ALT in Pediatric Brain Tumors Can Occur without *ATRX* Mutation and is Enriched in Patients with Pathogenic Germline MMR Variants

Jennifer L. Stundon[1], Heba Ijaz[1,2], Krutika S. Gaonkar[3,4,5], Rebecca S. Kaufman[1,4], Run Jin[3,5], Anastasios Karras[1], Zalman Vaksman[1,4], Jung Kim[6], Daniel P. Miller[3,5], Yiran Guo[3,5], Mariarita Santi[7], Marilyn Li[7], Gonzalo Lopez[1], Phillip J. Storm[3,5], Adam C. Resnick[3,5], Angela J. Waanders[8,9], Suzanne P. MacFarland[1,10], Douglas R. Stewart[6], Sharon J. Diskin[1,4,10,11], Jo Lynne Rokita[3,4,5*], Kristina A. Cole[1,10,11]

[1]Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, PA

[2]Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA

[3]Center for Data-Driven Discovery in Biomedicine, Children's Hospital of Philadelphia, Philadelphia, PA

[4]Department of Bioinformatics and Health Informatics, Children's Hospital of Philadelphia, Philadelphia, PA

[5]Division of Neurosurgery, Children's Hospital of Philadelphia, Philadelphia, PA

[6]Division of Cancer Epidemiology and Genetics, National Cancer Institute, Rockville, MD

[7]Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, Philadelphia, PA

[8]Division of Hematology, Oncology, NeuroOncology, and Stem Cell Transplant, Ann & Robert H Lurie Children's Hospital of Chicago, IL

[9]Department of Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, IL

[10]Department of Pediatrics, University of Pennsylvania, Philadelphia, PA

[11]Abramson Family Cancer Research Institute, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA

*Corresponding author: Jo Lynne Rokita (rokita@chop.edu)

Running Title: Genetic Associations with ALT in Pediatric HGAT

Author Contributions

Conceptualization: KAC, JLS, JLR

Methodology: JLS, KAC, KSG, JLR, SJD, MML

Software: KSG, RJ, DPM, JLR

Validation: JLR, SJD, RSK

Formal Analysis: JLS, KSG, DPM, RJ, JLR, RSK, ZV, SJD

Investigation: KSG, RJ, JLR, SJD, JK, RSK, ZV, HI, AK, MML

Resources: KAC, SJD, ACR, PJS

Data Curation: KSG, RJ, JLR, GL, SM, AJW

Writing - Original Draft: JLS, JLR, KAC

Writing - Review & Editing: SJD, MML, GL, KAC, JLS, JLR, DRS, JK

Visualization: JLS, HI, KAC, RJ, JLR

Supervision: KAC, JLR, SJD, DRS

Project Administration: KAC, JLR

Funding Acquisition: KAC, JLR, PJS, ACR, SJD

ABSTRACT

Background: To achieve replicative immortality, most cancers develop a telomere maintenance mechanism, such as reactivation of telomerase or alternative lengthening of telomeres (ALT). There are limited data on the prevalence and clinical significance of ALT in pediatric brain tumors, and ALT-directed therapy is not available.

Methods: We performed C-circle analysis (CCA) on 586 pediatric brain tumors that had corresponding tumor/normal whole genome sequencing through the Open Pediatric Brain Tumor Atlas (OpenPBTA). We detected ALT in 6.8% (n=40/586) of these tumors and completed additional validation by ultrabright telomeric foci *in situ* on a subset of these tumors. We used CCA to validate *TelomereHunter* for computational prediction of ALT status and focus subsequent analyses on high-grade astrocytic tumors (HGAT). Finally, we examined whether ALT is associated with recurrent somatic or germline alterations.

Results: ALT is common in pediatric HGAT (n=24/63, 38.1%), but occurs infrequently in other pediatric brain tumors (<3%). Somatic *ATRX* mutations occur in 50% of ALT+ HGAT and in 30% of ALT- HGAT. Rare pathogenic germline variants in mismatch repair (MMR) genes are significantly associated with an increased occurrence of ALT.

Conclusions: We demonstrate that *ATRX* is mutated in only a subset of ALT+ HGAT, suggesting other mechanisms of *ATRX* loss of function or alterations in other genes may be associated with the development of ALT in these patients. We show that germline variants in MMR are associated with development of ALT in patients with HGAT.

Keywords

Alternative lengthening of telomeres (ALT), Telomere, HGAT, mismatch repair, ATRX, pediatric brain tumors

Key Points:

ATRX alterations are frequent, but not required, for an ALT phenotype in HGATs

HGAT patients with germline mismatch repair variants have higher rate of ALT tumors

TelomereHunter is validated to predict ALT in high-grade astrocytic tumors

Importance of the Study

We performed orthogonal molecular and computational analyses to detect the presence of alternative lengthening of telomeres in a highly characterized cohort of pediatric brain tumors. We demonstrate that many high-grade astrocytic tumors utilize ALT without a mutation in *ATRX*, suggesting either loss of function of *ATRX* via an alternative mechanism or an alternate means of development of ALT. We show that germline variants in MMR genes are significantly associated with ALT in HGAT. Our work adds to the biological understanding of the development of ALT and provides an approach to stratify patients who may benefit from future ALT-directed therapies in this patient population.

Introduction

As human cells divide, telomeres become progressively shorter, leading to senescence [1]. This is known as the end replication problem, and human cancer must overcome this barrier in order to achieve replicative immortality [2]. In most human cancer cells, telomeres are maintained via telomerase reactivation, however, in about 10-15% of cancers, telomeres are lengthened by the recombination-based mechanism known as alternative lengthening of telomeres (ALT) [3]. ALT occurs most frequently in mesenchymal tumors and has been shown to occur frequently in pediatric high-grade astrocytic tumors (HGAT), neuroblastoma, osteosarcoma, as well as adult low-grade glioma and pancreatic neuroendocrine tumors [4-7]. ALT is rarely found in epithelial malignancies, likely because telomerase expression is less tightly regulated in epithelial cells [8]. While ALT has been shown to frequently occur in pediatric HGAT, previous studies have not included associated whole genome tumor and normal DNA and clinical data for these patients [9]. Additionally, ALT has been observed in some medulloblastomas and primitive neuroendocrine tumors [10].

ALT+ telomeres utilize homologous recombination to maintain telomere length and have a high level of telomeric DNA damage secondary to replication stress [12]. ALT can be identified in patient samples by measuring the presence of ultra-bright telomeric foci, C-circles that are formed during ALT telomere replication, or ALT-associated pro-myelocytic bodies [3,13,14]. Additionally, there are computational methods to identify and count telomere repeats from sequencing data [15-18], though previous studies have utilized limited pediatric brain tumors [19]. Collectively, these techniques measure the presence of DNA damage, replication stress, and altered telomeric content that are characteristic of ALT telomeres. The use of ALT as a telomere maintenance mechanism (TMM) has been associated with poor outcome in some cancers, such as neuroblastoma, though in other cancers including adult glioblastoma, patients with ALT+ tumors have a better prognosis when compared to patients with telomerase positive cancer [20,21]. It remains unclear whether there is a prognostic difference among pediatric patients with ALT+ HGAT compared to similar patients with ALT- tumors.

Loss of function mutations in the *ATRX* chromatin remodeling gene are strongly correlated with ALT positivity and one known role of *ATRX* is to inhibit ALT [22-25]. A recent study in pediatric neuroblastoma showed that reduced abundance of *ATRX*, either due to mutations or low protein expression, was only observed in

55% of ALT+ patients [20] and similarly, *ATRX* mutations were not required for activation of ALT in adult pancreatic neuroendocrine tumors and melanoma [26,27].

It is not known whether germline variants in DNA repair genes are associated with the development of ALT. Recent literature has suggested that loss of MMR function may have an important role in ALT activity in human cancer cell lines [28], though this association has not yet been demonstrated in primary human tumors. Additionally, it has been shown that loss of MMR function in yeast and mice is associated with telomerase-independent telomere lengthening and improved organismal survival and fitness [29,30], which supports a role for loss of the MMR pathway in promoting the development of telomerase independent telomere lengthening, such as ALT. Cancer predisposition syndromes such as constitutional MMR deficiency (CMMRD) and Lynch Syndrome (LS), as well as acquired somatic MMR gene alterations, are major mechanisms of MMR pathway loss of function. CMMRD is a very aggressive predisposition resulting from biallelic pathogenic germline variants in the MMR genes *PMS2* (60%), *MSH6* (20-30%), *MLH1*/*MLH2* (10-20%). LS has an autosomal dominant mode of inheritance and is caused by monoallelic pathogenic germline variants in the same MMR genes: *MSH2/MLH1* (80%), *MSH6* (13%), and *PMS2* (6%)[31].

