors, respectively (figure 2.2(B) and Appendix table 6.1).

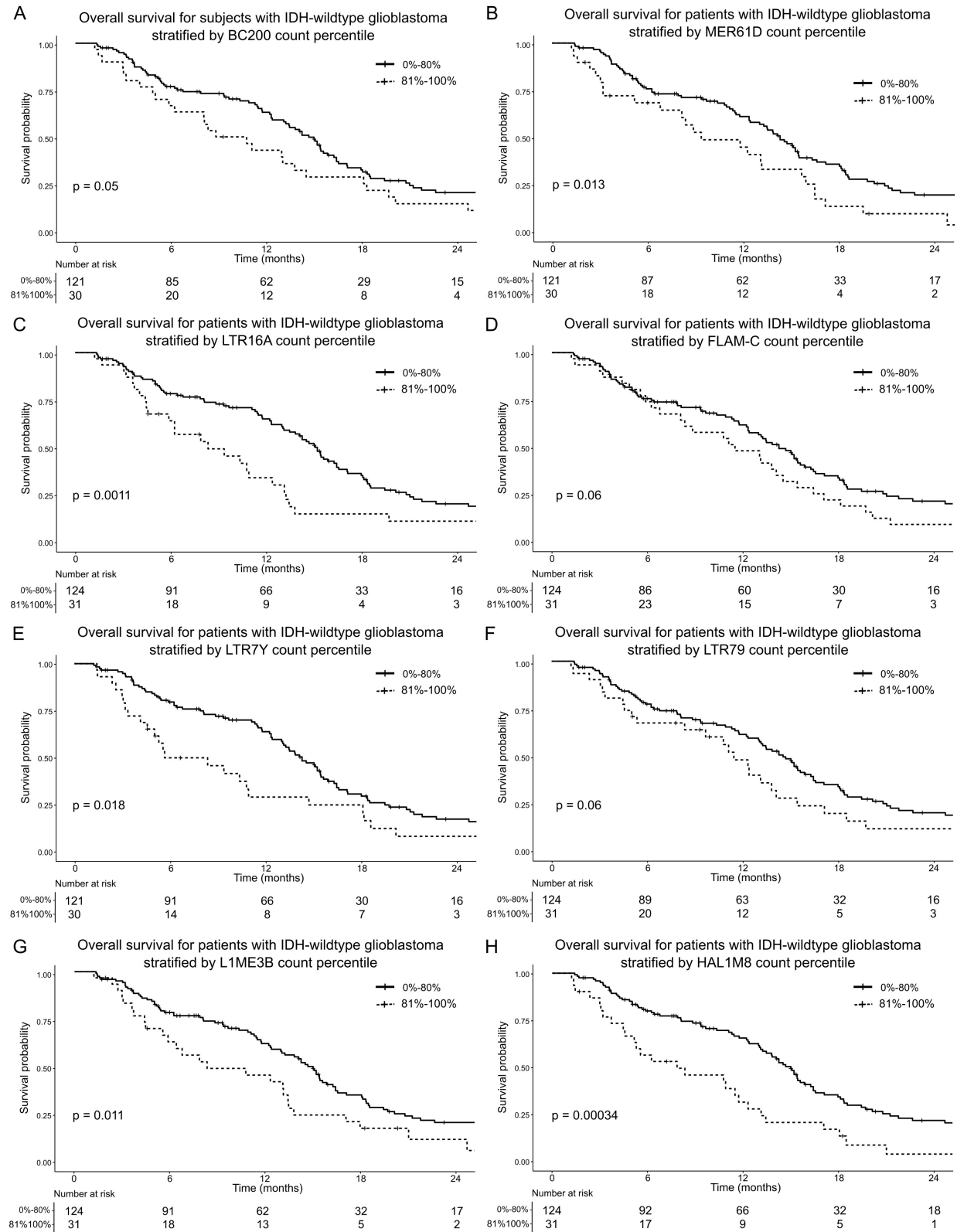


Figure 2.2 Kaplan-Meier curves stratified by count percentile of individual TEs. (A) BC200, (B) MER61D, (C) LTR16A, (D) FLAM-C, (E) LTR7Y, (F) LTR79, (G) L1ME3B, (H) HAL1M8

Fifty-six TEs in whole genome, 49 in introns, 43 in exons, and 59 in intragenic regions were only nominally significant before FDR correction ($q < 0.1$) and are further reported in Appendix table 6.1. Among expression of TEs in exons, significant associations between overexpression of TEs and survival were found. Specifically, L1ME3B (HR=2.27, 95%CI:1.50-3.44, $q=0.022$), HAL1M8 (HR=1.51, 95%CI:1.22-1.86, $q=$), and LTR79 (HR=1.58, 95%CI=1.24-2.01, $q=0.022$) were significantly associated with reduced survival rates among IDH-wildtype GBM subjects (figure 2.2 and Appendix table 6.3).

Similarly, LTR7Y expression within introns was found to be significantly associated with lower survival rates (HR=1.40, 95%CI=1.19-1.65, $q=0.0209$), which was consistent with whole genome analysis. Additionally, FLAM-C (HR=1.64, 95%CI:1.32-2.04, $q=0.0093$), exhibited an association with lower survival rates (figure 2.4, and Appendix table 6.2).

We utilized available molecular typing of known prognostic markers CDKN2A homozygous deletion and MGMT promoter methylation status. Of the 151 samples, 142 had known CDKN2A status, 122 had MGMT methylation status, 109 had both. Cox models including these markers individually or in combination did not significantly alter the results (figure 2.4). In order to maintain model parsimony and maximize sample size, these molecular factors were not included in the final analysis.

2.3.2 Gene Expression Tied to Transposable Element Abundance in Tumor

Pairwise expression analyses revealed a large number of genes correlated to the expression of our nominated TEs within GBM tumor samples. Of the considered 20000 genes, 7319 were significantly correlated (FDR corrected q -value < 0.05) across the whole genome with BC200, 17916 with MER61D, and 15441 with LTR7Y. With exonic specific expression, 7676 genes were correlated

Correlation matrix of nominated Transposable Elements by genomic region

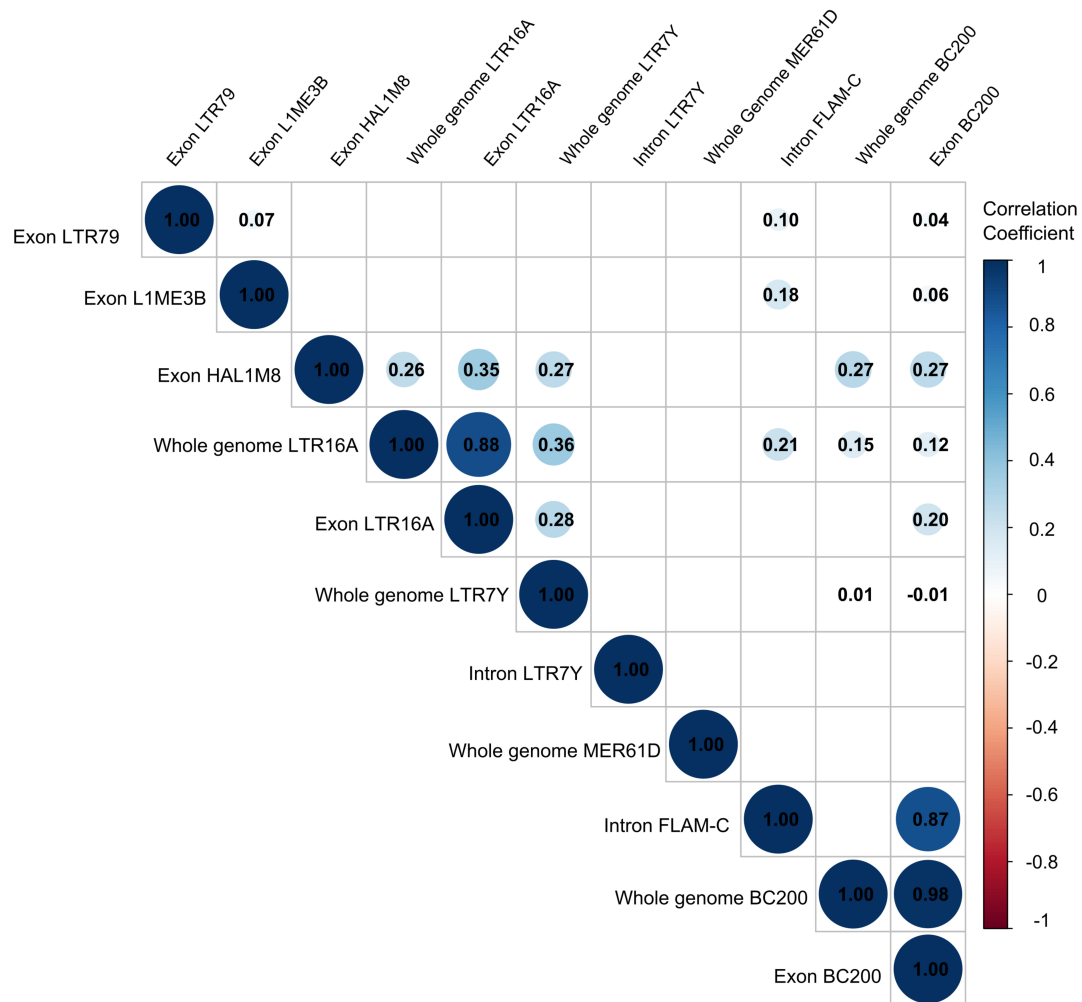


Figure 2.3 Correlation matrix of transposable elements by genomic region. Only statistically significant correlation coefficients ($p < 0.05$) are shown.

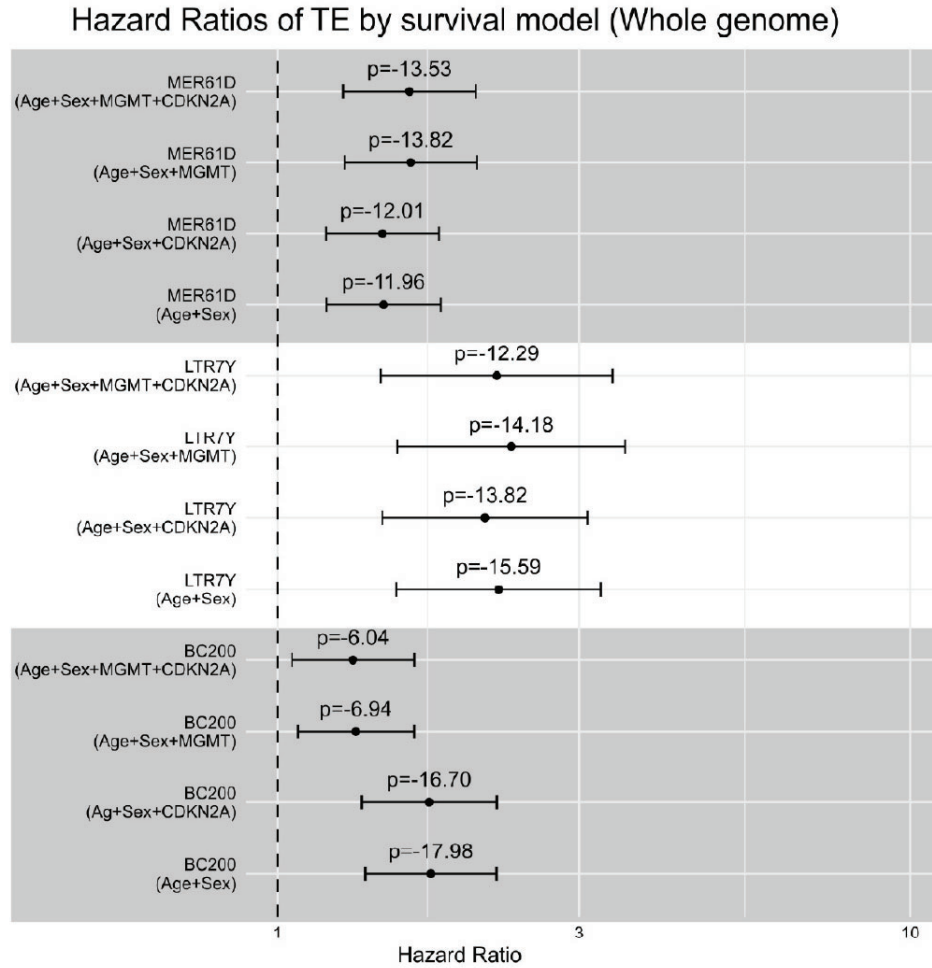


Figure 2.4 *Hazard Ratios of Transposable elements by survival model in Whole genome* Hazard Ratios of transposable elements by different survival models in whole genome. HRs and corresponding 95% CI for individual TEs (MER61D, LTR7Y, BC200) across different Cox-proportional hazard models. Models adjust for age, sex, MGMT promoter methylation status, and CDKN2A deletion. The p-value displayed corresponds to the log2 fold change.

with BC200, 3174 with L1ME3B, 2612 with LTR16A, 198 with LTR79 (Figure 2.5). No genes were associated with HAL1M8. Analysis within intronic regions showed 4095 genes correlated with FLAM-C and 2740 with LTR7Y.

Although BC200 has a specific location in the genome (Chromosome 2: 47,558,199 - 47,571,656), we observed significant pathway enrichment from correlated genes showing high expression that were correlated with BC200, regardless of the distance between BC200 and other associated genes. This pathway enrichment analysis showed that genes correlated with BC200

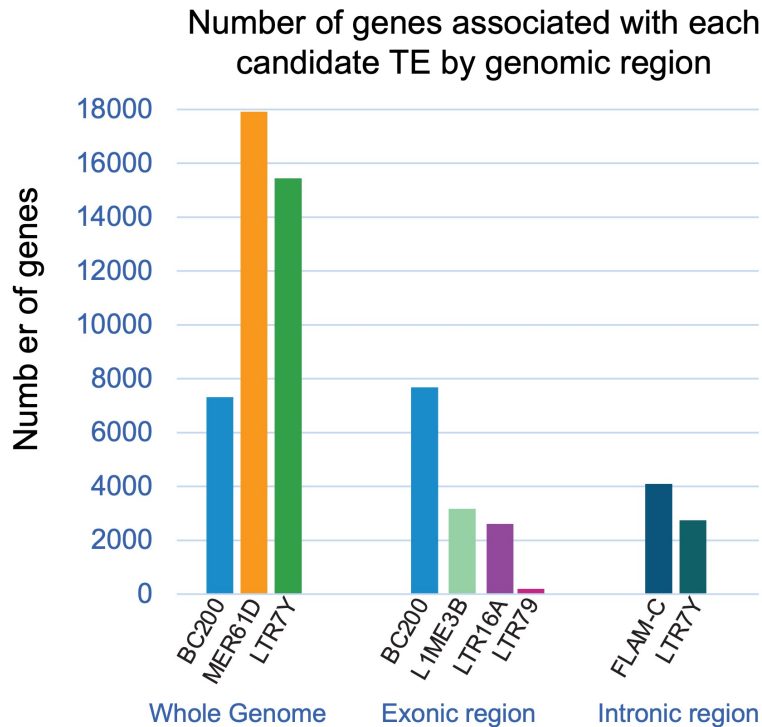


Figure 2.5 Number of genes associated with candidate TEs by genomic region

were enriched for thermogenesis pathway, coronavirus disease (COVID-19), chemical carcinogenesis reactive oxygen species, and ubiquitin-mediated proteolysis pathways with cluster counts of > 60 genes. GO analysis showed significant enrichments of genes associated with RNA processes and pathways (ribonucleoprotein complex biogenesis, RNA splicing, ncRNA processing, ribosome biogenesis, RNA splicing, and rRNA metabolism) with cluster counts of >150 genes. Genes correlated with whole-genome LTR7Y abundance were significantly associated with endocytosis, coronavirus disease (COVID-19), motor proteins, axon guidance, and Oxytocin signaling pathways with cluster counts of > 60 genes. GO analysis demonstrated enrichment for pathways related to cellular motility and structure (microtubule-based movement cilium organization, axonogenesis, regulation of neuron projection development, cilium assembly, and regulation of cellular component size) with cluster counts of >150 genes.

Genes correlated with MER61D were significantly enriched in pathways relating to axon guid-

ance, mTOR signaling pathway, ubiquitin-mediated proteolysis, spinocerebellar ataxia, glutamatergic synapse, phosphatidylinositol signaling system, and aldosterone synthesis and secretion pathways with cluster counts of > 60 genes in the disease pathway enrichment analysis. The GO enrichment analysis showed enrichment in pathways for brain cell and synapse structures and development (synapse organization, modulation of chemical synaptic transmission, regulation of trans-synaptic signaling, regulation of neuron projection development, dendrite development) RNA and DNA pathways (RNA splicing, double-strand break repair, DNA replication, protein polyubiquitination) with cluster counts of >150 genes.

LTR16A was significantly associated with processes related to the immune system. Disease pathway enrichment showed an association with Cytokine-cytokine receptor interaction, osteoclast differentiation, lysosome, coronavirus disease (COVID-19), tuberculosis, and chemokine signaling pathway with cluster gene counts of >40 genes. GO analysis reinforced these results showing strongly significant associations to immune processes such as immune response-activating signaling pathway, leukocyte mediated immunity, positive regulation of cytokine production mononuclear cell differentiation, adaptive immune response, and lymphocyte differentiation with cluster counts of >110 genes.

L1ME3B was associated with RNA processes such as ncRNA processing, ribonucleoprotein complex biogenesis, ribosome biogenesis, RNA splicing, rRNA metabolism, methylation, RNA modification with cluster counts of >50 genes. Genes correlated with LTR79 and HAL1M8 showed no specific pathway enrichments. Results are depicted in figure 2.6 (Whole genome), figure 2.7 (Exons), figure 2.8 (Introns).

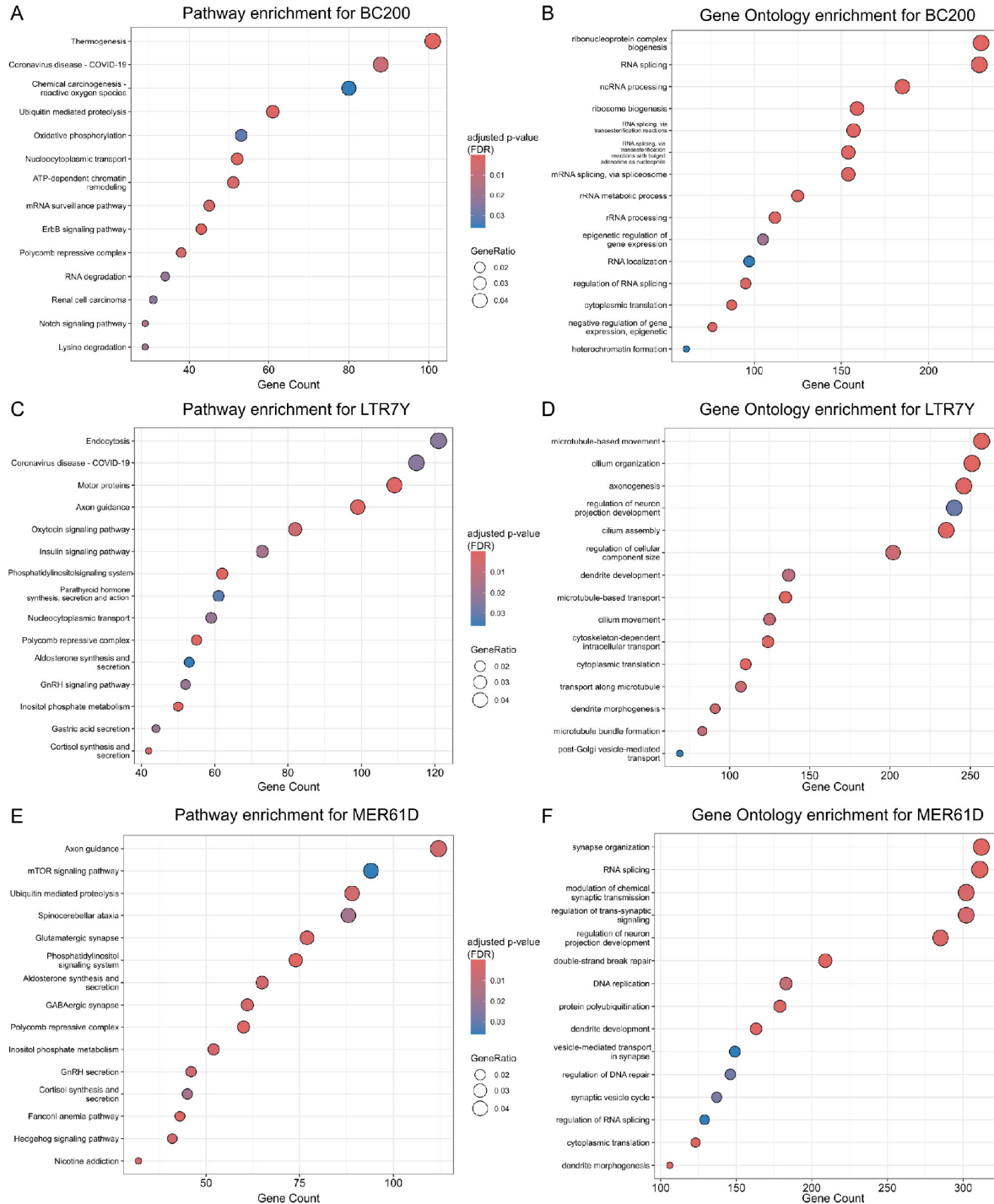


Figure 2.6 Pathway and Gene Ontology enrichment analysis for overexpressed TEs identified in the whole genome. (A) Pathway enrichment analysis for BC200, (B) Gene ontology enrichment analysis for BC200, (C) Pathway enrichment analysis for LTR7Y, (D) Gene ontology analysis for LTR7Y, (E) Pathway enrichment analysis for MER61D, (F) Gene ontology enrichment analysis for MER61D.

