

Genetic Studies Identify Critical Biomarkers and Refine the Classification of Malignant
Gliomas

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Pathology in the Graduate School
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ABSTRACT

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Abstract

Gliomagenesis is driven by a complex network of genetic alterations and while the glioma genome has been a focus of investigation for many years; critical gaps in our knowledge of this disease remain. The identification of novel molecular biomarkers remains a focus of the greater cancer community as a method to improve the consistency and accuracy of pathological diagnosis. In addition, novel molecular biomarkers are drastically needed for the identification of targets that may ultimately result in novel therapeutics aimed at improving glioma treatment. Through the identification of new biomarkers, laboratories will focus future studies on the molecular mechanisms that underlie glioma development. Here, we report a series of genomic analyses identifying novel molecular biomarkers in multiple histopathological subtypes of glioma and refine the classification of malignant gliomas. We have completed a large scale analysis of the WHO grade II-III astrocytoma exome and report frequent mutations in the chromatin modifier, alpha thalassemia mental retardation x-linked (*ATRX*), isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*), and mutations in tumor protein 53 (*TP53*) as the most frequent genetic mutations in low grade astrocytomas. Furthermore, by analyzing the status of recurrently mutated genes in 363 brain tumors, we establish that highly recurrent gene mutational signatures are an effective tool in stratifying homogeneous patient populations into distinct groups with varying outcomes, thereby

capable of predicting prognosis. Next, we have established mutations in the promoter of telomerase reverse transcriptase (*TERT*) as a frequent genetic event in gliomas and in tissues with low rates of self renewal. We identify *TERT* promoter mutations as the most frequently mutated gene in primary glioblastoma. Additionally, we show that *TERT* promoter mutations in combination with *IDH1* and *IDH2* mutations are able to delineate distinct clinical tumor cohorts and are capable of predicting median overall survival more effectively than standard histopathological diagnosis alone. Taken together, these data advance our understanding of the genetic alterations that underlie the transformation of glial cells into neoplasms and we provide novel genetic biomarkers and multi-gene mutational signatures that can be utilized to refine the classification of malignant gliomas and provide opportunity for improved diagnosis.

Dedication

This work is dedicated to all people whose lives are touched by brain tumors.

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1. Introduction to this dissertation

1.1 Non-exclusive distribution license

This dissertation contains reproductions of tables, figures, and excerpts from published articles. These tables, figures, and excerpts from published work are reproduced in accordance with the publishers policies as described in Appendix A. Work in this dissertation was primarily performed by the candidate but was aided in many cases by collaborators and colleagues as described in the Acknowledgements.

1.2 Gliomas are a major health challenge

Gliomas, or tumors arising from the glial cells in the brain, have been at the forefront of clinical cancer research for decades. Glial cells, primarily made up of astrocytes, oligodendrocytes, ependymal cells, and microglia, represent the non-neuronal supporting cells of the brain and are present in numbers 10-50 times greater than those of neurons (Kandel, Schwartz et al. 2000, Bear, Connors et al. 2001). Tumors of the CNS are named according to the histological features of their cells and are graded based on histological criteria established by the World Health Organization (WHO) (Louis, Ohgaki et al. 2007). Tumors are classified from grade I to grade IV, with low grade tumors (WHO grade I and II) traditionally representing well differentiated tumors often resulting in better prognosis. High grade tumors (WHO grade III and IV) typically represent tumors that are undifferentiated or contain anaplastic features and clinically, these tumors often result in poor prognosis. The work contained herewithin will

primarily assess the genetic profiles of four major histological subtypes of gliomas: astrocytomas, oligodendrogliomas, oligoastrocytomas, and glioblastomas.