Here, we assess the frequency of ALT, as well as clinical and molecular phenotypes associated with ALT, in a large cohort of pediatric brain tumors from the OpenPBTA, with detailed investigation of HGATs [32-34]. We validated the use of the computational algorithm, *TelomereHunter* [19], to predict ALT status. We demonstrate that *ATRX* is only mutated in a subset of ALT+ HGAT patients, and that presence of pathogenic germline variants in MMR genes is strongly associated with the development of ALT. This is important, as germline variants in MMR genes are seen in roughly 6% of patients with pediatric high-grade glioma [35], a common tumor observed in patients with LS and CMMRD [36]. By demonstrating that MMR variants are associated with ALT and that *ATRX* is only mutated in a subset of ALT+ pediatric high-grade glioma patients, we add to our understanding of the key molecular changes that are associated with ALT. Developing a greater understanding of the molecular drivers of ALT will be critical to the creation of ALT directed therapy.

Methods:

Pediatric brain tumor data and genomic analyses

TelomereHunter

Paired tumor and normal WGS BAMs (N = 940) from previously sequenced pediatric brain tumors were obtained by data access request to the Children's Brain Tumor Network (CBTN). The BAMs were used as paired inputs to *TelomereHunter* [19], which was run using default parameters to estimate telomere content. The ratio of telomere content in tumor compared to its normal was calculated and used for all downstream analyses. Using the C-circle assay molecular readout as a positive or negative ALT phenotype (N = 586 samples), we used the R package *cutpointr* [37] to determine a telomere ratio cutoff to assign samples as ALT+ while also estimating accuracy, sensitivity, and precision. To further validate receiver operating characteristic (ROC) curves, we randomly shuffled telomere ratio scores and plotted the ROC for shuffled scores.

Assessment of germline variant pathogenicity

Germline variants in genes included in the KEGG MMR gene set, plus *POLE* (Table S3) were first annotated using SnpEff v4.3t, ANNOVAR (06-07-2020. Variants with read-depth ≥ 15, variant allele fraction ≥ 0.20, and observed in < 0.1% across each population in the public control databases non-TCGA ExAC (exonic) or gnomAD 2.1.1 (non-exonic, splicing) were considered for further study. We retained variants annotated as Pathogenic/Likely Pathogenic (P/LP) in ClinVar (05-07-2022) or InterVar v2.2.2. All Pathogenic/Likely Pathogenic (P/LP) calls were manually reviewed by an interdisciplinary team, including clinicians and genetic counselors.

C-Circle Analysis

Tumor DNA and associated genomic and clinical data was obtained from the Children's Brain Tumor Network from individuals consented on the institutional review board approved CBTN protocol. C-circle analysis (CCA), which is a highly accurate read-out of ALT [38], was performed on tumor samples and controls as described previously [13]. Quantification of positivity was performed as described previously [39].

Tissue Microarray

Full methods detailing creation of tissue microarray, ATRX IHC staining and UBTF analysis are included in supplementary methods.

Statistical tests

Fisher exact tests were used to determine statistical significance for categorical variable. Mann-Whitney U testing was used to compare populations of two groups.

Results:

Frequency of ALT in pediatric brain tumors

To characterize ALT in pediatric brain tumors, we performed CCA on 586 tumors from unique patients which had corresponding sequencing data available in the OpenPBTA (**Figure 1A-B**). We found a low frequency of ALT in ATRT (N = 2/23, 8.7%), ependymoma (N =3/64, 4.7%), ganglioglioma (N = 1/41, 2.5%), medulloblastoma (N = 4/86, 4.7%), and low-grade astrocytoma (N = 4/203, 1.9%). ALT has not been previously described in ATRT or ependymoma [10]. In contrast, we confirmed that 38.1% of HGAT tumors (N = 24/63) have ALT. This is concordant with previous studies reporting that approximately 40% of pediatric HGAT utilize ALT [7,9,10]. We orthogonally validated the CCA by measuring ultra-bright telomeric foci (UBTF) via telomere FISH in 28 HGAT tumor. We show that 100% of tumors which were CCA positive were also positive for UBTF (N = 8), and only 2 (10%) of the remaining 20 tumors which were CCA negative were positive by UBTF (**Supplementary Table 1, Figure 2**). Since the majority of ALT tumors in our study were HGAT, we focused subsequent analyses on the HGAT cohort (N = 85: *N = 63 with CCA + UBTF, N = 20 with WGS only, N = 2 with WGS+UBTF*, denoted as the "primary analysis" cohort) shown in **Figure 1B**.

***TelomereHunter* accurately predicts an ALT phenotype in pediatric HGAT**

Computational methods of identification of ALT can provide rapid prediction of ALT in some patient tumors and may become important clinical tools as ALT-directed therapies are developed [19,40]. To determine whether a computational method of ALT identification could be utilized to detect ALT in pediatric brain cancer, we used *TelomereHunter* [19] to estimate telomere content from paired tumor/normal WGS (N = 940) and

calculated the tumor/normal telomere content ratio (**Figure 1 C-D** and **Supplementary Figure S1**). Using the molecular readout for HGAT patients on which CCA was performed (N = 63), we validated the utility of *TelomereHunter* to accurately stratify HGAT tumors by ALT status. We determined that a tumor/normal telomere content ratio of >1.0679 could identify ALT in HGAT (ROC = 0.95), achieving 90.59% accuracy, 93.75% sensitivity, and 88.68% specificity (**Figure 1C** and **1D**). This demonstrates that the use of *TelomereHunter*, which can be performed on any patient tumor with paired normal and/or tumor whole genome sequencing, can identify ALT in pediatric HGAT with high accuracy. We were additionally able to identify a tumor/normal telomere content ratio of >0.9963 for non-HGAT tumors (ROC = 0.66), though at a lower accuracy (76.97%), sensitivity (56.34%), and specificity (79.25%) (**Supplementary Figure S1, A-B**). This lower ROC may be the result of histology heterogeneity and/or the lower number of tumors in this group positive from CCA. However, randomized telomere content ratios (**Supplementary Figure S1C**) resulted in an expected diagonal (ROC = 0.53), suggesting the signal in both HGAT and non-HGAT groups is real. We found that an ALT phenotype was predicted for tumors across all histologies except for subependymal giant cell astrocytoma (**Supplementary Figure S1D**).

To expand the cohort of HGAT patients for downstream clinical and genomic analyses, we used *TelomereHunter* to assign ALT phenotypes to 22 HGAT previously sequenced tumors without sufficient DNA for CCA to increase the HGAT cohort size to N = 85, including 53 ALT+ and 32 ALT- patients (**Table 1**).

Older age is associated with a higher frequency of ALT

We analyzed the clinical status of patients with HGAT with and without ALT (**Table 1**). We demonstrate that amongst all HGAT patients in our cohort, patients with ALT+ HGAT were significantly older (11.06 years vs. 7.9 years, p= 0.007, **Table 1**). This suggests that there may be inherent biologic differences in tumors of patients with older ages that contribute to the development of ALT or that older patients may be more likely to develop tumors that are more frequently associated with ALT. For example, all patients with an *H3F3A* G35 mutation were ALT+, as has been previously reported [41]. The age range for these patients is 14.8-18.4 years, and this subtype of HGAT is known to be more common in adolescence and young adults [42]. We show no

difference between race or ethnicity when comparing ALT+ and ALT- patients. We further analyzed to assess differences in somatic mutations, mutational burden, and germline mutations (**Figure 2**).

Genomic landscape of ALT positive or negative HGATs

Depicted in **Figure 2** is an oncoprint of 85 pediatric HGAT tumors ordered by *TelomereHunter* tumor/normal telomere content ratio. Selected clinical demographics, molecular assay results, and genomic alterations are displayed. Notably, we observed high (predicted ALT+) tumor/normal telomere content ratios in all CCA positive cases except two tumors. Likewise, all samples positive for UBTF were predicted as ALT+ by *TelomereHunter*. Additionally, we show an inverse relationship between samples positive for ATRX protein and those positive for CCA and/or UBTF, consistent with previous work [9]. This inverse relationship also extends to somatic DNA alterations in *ATRX:* samples with somatic alterations in *ATRX* generally have loss of ATRX protein. We found slight enrichment of ALT in tumors with two of the most frequently occurring somatic alterations in pediatrics HGATs, *TP53* and *H3F3A* (p=0.027 and p=0.047, respectively). ALT was not enriched in *NF1* mutated tumors (**Supplementary Figure S2A**). Finally, we show that hypermutant and ultra-hypermutant tumors, as well as tumors with either germline or somatic MMR (**Table 2** and **Table S3**), were more likely to be ALT+ by CCA and/or *TelomereHunter*.

Somatic *ATRX* mutations occur in 50% of ALT-positive HGAT

We sought to determine the frequency of *ATRX* mutations in our patient cohort and whether any other recurrent somatic mutations occurred more frequently in ALT+ patient tumors. Somatic *ATRX* alterations were present in 50% of ALT+ HGAT patient tumors (N = 16/32, **Figure 3A**, **Supplementary Table 2**). *ATRX* mutations were rare in ALT- HGAT, and these were frequently variants of uncertain significance (N = 3/5). In contrast, mutations in *ATRX* in ALT+ HGAT were likely to be oncogenic (N = 13/16, **Figures 3A and Supplementary Figure S2, B-C).** We next compared the frequency of ALT in *ATRX* WT versus *ATRX* mutant HGAT. 52% of *ATRX* mutated HGAT (N = 23/44) are ALT+, as compared to 28% (N = 16/64) of *ATRX* WT tumors (p=0.0046, **Figure 3B**).