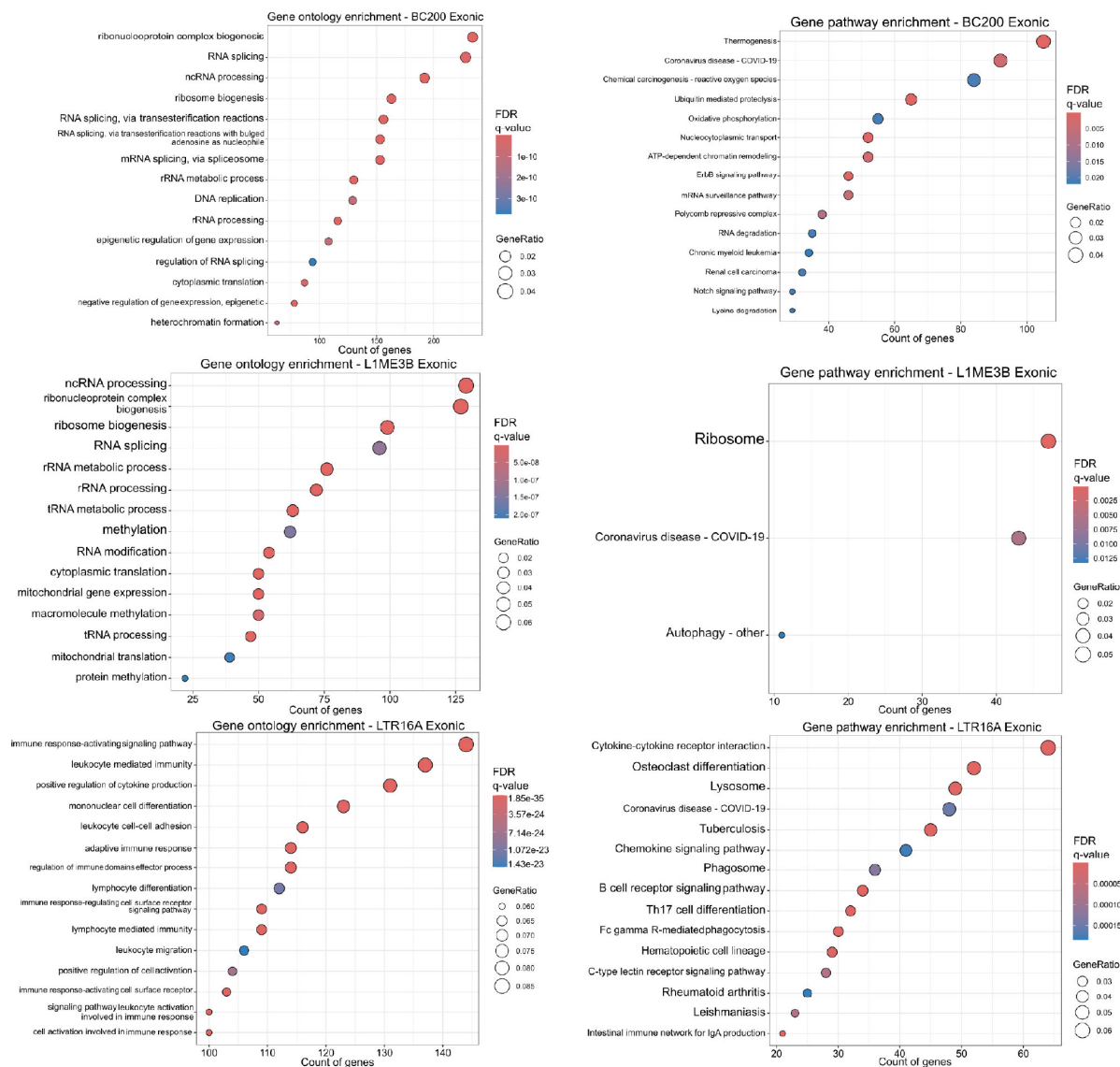


Figure 2.7 Pathway and Gene Ontology enrichment analysis for overexpressed TEs identified in exonic regions.

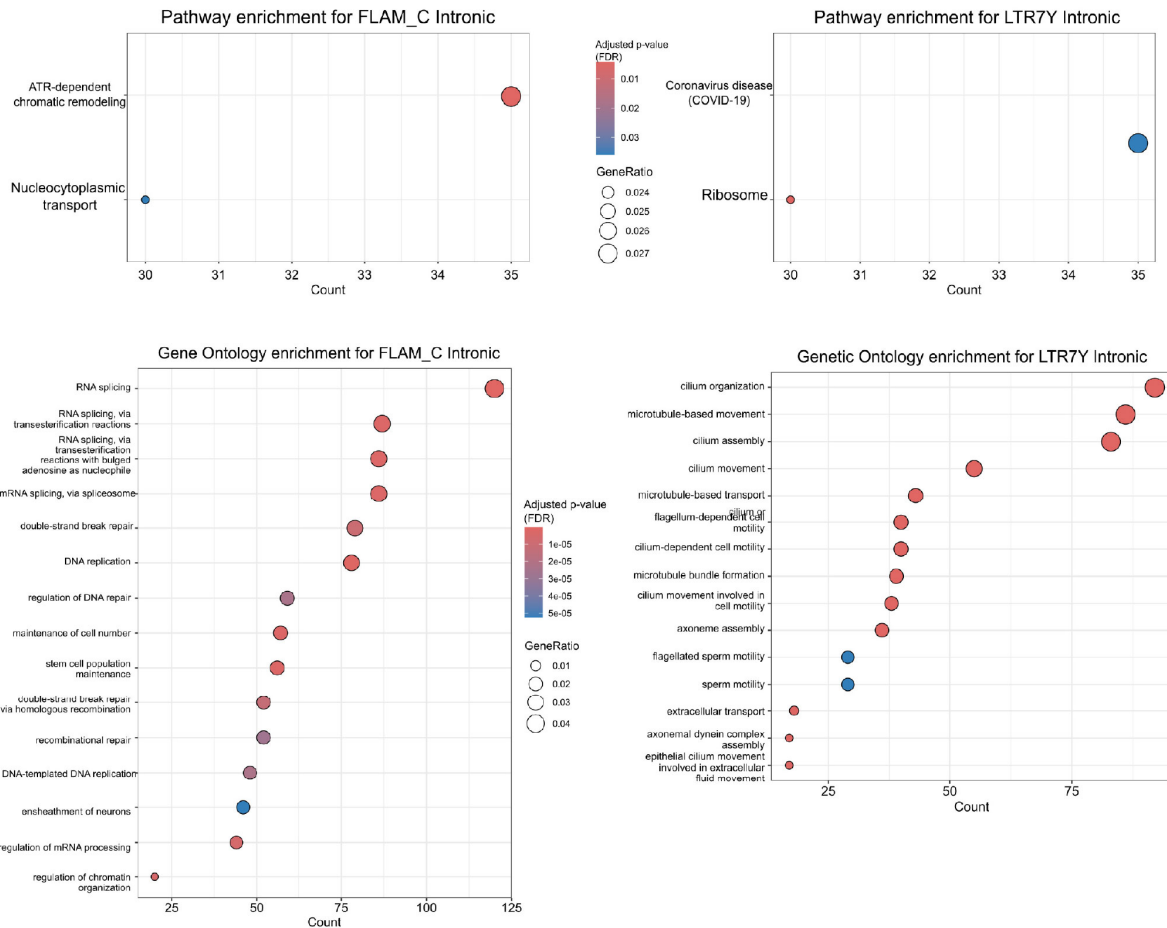


Figure 2.8 Pathway and Gene Ontology enrichment analysis for overexpressed TEs identified in intronic regions.

2.3.3 Overall dysregulation of TEs by Family and GBM

survival Overexpression dysregulation scores were calculated overall and for 48 distinct TE families. Analyses were stratified by expression levels at specific genomic regions (whole genome, intronic, exonic, intergenic). After FDR adjustment, there were no significant associations between TE family-specific dysregulation and survival.

2.4 Discussion

2.4.1 TE Associations with Glioblastoma Survival

Glioblastoma remains a malignancy with poor overall survival, and in the context of central nervous system tumors, it presents as a critical subject of study. The molecular heterogeneity of GBM drives the exploration of genetic and epigenetic alterations that may underpin the observed variability in patient outcomes.⁵⁵ In this study, we conducted a comprehensive analysis of transposable elements (TEs) in IDH-wildtype glioblastoma. By examining the association between the expression of TEs across various genomic regions and overall survival, we have identified several elements that appear to act as independent prognostic markers. These findings suggest that TEs could play a significant role in the progression of glioblastoma and subsequently, overall survival, and serve as potential therapeutic targets and as biomarkers.

Our analysis identified several TEs with significant associations with survival in glioblastoma patients. Notably, TEs members from the L1 (L1ME3B, and HAL1M8), ERV1 (LTR7Y), EVRVL (LTR16A, LTR79), Alu (FLAM-C), and siRNA (BC200) subfamilies were associated with decreased survival times when overexpressed in tumor samples across distinct genomic regions. In general, all TEs identified showed a reduction in survival time for subjects with TE overexpression, while no association was found between reduced expression and survival, supporting their role as prognostic markers in glioblastoma.

The lack of correlation between these TEs suggests that they may function independently within the tumor microenvironment, potentially influencing distinct pathways that contribute to the aggressive nature of IDH-wildtype glioblastoma. The relevance of these TEs is further highlighted

by their presence in the central nervous system and/or their association with cell-regulating genes as well as with various tumor types.

One major functional mechanism of TEs is the creation of new genomic insertions. For instance, Line-1 (L1) elements replicate by copying (reverse transcribing) their RNA into genomic DNA.^{45, 56} Although the genome harbors hundreds of thousands of LINE-1 fragments, most are inactive, existing as 3' fragments or containing mutated open reading frames (ORFs), rendering them incapable of retrotransposition.^{25, 34} In healthy human cells, transcription of retrotransposon sequences, like L1, are suppressed. As such, their normal activity has been shown to be predominant in developing germ cells and placental tissues.^{57, 58}

Suppression of retroelement activity not only prevents retrotransposition but also avoids disturbances in transcription caused by retroelement promoters, interferences from retroelement enhancers, activities of retroelement-encoded enzymes, and recombination between homologous elements. However, while dysregulated TE sequences can create genomic instability, they might also have beneficial, "symbiotic" effects. For example, silencing retrotransposons in somatic cells may help organize the genome into macro- and microdomains with different transcriptional activities.^{58, 59} Therefore, failure to silence retroelements in cancer cells could allow harmful retroelement activities and disrupt any beneficial effects.⁵⁹ DNA methylation, notably of the L1 promoter, is critical for silencing genomic L1-derived TEs. Hypomethylation of the LINE-1 promoter has been associated with various cancers, including non-small-cell lung cancer (NSCLS), colon cancer, esophageal squamous cell carcinoma, breast cancer, hepatocellular carcinoma, and ovarian cancer, correlating with genomic instability and poor prognosis.^{30, 32, 36, 60, 61} Progressive hypomethylation of retroelement promoters during cancer progression, particularly Alu and L1 loci, leads to elevated retroelement expression, further potentiated by stress and translational

inhibitors.^{31, 36} As shown in our pathway enrichment analysis, the nominated TEs are correlated with different cellular processes that are crucial for tumor cell maintenance and growth such as replication, cell structure, cell migration, and RNA processes. These cellular processes are consistent with previous functional studies that have shown that retroelements contribute to cancer through cell migration, and alterations in DNA repair pathways.^{22, 33, 56, 62, 63} Similarly, we found that TEs are correlated with other pathways that are implicated in glioblastoma cell migration, tumor progression, and resistance to therapy such as reactive oxygen species⁶⁴, ErBB⁶⁵, oxidative phosphorylation⁶⁶, and mTOR signaling pathways⁶⁷. In contrast to normal cells, most human cancers and cancer-derived cell lines exhibit increased endogenous full-length L1 mRNA expression due to promoter hypomethylation, influenced partly by the argonaute protein family.⁶⁸ Changes in methylation status, with a tendency towards hypomethylation in early stages of tumor malignancy are extensively documented.^{30, 31, 36, 60, 69}

Studies investigating the role of TEs, particularly L1 elements, in brain tumors have yielded inconclusive results in somatic cells.⁷⁰ While early studies emphasized germline expression of L1 elements, recent findings indicate ongoing expression in human somatic cells, including Leydig, Sertoli, and vascular endothelial cells, as well as hypomethylated L1 loci in normal human brain and skin tissues.^{71, 72} These observations support the notion of ongoing endogenous TE expression in healthy human somatic cells. Apart from L1 sequences, another significant class of autonomous TEs in humans includes long terminal repeat (LTR) sequences.⁷³ Structurally akin to exogenous retroviruses, these encompass endogenous retroviruses (ERVs), mammalian LTR transposons (MLTs), and shorter mammalian apparent LTR retrotransposon (MaLR) repeats. Among human ERVs, the HERV-K group has shown recent human activity, evidenced by a few polymorphic loci in human populations.^{38–40, 74–77}

Functional studies have demonstrated that ERV1 is localized to the distal portion of the long arm of human chromosome 18, a region of interest due to its proximity to the constitutive fragile site at band 121.3, which is associated with the 14;18 translocations observed in follicular lymphomas.⁷⁸ In our analysis, we identified LTR7Y, a member of the ERV1 subfamily- as being highly correlated with genes enriched in processes involving cell structure and cell mobility, both of which are crucial hallmarks of cancer. In the context of glioblastoma, we found that LTR7Y expression was correlated with the enrichment of pathways implicated in axonogenesis, axon guidance, and dendrite development. Previous studies have shown that bidirectional signaling between tumors and neurons propagates as a vicious cycle of proliferation, synaptic integration, and brain hyperactivity. This interaction may lead to the promotion of tumor progression and widespread infiltration, particularly as projection neurons located in the hemisphere contralateral to the primary glioblastoma tumor are involved.⁷⁹ This phenomenon contributes to the poor prognosis often associated with glioblastoma, reinforcing the potential significance of LTR7Y as a prognostic biomarker.

Furthermore, the pathway enrichment analyses revealed that these gene-TE correlations are not random but rather are enriched in pathways associated with critical cellular processes, including RNA processing, cellular motility, immune response, and neurodevelopment, many of which are implicated in cancer and other complex diseases. For instance, the enrichment of genes correlated with BC200 in pathways related to RNA processing and ubiquitin-mediated proteolysis suggests that BC200 may influence glioblastoma progression through dysregulation of RNA stability and protein turnover. BC200, also known as brain cytoplasmic RNA 1 (BCYRN1), is a non-coding RNA sense-transcribed from its gene locus on human chromosome 2, situated between the protein-coding genes for calmodulin 2 (CALM2) and epithelial cellular adhesion molecule (EPCAM). Interestingly, BC200 synthesis has been linked to two important transcription factors, c-MYC⁷² and estrogen

receptor alpha ($ER\alpha$), in lung and breast cancer cell lines, respectively.⁸⁰ Given that the MYC proto-oncogene is deregulated in many cancers, it remains to be seen if MYC drives BC200 expression in other cancers besides non-small cell lung cancer (NSCLC).

The specific case of BC200 is particularly intriguing because it represents one of the few transposable elements (TEs) that, over evolutionary time, acquired a regulatory function, evolving into the gene BCYRN1. As part of the class of long non-coding RNAs (lncRNAs), BC200 is conserved in humans, and initial studies identified it as being neural-specific and dendritic localized. A link to cancer was provided a few years later by a key study revealing BC200 RNA expression in a number of human tumors through Northern blot analysis and in situ hybridization.⁸¹ In that study, the authors found BC200 expression to be substantially increased in tumors such as those of the breast, cervix, esophagus, lung, ovary, parathyroid, and tongue, while it was undetectable in corresponding normal tissues and in other cancers, such as bladder, colon, and liver. More recent investigations suggest that BC200 has roles in cell migration, proliferation, and survival, indicating that BC200 directly contributes to cancer development and progression.^{82, 83}

Furthermore, unlike many lncRNAs that function primarily within the nucleus, BC200 is classified as a small, cytoplasmic RNA. Many of its molecular interactions involve proteins located in the cytoplasm. Additionally, BC200 is found primarily in the primate brain.⁸⁴ Among neural cells, BC200 is highly expressed in dendrites, where it is thought to play a role in local translational control. Given its expression in the nervous system, numerous studies have examined BC200's molecular interactions in neurons. This becomes particularly relevant in the context of glioblastoma, given that neuronal activity has been demonstrated to promote glioma growth through both paracrine signaling and other electrochemical synapses.⁸⁵

BC200 has also been implicated in Alzheimer's disease due to its altered expression. Specifically, elevated BC200 RNA expression was observed in brain regions associated with the disease, leading to the hypothesis that elevated BC200 RNA contributes to synaptodendritic deterioration in Alzheimer's neurons.⁸⁶ Furthermore, given BC200's many unique characteristics such as being a Pol II transcript, its mostly cytoplasmic localization, and its small size it is likely that BC200 occupies different functional roles compared to more well-characterized, cancer-associated lncRNAs, such as MALAT1 and HOTAIR, which have nuclear roles in gene regulation.⁸⁷ Previous work demonstrated that BC200 lncRNA was detectable in carcinomas of the breast, cervix, esophagus, lung, ovary, parotid, and tongue, while no expression was found in normal tissue through immunohistochemistry analyses of tissue sections.^{80, 81} While upregulation of BC200 RNA expression in cancer cells does not reflect an overall increase in Pol III transcription, there is a report that BC200 expression is responsive to c-MYC, and that c-MYC binds to the BC200 promoter region in NSCLC cells.⁸⁸

In the context of gliomas, a recent study found BC200 to be differentially expressed in glioma samples from patients.^{89, 90} However, unlike previous studies, this functional study showed that BC200 overexpression inhibited cell proliferation and migration in glioma cells, which was associated with poor patient outcomes, consistent with our findings in pathway enrichment analysis and survival analysis.⁸⁹ Conversely, BC200 depletion resulted in the opposite effect. Given its differential expression and widely studied functions, BC200 presents itself as a promising candidate for a prognostic biomarker and therapeutic target.

While BC200 plays a regulatory role in neural cells and gliomas, another transposable element, MER61D, interacts with cancer biology through the TP53 pathway, a key tumor suppressor gene. MER61D has been shown to be significantly affected by the depletion of p53 via copy loss,

suggesting that p53 controls TEs in a highly specific manner rather than universally regulating all TE subfamilies.³⁵ Additionally, MER61D retrotransposition may have contributed to the establishment of the TP53 binding landscape, particularly during early human development, where it supports cell proliferation and prevents cellular differentiation in trophoblasts by maintaining a pluripotent state. Furthermore, MER61 elements have expanded the TP53 binding network in primates, mediating cellular stress responses in lymphoblastoid cells and contributing to immune cell development.⁹¹

The importance of this lies in the regulatory activity of the TP53 gene, a tumor suppressor located on chromosome 17p13.1.⁹² TP53 is involved in crucial cellular processes such as cell cycle arrest, differentiation, apoptosis, and DNA damage repair, thereby protecting cells from tumorigenesis.⁹³ Mutations in TP53 occur in 50% of all human cancers, causing mutant p53 protein to accumulate in the nucleus. This mutant protein interacts with oncoproteins, inducing tumorigenesis.^{94, 95} The identification of MER61D's association with synapse organization and DNA repair pathways points to its involvement in maintaining the genomic integrity and cellular communication networks within glioblastoma cells. These functional insights align with the known characteristics of glioblastoma, including its resistance to DNA-damaging therapies and its reliance on robust cell signaling mechanisms for survival and proliferation.

Furthermore, LTR16A's correlation with genes involved in key immune system processes, such as the immune response-activating signaling pathway, cytokine production, immune cell adhesion, and leukocyte migration, suggests that this TE may play a role in modulating the tumor microenvironment.⁹⁶ Specifically, by influencing cytokine production, LTR16A could alter the balance of pro- and anti-inflammatory signals within the tumor, potentially creating an immunosuppressive microenvironment that hinders the recruitment and activation of immune cells.⁹⁷ In addition, the correlation with genes involved in immune cell adhesion and leukocyte migration hints at a possible

mechanism by which LTR16A could impede the infiltration of immune cells into the tumor. These immune-modulatory functions may contribute to glioblastoma's well-documented ability to evade immune surveillance and resist immune-mediated destruction, further complicating therapeutic interventions. Such findings raise the possibility that LTR16A influences immune escape mechanisms, a hallmark of cancer, and could serve as a potential target for therapies aiming to overcome immune resistance in glioblastoma. This finding is particularly intriguing given the current interest in immunotherapy for glioblastoma, as it raises the possibility that LTR16A could be targeted to enhance immune responses against the tumor. L1ME3B's association with RNA processing and modification pathways underscores its potential role in the regulation of gene expression at the post-transcriptional level. This TE could influence various aspects of tumor biology, including cell growth, survival, and adaptation to stress, making it a candidate for further investigation as a therapeutic target.