Diffuse astrocytoma's, tumors primarily derived from astrocytes, range from low to high grade tumors and are largely infiltrative (López, Cummings et al. 2001, Prados and American Cancer Society. 2002, Bigner, McLendon et al. 2006, Louis, Ohgaki et al. 2007). Pilocytic astrocytomas, WHO grade I tumors, do not progress to higher grade tumors and are thought of as favorable prognostic disease entities. Typically, these tumors appear in children or young adult populations and studies have shown pilocytic astrocytomas harbor genetic alterations distinct from WHO grade II-IV astrocytomas (Prados and American Cancer Society. 2002, Korshunov, Meyer et al. 2009, Jones, Hutter et al. 2013). WHO grade II-III tumors represent a unique subtype of astrocytomas, typically occurring in patients in their third decade of life. While these tumors have been understudied compared to the WHO grade IV glioblastomas, a series of mutations have been associated with increasing frequency during astrocytoma progression. Specifically, mutations in the tumor suppressor *TP53*, mutations in *PIK3CA*, deletions in *CDKN2A*, amplification of *CDK4*, and, overexpression of *PDGFR* have been reported in astrocytoma cohorts (Guha, Dashner et al. 1995, Ohgaki, Schauble et al. 1995, Watanabe, Sato et al. 1997, Ichimura, Bolin et al. 2000, Rasheed, Herndon et al. 2002, Broderick, Di et al. 2004, Martinho, Longatto-Filho et al. 2009).

Oligodendrogliomas are largely circumscribed, slow growing tumors, thought to derive from oligodendrocytes (López, Cummings et al. 2001, Prados and American Cancer Society. 2002, Bigner, McLendon et al. 2006, Louis, Ohgaki et al. 2007). Typically, these tumors are associated with a better overall prognosis and often respond to conventional therapies. Loss of chromosomal arms 1p and 19q are a frequent event in oligodendrogliomas, occurring in approximately 60-70% of patients, and is commonly used as the gold standard test for neuropathologists' in oligodendroglioma diagnosis (Reifenberger, Reifenberger et al. 1994, Bigner, Matthews et al. 1999, Smith, Perry et al. 2000, Jenkins, Blair et al. 2006, Vogazianou, Chan et al. 2010). Mutations in PIK3CA have also been reported as a frequent genetic event in high grade oligodendrogliomas (Broderick, Di et al. 2004). Patients whose tumors exhibit the characteristic loss of 1p and 19q often respond favorably to chemotherapy and radiotherapy. Furthermore, this chromosomal loss has been shown as a favorable prognostic marker among all gliomas (Cairncross, Ueki et al. 1998, Intergroup Radiation Therapy Oncology Group, Cairncross et al. 2006, Vogazianou, Chan et al. 2010). Oligoastrocytomas, contain histological features of both oligodendrogliomas and astrocytomas, presenting challenges to neuropathologists' as diagnoses among institutions may vary (López, Cummings et al. 2001, Prados and American Cancer Society. 2002, Louis, Ohgaki et al. 2007). As their histological name suggests, the molecular markers that define oligoastrocytomas remain challenging, as tumors often harbor events representative of both astrocytomas,

mutations in *TP53*, and oligodendrogliomas, loss of chromosomal arms 1p and 19q (Mueller, Hartmann et al. 2002, Kim, Nobusawa et al. 2010). A drastic need for molecular markers that are capable of delineating distinct subgroups of these tumors remains.

WHO grade IV astrocytomas, classified as glioblastoma, represent the most common primary malignant brain tumor in adults and the clinically most aggressive form of glioma. Glioblastomas are largely infiltrative, often invading surrounding tissue, and upon gross examination contain large necrotic regions (López, Cummings et al. 2001, Prados and American Cancer Society. 2002, Louis, Ohgaki et al. 2007).

Glioblastomas are commonly classified in two categories, primary or secondary. Primary glioblastomas are WHO grade IV tumor that arise de novo with no prior history upon presentation to the clinic, while secondary glioblastomas are tumors that progress from lower grade lesions.

In 2008, two studies undertook the challenge of profiling the glioblastoma genome, these studies were conducted by the large multi-institute network referred to as The Cancer Genome Atlas (TCGA) and a collaborative group of researchers from multiple universities including Duke University, Baylor College of Medicine, and Johns Hopkins University (Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008). While both studies unearthed novel and distinct findings, frequently mutated pathways emerged including, alterations in RTK/RAS/PI3K (88%), TP53 (87%), and RB (78%). Based on the mutational and molecular profiles of the tumors analyzed in these cohorts,

future studies have begun to classify glioblastomas based on expression profiles, identifying four major subtypes: neural, proneural, mesenchymal, and classical, in hopes of establishing molecular subtypes of glioblastoma that may prove use for future clinical stratification (Verhaak, Hoadley et al. 2010). These studies have provided a glimpse into the complex network of genetic abnormalities in glioblastoma, showing tumors are often targeted simultaneously by somatic mutation, copy number alterations, and changes in gene expression thus making them difficult to treat clinically. While these studies have contributed to the general knowledge of glioma genetics, a major challenge remaining is the lack of specific and actionable drug targets that can be applied to large glioma patient populations.