To gain a greater understanding of the *ATRX* biology in our cohort, we performed ATRX immunohistochemistry (N= 30) using the same antibody and conditions as our clinical laboratory, to assess for presence of absence of the ATRX protein on the HGAT brain tumor TMA. We demonstrate that 25% of the CCA positive HGAT tested (N = 3/12) in this cohort retained ATRX protein expression. Representative images of ALT+ patients with retained (**3E, 3F**) and lost ATRX expression are shown (**3G)** and full details are provided in **Supplementary Table 1**. Thus, we believe that neither *ATRX* mutation nor loss of ATRX protein expression should be used as a primary biomarker for ALT in HGAT, aligning with similar reports of ALT in neuroblastoma [43]. Additionally, we show that in the pediatric brain tumor population, ALT can occur independent of somatic *ATRX* alterations.

Germline variants in MMR genes are associated with ALT

We were specifically interested in whether germline variants in the mismatch repair (MMR) pathway may be associated with the development of ALT, as previous work in model organisms and in a human cancer cell lines have suggested a relationship between MMR and ALT [29,30,44-46]. Tumors with germline variants in MMR and/or POLE make up approximately 8% of patients with pediatric HGAT[35]. Additionally, germline MMR variants combined with acquired somatic mutations in MMR genes or *POLE* are known to result in ultra-hypermutated patient tumors[47]. We sought to understand whether there was a relationship between germline MMR variants and ALT in our cohort.

Using analysis of paired tumor and normal whole genome sequencing, we identified that 7% (N = 6/85) of patients with HGAT harbor heterozygous pathogenic germline variants in MMR genes (**Supplementary Tables 2 and 3**). Of the six HGAT tumors we identified with pathogenic or likely pathogenic germline MMR variants, five were ALT+, whereas 27/79 tumors without germline variants were ALT+ (p=0.02, **Table 2, Figure 2**). These results suggest that loss of function of the MMR pathway may be associated with ALT.

We additionally reviewed the pathology reports where available and identified one additional patient in our cohort with a self-reported *PMS2* variant who was not identified in the unbiased germline variant analysis. This patient had a negative CCA but was noted to have positive UBTF and a positive *TelomereHunter* score.

Two additional tumors from patients with clinically known MMR germline variants (LS and CMMRD) were analyzed on our TMA and were not part of the PBTA cohort. For both patients, UBTF was positive though only one patient had a positive CCA. TMA data for a patient with germline MMR is shown in **Figure 3F**.

Together, this demonstrates that most patients with a germline variant in MMR or a clinically-diagnosed MMR disorder, such as CMMRD or LS, have a higher frequency of ALT positive HGAT tumors as compared to patients without germline variants or clinically diagnosed MMR syndromes.

Tumor mutational burden is higher in ALT+ HGAT

Patients with germline MMR variants, particularly if biallelic, are known to have extremely high tumor mutational burden [48], and we replicate this in our cohort (**Table 2**). We sought to explore whether ALT is associated with increased TMB among the 85 HGAT patients in the PBTA. A small number of patients in our sample had extremely high levels of tumor mutational burden. We excluded patients with hypermutant or ultra-hypermutant status (>10 mutations/mb or >100 mutations/mb) and showed that ALT+ HGAT have a higher tumor mutational burden (**Figure 3C**, p=0.0022). This suggests that among patients with ALT+ HGAT, there may be tolerance for a greater level of DNA damage. Previously, somatic mutations in *ATRX* have been associated with higher TMB in pediatric HGAT, but the association between ALT and TMB has not been previously examined [49]. We therefore sought to determine whether our finding is independent of *ATRX* mutation status. Due to the close association of *ATRX* mutation with ALT status, we were not able to fully separate the effect of *ATRX* on tumor mutation. We demonstrate that among *ATRX* WT tumors, there is a significant increase in tumor mutational burden in ALT+ HGAT as compared to ALT- HGAT (p=0.0098, **Figure 3D**) suggesting that *ATRX* mutation alone does not account for the increase in TMB observed in ALT+ tumors.

Patients with ALT+ H3 K28M tumors may have a survival benefit

We assessed the impact of ALT status on overall survival (OS), first without any covariates and then by ALT status and histone H3 subtype. We did not find a significant effect of ALT status on OS in HGATs (p = 0.499). When stratifying by histone mutation status, there was no difference in OS in patients with H3 WT ALT + (median OS = 27.7 months) and H3 WT ALT- tumors (median OS = 28.1 months). However, using an

additive cox regression model, we observed a near-significant survival benefit (HR = 1.23, p = 0.0526) in patients with H3 K28M, ALT+ tumors (median OS = 14.4 months) compared to those with H3 K28M ALT- tumors (median OS = 9.4 months). H3 G35 mutations (median OS = 69.9 months) occurred exclusively in ALT+ patients (**Table S4**). Kaplan-Meier and forest plots are shown in **Supplementary Figure S3**.

Discussion

We analyzed primary patient tumors for ALT in 586 pediatric patient tumors using the gold standard CCA assay from a well-characterized pediatric brain tumor cohort. We identified ALT at low frequency in ATRT, ependymoma and low-grade astrocytic tumors. We used a cohort of 85 unique patients with HGAT to analyze the clinical, demographic, and molecular differences in ALT+ and ALT- HGAT and demonstrate that *ATRX* is mutated in 50% of ALT+ HGAT, ALT+ tumors have a higher mutational burden, and presence of pathogenic germline variants in MMR genes is strongly associated with the development of ALT. By correlating CCA and *TelomereHunter* output, we were able to further validate *TelomereHunter* as a reliable tool to predict ALT status using whole genome sequencing data, which may have important clinical implications when ALT directed therapies are available clinically.

Survival differences have been seen in other ALT+ and ALT- cancers and collecting additional samples may clarify our observed trend. Similar to what has been seen in adult GBM, the presence of ALT appears to have a protective feature and extend overall survival in patients with K28M HGAT [21]. It will be important to understand whether this is a true causal relationship, or whether the presence of ALT is associated with other changes that confer a more favorable outcome. For example, we have demonstrated that ALT+ HGAT have a higher tumor mutational burden, and it is possible that acquisition of specific mutations may improve overall survival, or that a high tumor mutational burden creates an instability in the tumor genome that favors a longer survival. Future work will include a larger patient analysis to determine if there are other key differences in ALT+ and ALT- HGAT patients that impact survival, focusing on the K28M HGAT.

In our primary analysis cohort, *ATRX* mutations occur in only 50% of our ALT+ HGAT patients, however, we failed to identify any other somatic mutations that may drive ALT. It is possible that ALT+ tumors

without *ATRX* mutations have changes to ATRX at the protein, RNA or transcriptional level that impact ATRX function. There may be less frequent mutations in certain classes of genes that are responsible for creating more accessible chromatin, similarly to *ATRX,* or there may be other changes that occur via a different pathway that promote development of ALT. While we sought to identify ALT in a relatively large population, we found only very low levels of ALT in the non-HGAT examined and thus were unable to make observations regarding the impact of ALT on the clinical outcomes of these patients or the mutational landscape. Future work will focus on larger cohorts of non-HGAT to determine the true frequency of ALT in these patients and to determine the clinical significance of ALT in these groups.

Our analysis relied primarily on the presence of C-circles to identify ALT. However, in a small subset of tumors, ALT may occur without C-circles [50]. Additionally, due to inherent tumor and microenvironment heterogeneity, it is possible that some areas of the tumor may be truly ALT+, whereas the areas from which the DNA extracted would not have sufficient c-circles to register as positive [38]. By orthogonally validating our CCA with measurement of UBTF, we partially address this concern.

While our data do not show any difference in clinical survival in patients with HGAT with or without ALT, ALT+ cancers are often treatment resistant cancers and novel therapies are needed for these patients [3]. ALT directed therapy remains an attractive target and may help sensitize ALT cancers to traditional cytotoxic chemotherapy [43]. By validating *TelomereHunter* with CCA, we have identified a computational tool that with additional verification and approval, may be clinically feasible to identify patients with ALT, which may be important as ALT-directed therapies become available.

We showed that *ATRX* loss or mutation only occurs in a subset of pediatric HGAT, which has two important clinical implications. First, *ATRX*-directed therapies may not be effective for a subset of ALT positive tumors for which alternative therapies will be needed. Second, there are likely other major pathways driving the development of ALT in our patient population and loss of ATRX function, as measured by loss of ATRX protein expression or *ATRX* mutation, cannot be used alone as a metric for determining ALT status in patients.