In contrast, Alu elements, though non-autonomous, can be mobilized in trans by L1-encoded proteins, further complicating the genomic landscape. Despite only a few copies being active, Alu germline activity is the highest of all human retrotransposons and does significantly contribute to genetic disease and population diversity.^{43, 98} This may explain our observation of an association between higher expression levels of Alu sequences in intronic regions of the genome and lower survival times in IDH-wildtype gliomas, which was not observed in exonic regions, as these are more actively transcribed. Although Alu is more frequently found in germ cells, somatic retrotransposition can contribute to cancer if the insert mutagenizes a gene necessary for cellular homeostasis such as a tumor suppressor.^{70, 99} Several reports have linked de novo Alu insertions to cancers, with some likely involving germline line insertions.⁹⁸ One notable example is de novo insertions associated with the NF1 gene.¹⁰⁰ In IDH-wildtype glioblastomas, NF1 loss of function plays a major

role in disease progression.^{101, 102}

2.4.2 Clinical Implications and Future Directions

The findings of this study have several important clinical implications. First, the identification of TEs as independent prognostic markers suggests that these TEs could be integrated into existing glioblastoma prognostic models to improve risk stratification and guide treatment decisions. Given the significant associations observed in multiple genomic regions, these TEs may also serve as potential therapeutic targets, particularly in the context of precision medicine approaches aimed at targeting specific epigenetic regulatory checkpoints that may be vulnerable in glioblastoma.

Pharmacological approaches aimed at activating an anti-viral response in tumors, based on the upregulation of transposable elements (TE) as “viral mimicry”, has recently become an active research topic with promising results.^{103, 104} Interestingly, a mammalian lineage-specific subset of ERVs act as interferon-inducible enhancers essential for the activation of the AIM2 inflammasome.¹⁰⁵ Furthermore, the robust gene expression correlations and pathway enrichment analyses provide a foundation for exploring the roles of these TEs in glioblastoma biology in a functional setting. Future studies should focus on elucidating the precise mechanisms by which these TEs contribute to tumor progression, including their potential interactions with known oncogenic genes and molecular pathways, and their impact on the tumor microenvironment. Treatment of glioblastoma cells with demethylating agents resulted in increased TE expression and antigenicity through the presentation of novel TE-derived peptides on class I MHC.³⁵ Chemotherapy-induced TE expression has been demonstrated to activate the MDA5 pathway leading to an inflammatory response required for hematopoietic regeneration.¹⁰⁶

Finally, our study underscores the need for further investigation into the role of TEs in cancer.

Our findings contribute to the growing body of evidence that TEs are active players in cancer biology, influencing gene expression, genomic instability, and ultimately, patient outcomes. Expanding our understanding of TE biology could lead to the development of novel biomarkers and therapeutic strategies across a range of cancers.

The consistency of the findings of the present study across genomic regions and the available literature further sheds light to the potential importance of TEs as prognostic markers and therapeutic targets.

2.4.3 Limitations

Despite the strengths of our study, there are several limitations that should be acknowledged. First, our analysis was limited to a single cohort of IDH-wildtype glioblastoma patients, and the findings should be validated in independent cohorts to ensure their generalizability. Additionally, while we focused on TEs with significant associations after FDR correction, the potential role of other nominally significant TEs should not be overlooked and may warrant further exploration in larger studies.

The observational nature of our study makes it difficult to establish causality between TE expression and glioblastoma survival. Functional studies are needed to determine whether the identified TEs play a direct role in tumorigenesis or are biomarkers of more severe disease. Future research should aim to integrate TE expression data with other genomic, epigenomic, demographic, and clinical information in the longitudinal setting to provide a more comprehensive understanding of glioblastoma biology.

2.5 Conclusions

This study underscores the significant role of TEs in the prognosis of IDH-wildtype glioblastoma. Our findings show that specific overexpression of TEs, including BC200, LTR7Y, MER61D, and L1ME3B, is strongly associated with reduced survival times. These elements are correlated with key oncogenic processes, such as cell proliferation, migration, immune evasion, and genomic instability, reinforcing their potential as biomarkers and therapeutic targets. Importantly, the pathway enrichment analyses identified specific TEs associated with critical pathways implicated in glioblastoma progression, including RNA processing, immune response modulation, and cellular motility. These findings suggest that TEs play a multifaceted role in IDH-wildtype glioblastoma, contributing to tumor growth, therapy resistance, and the ability to evade immune surveillance.

The discovery of TE influence on immune-related pathways, particularly LTR16A, opens new avenues for exploring how these elements could be targeted to enhance the effectiveness of emerging immunotherapies. Moreover, the observed correlation between TE overexpression and poor outcomes underscores the need to further investigate their role in tumor biology.

Future research should aim to explore the mechanistic roles of these TEs in glioblastoma, particularly their interactions with essential cellular pathways such as DNA repair, cell cycle regulation, and RNA processing. Further studies are needed to understand how activation of TEs triggers downstream effects that promote tumor heterogeneity and what is their specific role during chemoradiation. Additionally, longitudinal studies could explore the dynamic expression of TEs throughout tumor progression, providing insights into how their roles may evolve as the disease progresses.

Another area of interest is the tissue-specific expression and regulation of TEs. Investigating why specific TEs, like BC200 and LTR7Y, are highly expressed in glioblastoma and other cancers, while remaining inactive in normal tissues, could illuminate the underlying mechanisms driving TE activation in malignancies. Comparative analysis of TE activity in different brain tumors (i.e. IDH-mutant gliomas) or across various stages of glioblastoma could help determine whether TE expression profiles serve as early markers of disease progression or treatment resistance.

Further validation of these findings in larger, independent datasets will be essential to confirm the prognostic value of TEs across diverse populations. Such studies could help establish the robustness of TE expression as biomarkers and ensure their broader applicability in clinical settings. Finally, advancing computation models and bioinformatic tools to analyze TE expression in large datasets will be critical. These tools can help to refine the understanding of TE-driven regulatory networks and their broader impact on tumor biology. Expanding on the current knowledge of TE integration sites and their interactions with chromatin architecture will further elucidate the functional consequences of TE insertions in cancer genomes.

Longitudinal profiling of IDH-mutant astrocytomas reveals acquired RAS-MAPK pathway mutations associated with inferior survival

3.1 Introduction

“Astrocytoma, IDH-mutant” is a newly codified tumor type in the 2021 World Health Organization (WHO) Classification of Central Nervous Systems Tumors that encompasses a group of diffuse astrocytic gliomas with *IDH1* p.R132 or *IDH2* p.R172 mutation, biallelic TP53 inactivation, frequent *ATRX* inactivation associated with alternative lengthening of telomeres, absence of chromosomes 1p and 19q whole arm co-deletion, and a distinct DNA methylation profile.¹ The majority occur in young adults in the third to fifth decades of life, but they can also arise in adolescents and during later adulthood.^{107–109} They occur most commonly in the cerebral hemispheres, but can also arise in the cerebellum or brainstem.¹¹⁰ The majority are sporadic/spontaneous tumors, but they occasionally arise in the setting of specific tumor predisposition syndromes including Li-Fraumeni, Ollier, and Lynch due to constitutional mutations affecting the TP53, IDH1, and DNA mismatch repair genes (MSH2, MSH6, MLH1, and PMS2).^{111–113} The majority arise as low-grade tumors (CNS WHO grade 2) which demonstrate slow growth over time. However, IDH-mutant astrocytomas cannot be cured by surgical resection alone given their infiltrative growth that extends beyond what can be visualized by current radiologic methods and what can be safely resected in

most patients. While maximal safe resection has a proven survival benefit for these patients, over time IDH-mutant astrocytomas recur and can often transform to higher grade tumors (CNS WHO grade 3 or 4) with more rapid growth and spread, necessitating repeat surgical resection and/or adjuvant therapies including external beam radiation and chemotherapy, such as with the alkylating agent temozolomide.⁹ While they are associated with a markedly better survival compared with IDH-wildtype glioblastoma, IDH-mutant astrocytomas are malignant tumors that are not curable with the currently available therapies. However, new therapeutic approaches are on the horizon for affected patients including immunotherapies and the recently FDA-approved small molecule inhibitors (e.g. vorasidenib) of the mutant isocitrate dehydrogenase (IDH) enzymes encoded by the IDH1 and IDH2 genes.^{9, 114, 115}

IDH-mutant astrocytomas are genetically defined by an activating missense mutation at codon p.R132 in *IDH1* or the analogous codon p.R172 in *IDH2*, which is the earliest initiating oncogenic event in these tumors and fuels gliomagenesis by catalyzing formation of the 2-hydroxyglutarate oncometabolite that underlies the glioma CpG island methylator phenotype.^{4, 5} Additionally, mutational inactivation of the *TP53* tumor suppressor gene is an early oncogenic event that cooperates with the *IDH1/2* mutation to initiate the formation of an IDH-mutant astrocytoma.¹¹⁶ As the glioma then expands, inactivating mutation or deletion of the *ATRX* chromatin remodeling gene is selected for in most tumors that causes alternative lengthening of telomeres (ALT) to bypass cellular senescence.^{117, 118} In CNS WHO grade 2 IDH-mutant astrocytomas demonstrating low proliferative activity, absence of anaplasia, and absence of necrosis and microvascular proliferation, the trio of *IDH1/2*, *TP53*, and *ATRX* mutations is typically present without additional accompanying oncogenic alterations.⁵⁰ Over time, IDH-mutant astrocytomas acquire additional genetic aberrations that drive high-grade transformation, treatment resistance, and more rapid growth. The additional genetic

perturbations that fuel this high-grade transformation are multiple and have been identified through longitudinal profiling studies over the past several years.^{suzukiMutationalLandscapeClonal2015, 119–122} The most frequent additional genetic aberration is homozygous deletion of the *CDKN2A* tumor suppressor gene on chromosome 9p21.3, which encodes the cell cycle regulator p16INK4a. Multiple studies have documented that *CDKN2A* homozygous deletion is enriched in recurrent and high-grade IDH-mutant astrocytomas and correlates with inferior survival compared to IDH-mutant astrocytomas with intact *CDKN2A* alleles.^{123–126} As such, the presence of *CDKN2A* homozygous deletion was added as a prognostic grading factor for IDH-mutant astrocytoma in the 2021 WHO Classification, with those tumors harboring this genetic alteration assigned as CNS WHO grade 4 regardless of their histologic features.¹

Several additional genetic aberrations associated with high-grade transformation and inferior survival of IDH-mutant astrocytomas beyond *CDKN2A* homozygous deletion have been identified. These include aberrations of other cell cycle regulators, such as amplification of *CDK4* (which encodes cyclin-dependent kinase 4), amplification of *CCND2* (cyclin D2), and mutation or deletion of *RB1* (retinoblastoma tumor suppressor).^{127–130} The receptor tyrosine kinase genes *PDGFRA* (platelet-derived growth factor receptor alpha) and *MET* (hepatocyte growth factor receptor) are also frequently amplified and rearranged in recurrent and higher grade IDH-mutant astrocytomas, which are being tested as therapeutic targets in this patient population.^{127, 131–134} Acquisition of *MYCN* amplification leading to overexpression of the encoded Myc family transcription factor occurs in a small subset of recurrent IDH-mutant astrocytomas that is associated with poor prognosis.¹³⁵ Mutations of *PIK3CA* and *PIK3R1* driving hyperactivation of the PI3-kinase-Akt-mTOR signaling pathway were also recently found to occur in a subset of IDH-mutant astrocytomas, particularly recurrent and/or higher grade tumors associated with inferior survival.¹³⁶ Increased burden of

chromosomal copy number aberrations across the genome has also been correlated with worse survival among patients with IDH-mutant astrocytomas.^{123, 137, 138} Lastly, recurrent IDH-mutant astrocytomas that develop somatic hypermutation following chemotherapy with the alkylating agent temozolomide are associated with malignant transformation, poor prognosis, and increased risk of cerebrospinal fluid dissemination.^{122, 139–141}

To further investigate the molecular events that drive tumor progression and treatment resistance, we performed comprehensive histopathologic assessment and targeted genomic sequencing on 205 IDH-mutant astrocytoma tumor specimens from 172 consecutive patients inclusive of all CNS WHO grades and disease timepoints as part of a sponsored glioma precision medicine program at our institution. We identified the RAS-MAPK signaling pathway as a novel target of frequent oncogenic alterations in recurrent IDH-mutant astrocytomas associated with high-grade transformation and inferior survival. We speculate that these RAS-MAPK pathway mutations drive treatment resistance during progression of IDH-mutant astrocytomas and may serve as a potential therapeutic target for affected patients using specific inhibitors.

3.2 Methods

3.2.1 Patient cohort and tumor samples

The study cohort was composed of 205 IDH-mutant astrocytoma tumor specimens from 172 patients who were treated at the University of California, San Francisco (UCSF) Medical Center between 2015 and 2023. All patients had tumors pathologically confirmed as “Astrocytoma, IDH-mutant” according to the 2021 WHO Classification of Central Nervous System Tumors based on the combination of histopathologic features and targeted next-generation DNA sequencing (NGS)

including assessment of gene mutations, fusions and other structural variants, and chromosomal copy number profiles. Specifically, all tumors were diffuse astrocytic gliomas located supratentorially in the cerebral hemispheres and were confirmed to be IDH-mutant with either *IDH1* p.R132 or *IDH2* p.R172 missense mutations detected by NGS. All tumors lacked whole arm co-deletion of chromosomes 1p and 19q and were histone H3-wildtype (e.g. lacked p.K27 or p.G34 mutation in H3-3A, H3-3B, H3C2, and H3C3). In total, 205 tumor specimens from the 172 included patients were examined by histopathology and genomic profiling. This included 95 initial treatment-naïve tumor specimens from an initial diagnostic surgical resection, and 110 recurrent tumor specimens from a subsequent surgical resection following either observation or adjuvant therapy. For 24 patients, there were multiple longitudinal tumor specimens that were genomically profiled. While a subset of patients was treated with an IDH mutant small molecule inhibitor (either vorasidenib, ivosidenib, or enasidenib) at time of disease progression (n=13), no patients in this cohort were treated with an IDH mutant small molecule inhibitor in the adjuvant setting following the initial diagnostic surgery, and all genomic profiling was performed prior to therapy with IDH mutant small molecule inhibitors. This study was approved by the institutional review board of the University of California, San Francisco. As part of routine clinical practice at UCSF, all patients included in the study cohort signed an informed consent waiver to contribute de-identified data to scientific research projects.

3.2.2 Histology and immunohistochemistry

All tumors were pathologically confirmed as IDH-mutant astrocytoma based on a combination of immunoreactivity for *IDH1* R132H mutant protein, NGS results showing presence of *IDH1* p.R132 or *IDH2* p.R172 variants, and chromosomal copy number status showing absence of whole arm co-deletion of chromosomes 1p and 19q. Detailed pathologic examination was performed retrospec-

tively to investigate histologic and immunohistochemical features of the IDH-mutant astrocytoma cohort, including both histologic grading based on the 2016 WHO Classification and integrated grading based on the 2021 WHO Classification. Immunohistochemistry was performed on whole formalin-fixed, paraffin-embedded tissue sections using the following antibodies: *IDH1* R132H mutant protein (Dianova, clone H09, 1:500 dilution, ER2 antigen retrieval), p53 (Biocare, clone DO-7, 1:100 dilution, ER2 antigen retrieval), *ATRX* (Sigma, polyclonal, 1:100 dilution), histone H3 K27M mutant protein (RevMAb Biosciences, clone RM192, 1:600 dilution), histone H3 lysine 27 trimethylation (Cell Signaling, clone C36B11, 1:50 dilution, ER2 antigen retrieval), histone H3.3 G34R mutant protein (RevMAb Biosciences, clone RM240, 1:100 dilution), Ki-67 (Dako, clone Mib1, 1:50 dilution, ER2 antigen retrieval), and phosphorylated p44/42 ERK1/2 Thr202/Tyr204 (Cell Signaling, clone D13.14.4E, 1:10,000 dilution). Tissue sections used for phospho-ERK1/2 immunohistochemistry included both a subset of the tumors with oncogenic RAS-MAPK alterations (n=12), adjacent uninvolved cortex from tumors with oncogenic RAS-MAPK alterations (n=10), and IDH-mutant astrocytomas lacking identifiable RAS-MAPK pathway alterations (RAS-MAPK wildtype, n=6). Immunostaining was performed in Leica Bond-Max or Ventana BenchMark Ultra automated stainers. Diaminobenzidine was used as the detection chromogen, followed by hematoxylin counterstain.

3.2.3 Targeted next-generation DNA sequencing

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tumor tissue of 205 longitudinal IDH-mutant astrocytoma tumor specimens from the 172 patients using the QIAamp DNA FFPE Tissue Kit (Qiagen). Tumor tissue was selectively scraped from unstained slides or punched from formalin-fixed, paraffin-embedded blocks using biopsy punches (Integra Miltex Instruments) to enrich for high tumor content. For a subset of patients, n=49 as indicated, a constitutional DNA sample was extracted from a buccal swab or peripheral blood specimen using the QIAamp

DNA Blood Midi Kit (Qiagen) and simultaneously sequenced to enable accurate discrimination of germline/constitutional versus somatic origin of variants. Capture-based next-generation DNA sequencing was performed using the UCSF500 NGS panel as previously described that targets all coding exons of 529 cancer-related genes, select introns and upstream regulatory regions of 73 genes to enable detection of structural variants including gene fusions, and DNA segments at regular intervals along each chromosome to enable genome-wide copy number and zygosity analysis, with a total sequencing footprint of 2.8 Mb.^{142–146} Multiplex library preparation was performed using the KAPA Hyper Prep Kit (Roche) according to the manufacturer's specifications using 200 ng of sample DNA. Hybrid capture of pooled libraries was performed using a custom oligonucleotide library (Integrated DNA Technologies). Captured libraries were sequenced as paired-end reads on an Illumina NovaSeq 6000 instrument. Sequence reads were mapped to the reference human genome build GRCh37 (hg19) using the Burrows-Wheeler aligner (BWA). Recalibration and deduplication of reads was performed using the Genome Analysis Toolkit (GATK). Coverage and sequencing statistics were determined using Picard CalculateHsMetrics and Picard CollectInsertSizeMetrics. Single nucleotide variant and insertion/deletion mutation calling was performed with Mutect2, FreeBayes, Unified Genotyper, and Pindel. Structural rearrangement calling was performed with Delly. Single nucleotide variants, insertions/deletions, and structural variants were visualized and verified using Integrative Genome Viewer. Genome-wide copy number and zygosity analysis was performed by CNVkit¹⁴⁷ and visualized using Nexus Copy Number (Biodiscovery). Microsatellite instability determination was performed with MSIsensor2 analysis of mononucleotide and dinucleotide repeats.¹⁴⁸ Astrocytomas were deemed positive for microsatellite instability when $\geq 10\%$ of the 86 microsatellites assessed by the UCSF500 NGS panel were unstable. Somatic tumor mutation burden (TMB) was determined by calculating the number of somatic mutations in the coding regions of genes in the UCSF500 NGS panel, counting both single nucleotide vari-

ants and short indels, divided by the total coding footprint of the assay (1.5 Mb). For patients with paired tumor-normal sequencing performed, TMB was calculated using only the confirmed somatic mutations. For patients with tumor-only sequencing performed, TMB was calculated by removing known germline variants present at $\geq 0.001\%$ frequency in human population datasets (ExAC, gnomAD, and NHLBI-ESP6515). Hypermutation was defined as those tumors with TMB values of ≥ 10 somatic mutations per Mb based on this assay. Temozolomide-induced hypermutation corresponding to single base substitution mutational signature 11 from the COSMIC Human Cancer Mutational Signatures Database version 3.4 was defined as those recurrent astrocytomas demonstrating somatic hypermutation acquired following chemotherapy with temozolomide characterized by a predominance of C>T/G>A transitions.^{122, 141, 149, 150} Each of the confirmed somatic variants from paired tumor-normal sequencing or filtered variants from tumor-only sequencing analysis was reviewed for predicted oncogenicity/pathogenicity using known cancer genomics data in the COSMIC (<https://cancer.sanger.ac.uk/cosmic>) and cBioPortal (<https://www.cbioportal.org/>) databases, location within encoded protein, and predicted functional effect based on mutation type (e.g. missense, nonsense, frameshift, splice site).

3.2.4 RAS-MAPK pathway genetic analysis

Somatic genetic alterations either known or predicted to cause activation of the RAS-MAPK signaling pathway were identified in the targeted DNA sequencing data from the IDH-mutant astrocytoma cohort. RAS-MAPK pathway genes evaluated for genetic alterations included *KRAS*, *NRAS*, *BRAF*, *NF1*, *SPRED1*, *PTPN11*, and *LZTR1*. This analysis included assessment for focal amplification events, activating missense variants, or structural variants/fusions targeting the oncogenic components *KRAS*, *NRAS*, *BRAF*, and *PTPN11*, as well as assessment for focal deletion events, truncating indel mutations or structural variants, or deleterious missense variants target-

ing the negative regulatory components *NF1*, *SPRED1*, and *LZTR1*.^{151–153} For survival analysis, patients were classified as RAS-MAPK wildtype or RAS-MAPK altered based on the presence or absence of known or likely oncogenic/pathogenic variants targeting these key genes: *KRAS*, *NRAS*, *BRAF*, *NF1*, *SPRED1*, *PTPN11*, and/or *LZTR1*.

3.2.5 Statistical analysis

All analyses were conducted using the statistical software R version 4.2.3.⁵² Patient demographics and tumor characteristics were summarized using descriptive statistics. Student's t-test, Fisher's exact test, and Chi-squared tests were employed to compare continuous and categorical variables between patient groups stratified by RAS-MAPK alterations. Overall survival from initial surgery was defined as the time from the initial diagnostic surgical procedure to death. Survival from surgically treated recurrence was defined as the time from repeat resection for pathologically confirmed viable recurrent IDH-mutant astrocytoma until death. Kaplan-Meier plots were used to estimate survival times stratified by clinical and molecular variables, with censoring performed for patients alive at last clinical follow-up. Median estimated survival with 95% confidence intervals (CI) and exact p-values were calculated by log-rank test. Univariate and multivariate Cox proportional hazards regression models were used to evaluate the associations between clinical and molecular factors with survival outcomes. Multivariate models were constructed using variables determined *a priori*.