The identification of frequent mutations in isocitrate dehydrogenase 1 (*IDH1*), a gene which catalyzes the oxidative decarboxylation of isocitrate to α -ketoglutarate, were originally identified in GBMs (Geisbrecht and Gould 1999, Parsons, Jones et al. 2008). Remarkably, all of the identified mutations in *IDH1* occurred at the same hotspot residue, arginine 132, most resulting in a change to histidine (R132H). Tumors bearing these R132H mutations appear to represent a biologically distinct subgroup comprising secondary GBM and primary GBMs with a protracted clinical course (Parsons, Jones et al. 2008). Further investigation has led to the identification of mutations in *IDH1* and *IDH2* as the most frequently mutated gene in low grade gliomas and these mutations are associated with younger age at diagnosis and improved survival (Balss, Meyer et al.

2008, Yan, Parsons et al. 2009). Specifically, *IDH1* and *IDH2* was mutated in over 79% of grade II-III astrocytomas, 100% of grade II-III oligoastrocytomas, and 89% of grade II-III oligodendrogliomas (Yan, Parsons et al. 2009). A functional consequence of the *IDH1/2* mutations is a neomorphic enzymatic activity that results in the production of 2-hydroxyglutarate, which has been implicated as an oncometabolite in brain tumors and leukemia (Dang, White et al. 2009, Reitman and Yan 2010, Ward, Patel et al. 2010). The significance of the *IDH1* and *IDH2* mutations remains under investigation today as more evidence of IDH mutations in numerous subtypes of cancers is revealed (Sjoblom, Jones et al. 2006, Kang, Kim et al. 2009, Mardis, Ding et al. 2009, Ward, Patel et al. 2010, Amary, Bacsi et al. 2011, Pansuriya, van Eijk et al. 2011, Borger, Tanabe et al. 2012). Recurrent mutations in *IDH1* R132 and its homologous residue *IDH2* R172 have provided promise for their use as an actionable biomarker and potential impact as a prognostic tool (Yen, Bittinger et al. 2010). The frequency and specificity of these mutations have led us to investigate its potential in combination with other genetic alterations as a biomarker in major subtypes of glioma.

Gliomas represent a major health challenge as more than 14,000 new cases are diagnosed each year in the United States (Wen and Kesari 2008). Despite years of research including advances in genetic profiles, surgery, radiation, and chemotherapy, prognosis ultimately remains dismal, typically measured in months (Wen and Kesari 2008, Dolecek, Propp et al. 2012). Genetic studies, including microarray analysis, whole

exome sequencing, whole genome sequencing, epigenetic profiling, and RNAseq, aimed at elucidating the fundamental genetic aberrations that result in the transformation of glial cells into neoplastic tissue has remained a priority of the larger brain tumor scientific community. A drastic need for novel molecular biomarkers that can be translated into new diagnostic and pharmacologic therapies for gliomas patients remains. Novel molecular biomarkers specific to gliomas will not only refine their classification but will aid the greater neuro-oncology community to provide diagnostic tools that will standardize brain tumor diagnoses among institutions. Studies focusing on the identification on novel alterations in gliomas will be critical to improving management of this deadly disease.

2. The Genetic Landscape of Anaplastic Astrocytoma

2.1 Introduction

“Cancer is, in essence, a genetic disease” (Vogelstein and Kinzler 2004) where cancer cells undergo continuous acquisition of mutations that can manifest in many forms including, but not limited to, single base pair mutations, deletions, amplifications, and translocations (Vogelstein and Kinzler 2004, Stratton, Campbell et al. 2009). Recently, advances in genomic technologies have permitted the interrogation of the glioma genome at a rapid pace. Studies have focused on copy number alterations, changes in gene expression, sequence alterations, and methylation patterns, all of which contribute to the evolving picture of refining glioma classification (Phillips, Kharbanda et al. 2006, Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008, Ohgaki and Kleihues 2009, Noushmehr, Weisenberger et al. 2010). The grade II tumors, which include diffuse astrocytomas (A2) and well-differentiated oligodendrogliomas (O2), are slow growing and tend to progress into grade III tumors. The grade III tumors include anaplastic astrocytomas (A3) and anaplastic oligodendrogliomas (O3), and are faster growing and more aggressive. These tumors often invade neighboring tissue and are able to progress into grade IV secondary glioblastoma multiforme (GBM). Primary GBM is a genetically and clinically unique disease which arises de novo. In clinics, it is often very difficult to distinguish a GBM from a contrast-enhancing A3 lesion by magnetic resonance imaging or histopathology alone. In addition to these purely astrocytic

tumors, there are also grade II and grade III oligoastrocytomas which present with histocytological appearances of oligodendrogliomas and astrocytomas, which can also progress to GBMs.