By demonstrating an association between ALT and loss of MMR function in pediatric HGAT patients, we may have identified an area for potential therapeutic targets to disrupt the function of ALT and lead to tumor senescence, though this finding ought to be validated in larger cohorts as they become available. Our future work will continue to focus on identifying the key molecular changes that drive the development of ALT in pediatric brain tumor patients. We will validate the association between loss of MMR function and development of ALT, and work to understand whether loss of MMR function creates a permissive environment that promotes the development of ALT. Once this has been established, we will work to identify key targets that are essential for the ongoing ALT in these patients, with the goal of developing novel targeted therapies that disrupt ALT, sensitize cells to traditional cytotoxic chemotherapy and promote tumoral senescence. Our future work will additionally focus on elucidating the molecular differences in *ATRX* mutant versus *ATRX* wild-type ALT+ HGAT.

Funding

This work was supported by NIH grant U2C-CA233285 (KAC), NIH 5T32CA009615-30 (JLS), NIH 2K12HD043245-16 (JLS), NIH R03-CA23036 (SJD), NIH Contract No. HHSN261200800001E (SJD), an Alex's Lemonade Stand Foundation Young Investigator Award (JLR), the Matthew Larson Foundation (KAC), the Division of Neurosurgery at the Children's Hospital of Philadelphia (PJS, ACR), and the Intramural Research Program of the Division of Cancer Epidemiology and Genetics of the National Cancer Institute.

Conflicts of Interest

Dr. Angela J. Waanders is a member of the Scientific Advisory boards for Alexion and DayOne Biopharmaceuticals.

Acknowledgements

We would like to thank the patients and families who have donated tissue for this research and we would like to acknowledge both the Children's Brain Tumor Network and the Pacific Pediatric Neuro-Oncology Consortium for collecting, sequencing, and harmonizing the genomic and clinical data used for this study.

Figure Legends

Figure 1. ALT is more prevalent in pediatric HGATs than other CNS tumors and can be computationally determined.

(**A**) C-circle analysis was completed for PBTA primary tumor samples from unique pediatric patients (N = 586). Tumor abbreviations: ETMR = Embryonal tumor with multilayered rosettes, GCT = germ cell tumor, GNT = glioneuronal tumor, OD = oligodendroglioma, PB = pineoblastoma, LGAT = low grade astrocytic tumor, GG = ganglioglioma, CP = craniopharyngioma, EP= ependymoma, MB = medulloblastoma, CP= choroid plexus tumor, ATRT = atypical teratoid rhabdoid tumor, HGAT = high grade astrocytic tumor. Benign lesions, non-primary brain tumors, and duplicate samples for a single patient were excluded from this analysis. HGAT represents 10.8% of tumors analyzed, but 60% of ALT+ tumors. (**B**) Representation of the HGAT subset (N = 85) on which paired tumor/normal WGS was performed. There was sufficient DNA available to perform C-circle assay on 63 samples and ultra-bright telomeric foci analysis on 24 samples. (**C**) Using the C-circle assay data as the "truth" set for an ALT phenotype, we used the R package *cutpointr* to determine the optimal tumor/normal telomere content ratio cutpoint for determining ALT +/- status. Shown are density plots for ALT + or - HGAT samples at a cutpoint ratio of 1.0679 (x-intercept). (**D**) This cutpoint enabled a 90.59% accuracy, 93.75% sensitivity and 88.68% specificity, shown with the receiver operating characteristic (ROC).

Figure 2. Molecular phenotypes and genomic alterations of pediatric HGAT tumors by ALT status.

Annotations for sex (estimated from germline WGS), tumor phase of therapy, *TelomereHunter* telomere ratio (tumor v. normal), C-circle assay, ultrabright telomeric foci assay and ATRX immunohistochemistry are shown.

TMB is annotated for hypermutant (100 Mut/Mb > TMB >= 10 Mut/Mb) and ultra-hypermutant (TMB >= 100 Mut/Mb) tumors. Positivity for variants in germline and/or mutations in somatic mismatch repair (MMR) genes (listed in **Table S3**) is annotated above the individual somatic mutations in TP53, H3F3A, ATRX, and NF1.

Figure 3. ALT+ HGATs are significantly enriched for *ATRX* mutations and have a higher tumor mutation burden.

(**A**) HGAT patients with ALT are more likely to have *ATRX* mutations ($p < 0.001$, N = 16/32 ALT+, N = 8/53 ALT-). *ATRX* mutations in ALT+ HGAT are more likely to be likely oncogenic mutations (N = 13/16) compared to mutations in ALT- HGAT (N = 2/5). (**B**). Mutations in *ATRX* are significantly associated with ALT ($p < 0.001$). (**C**) ALT+ HGATs have a higher TMB than ALT- HGATs (p=0.0038). (**D**) *ATRX* WT ALT+ tumors have a higher TMB as compared to *ATRX* WT ALT- HGAT tumors (p=0.0098). HGAT tumors may be ALT positive with (**E**) or without (**F-H**) ATRX protein expression *in situ*. Left and middle panels: Representative images of multiplex immunofluorescence of UBTF (red), ATRX protein (yellow) or both within DAPI stained nuclei (dark blue) of ALT+ HGAT tissues from 4 patient tumors (**E**: 7316-158; **F**: 7316-3058; **G**: 7316-3765; **H**: 7316-114). Right panels: representative H&E images and ATRX IHC, noting that in E (tumor 7316-158) ATRX protein expression is absent in tumor nuclei (blue) with positive ATRX staining in non-tumor nuclei. The remaining ALT+ HGAT tumors (**F-H**) demonstrate ATRX protein staining.

Table Legends

Table 1. Demographics and clinical information for patients with HGAT, separated by ALT status. Patients with ALT+ HGAT are older and no differences in sex, race, ethnicity, tumor location or survival were noted. Mutations in *ATRX* were more common in ALT+ tumors and TMB is higher in ALT+ tumors. Germline variants in MMR genes are associated with ALT+ status (p=0.02).

Table 2. Pediatric HGAT patients with pathogenic germline variants in MMR pathway genes have tumors enriched for ALT

Listed are the seven patients from our primary analysis HGAT cohort (N = 85) and two patients from a clinical validation cohort in which we found a predicted pathogenic (P) or likely pathogenic (LP) germline variant in an MMR pathway gene. Somatic MMR and *ATRX* alterations predicted to be oncogenic are listed. *TelomereHunter* ratios, TMB, C-Circle, and UBTF are also shown. *Note: patient C641691 had a self-reported *PMS2* germline variant and was not included in statistical calculations.

References:

1. Lundblad V. The end replication problem: More than one solution. *Nature Medicine.* 1997; 3(11):1198-1199.

2. Hanahan D, Robert. Hallmarks of Cancer: The Next Generation. *Cell.* 2011; 144(5):646-674.

3. Dilley RL, Greenberg RA. ALTernative Telomere Maintenance and Cancer. *Trends Cancer.* 2015; 1(2):145-156.

4. Barthel FP, Wei W, Tang M, et al. Systematic analysis of telomere length and somatic alterations in 31 cancer types. *Nat Genet.* 2017; 49(3):349-357.

5. Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. *Nature Reviews Genetics.* 2010; 11(5):319-330.

6. Claude E, Decottignies A. Telomere maintenance mechanisms in cancer: telomerase, ALT or lack thereof. *Curr Opin Genet Dev.* 2020; 60:1-8.

7. Heaphy CM, Subhawong AP, Hong SM, et al. Prevalence of the alternative lengthening of telomeres telomere maintenance mechanism in human cancer subtypes. *Am J Pathol.* 2011; 179(4):1608-1615.

8. Henson JD, Neumann AA, Yeager TR, Reddel RR. Alternative lengthening of telomeres in mammalian cells. *Oncogene.* 2002; 21(4):598-610.

9. Abedalthagafi M, Phillips JJ, Kim GE, et al. The alternative lengthening of telomere phenotype is significantly associated with loss of ATRX expression in high-grade pediatric and adult astrocytomas: a multi-institutional study of 214 astrocytomas. *Modern Pathology.* 2013; 26(11):1425-1432.

10. Mangerel J, Price A, Castelo-Branco P, et al. Alternative lengthening of telomeres is enriched in, and impacts survival of TP53 mutant pediatric malignant brain tumors. *Acta Neuropathologica.* 2014; 128(6):853-862.

11. Tabori U, Vukovic B, Zielenska M, et al. The role of telomere maintenance in the spontaneous growth arrest of pediatric low-grade gliomas. *Neoplasia.* 2006; 8(2):136-142.

12. Sobinoff AP, Pickett HA. Alternative Lengthening of Telomeres: DNA Repair Pathways Converge. *Trends Genet.* 2017; 33(12):921-932.

13. Henson JD, Lau LM, Koch S, Martin La Rotta N, Dagg RA, Reddel RR. The C-Circle Assay for alternative-lengthening-of-telomeres activity. *Methods.* 2017; 114:74-84.