3.3 Results

3.3.1 IDH-mutant astrocytoma patient cohort

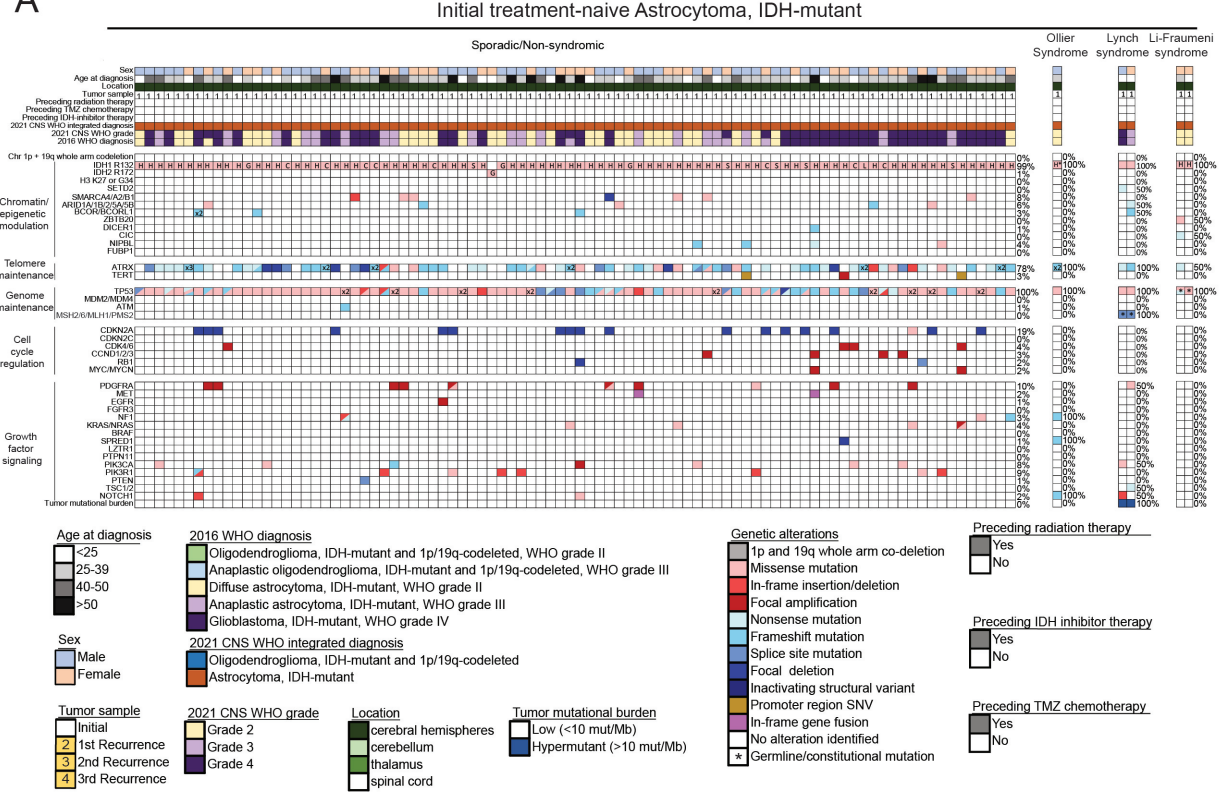
The study cohort consisted of 172 patients (61% male, 39% female) with pathologically confirmed IDH-mutant astrocytoma who underwent surgical resection of one or more longitudinal tumor specimens that were genomically profiled (Table 3.1). In total, 205 tumor specimens were included, consisting of 95 treatment-naïve tumors from an initial diagnostic surgical resection and 110 recurrent tumors from a subsequent surgical resection following either observation or adjuvant therapy. The median age at initial diagnosis for the 172 patients was 33.2 years (range 10.1-67.0 years). The tumors were uniformly supratentorial located in the cerebral hemispheres. Dependent on clinical factors including residual disease after resection and tumor grade, a subset of patients was observed by follow-up MR imaging without adjuvant therapy, while others received adjuvant external beam radiation and/or adjuvant cycles of chemotherapy with temozolomide. While a subset of patients was treated with an IDH mutant small molecule inhibitor (either vorasidenib, ivosidenib, or enasidenib) at time of disease progression (n=13), no patients in this cohort were treated with an IDH mutant small molecule inhibitor in the adjuvant setting following the initial diagnostic surgery, and all genomic profiling was performed prior to therapy with IDH mutant small molecule inhibitors. The median overall survival from initial diagnostic surgery was 11.8 years (95% CI: 9.9-15.5 years), and the median follow-up time was 5.5 years. Individual genetic alterations known to be associated with inferior survival in IDH-mutant astrocytomas including *CDKN2A* homozygous deletion and *PDGFRA* amplification were observed to correlate with worse survival in this patient cohort (data not shown). Among 81 genomically profiled recurrent IDH-mutant astrocytomas following preceding treatment with temozolomide, 14 tumors (17%) demonstrated somatic

hypermethylation composed almost entirely of C>T/G>A transitions corresponding with single base substitution (SBS) signature 11 in the COSMIC Human Cancer Mutational Signatures Database version 3.4 and harbored a high frequency (79%) of mismatch repair genes mutations involving *MSH2*, *MSH6*, *MLH1*, or *PMS2*.

3.3.2 Clinical subtypes of IDH-mutant astrocytomas

Among the 172 patients, 49 patients underwent simultaneous sequencing of a matched constitutional DNA sample extracted from peripheral blood or buccal swab, as dictated by either patient request or clinical indication based on personal or family history of malignancy. Five patients (3%) had confirmed constitutional variants (two in *TP53*, one in *IDH1*, two in *MSH2*), diagnostic of IDH-mutant astrocytoma arising in the setting of Li-Fraumeni, Ollier, and Lynch syndromes, respectively. See appendix 6.1 for detailed description of these five patients with syndromic IDH-mutant astrocytomas. The other 44 patients lacked deleterious constitutional variants in genes known to be associated with glioma predisposition including *TP53*, *CDKN2A*, *NF1*, *IDH1*, and the mismatch repair genes (*MSH2*, *MSH6*, *MLH1*, and *PMS2*), with all identified oncogenic mutations in the tumor specimens confirmed to be somatic (i.e. tumor acquired), and were thus designated as having sporadic non-syndromic IDH-mutant astrocytomas. The other 123 patients had likely sporadic tumors as well, but did not have constitutional testing performed for definitive confirmation. A Kaplan-Meier plot of survival for the patients with IDH-mutant astrocytomas stratified by clinical subtype as sporadic/non-syndromic versus arising in these different syndromes is shown in Figure 3.1(B) to provide visualization of outcome lengths (no statistical comparison performed given small cohort sizes).

A



B

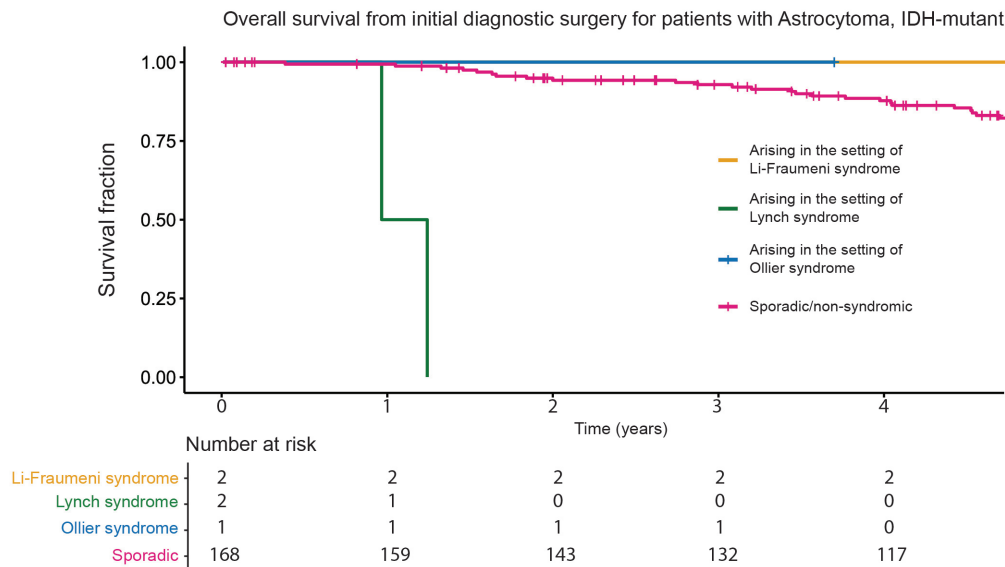


Figure 3.1 IDH-mutant astrocytomas can be classified into multiple clinical subtypes as either sporadic (non-syndromic) or arising in the setting of Li-Fraumeni syndrome, Lynch syndrome, or Ollier syndrome. (A) Oncoprint summary plot of the 95 patients with initial treatment-naïve IDH-mutant astrocytoma stratified by subtypes. (B) Kaplan-Meier plot of overall survival from initial diagnostic surgery for the total cohort of 172 patients with astrocytoma, IDH-mutant stratified by subtypes.

3.3.3 Novel RAS-MAPK pathway alterations in IDH-mutant astrocytomas associated with high-grade features

Through genomic profiling of 205 IDH-mutant astrocytomas from 172 patients, we identified genetic alterations of the RAS-MAPK signaling pathway in 27 tumors (13%) that has not been previously identified as a target of frequent genetic alterations in this tumor type (Table 3.1, Figures 3.1-3.4). These were all genetic alterations either known or likely to be oncogenic and activating of RAS-MAPK signaling and involved multiple genes in the pathway including *KRAS*, *NRAS*, *BRAF*, *NF1*, *SPRED1*, and *LZTR1*, often in a mutually exclusive manner with each tumor usually harboring an event in one of the genes. No IDH-mutant astrocytomas in this cohort had activating mutations within *PTPN11*, a RAS-MAPK pathway gene that is a frequent target of activating mutations in other glioma tumor types including IDH-wildtype glioblastoma and rosette-forming glioneuronal tumor (RGNT).^{143, 154} The identified variants within the RAS-MAPK pathway in this cohort of IDH-mutant astrocytomas were diverse and included known oncogenic missense mutations in *KRAS* and *NRAS* (n=8, including p.G12D, p.G12V, p.G12A, p.G13D, p.G13S, p.D33E, p.K117N, p.A146T), focal high-level amplification of *KRAS* (n=3), known oncogenic missense mutations in *BRAF* (n=2, p.G464E, p.D594G), in-frame *BRAF* gene fusion (n=1), truncating (nonsense, frameshift, splice site) mutations or known deleterious missense mutations in *NF1* and *LZTR1* (n=17 and 4, respectively), and focal homozygous/biallelic deletion or truncating frameshift mutation of *SPRED1* (n=2). The *BRAF* gene fusion linked the N-terminal exons 1-17 of *PTPRZ1* with the C-terminal exons 11-18 of *BRAF* that encode the serine/threonine kinase domain. *PTPRZ1* is a rare fusion partner of *BRAF* that has been previously encountered in a pilocytic astrocytoma.¹⁵⁵ All variants in RAS-MAPK genes were either confirmed as somatic by paired tumor-normal sequencing or by longitudinal profiling of multiple temporally-spaced tumor specimens with variants private to

individual tumor specimens, or they were present in tumor specimens analyzed as tumor-only at variant allele frequencies consistent with probable somatic origin.

Table 3.1. Study cohort of patients with sporadic IDH-mutant astrocytomas stratified by presence/absence of likely oncogenic alterations within RAS-MAPK pathway genes (*KRAS*, *NRAS*, *BRAF*, *NF1*, *SPRED1*, *PTPN11*, and *LZTR1*)

n (%)	Initial treatment-naïve sporadic IDH-mutant astrocytoma				Recurrent sporadic IDH-mutant astrocytoma			
	Total 90	RAS-MAPK pathway mutant 8 (9)	RAS-MAPK pathway wildtype 82 (91)	p-value	Total 109	RAS-MAPK pathway mutant 18 (17)	RAS-MAPK pathway wildtype 91 (83)	p-value
Sex, n (%)				0.48				0.83
Female	40 (44)	5 (63)	35 (43)	—	35 (32)	6 (33)	29 (32)	—
Male	50 (56)	3 (37)	47 (57)	—	74 (68)	12 (67)	62 (68)	—
Age at initial diagnosis, mean (SD)	35.7 (12.5)	39.7 (7.1)	35.3 (12.8)	0.15	33.8 (8.5)	32.9 (10.0)	34 (8.2)	0.65
Tumor location, n (%)				1				1
Cerebral hemispheres	90 (100)	8 (100)	82 (100)		109 (100)	18 (100)	91 (100)	
Brainstem/spinal cord	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	0 (0)	
Cerebellum	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	0 (0)	
Lobe location, n (%)				0.66				0.61
Frontal	54 (60)	3 (38)	51 (62)		51 (47)	6 (33)	45 (50)	
Temporal	12 (13)	2 (26)	10 (12)		26 (24)	5 (28)	21 (23)	
Parietal	10 (11)	1 (12)	9 (11)		18 (17)	2 (11)	14 (15)	
Occipital	1 (1)	0 (0)	1 (1)		2 (2)	0 (0)	1 (1)	
Frontotemporal	9 (10)	1 (12)	8 (10)		9 (8)	2 (11)	6 (7)	
Frontoparietal	3 (3)	1 (12)	2 (2)		1 (1)	0 (0)	1 (1)	
Parietooccipital	1 (1)	0 (0)	1 (1)		0 (0)	0 (0)	0 (0)	
Temporoparietal	0 (0)	0 (0)	0 (0)		1 (1)	3 (17)	2 (2)	
Temporooccipital	0 (0)	0 (0)	0 (0)		1 (1)	0 (0)	1 (1)	
Histologic grade per 2016 WHO classification, n (%)				0.39				0.33
Grade II	30 (33)	1 (13)	29 (35)		17 (16)	2 (11)	15 (17)	
Grade III	22 (24)	3 (37)	19 (23)		22 (20)	2 (11)	20 (22)	
Grade IV	38 (42)	4 (50)	34 (42)		70 (64)	14 (78)	56 (61)	
2021 CNS WHO classification grade, n (%)				0.42				0.31
Grade 2	30 (33)	1 (12)	29 (35)		13 (12)	1 (5)	12 (13)	
Grade 3	19 (21)	2 (25)	17 (21)		20 (18)	2 (11)	18 (20)	
Grade 4	41 (46)	5 (63)	36 (44)		76 (70)	15 (84)	61 (67)	
Extent of resection at initial surgery, n (%)				0.69				0.69
Biopsy only	6 (7)	0 (0)	6 (7)		7 (6)	2 (11)	5 (5)	
Subtotal	57 (63)	5 (63)	52 (63)		21 (19)	3 (17)	18 (20)	
Gross total	27 (30)	3 (37)	24 (30)		81 (74)	13 (72)	68 (75)	
Adjuvant radiation after initial surgery, n (%)				0.41				0.61
Yes	50 (64)	6 (86)	44 (62)		63 (60)	10 (56)	53 (61)	
No	28 (36)	1 (14)	27 (38)		42 (40)	8 (44)	34 (39)	
Unknown	12	1	11		4	0	4	
Adjuvant temozolomide after initial surgery, n (%)				0.25				0.61
Yes	49 (63)	6 (86)	43 (60)		63 (60)	10 (56)	53 (61)	
No	29 (37)	1 (14)	28 (40)		42 (40)	8 (44)	34 (39)	
Unknown	12	1	11		4	0	4	
Overall survival from initial surgery (years), median (95% CI)	8.0 (7.5-NA)	8.0 (NA-NA)	12.1 (7.5-NA)	0.59	N/A	N/A	N/A	
Overall survival from surgically treated recurrence (years), median (95% CI)	N/A	N/A	N/A		3.2 (2.8-4.9)	1.2 (0.8-NA)	3.5 (2.6-5.1)	0.01

We performed immunohistochemistry for phosphorylated Thr202 and Tyr204 isoforms of ERK1/2 on IDH-mutant astrocytomas with and without RAS-MAPK pathway mutations to further investigate the status of the signaling pathway downstream of these genetic events (Figure 3.4). We observed minimal to absent phospho-ERK1/2 labeling in normal cortex and IDH-mutant astrocytomas with no identifiable RAS-MAPK pathway genetic alterations (RAS-MAPK wildtype). In

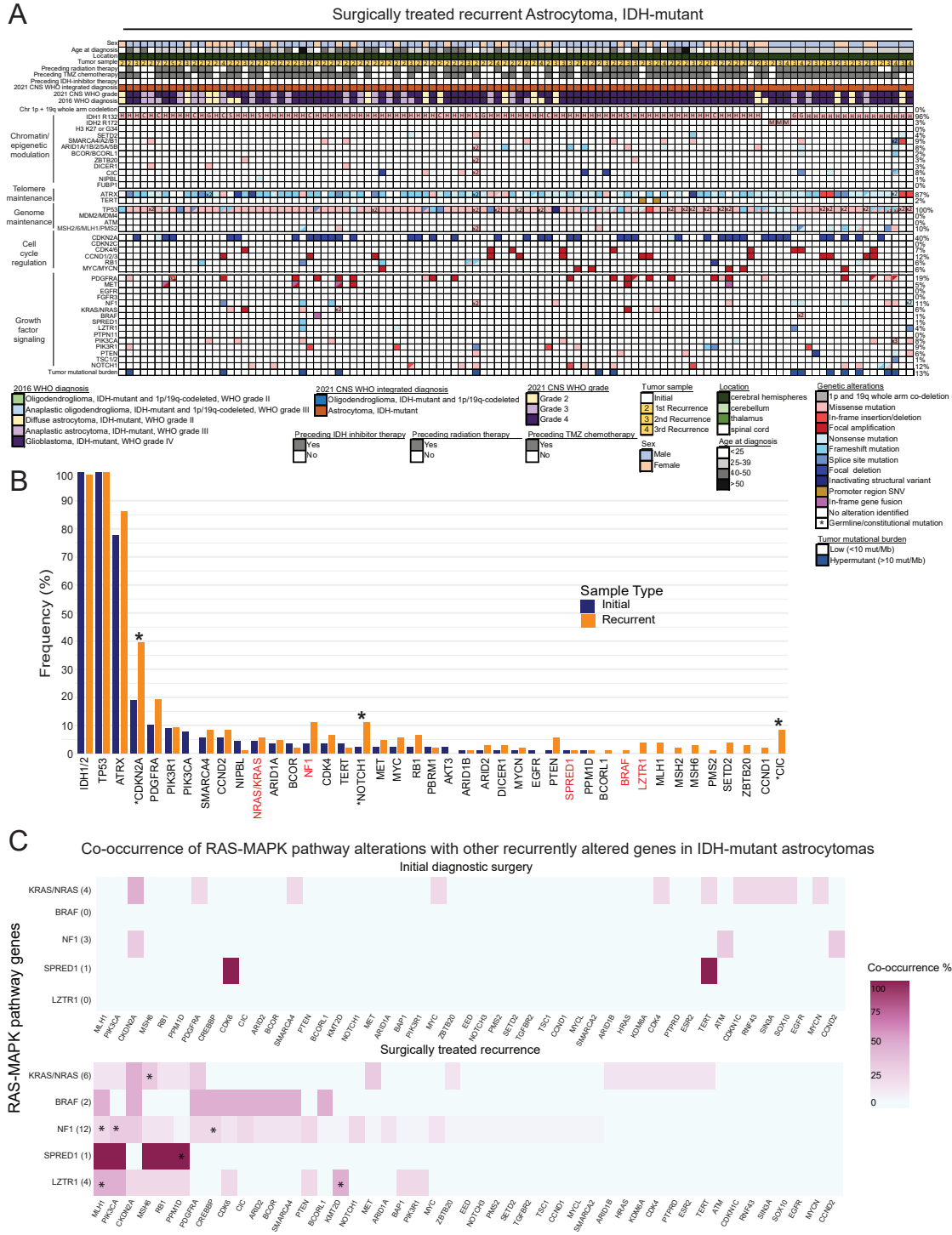


Figure 3.2 Recurrent IDH-mutant astrocytomas are enriched with *CDKN2A* homozygous deletion and diverse RAS-MAPK pathway oncogenic alterations. (A) Oncoprint summary plot for the 110 surgically treated recurrent IDH-mutant astrocytomas which were all sporadic/non-syndromic. (B) Bar plot of oncogenic alteration frequency for the most commonly altered genes in sporadic IDH-mutant astrocytomas segregated by 90 initial treatment-naïve tumors vs. 110 surgically treated recurrent tumors. Asterisks denote those individual genes with significantly ($p < 0.05$) increased alteration frequency at recurrence. (C) Co-occurrence heatmap of RAS-MAPK pathway alterations with other recurrently altered genes in sporadic IDH-mutant astrocytomas segregated by initial treatment-naïve tumors vs. surgically treated recurrent tumors. Asterisks denote significantly ($p < 0.01$) co-occurring genetic alterations by Fisher's exact test.

contrast, we observed robust phospho-ERK1/2 positivity in IDH-mutant astrocytomas containing the diverse oncogenic alterations within the RAS-MAPK component genes. This finding provides protein-level evidence that these RAS-MAPK genetic alterations were causing activation of this mitogenic signaling pathway in a subset of IDH-mutant astrocytomas.