The fatal nature of GBMs together with the availability of only a few minimally efficacious FDA approved treatment modalities led to the ambitious undertaking of sequencing the GBM genome in the hopes of finding unique genetic alterations to help classify tumors and identify potential therapeutic targets (Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008, Sturm, Witt et al. 2012, Frattini, Trifonov et al. 2013). While this pursuit has proved fruitful in identifying several key genes involved in GBM tumorigenesis, relatively little has been done for the genome wide sequencing of progressive astrocytomas, including A2s, A3s, and secondary GBMs. In an effort to establish the genetic landscape of progressive astrocytomas, we have sequenced the exome of a total of 57 gliomas, 30 of which were progressive astrocytomas or oligoastrocytomas (A2, n=7; A3, n=16; OA2, n = 2; OA3, n = 4; secondary GBM, n=1) and 27 of which were primary GBMs. Our study revealed that mutations in *IDH1*, *ATRX*, and *TP53* are the most frequent genetic alterations in progressive astrocytomas. Novel alterations, including recurrent mutations in *PIK3R1* and Notch family genes (*NOTCH1*, *NOTCH2*, *NOTCH4*, *NOTCH2NL*) are also present in the *IDH1*-mutated astrocytomas. Additionally, our data further supports that on a genetic level, primary GBMs displayed distinct mutation spectrums differing from those of progressive astrocytomas.

2.2 Methods

2.2.1 Sample Collection and Processing

Tumor samples and corresponding clinical information were obtained with consent and Institutional Review Board approval from the Preston Robert Tisch Brain Tumor Center BioRepository at Duke University in accordance with the Health Insurance Portability and Accountability Act. Tissue sections were reviewed by board certified neuropathologists to confirm diagnosis and to ensure sections contain $\geq 95\%$ tumor cells. DNA was extracted from snap frozen tumors and normal blood in 16 grade III astrocytomas, 7 grade II astrocytomas, 2 grade II Oligoastrocytomas, 4 grade III Oligoastrocytomas, and 28 glioblastomas and processed for exome sequencing. Secondary GBM designates tumors which were resected > 1 year after a prior diagnosis of a low grade glioma (Grade II-III).

2.2.2 Methods for Cancer Genome Analysis

Genomic purification, library construction, exome capture, next generation sequencing, and bioinformatic analyses of tumor and normal samples were performed at Personal Genome Diagnostics (Baltimore, MD). In brief, genomic DNA from tumor and normal samples were fragmented and used for Illumina TruSeq library construction (Illumina, San Diego, CA). Exonic regions were captured in solution using the Agilent SureSelect 51 Mb kit (version 4) according to the manufacturer's instructions (Agilent, Santa Clara, CA). Paired-end sequencing, resulting in 100 bases from each end of the fragments, was performed using a HiSeq 2000 Genome Analyzer (Illumina, San Diego,

CA). The tags were aligned to the human genome reference sequence (hg18) using the Eland algorithm of CASAVA 1.7 software (Illumina, San Diego, CA). The chastity filter of the BaseCall software of Illumina was used to select sequence reads for subsequent analysis. The ELAND algorithm of CASAVA 1.7 software (Illumina, San Diego, CA) was then applied to identify point mutations and small insertions and deletions. Known polymorphisms recorded in dbSNP were removed from the analysis. Potential somatic mutations were filtered and visually inspected as described previously (Jones, Wang et al. 2010).

2.2.3 Mutation Validation

Genes which contain mutations in 3 or more tumors were selections for mutational validation utilizing Sanger sequencing technologies as described previously (Sjoblom, Jones et al. 2006), accounting for 255 mutations. All PCR Primers were designed using Primer3 to generate PCR products of 300-500 bases.