14. Henson JD, Reddel RR. Assaying and investigating Alternative Lengthening of Telomeres activity in human cells and cancers. *FEBS Lett.* 2010; 584(17):3800-3811.

15. Lee M, Teber ET, Holmes O, et al. Telomere sequence content can be used to determine ALT activity in tumours. *Nucleic Acids Res.* 2018; 46(10):4903-4918.

16. Nersisyan L, Arakelyan A. Computel: Computation of Mean Telomere Length from Whole-Genome Next-Generation Sequencing Data. *PLOS ONE.* 2015; 10(4):e0125201.

17. Farmery JHR, Smith ML, Lynch AG. Telomerecat: A ploidy-agnostic method for estimating telomere length from whole genome sequencing data. *Scientific Reports.* 2018; 8(1).

18. Ding Z, Mangino M, Aviv A, Spector T, Durbin R. Estimating telomere length from whole genome sequence data. *Nucleic Acids Research.* 2014; 42(9):e75-e75.
19. Feuerbach L, Sieverling L, Deeg KI, et al. TelomereHunter – in silico estimation of telomere content and composition from cancer genomes. *BMC Bioinformatics.* 2019; 20(1).
20. Hartlieb SA, Sieverling L, Nadler-Holly M, et al. Alternative lengthening of telomeres in childhood neuroblastoma from genome to proteome. *Nature Communications.* 2021; 12(1).
21. McDonald KL, McDonnell J, Muntoni A, et al. Presence of Alternative Lengthening of Telomeres Mechanism in Patients With Glioblastoma Identifies a Less Aggressive Tumor Type With Longer Survival. *Journal of Neuropathology & Experimental Neurology.* 2010; 69(7):729-736.
22. Brosnan-Cashman JA, Yuan M, Graham MK, et al. ATRX loss induces multiple hallmarks of the alternative lengthening of telomeres (ALT) phenotype in human glioma cell lines in a cell line-specific manner. *PLOS ONE.* 2018; 13(9):e0204159.
23. Graham MK, Kim J, Da J, et al. Functional Loss of ATRX and TERC Activates Alternative Lengthening of Telomeres (ALT) in LAPC4 Prostate Cancer Cells. *Molecular Cancer Research.* 2019; 17(12):2480-2491.
24. Haase S, Garcia-Fabiani MB, Carney S, et al. Mutant ATRX: uncovering a new therapeutic target for glioma. *Expert Opin Ther Targets.* 2018; 22(7):599-613.
25. Napier CE, Huschtscha LI, Harvey A, et al. ATRX represses alternative lengthening of telomeres. *Oncotarget.* 2015; 6(18):16543-16558.
26. De Nonneville A, Reddel RR. Alternative Lengthening of Telomeres is not synonymous with mutations in ATRX/DAXX: Cold Spring Harbor Laboratory; 2020.
27. Sieverling L, Hong C, Koser SD, et al. Genomic footprints of activated telomere maintenance mechanisms in cancer. *Nature Communications.* 2020; 11(1).
28. Sakellariou D, Bak ST, Isik E, et al. MutSβ regulates G4-associated telomeric R-loops to maintain telomere integrity in ALT cancer cells. *Cell Reports.* 2022; 39(1):110602.
29. Martinez P, Siegl-Cachedenier I, Flores JM, Blasco MA. MSH2 deficiency abolishes the anticancer and pro-aging activity of short telomeres. *Aging Cell.* 2009; 8(1):2-17.
30. Rizki A, Lundblad V. Defects in mismatch repair promote telomerase-independent proliferation. *Nature.* 2001; 411(6838):713-716.
31. Aronson M, Colas C, Shuen A, et al. Diagnostic criteria for constitutional mismatch repair deficiency (CMMRD): recommendations from the international consensus working group. *J Med Genet.* 2022; 59(4):318-327.
32. Ijaz H, Koptyra M, Gaonkar KS, et al. Pediatric high-grade glioma resources from the Children's Brain Tumor Tissue Consortium. *Neuro-Oncology.* 2020; 22(1):163-165.
33. Petralia F, Tignor N, Reva B, et al. Integrated Proteogenomic Characterization across Major Histological Types of Pediatric Brain Cancer. *Cell.* 2020; 183(7):1962-1985.e1931.
34. Shapiro JA, Savonen CL, Bethell CJ, et al. *An open pediatric brain tumor atlas*: Manubot;2022.
35. Johnson A, Severson E, Gay L, et al. Comprehensive Genomic Profiling of 282 Pediatric Low- and High-Grade Gliomas Reveals Genomic Drivers, Tumor Mutational Burden, and Hypermutation Signatures. *Oncologist.* 2017; 22(12):1478-1490.
36. Wimmer K, Kratz CP, Vasen HF, et al. Diagnostic criteria for constitutional mismatch repair deficiency syndrome: suggestions of the European consortium 'care for CMMRD' (C4CMMRD). *J Med Genet.* 2014; 51(6):355-365.
37. Thiele C, Hirschfeld G. cutpointr: Improved Estimation and Validation of Optimal Cutpoints in R. *Journal of Statistical Software.* 2021; 98(11):1 - 27.
38. Idilli AI, Segura-Bayona S, Lippert TP, Boulton SJ. A C-circle assay for detection of alternative lengthening of telomere activity in FFPE tissue. *STAR Protoc.* 2021; 2(2):100569.
39. Fogli A, Demattei M-V, Corset L, et al. Detection of the alternative lengthening of telomeres pathway in malignant gliomas for improved molecular diagnosis. *Journal of Neuro-Oncology.* 2017; 135(2):381-390.
40. Recagni M, Bidzinska J, Zaffaroni N, Folini M. The Role of Alternative Lengthening of Telomeres Mechanism in Cancer: Translational and Therapeutic Implications. *Cancers (Basel).* 2020; 12(4).

41. Minasi S, Baldi C, Gianno F, et al. Alternative lengthening of telomeres in molecular subgroups of paediatric high-grade glioma. *Child's Nervous System.* 2021; 37(3):809-818.
42. Picart T, Barritault M, Poncet D, et al. Characteristics of diffuse hemispheric gliomas, H3 G34-mutant in adults. *Neuro-Oncology Advances.* 2021; 3(1).
43. Koneru B, Farooqi A, Nguyen TH, et al. ALT neuroblastoma chemoresistance due to telomere dysfunction-induced ATM activation is reversible with ATM inhibitor AZD0156. *Sci Transl Med.* 2021; 13(607).
44. Siegl-Cachedenier I, Munoz P, Flores JM, Klatt P, Blasco MA. Deficient mismatch repair improves organismal fitness and survival of mice with dysfunctional telomeres. *Genes Dev.* 2007; 21(17):2234-2247.
45. Barroso-González J, García-Expósito L, Galaviz P, et al. Anti-recombination function of MutSα restricts telomere extension by ALT-associated homology-directed repair. *Cell Reports.* 2021; 37(10):110088.
46. Barroso-González J, García-Expósito L, Galaviz P, et al. Anti-recombination function of MutSα restricts telomere extension by ALT-associated homology-directed repair. *Cell Rep.* 2021; 37(10):110088.
47. Campbell BB, Light N, Fabrizio D, et al. Comprehensive Analysis of Hypermutation in Human Cancer. *Cell.* 2017; 171(5):1042-1056.e1010.
48. Miklja Z, Pasternak A, Stallard S, et al. Molecular profiling and targeted therapy in pediatric gliomas: review and consensus recommendations. *Neuro Oncol.* 2019; 21(8):968-980.
49. Koschmann C, Calinescu A-A, Nunez FJ, et al. ATRX loss promotes tumor growth and impairs nonhomologous end joining DNA repair in glioma. *Science Translational Medicine.* 2016; 8(328):328ra328-328ra328.
50. Zhang J-M, Yadav T, Ouyang J, Lan L, Zou L. Alternative Lengthening of Telomeres through Two Distinct Break-Induced Replication Pathways. *Cell Reports.* 2019; 26(4):955-968.e953.