Among the 26 sporadic non-syndromic IDH-mutant astrocytomas with likely oncogenic variants affecting the RAS-MAPK signaling pathway, 8 were in the cohort of 90 initial treatment-naïve tumors (9% frequency, Figure 3.1A) and 18 were in the cohort of 109 recurrent tumors (17% frequency, Figure 3.2A-B). The oncogenic RAS-MAPK pathway mutations frequently co-occurred with *CDKN2A* homozygous deletion and *PIK3CA* mutation (Figure 3.2A and 3.2C). Longitudinal genomic profiling of multiple tumor samples from individual patients revealed that these RAS-MAPK pathway mutations occurred during tumor progression after the initiating truncal *IDH1*, *TP53*, and *ATRX* mutational events (Figure 3.3). For example in patient #146, a private *NF1* truncating nonsense mutation was newly acquired in the second recurrence tumor specimen that was not present in the prior first recurrence (Figure 3.3B). In patient #64, both the second and third recurrence tumor specimens harbored *NF1* truncating nonsense mutations that were two different early-stop mutations affecting divergent codons in the *NF1* gene, thereby indicating these mutations were private and newly acquired in each of the tumor specimens. These *NF1* mutations were accompanied by additional private *LZTR1* splice site mutation in the second recurrence and two private *BRAF* missense mutations in the third recurrence (Figure 3.3B).

There were no differences in sex, age at diagnosis, or tumor location among the patients whose tumors did or did not have RAS-MAPK pathway alterations (Table 3.1). Nearly all IDH-mutant astrocytomas with RAS-MAPK pathway alterations had high-grade histologic features (Table 3.1). Among the 8 initial treatment-naïve tumors, 4 had either necrosis or microvascular proliferation

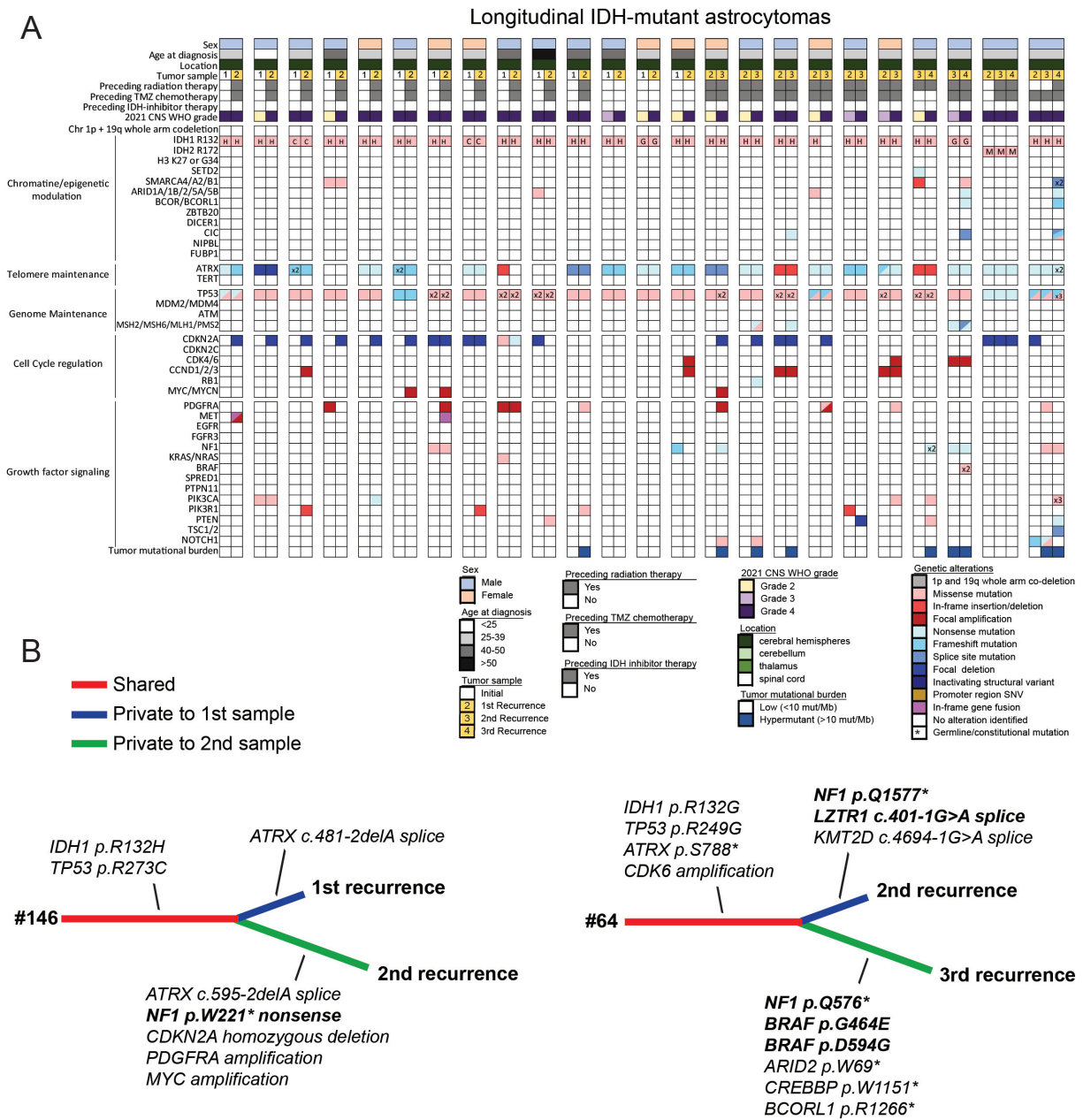


Figure 3.3 Longitudinal genomic profiling of IDH-mutant astrocytomas reveals acquisition of CDKN2A homozygous deletion and diverse RAS-MAPK pathway oncogenic alterations in recurrent high-grade tumors. (A) Oncoprint summary plot of the 50 sporadic IDH-mutant astrocytomas from 24 patients that were longitudinally profiled. (B) Genetic evolution dendrograms for patients with longitudinally profiled IDH-mutant astrocytoma demonstrating new acquisition of RAS-MAPK alterations at recurrence.

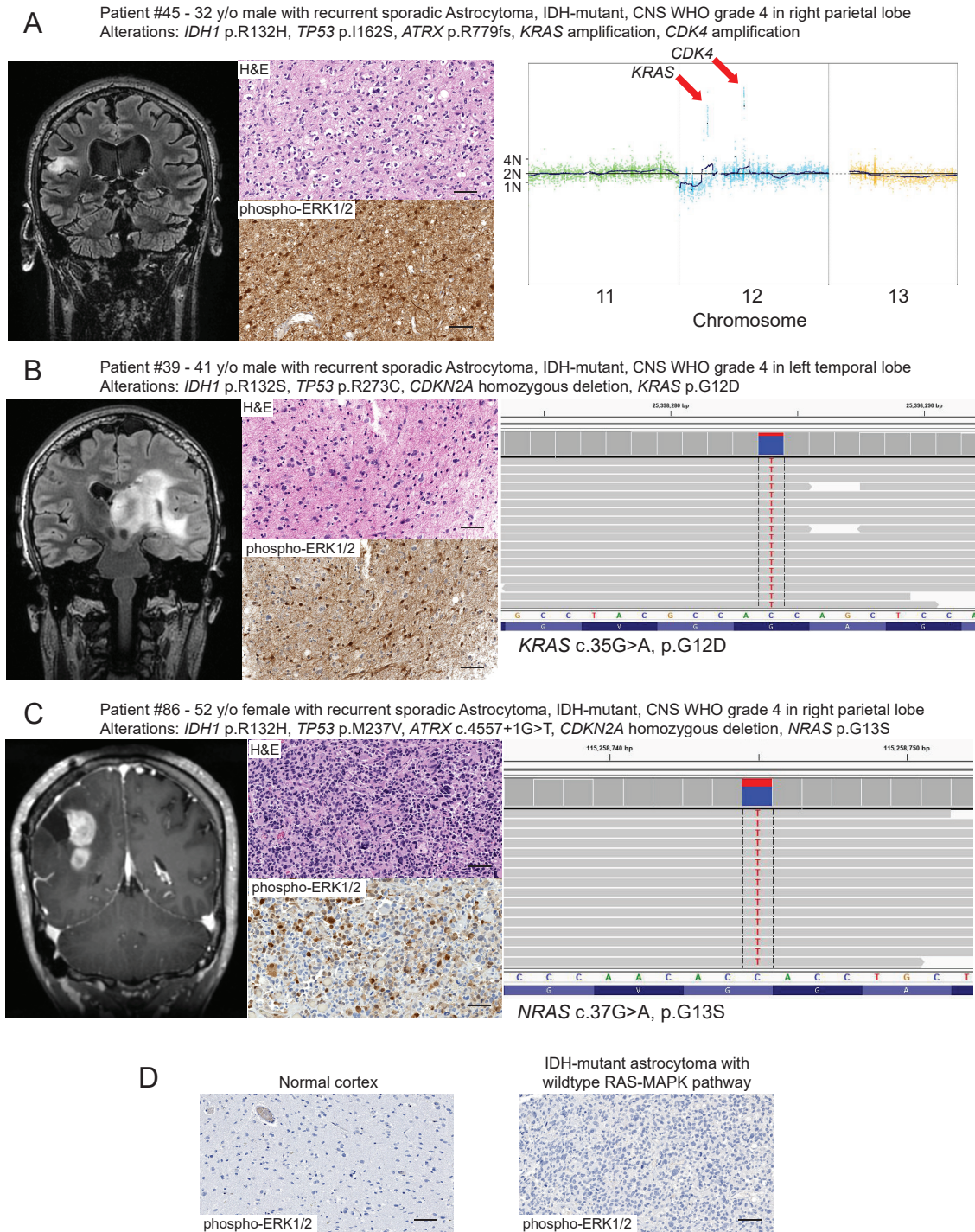


Figure 3.4 Diverse RAS-MAPK pathway oncogenic alterations in recurrent high-grade sporadic IDH-mutant astrocytomas. Shown are three representative patients, including a coronal T1 post-contrast MR image, hematoxylin and eosin-stained histology image, phospho-ERK1/2 Thr202/Tyr204 immunostaining, and a snapshot of the oncogenic RAS-MAPK alterations. (A) Focal high-level amplification of the *KRAS* oncogene on chromosome 12p12.1. (B) *KRAS* p.G12D known activating hotspot missense mutation. (C) *NRAS* p.G13S known activating hotspot missense mutation. (D) Phospho-ERK1/2 Thr202/Tyr204 immunostaining of normal cortex and an IDH-mutant astrocytoma with wildtype RAS-MAPK pathway.

(WHO grade IV per 2016 WHO Classification criteria) and 3 had anaplasia and increased mitotic activity only (WHO grade III per 2016 WHO Classification criteria). Only 1 tumor lacked appreciable high-grade histologic features. Among the 18 recurrent tumors, 14 had either necrosis or microvascular proliferation (WHO grade IV per 2016 WHO Classification criteria) and 4 had anaplasia and increased mitotic activity only (WHO grade III per 2016 WHO Classification criteria), with only 2 tumors lacking appreciable high-grade histologic features. When accounting for the molecularly integrated 2021 WHO Classification criteria that includes *CDKN2A* homozygous deletion into the grading scheme, only 1 initial treatment-naïve tumor remained at CNS WHO grade 2 and only 1 recurrent tumor remained at CNS WHO grade 2, with the other 24 tumors all assigned as high-grade (20 as CNS WHO grade 4 and 4 as CNS WHO grade 3). Thus, there was a substantial enrichment of RAS-MAPK oncogenic alterations in high-grade IDH-mutant astrocytomas in this cohort ($p < 0.05$ by Chi-squared test), with the majority being CNS WHO grade 4 based on co-occurring *CDKN2A* homozygous deletion and/or necrosis and microvascular proliferation histologically.

3.3.4 Recurrent IDH-mutant astrocytomas with oncogenic RAS-MAPK pathway alterations are associated with inferior survival

Among the 90 patients with initial treatment-naïve IDH-mutant astrocytomas that were studied, the median overall survival from initial surgery was 8.0 years for those with RAS-MAPK pathway mutation versus 12.1 years for those without RAS-MAPK pathway mutation. However, likely due to the relative rarity of oncogenic RAS-MAPK pathway mutations in initial resection specimens of IDH-mutant astrocytomas (only 8 patients in this cohort), this survival difference was not statistically significant ($p = 0.59$; Table 3.1). Among the 109 recurrent IDH-mutant astrocytomas that were studied, the median survival from surgically-treated recurrence was 1.2 years for those with RAS-MAPK pathway mutation versus 3.5 years for those without RAS-MAPK pathway mutation ($p =$

0.01; Table 3.1, Figure 3.5A). Among the 76 recurrent IDH-mutant astrocytomas designated as CNS WHO grade 4 based on the 2021 WHO Classification, the median survival from surgically-treated recurrence was 1.0 years for those with RAS-MAPK pathway mutation versus 3.1 years for those without RAS-MAPK pathway mutation ($p < 0.01$; Figure 3.6A). Among the 70 recurrent IDH-mutant astrocytomas designated as WHO grade IV based on histologic features only using the 2016 WHO Classification criteria, the median survival from surgically-treated recurrence was 1.1 years for those with RAS-MAPK pathway mutation versus 3.1 years for those without RAS-MAPK pathway mutation ($p < 0.01$; Figure 3.6B).

Among the 43 recurrent IDH-mutant astrocytomas harboring *CDKN2A* homozygous deletion, the presence of oncogenic RAS-MAPK pathway alteration was associated with inferior survival with median survival from surgically-treated recurrence of 0.9 years versus 2.8 years ($p < 0.001$; Figure 3.5B). Among the 18 patients with recurrent IDH-mutant astrocytomas harboring oncogenic RAS-MAPK pathway alterations, the presence of *CDKN2A* homozygous deletion was associated with a trend towards inferior survival with median survival from surgically-treated recurrence of 0.9 years versus 1.9 years ($p = 0.08$; Figure 3.5C). Multivariate analysis demonstrated that oncogenic RAS-MAPK pathway alterations were independently associated with inferior survival for patients with surgically-treated recurrent IDH-mutant astrocytoma (hazard ratio = 2.83, $p < 0.01$; Figure 3.5D).

3.4 Discussion

Here we confirm recent reports demonstrating that while the vast majority of IDH-mutant astrocytomas are sporadic/spontaneous tumors, a small subset of patients (3% in this cohort) have tumors arising in the setting of specific tumor predisposition syndromes (Li-Fraumeni syndrome,

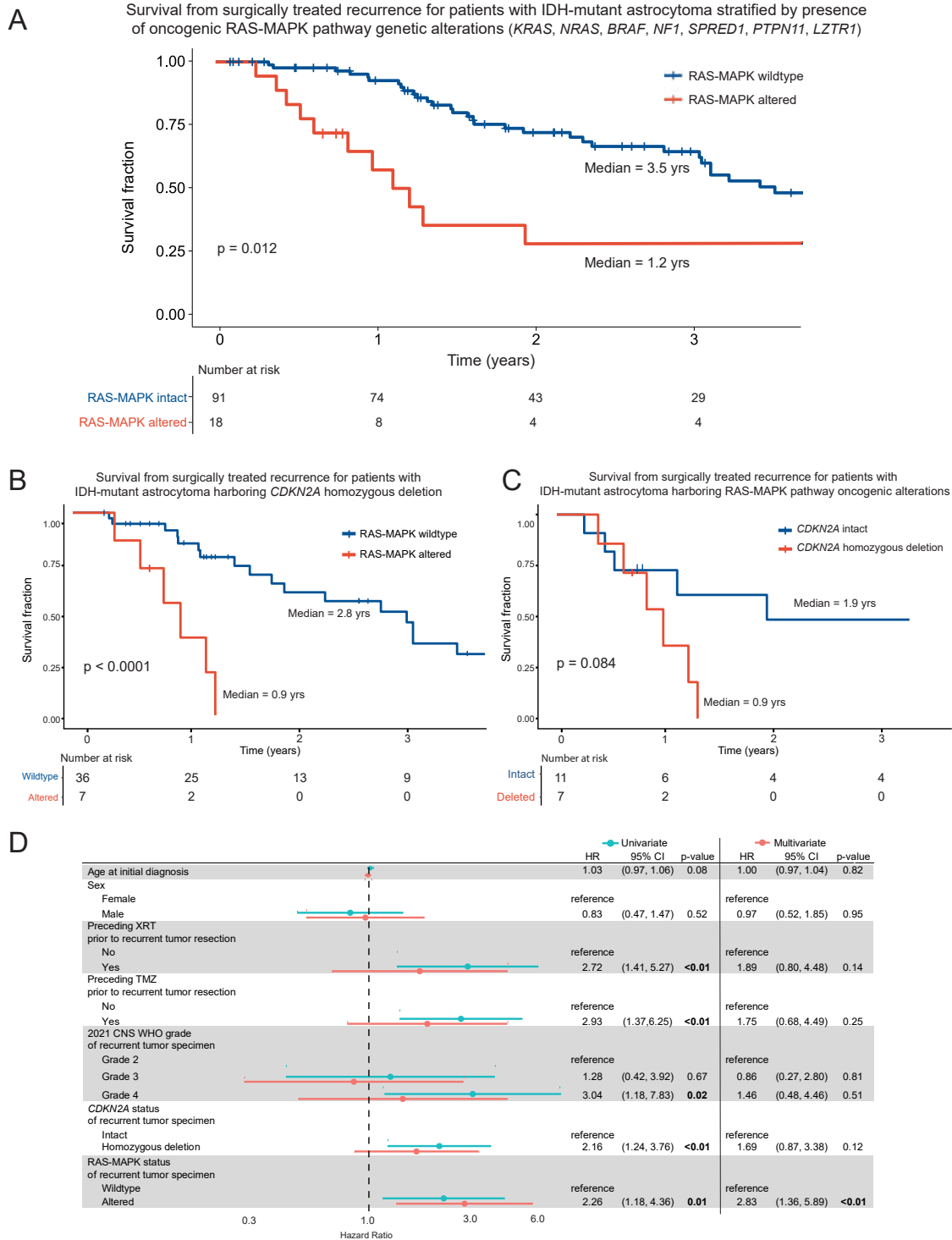


Figure 3.5 Oncogenic RAS-MAPK pathway genetic alterations are associated with inferior survival in patients with recurrent IDH-mutant astrocytoma. (A) Kaplan-Meier plot showing survival from surgically treated recurrence for 109 IDH-mutant astrocytomas stratified by presence or absence of oncogenic RAS-MAPK pathway alterations involving *KRAS*, *NRAS*, *BRAF*, *NF1*, *SPRED1*, *PTPN11*, or *LZTR1*. (B) Kaplan-Meier plot showing survival from surgically treated recurrence for 43 IDH-mutant astrocytomas harboring *CDKN2A* homozygous deletion by presence or absence of oncogenic RAS-MAPK pathway alterations. (C) Kaplan-Meier plot showing survival from surgically treated recurrence for 18 IDH-mutant astrocytomas harboring oncogenic RAS-MAPK pathway alteration stratified by presence or absence of *CDKN2A* homozygous deletion. (D) Univariate and multivariate Cox proportional hazard regression analysis of overall survival from surgically treated recurrence for patients with recurrent IDH-mutant astrocytoma (n=109). 54

Ollier syndrome, and Lynch syndrome) due to constitutional pathogenic variants in the *TP53*, *IDH1*, and mismatch repair genes (*MSH2*, *MSH6*, *MLH1*, and *PMS2*).^{111, 112} These constitutional variants can either be inherited in the germline or arise de novo during embryogenesis associated with constitutional mosaicism. Paired tumor-normal sequencing enabling accurate discrimination of the somatic versus constitutional/germline origin of identified DNA sequence variants is useful for detecting these patients with IDH-mutant astrocytomas arising due to underlying tumor predisposition syndromes. This identification of patients with underlying tumor predisposition syndromes can enable genetic counseling about future cancer risk to affected family members including siblings and offspring, as well as enable potentially life-saving cancer surveillance such as colonoscopy, screening MRI, and emerging cell-free DNA screening.^{156, 157}

Furthermore, IDH-mutant astrocytomas arising in the setting of these different tumor predisposition syndromes may be biologically different than their sporadic counterparts and warrant different prognostic counseling and treatment regimens. For example, IDH-mutant astrocytomas arising in patients with constitutional/germline mismatch repair gene mutation (i.e. Lynch syndrome) have been demonstrated to compose a unique DNA methylation cluster distinct from sporadic IDH-mutant astrocytomas with intact mismatch repair activity and are associated with poor prognosis compared to patients with sporadic IDH-mutant astrocytomas.¹¹² These unique tumors have been termed “primary mismatch repair deficient IDH-mutant astrocytoma” and should likely be considered a distinct subtype of IDH-mutant astrocytoma moving forward, for which the clinical utility of temozolomide versus other chemotherapeutic agents including immune checkpoint blockade needs to be tested. Both of the two patients in this cohort with IDH-mutant astrocytomas arising in the setting of Lynch syndrome due to heterozygous constitutional *MSH2* inactivating mutations had poor outcomes (survival less than 2 years from initial diagnosis), similar to that reported in this prior

study.¹¹² In contrast, both of the patients in this cohort with IDH-mutant astrocytomas arising in setting of Li-Fraumeni syndrome due to heterozygous constitutional TP53 mutations had favorable survivable and remained alive at last clinical follow-up at 5 and 7 years after initial glioma diagnosis. This is similar to a prior study demonstrating favorable outcome of IDH-mutant astrocytomas arising in the setting of Li-Fraumeni syndrome compared to other patients (usually pediatric) with IDH-wildtype gliomas arising in the setting of Li-Fraumeni syndrome.¹¹¹ We also document the genetic composition of an IDH-mutant astrocytoma arising in the setting of Ollier disease due to constitutional mosaicism for IDH1 p.R132H mutation, revealing additional somatic *TP53*, *ATRX*, *NF1*, *SPRED1*, and *NOTCH1* oncogenic/pathogenic mutations. It remains uncertain why a subset of IDH-mutant astrocytomas arise in the setting of these specific tumor predisposition syndromes, but have not been observed to occur in the setting of other syndromes where primary brain tumors frequently occur such as neurofibromatosis type 1 (NF1) and familial melanoma-astrocytoma syndrome (now termed *CDKN2A*-related tumor predisposition syndrome).

Genetic events underlying malignant progression and high-grade transformation for IDH-mutant astrocytomas have been incompletely defined. Prior longitudinal genomic profiling studies have documented that *IDH1*, *TP53*, and *ATRX* are the earliest mutational events in IDH-mutant astrocytomas that give rise to a low-grade astrocytoma (CNS WHO grade 2).^{suzukiMutationalLandscapeClonal2015, 122} The accumulation of additional genetic perturbations affecting cell cycle regulation (e.g. *CDKN2A* homozygous deletion, *CDK4* amplification) and growth factor signaling (e.g. *PDGFRA* amplification, *PTPRZ1::MET* fusion) have been identified as genetic perturbations that are found in IDH-mutant astrocytomas at time of recurrence and high-grade transformation and are associated with inferior survival compared to IDH-mutant astrocytomas lacking these additional genetic alterations.^{123, 124, 126, 127, 131, 132, 134}

Here we identified novel genetic alterations driving activation of the RAS-MAPK mitogenic signaling pathway in a substantial subset of IDH-mutant astrocytomas, which were enriched in those tumors with high-grade histologic features and also in recurrent tumors compared to initial treatment-naïve tumors (17% vs. 9%). Genetic evolution analysis of multiple longitudinal tumor samples from individual patients revealed that these RAS-MAPK pathway mutations occurred during tumor progression subsequent to the earlier initiating *IDH1*, *TP53*, and *ATRX* mutational events (Figure 3.3).

Immunohistochemistry for phospho-ERK1/2 demonstrated evidence supporting RAS-MAPK signaling pathway activation in IDH-mutant astrocytomas with the diverse oncogenic alterations in RAS-MAPK pathway genes (Figure 3.4). Together, these findings indicate that the RAS-MAPK mutational events are likely to be an underlying contributor of high-grade transformation and treatment resistance for IDH-mutant astrocytomas, probably in cooperation with other such events that have been previously identified such as *CDKN2A* deletion and *PDGFRA* amplification. While each individual RAS-MAPK gene was only altered in a small percentage of tumor specimens, the cumulative frequency of oncogenic RAS-MAPK pathway alterations in this cohort of recurrent IDH-mutant astrocytomas was nearly 20%, potentially explaining why prior longitudinal genomics studies of IDH-mutant gliomas have not focused on these RAS-MAPK events. Notably, RAS-MAPK pathway mutations are known hallmarks of other CNS tumor types (e.g. BRAF mutation/fusion in ganglioglioma and pilocytic astrocytoma, *KRAS* mutation in tectal glioma, *NF1* mutation/deletion in gliomas arising in the setting of neurofibromatosis type 1 and a subset of sporadic IDH-wildtype glioblastomas).^{6, 146, 158–160} However, this is the first study to our knowledge to identify and focus on the significance of RAS-MAPK pathway mutations in IDH-mutant astrocytomas.

Our analysis demonstrated that patients whose recurrent IDH-mutant astrocytomas harbored

oncogenic RAS-MAPK pathway alterations had significantly worse survival compared to those with RAS-MAPK wildtype tumors. The combination of oncogenic RAS-MAPK pathway mutation and *CDKN2A* homozygous deletion was associated with markedly poor outcomes beyond *CDKN2A* deletion in isolation, with multivariate analysis demonstrating that RAS-MAPK pathway mutation was an independent prognostic factor after adjusting for other relevant clinical and molecular variables. These findings suggest that oncogenic RAS-MAPK pathway alterations are a significant contributor to the malignant transformation and poor prognosis of patients with recurrent IDH-mutant astrocytomas, underscoring their potential as a valuable prognostic biomarker and potential therapeutic target in this patient population.

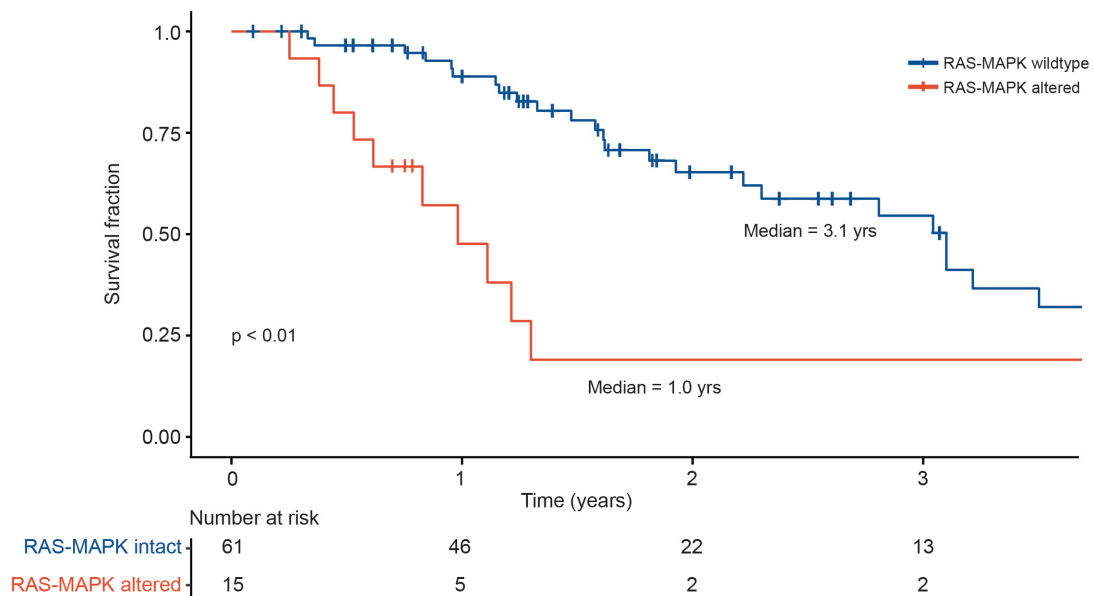
In addition to the novel RAS-MAPK pathway genetic alterations, our longitudinal genomic analysis also identified an increased frequency of likely oncogenic alterations targeting the *NOTCH1* and *CIC* tumor suppressor genes in recurrent versus initial treatment-naïve IDH-mutant astrocytomas (Figure 3.2B). Interestingly, while *NOTCH1* and *CIC* are more well recognized as recurrently mutated genes in oligodendrogliomas, our finding of *NOTCH1* and *CIC* mutations in a small subset of recurrent IDH-mutant astrocytomas indicates that these two genes may also play a role in the progression and treatment response of astrocytomas.

Notably, this study was conducted at a single institution and validation of these findings in additional independent patient cohorts will be essential to confirm the ultimate frequency and prognostic significance of RAS-MAPK alterations in IDH-mutant astrocytomas. Further research using cell or xenograft models of IDH-mutant gliomas expressing RAS-MAPK pathway mutations will help elucidate the specific mechanisms by which these alterations drive tumor aggressiveness and therapeutic vulnerability. At present, no predictive biomarkers have been identified for reliably determining which patients will acquire RAS-MAPK pathway mutation during their disease course,

but this may potentially be elucidated through further multi-omic profiling studies examining for DNA methylation or gene expression differences between initial IDH-mutant astrocytoma tumor specimens that do or do not subsequently acquire these alterations at recurrence.

In summary, we have identified a new critical role of RAS-MAPK pathway alterations in the progression of IDH-mutant astrocytomas. Our findings reveal that these RAS-MAPK pathway mutations are typically absent in the initial resection of a low-grade IDH-mutant astrocytoma and are acquired later during gliomagenesis in predominantly high-grade and recurrent tumors. They frequently co-occur with other key oncogenic events known to correlate with inferior survival in IDH-mutant astrocytomas including *CDKN2A* homozygous deletion and *PIK3CA* mutation. Nonetheless, we found in multivariate analysis that these oncogenic RAS-MAPK pathway mutations are independently correlated with inferior survival in patients with recurrent IDH-mutant astrocytoma and should therefore be considered as a potential prognostic determinant for future molecularly-integrated grading schemes for IDH-mutant astrocytomas. Future studies are needed to determine how to best treat such IDH-mutant astrocytomas with RAS-MAPK pathway mutations, considering that activation of this mitogenic signaling pathway may be driving resistance to conventional radiation and temozolomide chemotherapy and also be associated with sensitivity to particular targeted therapy agents such as *RAS* or *MEK* inhibitors. Furthermore, the molecular determinants of sensitivity and resistance to the newly FDA-approved IDH mutant small molecule inhibitors remains to be determined, and whether these RAS-MAPK pathway mutations determine which patients will respond successfully or who might need combinatorial multi-agent treatment will be important to investigate.

A Survival from surgically treated recurrence for patients with CNS WHO grade 4 (2021 WHO Classification) IDH-mutant astrocytoma stratified by presence of oncogenic RAS-MAPK pathway genetic alterations (*KRAS*, *NRAS*, *BRAF*, *NF1*, *SPRED1*, *PTPN11*, *LZTR1*)



B Survival from surgically treated recurrence for patients with WHO grade IV (2016 WHO Classification) IDH-mutant astrocytoma stratified by presence of oncogenic RAS-MAPK pathway genetic alterations (*KRAS*, *NRAS*, *BRAF*, *NF1*, *SPRED1*, *PTPN11*, *LZTR1*)

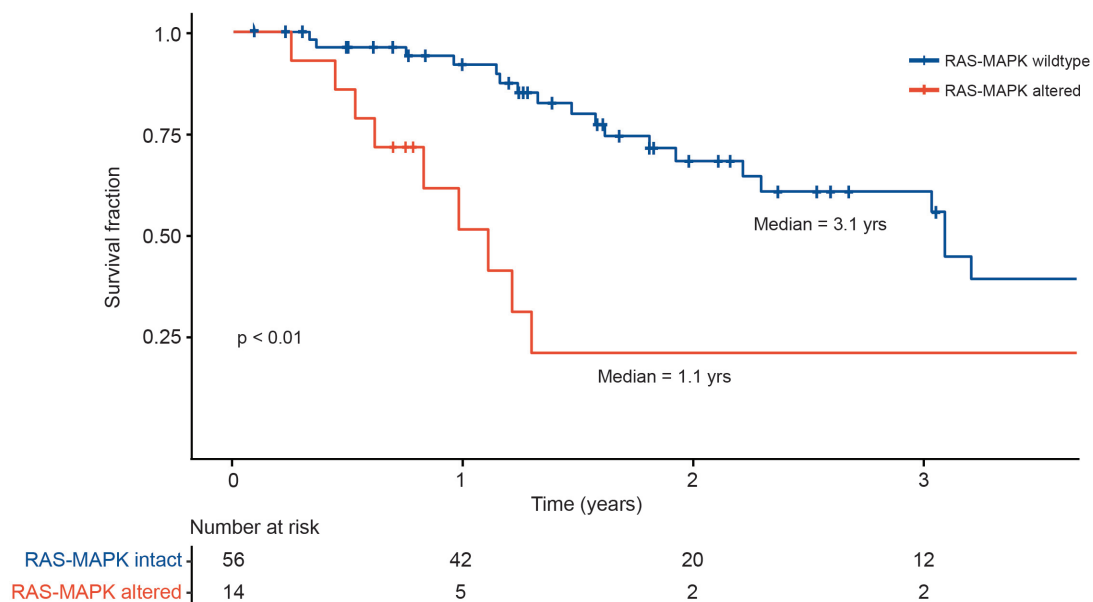


Figure 3.6 Kaplan-Meier plots showing survival from surgically treated recurrence for IDH-mutant astrocytomas of CNS WHO grade 4 by 2021 WHO Classification (A) or WHO grade IV by 2016 WHO Classification (B) stratified by presence or absence of oncogenic RAS-MAPK pathway alterations involving *KRAS*, *NRAS*, *BRAF*, *NF1*, *SPRED1*, *PTPN11*, or *LZTR1*.

Peripheral immune cell dynamics of patients with IDH-mutant gliomas after surgical resection

4.1 Introduction

Gliomas account for approximately 30% of all central nervous system tumors and 80% of malignant brain tumors, with isocitrate dehydrogenase (IDH)-mutant gliomas being the most common malignant primary brain tumors in adults under 50 years of age.² Under the World Health Organization (WHO) Classification of Central Nervous System Tumors, IDH-mutant gliomas are comprised of astrocytoma and oligodendroglioma.¹

Together, IDH-mutant gliomas are associated with a 10-year overall survival rate of 41.8%, representing a significant cause of morbidity and mortality in this population. Surgical intervention remains the gold standard for treatment, with maximal surgical resection playing a pivotal role in determining overall survival outcomes.^{1, 161, 162}

In gliomas, the immune system plays a dual role, acting both as a barrier to tumor progression and as a major contributor to the immunosuppressive tumor microenvironment.^{163, 164} Gliomas evade immune surveillance through mechanisms such as T-cell exhaustion, regulatory T-cell recruitment, and secretion of immunosuppressive cytokines.^{165–168} These mechanisms not only drive tumor progression but also limit the effectiveness of immune-based therapies.^{169, 170}

Evidence suggests that variations in peripheral immune cell populations may serve as predic-

tors of overall survival in glioma patients.^{171–173} For instance, Molinaro et al. demonstrated that patients younger than 58 years with low CD4 T-cell proportions exhibit poor survival outcomes.¹⁷⁴ While these findings have advanced our understanding of peripheral immune cell dynamics, they are typically derived from single post-diagnosis blood samples, providing limited insight into the longitudinal changes in immune responses. Closing this knowledge gap is essential for understanding how peripheral immune changes over time influence clinical outcomes such as survival and treatment efficacy.

Studying immune responses in glioma patients is inherently complex due to the substantial heterogeneity in immune cell composition and function, shaped by tumor genetics, location, and treatment history.¹⁷⁵ Despite surgery being the cornerstone of treatment and diagnosis, the effects of surgical resection on peripheral immune cell populations in IDH-mutant gliomas remain particularly understudied. As evidence shows, surgery has the potential to modulate both the tumor microenvironment and systemic immune responses, yet the specific changes it induces and their clinical relevance remain unclear.^{176, 177} Clarifying these effects is crucial, as surgery-induced peripheral immune modulation could have profound implications for disease progression and the success of subsequent therapies.

Understanding these changes is especially important in light of the growing emphasis on immunotherapy for glioma patients.^{169–171} Insights into longitudinal patterns of peripheral immune cell changes could guide the timing and design of immunotherapeutic interventions, ensuring they are deployed in contexts that maximize their efficacy.

Herein, we address these critical knowledge gaps by focusing on peripheral blood immune cell dynamics utilizing data from the ongoing University of California, San Francisco (UCSF) Immune

Profiles Study (IPS) to characterize the change in immune cell counts of subjects with IDH-mutant glioma across the time spanning surgical resection, prior to chemo radiation, while accounting for the influence of other key covariates – most notably including dexamethasone administration – providing the first longitudinal perspective on immune cell dynamics.

4.2 Methods

4.2.1 Patient cohort and blood samples

Pre-surgery and post-surgery blood samples were collected from subjects enrolled in the IPS. One hundred and sixty-eight subjects with newly diagnosed or recurrent IDH-mutant cases with tumor types classified as IDH-mutant astrocytomas or IDH-mutant oligodendrogliomas were included. All subjects underwent subtotal or gross total surgical resection between 2018 and 2022 at the University of California, San Francisco. Blood samples included in this study were collected at two timepoints: (1) pre-surgery and (2) post-surgery but prior to chemotherapy/radiation treatment for a total of 263 samples. Clinical data, including age at the time of surgery, sex, tumor diagnosis, and dexamethasone exposure at each blood draw were abstracted from the electronic medical record (EHR). Dexamethasone exposure was defined as administration within 5 days prior to the blood draw.

The IPS was approved by the University of California, San Francisco Institutional Review Board, and informed consent was obtained from all study participants.

4.2.2 DNA methylation array

DNA was extracted from anticoagulated whole blood within 24 hours of blood draw and archived at -80°C as previously described.¹⁷⁸ All samples and array experiments were performed blinded to clinicopathologic variables. Cell count and cell deconvolution have been described previously.¹⁷⁹ Briefly, cell deconvolution corrects for inter-individual variability, cell lineage relationships (e.g., shifts in the myeloid-to-lymphoid ratio), and the impact of prominent methylation differences across general cell subpopulations. Cell counts were derived using the Illumina HumanMethylation EPIC (EPIC) array, which interrogates over 860,000 CpG sites, including regions critical for hematopoietic development and differentiation, such as enhancers and DNase hypersensitive sites (DHS).¹⁸⁰ A custom algorithm was applied to deconvolve blood cell proportions using the EPIC array.¹⁷⁹ This method leverages DNA methylation data from antibody bead-sorted immune cell types, including neutrophils, B cells, monocytes, NK cells, CD4⁺ T cells, and CD8⁺ T cells. An iterative algorithm for Identifying Optimal Libraries (IDOL), based on leukocyte-specific differentially methylated regions (L-DMRs), was employed to enhance the accuracy and efficiency of cell composition estimates obtained through cell mixture deconvolution.¹⁸¹

DNA methylation-based immune profiles are highly accurate and reproducible. The proportions of each cell were estimated for each sample using the function “estimate CellCounts2” in the FlowSorted Blood EPIC Bioconductor package.¹⁸⁰ Absolute cell counts for cell types were then obtained by multiplying cell type proportions by each sample’s total white cell count. Absolute cell counts of 15 immune cell types were estimated: Basophils, total B-cells, memory B-cells, naive B-cells, total CD4 cells, memory CD4 cells, naive CD4 cells, total CD8 cells, memory CD8 cells, naive CD8 cells, eosinophils, monocytes, natural killer (NK) cells, T regulatory (T regs) cells, and neutrophils. Figure 4.1 depicts the blood sample processing.

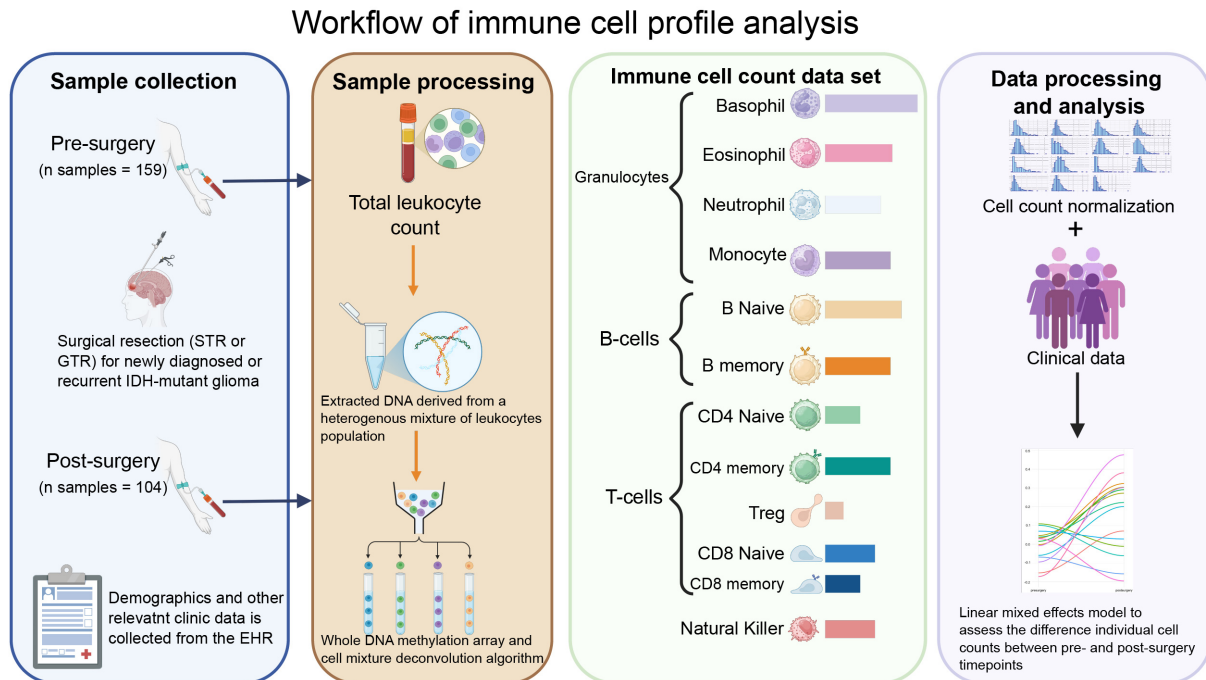


Figure 4.1 *Workflow of blood sample processing.* Each panel depicts a different stage of sample processing. From left to right: Blood sample and data collection; Sample processing using the DNA methylation array and cell mixture deconvolution algorithm; output from the cell classifier; computational processing of cell count and integration of clinical data

4.2.3 Statistical methods

The primary variable of interest were the absolute counts of each of the 15 cell types from blood samples of subjects with a confirmed diagnosis of newly diagnosed or recurrent IDH-mutant glioma, as measured at pre- and post-surgical timepoints. The outcome, represented by each cell count, was centered around the mean of the 263 samples and rescaled to reflect count changes in one standard deviation unit. All analyses were performed using R version 4.2.3.⁵² Linear mixed effects modeling was performed using the lme4 package.¹⁸² Visualizations were processed using the ggplot2 package.⁵³

4.2.4 Modeling approach

For each transformed immune cell type, a linear mixed-effects model was fit to examine cell count changes between pre- and post-surgical periods. This difference was modeled using a binary variable indicating the period when the blood sample was drawn. The models included a random intercept for each subject and fixed effects for tumor histology (IDH-mutant oligodendroglioma or astrocytoma), tumor recurrence status (newly diagnosed vs. recurrent), dexamethasone use within five days of the blood draw (yes/no), age at the time of surgery, and sex. Statistically significant associations with cell counts were defined by a two-sided p-value ≤ 0.05 .