Figure 1

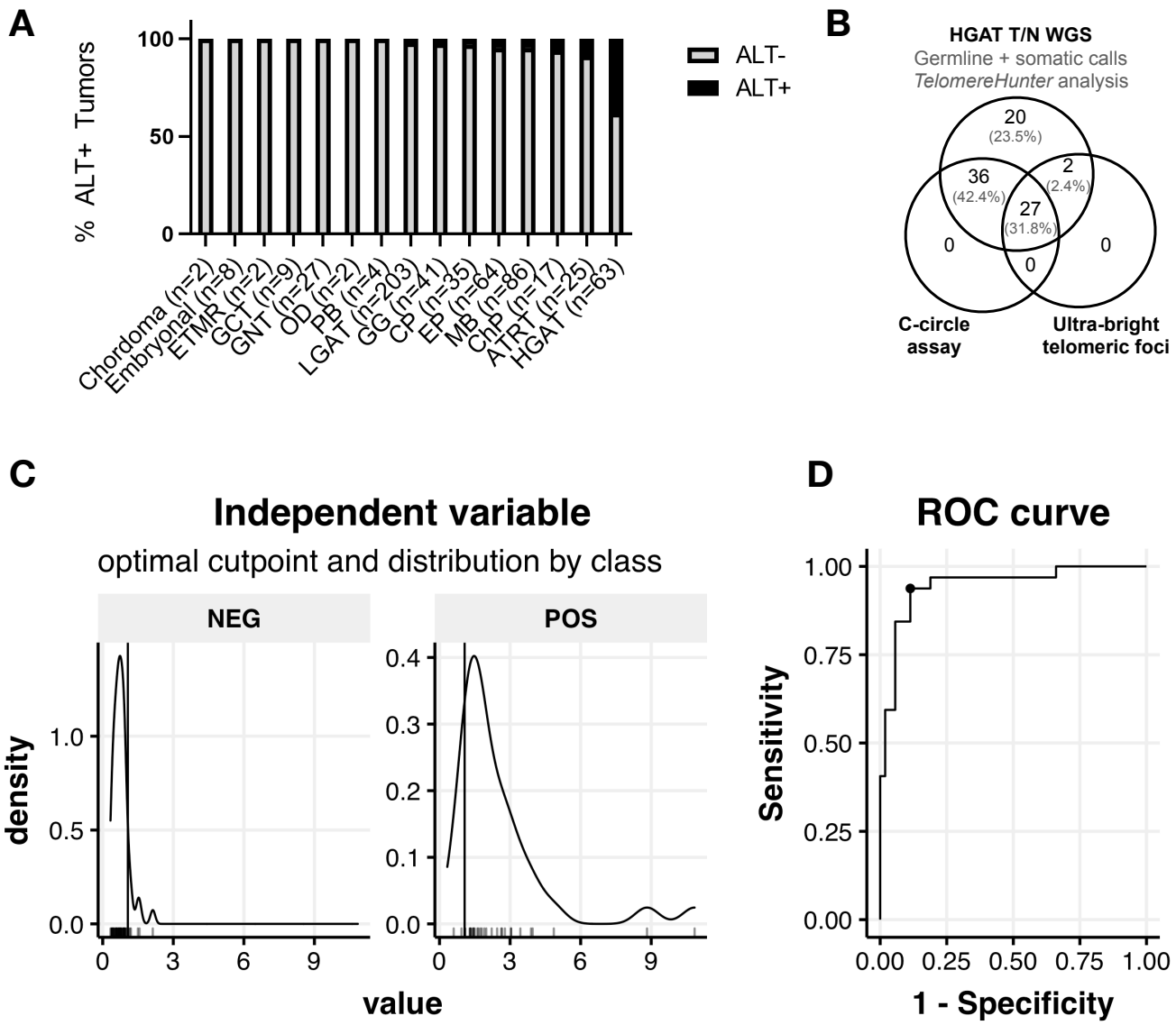

A
% ALT+ Tumors
100
50
0
ALT-
ALT+
Chordoma (n=2)
Embryonal (n=8)
ETMR (n=2)
GCT (n=9)
GNT (n=27)
OD (n=2)
PB (n=4)
LGAT (n=203)
GG (n=41)
CP (n=35)
EP (n=64)
MB (n=86)
ChP (n=17)
ATRT (n=25)
HGAT (n=63)
B
HGAT T/N WGS
Germline + somatic calls
TelomereHunter analysis
20
(23.5%)
36
(42.4%)
2
(2.4%)
27
(31.8%)
0
0
0
C-circle assay
Ultra-bright telomeric foci
C
Independent variable
optimal cutpoint and distribution by class
NEG
POS
density
value
D
ROC curve
Sensitivity
1 - Specificity

Figure 2

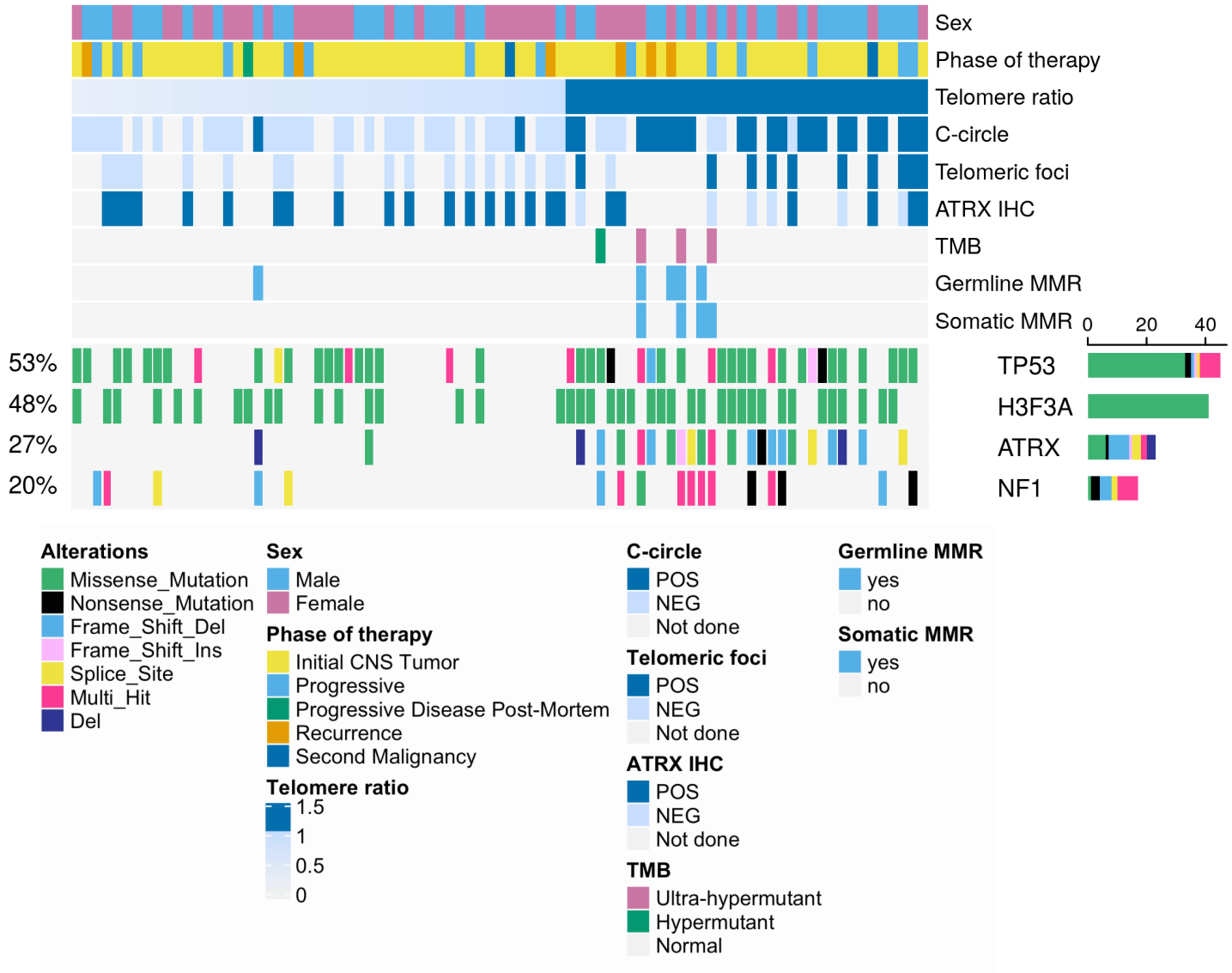
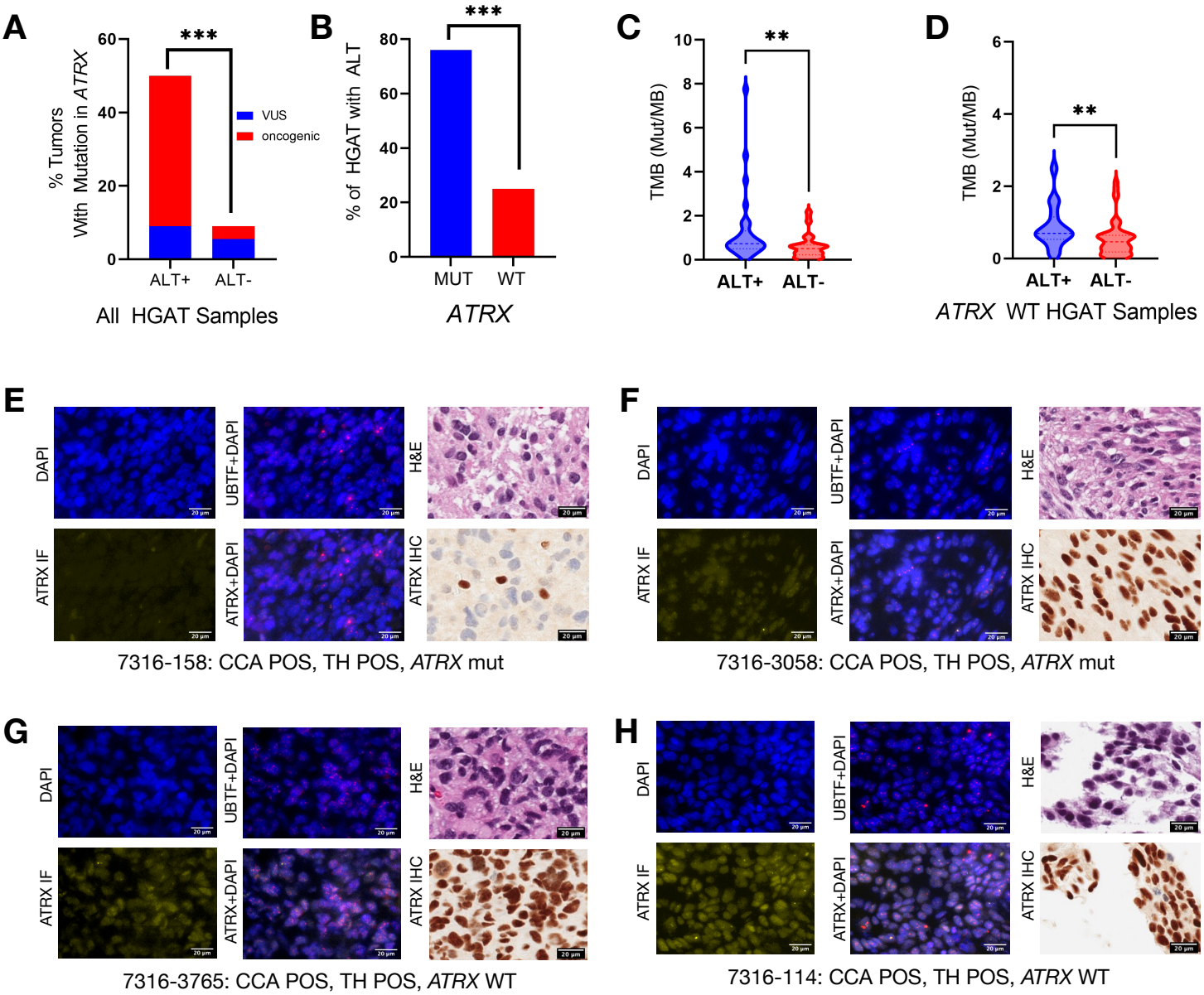