4.2.5 Subgroup analysis

Dexamethasone (DEX) is a well-established biologic modulator of immune cell counts, and common in perioperative management. As such, as an exploratory aim, we sought to assess the effect of dexamethasone use on cell counts. Along with adjustment for dexamethasone use in the previously described model, we assessed whether the significant differences immune cell counts from pre- to post-surgery were consistent based on four distinct patterns of dexamethasone administration: subjects who received dexamethasone at both pre- and post-surgery blood draws (“DEX pre/post”), dexamethasone only at pre-surgery (“DEX pre only”), dexamethasone only at post-surgery (“DEX post only”), and subjects who were not treated with dexamethasone at either timepoint (“No DEX”). The estimates of change of transformed cell counts from pre- to post-surgery were obtained using the same linear mixed effects model described above applied to each subgroup individually. Given that each subgroup was restricted to a DEX use pattern, the dexamethasone use variable was excluded from the model. Significant differences in the effect of surgery by dexamethasone usage pattern were assessed using a Wald test.

4.3 Results

4.3.1 IDH-mutant glioma patient cohort

The study cohort included 168 subjects (46% female, 53% male) with a confirmed diagnosis of either newly diagnosed (47%) or recurrent (53%) IDH-mutant glioma. Blood samples were collected at pre-surgical (n = 159) and/or post-surgical (n = 104) timepoints, with 95 subjects having both pre- and post-surgery timepoints available. The median age at time of surgery was 40 years (interquartile range [IQR], 33–50 years). Of the total samples, 139 (53%) belonged to subjects diagnosed with IDH-mutant oligodendroglioma, while 124 (47%) belonged to subjects diagnosed with IDH-mutant astrocytoma. At the pre-surgical timepoint, 30 subjects (18.1%) were recently exposed to dexamethasone, while 71 subjects (44%) were exposed post-surgery. The median time between pre-surgical blood draw to surgery (day 0) was 1 day (IQR: -2 to -1 days), and the median time between surgery and post-surgical blood draw was 14 days (IQR: 13 to 21 days). Table 4.1 provides a detail summary of the cohort characteristics. All post-surgical blood draws were taken prior to the beginning of chemotherapy and/or radiation treatment. Distribution of absolute counts at pre- and post-surgical periods are shown in figure 4.2.

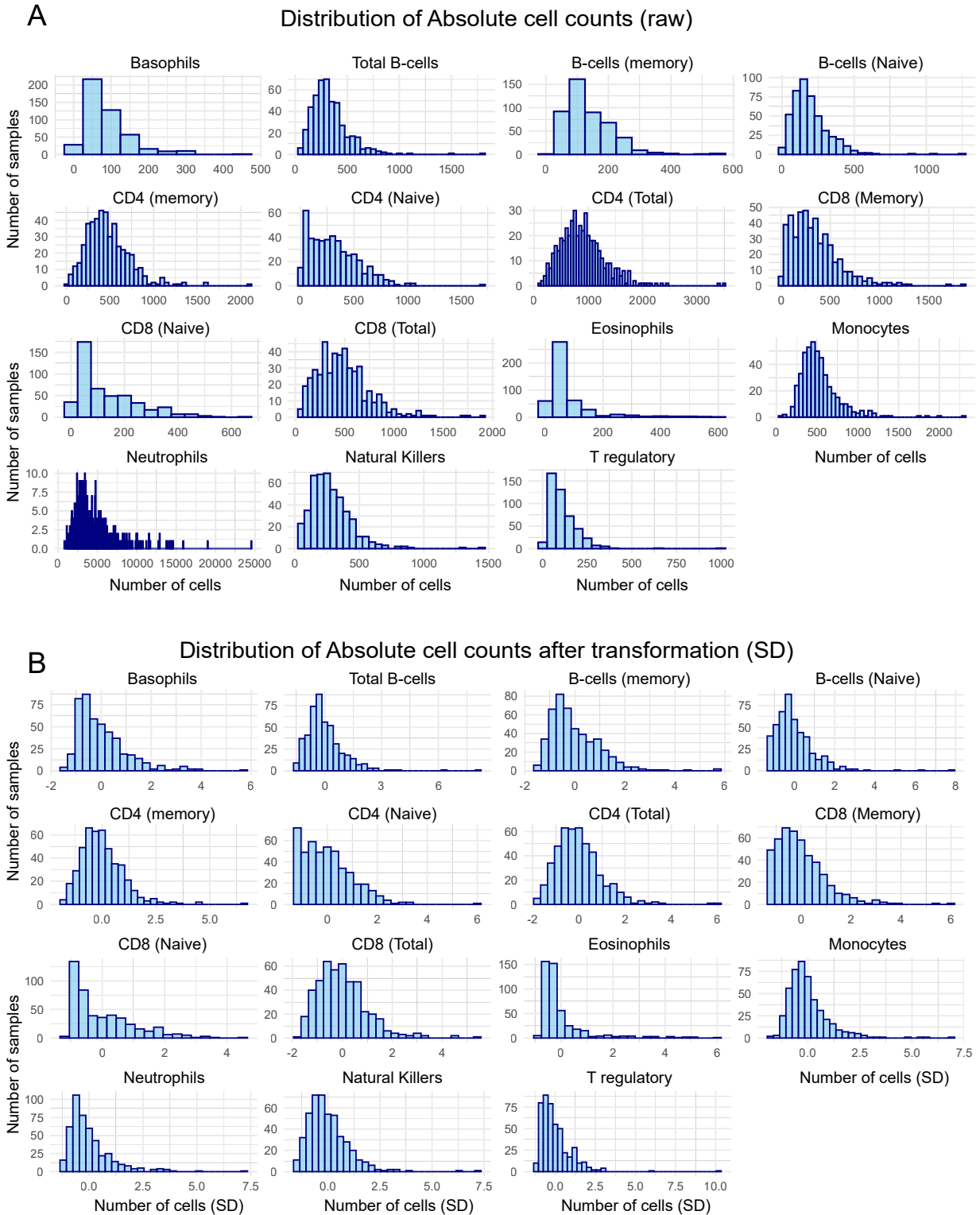


Figure 4.2 Cell count distribution across time points. (A) Distribution of absolute cell count across time points prior to transformation. (B) Distribution of absolute cell count across time points after transformation to represent changes in standard deviations from the population mean of each individual cell.

Table 4.1. Cohort Characteristics

	Pre-surgery (N=159)	Post-surgery (N=104)	Overall (N=263)
Age (mean) [IQR]	42.1 [34.0, 50.5]	41.4 [33.0, 48.0]	41.8 [33.0, 50.0]
Sex n (%)			
Female	75 (47.2)	47 (45.2)	122 (46.4)
Male	84 (52.8)	57 (54.8)	141 (53.6)
Race (caucasian) n (%)			
No	30 (18.9)	17 (16.3)	47 (17.9)
Yes	129 (81.1)	87 (83.7)	216 (82.1)
Type of Tumor n (%)			
IDH-mutant oligodendroglioma	85 (53.5)	54 (51.9)	139 (52.9)
Grade 2	49 (30.8)	31 (29.8)	80 (30.4)
Grade 3	36 (22.6)	23 (22.1)	59 (22.4)
IDH-mutant astrocytoma	74 (46.5)	50 (48.1)	124 (47.1)
Grade 2	33 (20.8)	24 (23.1)	57 (21.7)
Grade 3	23 (14.5)	14 (13.5)	37 (14.1)
Grade 4	18 (11.3)	12 (11.5)	30 (11.4)
Tumor recurrence n (%)			
Newly diagnosed	79 (49.7)	45 (43.3)	124 (47.1)
Recurrent	80 (50.3)	59 (56.7)	139 (52.9)
Type of surgery n (%)			
Subtotal resection	73 (45.9)	49 (47.1)	122 (46.4)
Gross total resection	86 (54.1)	55 (52.9)	141 (53.6)
DEX exposure at draw n (%)			
No	129 (81.1)	33 (31.7)	162 (61.6)
Yes	30 (18.9)	71 (68.3)	101 (38.4)
Days to pre-surgical blood draw (median) [IQR]	-1 [-2, -1]	N/A	N/A
Days to post-surgical blood draw (median) [IQR]	N/A	19 [13, 21]	N/A

4.3.2 Effect of surgery on absolute cell count

A statistically significant difference in cell counts post-surgery compared with pre-surgery was observed in basophils with an increase of 0.5 standard deviations (95% CI: 0.29, 0.65) above the sample's mean ($p < 0.001$) and regulatory T-cells (Treg) with an increase of 0.26 standard deviations (95% CI: 0.009, 0.52) above the sample's mean ($p = 0.043$). We observed no significant difference between pre- and post-surgery counts for the other 13 cell types, when adjusting for all available relevant covariates. Figure 4.3 summarizes the estimated change in cell counts between pre- and

post-surgical periods on each individual cell.

Change in cell counts from pre- to post-surgery timepoints

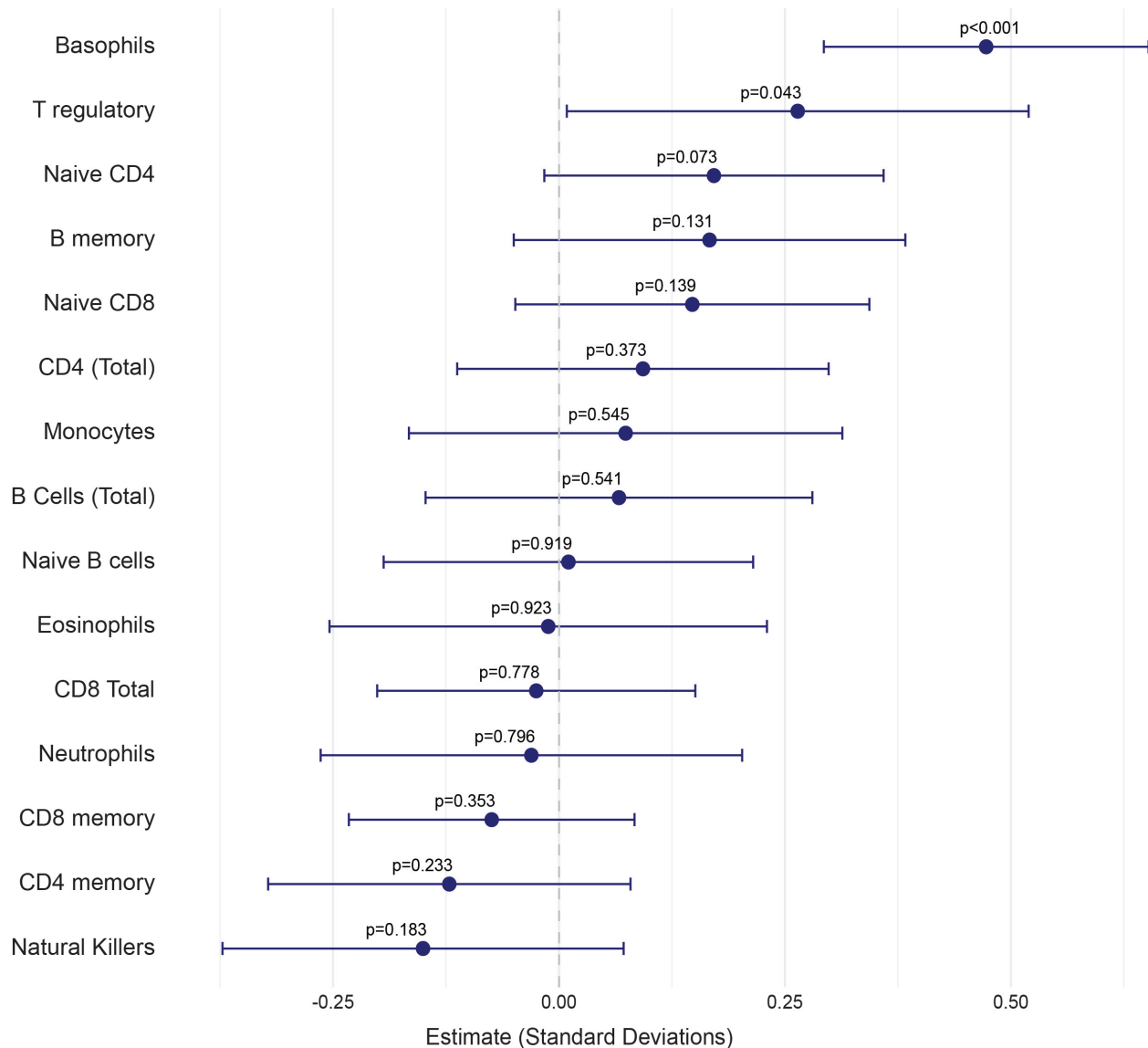


Figure 4.3 Cell count change from pre- to post-surgical periods in standard deviations Estimated cell count change from pre-surgery to post-surgery in standard deviations as estimated by the linear mixed effects model.

4.3.3 Subgroup analysis by dexamethasone usage patterns

Subgroup analysis was conducted on the four distinct subgroups based on dexamethasone administration patterns: DEX pre/post (n=13), DEX pre only (n=17), DEX post only (n=58), and No

DEX (n=80).

Key findings from these subgroup analyses are illustrated in figure 4.4. Changes in immune cell dynamics between pre- and post-surgery blood draws varied markedly between subgroups, highlighting the nuanced interplay between dexamethasone use and immune modulation. Notably, most cells showed an increased count between pre- and post-surgery among the DEX post only group with the exception of natural killer cells (NK) that showed a significant reduction. In contrast, the No DEX group showed significant cell count increase in basophils and significant cell count decrease in CD4 total and memory CD4 at post-surgery vs pre-surgery.

4.4 Discussion

This study provides insights into the peripheral immune landscape of IDH-mutant glioma patients, highlighting the impact of surgery and dexamethasone administration on circulating immune cell populations. Our findings underscore the complex interplay between surgical intervention, dexamethasone exposure, and immune modulation in glioma patients. Here, we contextualize these results within the existing literature, discuss potential biological mechanisms and outline their clinical implications.

4.4.1 Immune Effects of Regulatory T cells (Treg) in Glioma and Surgery

Regulatory T cells (Tregs), a subset of CD4⁺ T cells, maintain immune homeostasis and tissue repair, influencing both immune modulation and wound healing during surgical interventions like tumor resections.^{183–187} While they mitigate inflammation and prevent autoimmunity, Tregs can also hinder effective anti-tumor immunity, a challenge notably observed in gliomas where they contribute to tumor-associated immunosuppression.^{188, 189} Preclinical studies reveal that Treg

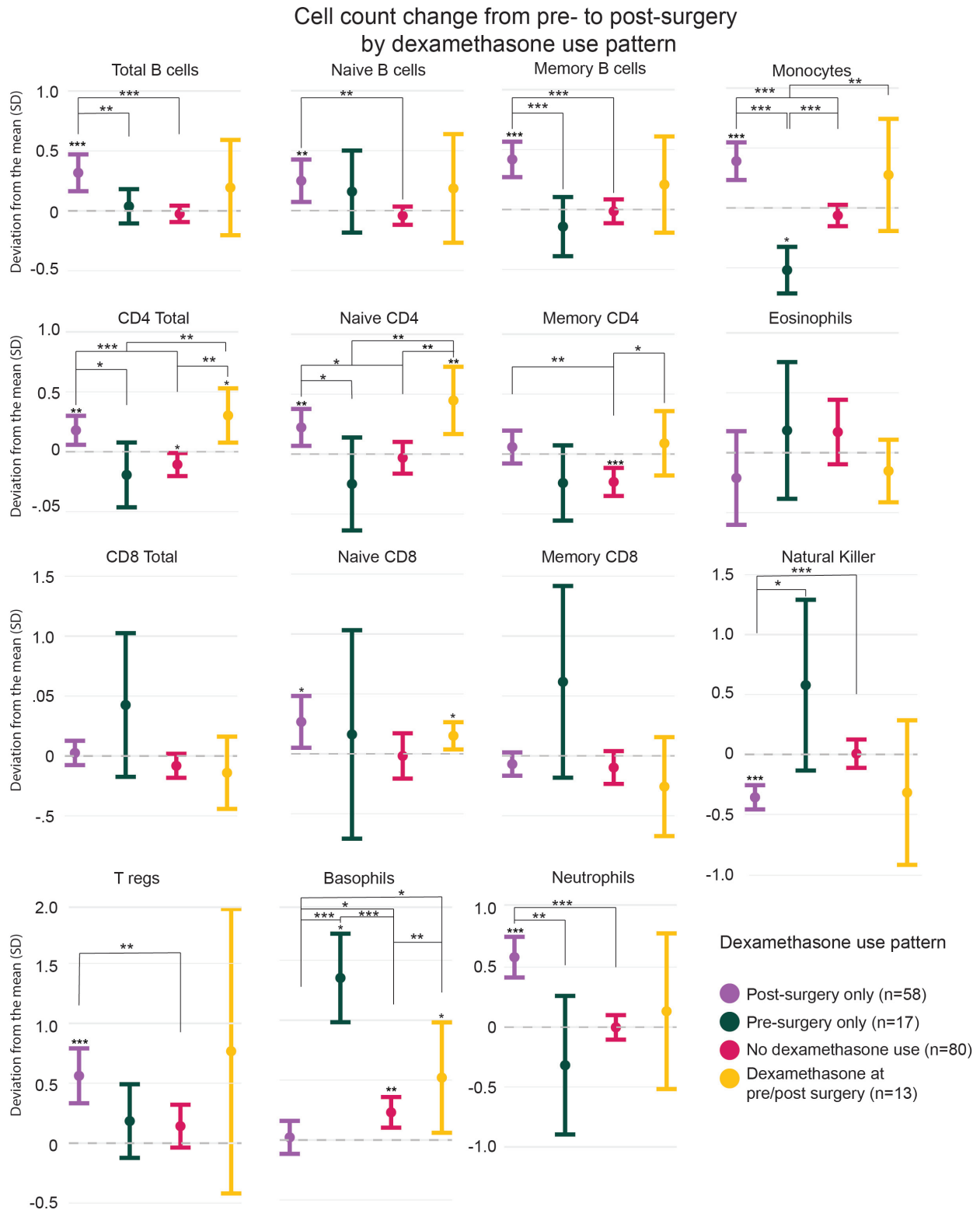


Figure 4.4 Comparison of estimated surgery effects across subgroups defined by dexamethasone administration patterns. Estimate was obtained using a linear mixed effects model with a subject's random intercept and fixed effects for age, sex, tumor histology, recurrence number Subgroup comparisons were conducted using the Wald test. Asterisks indicate levels of statistical significance: (*) p-value < 0.05, (**) p-value < 0.01, (***) p-value < 0.001.

depletion enhances tumor rejection and prolongs survival in gliomas.¹⁸⁹ However, clinical evidence shows that glioma patients experience compromised immune responses, marked by increased Tregs and reduced CD4+ T cell function.^{163, 189–191}

Our findings indicate a significant rise in Tregs post-surgically, suggesting an immunoregulatory response to the surgical procedure. However, this increase may also signify glioma-driven mechanisms inhibiting tumor-reactive effector T cells.^{192, 193} Understanding the dual role of Tregs in immune regulation and tumor-driven immunosuppression is crucial for developing strategies that balance immunosuppression with effective anti-tumor responses. Future research should explore methods that modulate Treg activity without compromising the body's ability to repair tissue or protect against autoimmunity, improving outcomes for glioma patients.

4.4.2 Immune effects of basophils in glioma and surgery

The role of basophils in gliomas is less understood than that of Tregs, yet they are recognized as key regulators of systemic adaptive immunity (40). Their capacity to bind antigens via IgE and IgG antibodies highlights their central role in humoral immune responses, potentially supporting B cells and influencing CD4+ T helper cell activity to promote a Th2 response, critical for granulocyte activation and tissue repair.^{194, 195}

Post-surgery, we observed increased basophils, likely a response to tissue injury and inflammation. Beyond aiding in repair, basophils sustain long-lived plasma cells, crucial for immunological memory, suggesting their importance in immune resilience during recovery.¹⁹⁶ Emerging evidence highlights the complex interaction between basophils, the tumor microenvironment (TME), and cancer progression, with implications that heightened basophil activity may correlate with improved outcomes among patients with glioma outcomes.^{197–204} Notably, basophils may recruit

tumor-specific CD8⁺ T cells by producing chemokines like CCL2 and CCL4, possibly supporting anti-tumor immunity.²⁰⁴

While our findings indicate a rise in basophils post-surgery as part of the innate immune response, their presence in the TME may have prognostic and therapeutic implications for glioma patients. Future studies should explore how basophils interact with other cells within the TME to foster effective anti-tumor responses. The simultaneous increase of basophils and Tregs post-surgery highlights the complexity of the immune landscape in glioma patients, underscoring the need for a nuanced understanding of immune cell dynamics in shaping outcomes.

4.4.3 Dexamethasone-driven immune patterns

Dexamethasone, a synthetic glucocorticoid for managing cerebral edema in glioma patients, significantly affects immune cell populations by promoting immunosuppressive subsets like Tregs and reducing cytotoxic cells such as NK cells and basophils^{205–207}. These immune alterations can hinder tumor surveillance and interfere with immunotherapy efficacy by downregulating pro-inflammatory cytokines and impairing dendritic and T-cell activity^{208, 209}. Our observations highlight potential infectious complications from dexamethasone-induced CD4⁺ lymphopenia and suggest poor survival with CD4⁺ counts below 200 cells/mm³²¹⁰.