Sex
Phase of therapy
Telomere ratio
C-circle
Telomeric foci
ATRX IHC
TMB
Germline MMR
Somatic MMR
53%
48%
27%
20%
0
20
40
TP53
H3F3A
ATRX
NF1
Alterations
Missense_Mutation
Nonsense_Mutation
Frame_Shift_Del
Frame_Shift_Ins
Splice_Site
Multi_Hit
Del
Sex
Male
Female
Phase of therapy
Initial CNS Tumor
Progressive
Progressive Disease Post-Mortem
Recurrence
Second Malignancy
Telomere ratio
1.5
1
0.5
0
C-circle
POS
NEG
Not done
Telomeric foci
POS
NEG
Not done
ATRX IHC
POS
NEG
Not done
TMB
Ultra-hypermutant
Hypermutant
Normal
Germline MMR
yes
no
Somatic MMR
yes
no

Figure 3


A
***
% Tumors With Mutation in ATRX
60
40
20
0
VUS
oncogenic
ALT+
ALT-
All HGAT Samples
B
***
% of HGAT with ALT
80
60
40
20
0
MUT
WT
ATRX
C
**
TMB (Mut/MB)
10
8
6
4
2
0
ALT+
ALT-
D
**
TMB (Mut/MB)
6
4
2
0
ALT+
ALT-
ATRX WT HGAT Samples
E
DAPI
UBTF+DAPI
H&E
ATRX IF
ATRX+DAPI
ATRX IHC
7316-158: CCA POS, TH POS, ATRX mut
F
DAPI
UBTF+DAPI
H&E
ATRX IF
ATRX+DAPI
ATRX IHC
7316-3058: CCA POS, TH POS, ATRX mut
G
DAPI
UBTF+DAPI
H&E
ATRX IF
ATRX+DAPI
ATRX IHC
7316-3765: CCA POS, TH POS, ATRX WT
H
DAPI
UBTF+DAPI
H&E
ATRX IF
ATRX+DAPI
ATRX IHC
7316-114: CCA POS, TH POS, ATRX WT

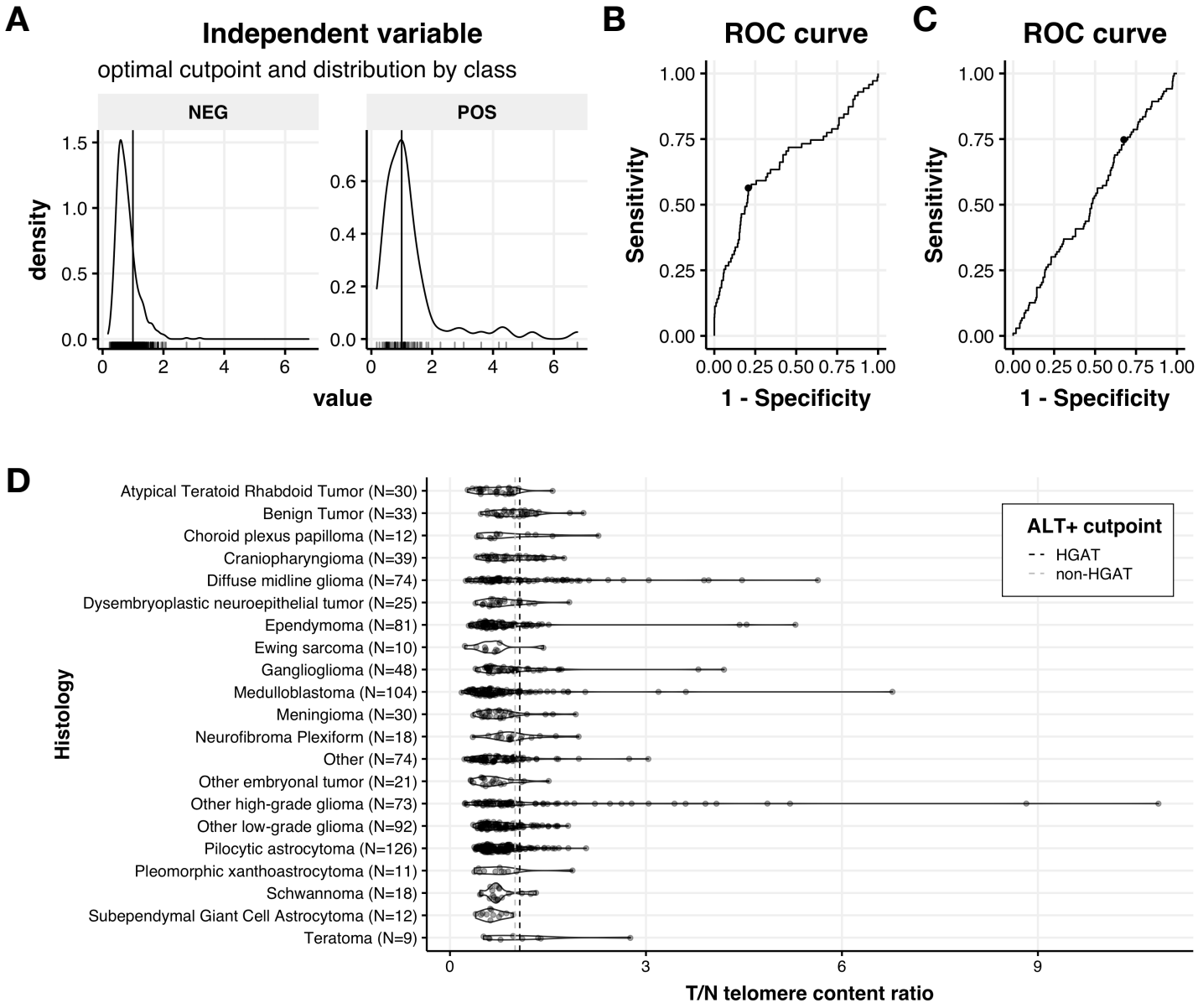


Figure S1. TelomereHunter performance on non-HGAT tumors in the PBTA. Using the C-circle assay data as the "truth" set for determine an ALT phenotype, we used the R package *cutpointr* to determine the optimal telomere ratio cutpoint for determining ALT +/- status. (**A**) Density plots for ALT + or - non-HGAT samples with a cutpoint of 0.9963 (x-intercept). (**B**) This cutpoint enabled an AUC = 0.66, 77% accuracy, 56.3% sensitivity, and 79.3% specificity, compared to shuffled telomere ratios (**C**), which resulted in an AUC = 0.526, 37.9% accuracy, 74.6% sensitivity, and 32.4% specificity. (**D**) Violin and sina plots of tumor/Normal (T/N) telomere content ratios across PBTA histologies. Cutpoints for ALT positivity are drawn as x-intercepts.