The dose-dependent effects of dexamethasone remain unclear regarding duration of exposure; exploring a dose threshold and “washout period” could balance therapeutic benefits with immune function impact. Subgroup analysis shows that both dexamethasone timing and administration influence immune response to surgery, potentially amplifying immune responses or altering immunomodulatory effects.

Post-surgical dexamethasone use may mitigate immune suppression and promote reparative pathways, though further randomized studies are needed for broader implications. Understanding dexamethasone's interactions with immune populations is vital for developing accurate outcome models and treatment strategies.

Of note, this study was conducted at a single-institution scope and non-randomized design, affecting comparability and generalizability. Despite these, the study contributes critically to understanding immune dynamics in glioma, using a novel deconvolution algorithm on methylation data to provide longitudinal insights.

In conclusion, this study establishes the basis for the study of immune cell dynamics in the context of IDH-mutant glioma. It emphasizes the impact that surgical resection and dexamethasone administration on peripheral immune cell counts, underlining the need for further research to inform perioperative management and improve outcomes such as response to therapy and overall survival.

Conclusion

This dissertation advances our understanding of the molecular and genetic factors driving survival of patients with glioma. Through integrative molecular epidemiology approaches, we identified novel prognostic biomarkers and potential therapeutic targets in both IDH-wildtype glioblastoma and IDH-mutant astrocytomas, offering insights that could refine clinical decision-making and improve patient outcomes.

Our study on transposable elements (TEs) in IDH-wildtype glioblastoma demonstrated that specific TEs, including BC200, LTR7Y, and MER61D, are significantly associated with reduced survival. These findings underscore the role of TEs in tumor biology, particularly their involvement in genomic instability, immune evasion, and transcriptional regulation. The observed TE dysregulation highlights their potential as both biomarkers and therapeutic targets, providing a foundation for future research into TE-directed therapies.

In IDH-mutant astrocytomas, our longitudinal profiling revealed acquired RAS-MAPK pathway mutations associated with high-grade transformation and inferior survival. The identification of recurrent alterations in KRAS, NRAS, BRAF, NF1, and LZTR1 suggests that these mutations contribute to disease progression and may serve as actionable therapeutic targets. These results provide critical insights into the molecular evolution of IDH-mutant gliomas, emphasizing the importance of early molecular surveillance and targeted intervention.

Collectively, these studies contribute to the growing body of research aimed at enhancing glioma classification, improving prognostic modeling, and identifying novel therapeutic avenues. Future

research should focus on validating these findings in independent cohorts, exploring functional mechanisms, and investigating potential interventions that target TEs and RAS-MAPK alterations. Additionally, integrating these molecular insights with patient-centered clinical decision tools could further refine personalized treatment strategies in neuro-oncology.

By bridging molecular genetics with clinical applications, this work lays the groundwork for more precise diagnostics, better risk stratification, and the development of novel therapeutic approaches in gliomas. As the field of neuro-oncology progresses, continued advancements in genomic technologies and targeted therapies will be crucial in transforming the landscape of glioma management and improving patient survival.

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Appendices

6.1 RAS-MAPK pathway mutations associated with inferior survival.

Syndromic cases

Two patients had deleterious/pathogenic missense variants in the *TP53* gene in the constitutional DNA sample diagnostic of IDH-mutant astrocytomas arising in the setting of Li-Fraumeni syndrome. Patient #135 is an 11-year-old female who underwent biopsy of a multifocal IDH-mutant astrocytoma from the left cerebrum with CNS WHO grade 2 histologic features who had a deleterious/pathogenic *TP53* p.R248Q missense mutation in the constitutional DNA sample at approximately 50% variant allele frequency consistent with representing a variant of germline origin. She also had osteosarcoma centered in the right tibia diagnosed at 9 years of age and a sister with choroid plexus carcinoma. Her IDH-mutant astrocytoma demonstrated somatic inactivation of the remaining *TP53* allele due to a separate focal deletion of the 5' coding exons, as well as somatic *IDH1* p.R132H mutation. She was followed by surveillance and was subsequently treated with laser interstitial thermal therapy (LITT) four years after the initial biopsy for progressive disease. She remained alive at last clinical follow-up at 15 years of age and 4.9 years after initial glioma diagnosis. Patient #51 is a 28-year-old female who underwent resection of an IDH-mutant astrocytoma from the left temporal lobe of the brain with CNS WHO grade 2 histologic features. She also had a separate mass lesion in the fourth ventricle of the brain favored to represent an ependymoma. Her constitutional DNA sample extracted from a buccal swab specimen demonstrated a deleterious/pathogenic *TP53* missense variant (p.R248Q) at an allele frequency of 26%, consistent with constitutional/somatic mosaicism. The IDH-mutant astrocytoma demonstrated somatic inactivation

tion of the remaining *TP53* allele via a separate p.R306* nonsense mutation, along with additional somatic *IDH1* p.R132H mutation, *ATRX* p.S819* nonsense mutation, and *CIC* p.R422* nonsense mutation. One year after initial surgical resection, she had local recurrence for which she underwent additional resection and subsequent therapy with the IDH mutant small molecule inhibitor ivosidenib. Further disease progression 6 years later resulted in additional resection and treatment with vorasidenib. She remains alive at last clinical follow-up at 7 years after initial diagnosis.

One patient (#155) with a history of multiple chondromas had the *IDH1* p.R132H variant present in multiple constitutional DNA samples at less than 50% variant allele frequency diagnostic of IDH-mutant astrocytoma arising in the setting of Ollier syndrome. This patient was a 32-year-old male who underwent resection of an IDH-mutant astrocytoma from the left frontal lobe of the brain with CNS WHO grade 2 histologic features. The *IDH1* p.R132H variant was detected at 6% allele frequency in a constitutional DNA sample extracted from peripheral blood and at 3% allele frequency in a buccal swab specimen. The tumor demonstrated the identical *IDH1* p.R132H oncogenic mutation at 42% allele frequency, along with somatic *TP53* p.Y163C mutation, two *ATRX* frameshift (p.E886fs and p.I360fs) mutations, *NF1* p.1273fs frameshift mutation, *SPRED1* p.v309fs frameshift mutation, and *NOTCH1* p.S990fs frameshift mutation. Two years after initial resection he was treated with the IDH mutant small molecule inhibitor vorasidenib due to disease progression. One year later he underwent surgical resection followed by radiation and adjuvant temozolomide due to further disease progression. He remains alive at last clinical follow-up at 5 years after initial glioma diagnosis.

Two patients had deleterious/pathogenic splice site variants in the *MSH2* gene in the constitutional DNA sample diagnostic of IDH-mutant astrocytomas arising in the setting of Lynch syndrome. Patient #105 is a 17-year-old male who underwent resection of an IDH-mutant as-

trocytoma from the right temporoparietal lobes of the brain with CNS WHO grade 4 histologic features. He had a deleterious/pathogenic *MSH2* c.366+1G>A splice site mutation in the constitutional DNA sample at approximately 50% variant allele frequency consistent with germline origin of the variant, accompanied by somatic loss of the remaining wildtype allele in the tumor. Additionally, the tumor demonstrated somatic hypermutation with approximately 40 mutations per Mb, was microsatellite unstable, and contained oncogenic/pathogenic mutations including *IDH1* p.R132H, *TP53* p.R267W and p.R248W missense mutations, and *ATRX* p.R444* nonsense mutation. After resection, he was treated with radiation, temozolomide, and immune checkpoint blockade therapy with pembrolizumab. He developed local recurrence at 8 months and disseminated disease in the cerebellum at 11 months after initial diagnosis, and died of disease at 12 months after initial diagnosis. Patient #166 is an 18-year-old female who underwent resection of an IDH-mutant astrocytoma from the left temporal lobe of the brain with CNS WHO grade 3 histologic features. She had a truncating pathogenic *MSH2* p.F523fs in the constitutional DNA sample at approximately 50% variant allele frequency consistent with germline origin of the variant, accompanied by a somatic splice site mutation c.366+1G>A inactivating the remaining *MSH2* allele in the tumor. Additionally, the tumor demonstrated somatic hypermutation with approximately 50 mutations per Mb, was microsatellite unstable, and contained oncogenic/pathogenic mutations including *IDH1* p.R132H, *TP53* p.R273H and p.P152L missense mutations, *ATRX* p.V957fs frameshift mutation. Following the initial diagnostic biopsy, she was treated with external beam radiation and adjuvant immune checkpoint blockade therapy with nivolumab and ipilimumab, and died of progressive disease at 14.9 months after diagnosis.

Table 6.1. Age- and sex-Adjusted Hazard Ratios for mortality associated with whole genome TE abundance. Derived from Cox proportional-hazards regression. Asterisks represent statistically significant associations.

RE	Repeat Family	Hazard ratio	Standard Error	FDR adjusted q-value
BC200	scRNA	1.75	0.12	0.0042*
LTR7Y	ERV1	2.24	0.19	0.0111*
FLAM_C	Alu	2.03	0.18	0.0324*
LTR16A	ERV1	3.05	0.29	0.0391*
MER61D	ERV1	1.48	0.11	0.0548
L1ME3B	L1	2.89	0.33	0.2205
Tigger1	TcMar-Tigger	0.13	0.66	0.2734
MER110	ERV1	0.03	1.20	0.3539
LTR38	ERV1	2.04	0.25	0.4471
Tigger4	TcMar-Tigger	2.55	0.33	0.4471
MER65A	ERV1	2.74	0.35	0.4471
L1MC5	L1	0.12	0.75	0.4471
L1MC5	L1	0.12	0.75	0.4471
MER85	PiggyBac	1.60	0.17	0.4471
L1PA11	L1	0.43	0.31	0.4534
LTR86B2	ERV1	1.91	0.24	0.4534
L1MB7	L1	2.05	0.27	0.4534
LTR37A	ERV1	4.06	0.52	0.4534
MER41C	ERV1	1.86	0.23	0.4743
U13	snRNA	1.95	0.25	0.4808
Tigger2	TcMar-Tigger	0.26	0.52	0.4988
LTR45C	ERV1	1.62	0.19	0.4988
LTR37.int	ERV1	2.18	0.31	0.4988
Cheshire	hAT-Charlie	0.41	0.35	0.4988
L1PB4	L1	1.92	0.26	0.4988
MER50	ERV1	3.91	0.56	0.6196
MER61E	ERV1	1.93	0.27	0.6377
MER68	ERV1	1.41	0.14	0.6601
LTR12	ERV1	2.10	0.31	0.6601
LTR2	ERV1	2.12	0.32	0.6601
Kanga1a	TcMar-Tc2	1.89	0.27	0.6601
LTR16B2	ERV1	0.40	0.40	0.6601
MER61F	ERV1	1.47	0.17	0.6601
MER113	hAT-Charlie	2.05	0.31	0.6601
MLT1F1	ERV1-MaLR	0.49	0.31	0.6601
Merlin1_HS	Merlin	0.74	0.13	0.6601
LTR19B	ERV1	1.79	0.26	0.7401
MER66.int	ERV1	0.46	0.35	0.7461
ERV1.E.int	ERV1	0.29	0.56	0.7461
MLT1E1A	ERV1-MaLR	0.36	0.46	0.7721
L1M3de	L1	0.61	0.23	0.7721
L1MA5	L1	0.55	0.28	0.7721
LTR77	ERV1	1.35	0.14	0.7721
FAM	Alu	1.36	0.14	0.7721
LTR25.int	ERV1	0.39	0.44	0.7721
LTR53	ERV1	1.84	0.29	0.7721
MER129	LTR	1.24	0.10	0.7721
HUERS.P2.int	ERV1	1.52	0.20	0.8209
AluYi6_4d	Alu	0.70	0.17	0.8242
MER97d	hAT-Tip100	0.70	0.17	0.8444
LTR29	ERV1	1.91	0.31	0.8444
UCON34	Unknown	1.26	0.11	0.9080
LTR53.int	ERV1	1.50	0.20	0.9080
MSTB1.int	ERV1-MaLR	0.58	0.27	0.9080
MER134	DNA	1.25	0.11	0.9080
L1MCc	L1	0.62	0.24	0.9125
LTR1F	ERV1	1.54	0.22	0.9130

Table 6.2. Age- and sex-Adjusted Hazard Ratios for mortality associated with intronic TE abundance. Derived from Cox proportional-hazards regression. Asterisks represent statistically significant associations.

RE	Repeat Family	Hazard ratio	Standard Error	FDR adjusted q-value
FLAM_C	Alu	1.64	0.11	0.0093*
LTR7Y	ERV1	1.40	0.08	0.0210*
FordPrefect_a	hAT-Tip100	1.39	0.09	0.0607*
L1MB7	L1	1.56	0.14	0.3198
L1MB2	L1	4.59	0.50	0.4220
MER50B	ERV1	1.50	0.14	0.4220
AluYi6_4d	Alu	0.68	0.14	0.4220
MER50.int	ERV1	1.63	0.17	0.4220
LTR54	ERV1	1.47	0.14	0.4220
MER68	ERVL	1.31	0.10	0.4220
LTR16A	ERVL	2.32	0.30	0.4220
L4_A_Mam	RTE-X	0.44	0.31	0.5510
HERVK.int	ERVK	1.32	0.11	0.5925
MER89	ERV1	1.55	0.17	0.5925
LTR27D	ERV1	0.70	0.14	0.5925
LTR40a	ERVL	0.56	0.22	0.5925
LTR2B	ERV1	1.40	0.13	0.5925
LTR26B	ERV1	1.29	0.10	0.6262
LTR65	ERV1	1.48	0.16	0.6349
MamTip3	hAT-Tip100	1.44	0.15	0.6668
MER66.int	ERV1	0.72	0.14	0.6668
LTR19B	ERV1	1.51	0.17	0.6668
MER47B	TcMar-Tigger	0.62	0.20	0.7094
SVA_B	SVA	1.33	0.12	0.7094
MER33	hAT-Charlie	1.33	0.12	0.7094
MamGypLTR1d	Gypsy	1.32	0.12	0.7250
Tigger16a	TcMar-Tigger	1.53	0.19	0.7646
L1MDb	L1	1.48	0.17	0.7684
MER92A	ERV1	1.24	0.10	0.7852
Cheshire	hAT-Charlie	0.64	0.20	0.7852
LTR9A1	ERV1	1.26	0.11	0.7870
MER96B	hAT-Tip100	1.50	0.18	0.8350
Kanga2_a	TcMar-Tc2	0.61	0.23	0.9233
MLT1H1	ERVL-MaLR	1.30	0.12	0.9522
L1ME4c	L1	0.60	0.24	0.9522
MER63D	hAT-Blackjack	0.72	0.16	0.9522
ERV24B_Prim.int	ERV1	0.69	0.18	0.9522
LTR22A	ERVK	0.70	0.17	0.9522
L1MA6	L1	0.55	0.29	0.9522
LTR41	ERVL	0.68	0.19	0.9522
LTR58	ERV1	1.31	0.14	0.9522
MER110.int	ERV1	1.33	0.14	0.9522
LTR40c	ERVL	0.69	0.18	0.9522
MER61E	ERV1	1.28	0.12	0.9522
Tigger16b	TcMar-Tigger	0.73	0.15	0.9522
FordPrefect	hAT-Tip100	0.73	0.16	0.9522
MSTB2	ERVL-MaLR	0.62	0.24	0.96
MLT1.int	ERVL-MaLR	1.22	0.10	0.9549
MamGypLTR1c	Gypsy	0.74	0.15	0.9549

Table 6.3. Age- and sex-Adjusted Hazard Ratios for mortality associated with exonic TE abundance. Derived from Cox proportional-hazards regression. Asterisks represent statistically significant associations.

RE	Repeat Family	Hazard ratio	Standard Error	FDR adjusted q-value
BC200	scRNA	1.53	0.10	0.0211*
L1ME3B	L1	2.27	0.21	0.0221*
HAL1M8	L1	1.51	0.11	0.0221*
LTR16A	ERV1	1.51	0.11	0.0221*
LTR79	ERV1	1.58	0.12	0.0221*
L1ME3E	L1	1.51	0.13	0.1060
L1MA10	L1	1.51	0.13	0.1452
MER87	ERV1	1.48	0.13	0.1452
MSTB	ERV1-MaLR	2.56	0.34	0.3788
L1ME2z	L1	1.53	0.16	0.3788
MER54B	ERV1	1.43	0.13	0.3861
Tigger4	TcMar-Tigger	1.52	0.16	0.4148
LTR16C	ERV1	1.59	0.18	0.4148
U13_	snRNA	1.25	0.09	0.4339
L1MEa	L1	1.27	0.10	0.4484
MER21C	ERV1	1.50	0.16	0.4484
L1M7	L1	0.73	0.13	0.5142
Charlie16a	hAT-Charlie	1.47	0.17	0.5142
LTR58	ERV1	1.54	0.19	0.5142
Tigger2a	TcMar-Tigger	1.57	0.19	0.5142
HERVH.int	ERV1	1.25	0.10	0.5142
L1PA4	L1	1.28	0.11	0.5142
Tigger2	TcMar-Tigger	0.68	0.17	0.5142
MamGypLTR3a	Gypsy	1.33	0.13	0.5142
LTR77	ERV1	1.30	0.12	0.5142
LTR24	ERV1	1.45	0.17	0.5142
MLT2B2	ERV1	0.73	0.15	0.5142
MER52A	ERV1	1.30	0.12	0.5142
Tigger10	TcMar-Tigger	1.23	0.09	0.5142
MER4D1	ERV1	0.70	0.16	0.5142
LTR1A2	ERV1	1.37	0.15	0.5142
L1MC5	L1	0.55	0.27	0.5142
MER87B	ERV1	1.21	0.09	0.5142
LTR40b	ERV1	0.74	0.14	0.5142
MER39	ERV1	1.28	0.12	0.5142
MER9a3	ERV1	0.70	0.17	0.5142
HERVIP10B3.int	ERV1	0.75	0.13	0.5142
MER4A	ERV1	1.26	0.11	0.5142
Charlie13a	hAT-Charlie	1.23	0.10	0.5142
ERV1.E.int	ERV1	0.65	0.21	0.5142
LTR16D2	ERV1	1.24	0.10	0.5142
MLT1F	ERV1-MaLR	0.68	0.19	0.6245
MER44B	TcMar-Tigger	1.25	0.11	0.6734

Table 6.4. Age- and sex-Adjusted Hazard Ratios for mortality associated with intergenic TE abundance. Derived from Cox proportional-hazards regression. Asterisks represent statistically significant associations.

RE	Repeat Family	Hazard ratio	Standard Error	FDR adjusted q-value
MER65A	ERV1	2.77	0.31	0.3723
L1ME3B	L1	2.07	0.22	0.3723
LTR37.int	ERV1	2.61	0.30	0.3723
MER61D	ERV1	1.36	0.10	0.3723
LTR7Y	ERV1	1.71	0.17	0.3723
LTR47B2	ERVL	1.94	0.22	0.3723
U13	snRNA	1.92	0.23	0.6232
MER110	ERV1	0.06	1.01	0.6232
MER50	ERV1	4.81	0.56	0.6232
MLT1F1	ERVL-MaLR	0.30	0.44	0.6434
MER4D1	ERV1	3.90	0.51	0.6514
LTR12	ERV1	2.02	0.27	0.6514
LTR14A	ERVK	1.58	0.18	0.6514
LTR61	ERV1	1.69	0.20	0.6514
L1M3de	L1	0.58	0.21	0.6514
MER41C	ERV1	1.92	0.26	0.6514
MER11C	ERVK	1.66	0.20	0.6514
L1PA11	L1	0.48	0.29	0.6514
MER65C	ERV1	1.43	0.14	0.6514
MLT1F	ERVL-MaLR	2.61	0.38	0.6514
L1PB4	L1	1.57	0.18	0.6514
MER66.int	ERV1	0.51	0.28	0.6724
LTR38	ERV1	1.71	0.23	0.7566
UCON34	Unknown	1.31	0.11	0.7566
MER97d	hAT-Tip100	0.70	0.15	0.7566
LTR86B2	ERVL	1.58	0.19	0.7566
MER85	PiggyBac	1.42	0.15	0.7959
MER124	DNA	1.74	0.24	0.8238
MER34B	ERV1	2.11	0.33	0.8557
L1MB3	L1	9.09	0.98	0.8557
LTR2	ERV1	2.34	0.39	0.8827
LTR37A	ERV1	3.01	0.50	0.8827
ERVL.E.int	ERVL	0.24	0.66	0.8827
MER21A	ERVL	2.57	0.44	0.8827
HERVK14C.int	ERVK	0.59	0.24	0.8827
LTR16B2	ERVL	0.51	0.32	0.8827
LTR33A_	ERVL	1.81	0.28	0.8827
Tigger1	TcMar-Tigger	0.40	0.43	0.8827
HUERS.P2.int	ERV1	1.47	0.18	0.8827
L2.3_Crp	L2	0.50	0.33	0.8827
MER90a	ERV1	0.45	0.38	0.8827
L1M3e	L1	0.74	0.15	0.8827
SVA_D	SVA	2.20	0.38	0.8827
L1PA10	L1	0.48	0.35	0.8827
L1MC5	L1	0.33	0.54	0.8827
MER44C	TcMar-Tigger	0.52	0.32	0.8827
FLAM_C	Alu	5.29	0.81	0.8827
HERVL18.int	ERVL	0.46	0.38	0.8827
MamGypLTR1b	Gypsy	1.61	0.23	0.8827
LTR60	ERV1	0.74	0.15	0.8827

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