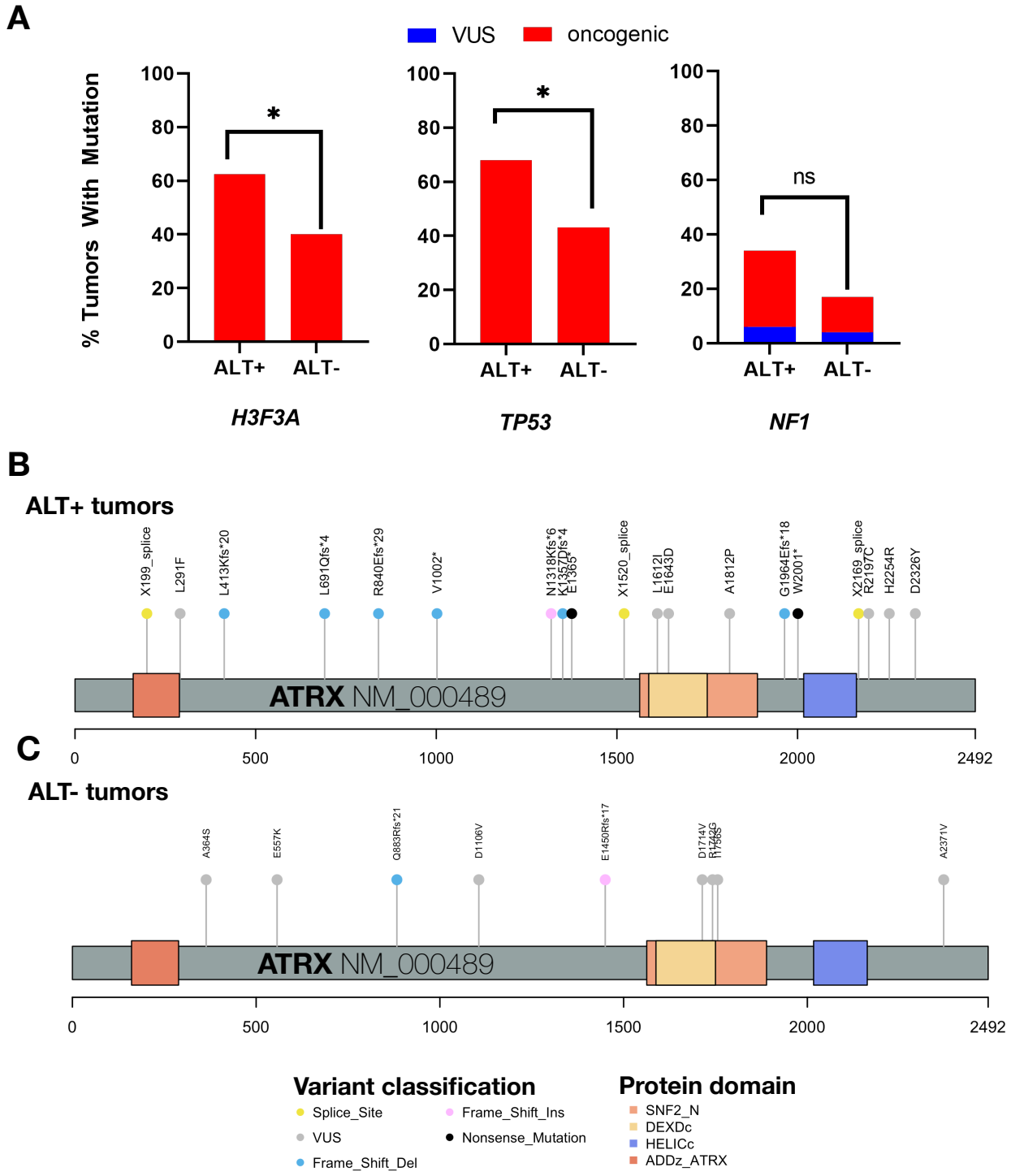


Figure S2. ALT+ HGATs have a higher prevalence of likely oncogenic ATRX mutations and higher TMB. (**A**) Barplots of ALT+/- tumors with somatic mutations in *H3F3A, TP53, or NF1.* Lollipop diagrams of protein changes due to *ATRX* mutations in ALT+ (**B**) or ALT- (**C**) HGAT tumors. Colored variant classifications were all categorized as “likely oncogenic”, while those in grey were categorized as “VUS”, by oncoKB. The ATRX protein and its domains are also labeled. Of note, each mutation was unique to one sample.

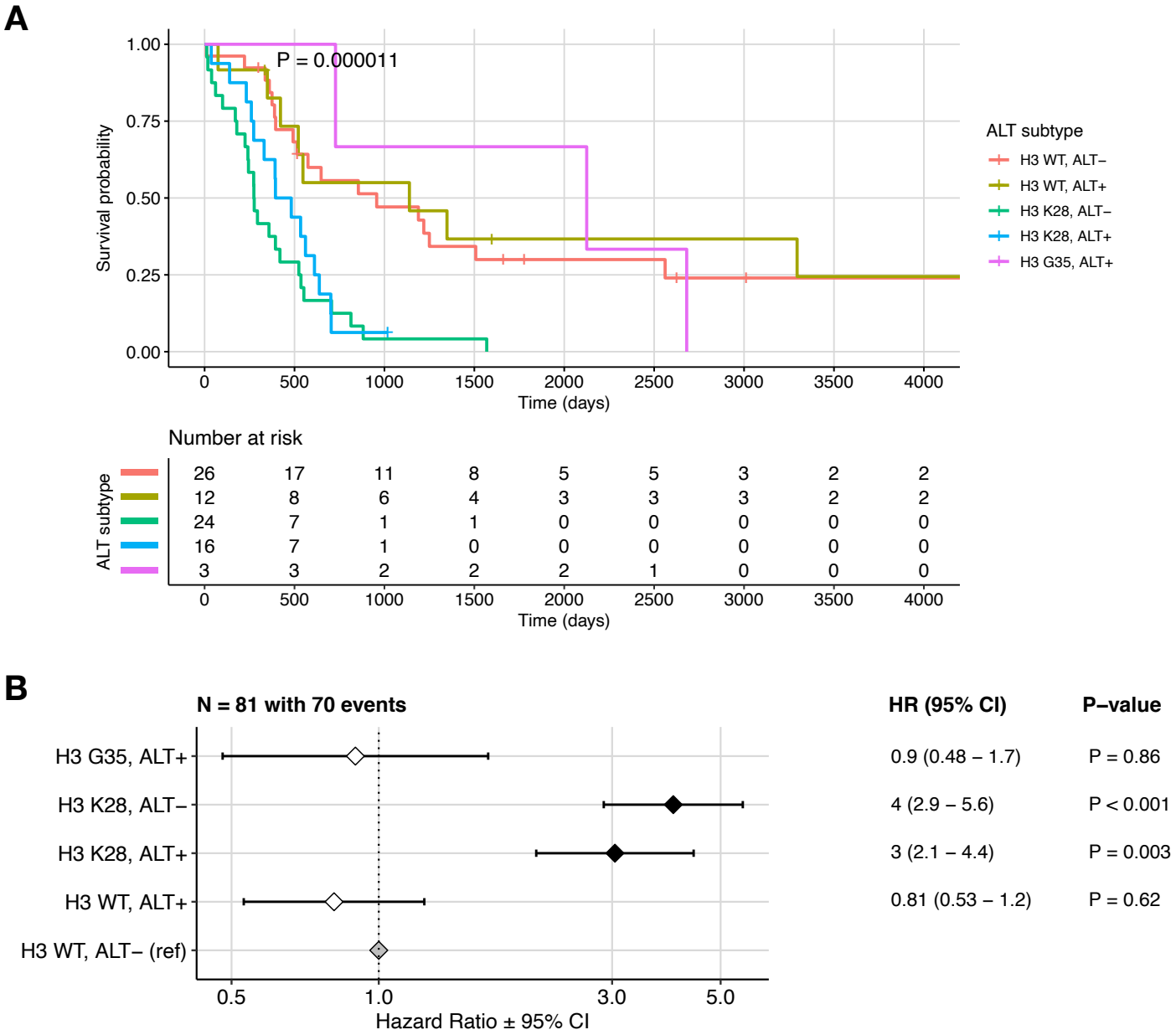


Figure S3. ALT status alone is not a prognostic indicator for pediatric HGATs. (**A**) There is a significant prognostic risk for H3 K28-mutant HGATs, compared to H3 wild-type (WT) or H3 G35-mutant tumors (Kaplan-Meier log-rank test p = 1.1e-5). Cox regression was performed on the same subgroups. Hazard Ratios (HR), 95% confidence intervals (CI), and p-values are plotted in (**B**) versus the reference H3 WT, ALT- tumors.