2.3 Results

2.3.1 Exome sequencing of astrocytic tumors

To establish the genetic landscape of progressive astrocytomas, we sequenced matched tumor and normal blood pairs for 57 progressive astrocytomas and GBM exomes from DNA samples collected at the Preston Robert Tisch Brain Tumor Center at Duke University (Table 1). Utilizing the Agilent SureSelect Exome platform, libraries on average yielded 18.2G bases with 94.5% of targeted regions represented by at least 10

high quality reads (Table 2). Our sample cohort including 23 A2s and A3s, 6 OA2s and OA3s, and 28 GBMs identified 2,003 somatic mutations and 645 copy number alterations. To assess the accuracy of our mutation calling criteria, 255 mutations were selected for verification, accounting for all genes that were mutated in 3 or more tumors. 92% of these mutations could be successfully amplified via Sanger sequencing, of these, 95% were verified.

Table 1: Clinical Characteristics of Exome Sequencing Cohort (n=57)

Case ID	Age	Gender	Survival (Months)	Tumor grade	Diagnosis	Survival Status at Last Follow-Up
P37	26.36	Male	25	3	Astrocytoma	Alive
P81	35.61	Female	65	3	Astrocytoma	Deceased
P82	32.2	Female	35	3	Astrocytoma	Deceased
P83	38.2	Male	51	3	Astrocytoma	Deceased
P84	39.3	Male	66	3	Astrocytoma	Deceased
P103	7.61	Male	164	2	Astrocytoma	Alive
P104	51.69	Male	9	2	Astrocytoma	Deceased
P106	30.21	Female	73	2	Astrocytoma	Deceased
P107	5.8	Female	147	2	Astrocytoma	Alive
P108	35.28	Male	20	2	Astrocytoma	Alive
P109	0.73	Male	112	2	Astrocytoma	Alive
P110	30.66	Male	56	2	Astrocytoma	Alive
P111	38.09	Male	6	3	Astrocytoma	Alive
P112	34.31	Male	12	3	Astrocytoma	Alive
P114	61.05	Male	37	3	Astrocytoma	Deceased
P115	35.66	Male	42	3	Astrocytoma	Deceased
P116	56.41	Male	35	3	Astrocytoma	Deceased
P117	32	Female	29	3	Astrocytoma	Deceased
P118	46.72	Male	206	3	Astrocytoma	Deceased
P119	25.53	Male	167	3	Astrocytoma	Deceased
P121	57.59	Male	14	3	Astrocytoma	Deceased
P122	55.2	Male	22	3	Astrocytoma	Deceased
P123	29.61	Male	23	3	Astrocytoma	Deceased

P124	36.11	Male	0.2	4	Glioblastoma	Unknown
P125	38	Male	53.1	2	Oligoastrocytoma	Alive
P126	34	Male	10.5	2	Oligoastrocytoma	Alive
P127	65	Male	62.7	3	Oligoastrocytoma	Alive
P128	34	Female	31.4	3	Oligoastrocytoma	Alive
P129	32	Male	24.97	3	Oligoastrocytoma	Alive
P130	42.04	Male	1	3	Oligoastrocytoma	Deceased
P132	30	Female	27	4	Glioblastoma	Alive
P133	28	Male	50	4	Glioblastoma	Deceased
P134	31	Male	19	4	Glioblastoma	Alive
P141	36	Male	46	4	Glioblastoma	Deceased
P142	62	Male	13	4	Glioblastoma	Deceased
P143	44	Female	23	4	Glioblastoma	Deceased
P144	63	Female	14	4	Glioblastoma	Deceased
P145	68	Female	10	4	Glioblastoma	Deceased
P146	48	Male	14	4	Glioblastoma	Deceased
P147	44	Male	9	4	Glioblastoma	Deceased
P148	42	Male	28	4	Glioblastoma	Deceased
P149	59	Male	10	4	Glioblastoma	Deceased
P150	51	Male	20	4	Glioblastoma	Deceased
P151	44	Male	1	4	Glioblastoma	Deceased
P152	46	Male	22	4	Glioblastoma	Deceased
P153	58	Male	8	4	Glioblastoma	Deceased
P154	66	Male	7	4	Glioblastoma	Deceased
P155	57	Male	23	4	Glioblastoma	Deceased
P156	54	Male	16	4	Glioblastoma	Deceased
P157	79	Male	25	4	Glioblastoma	Deceased
P158	65	Female	31	4	Glioblastoma	Deceased
P159	62	Male	2	4	Glioblastoma	Deceased
P160	80	Female	4	4	Glioblastoma	Deceased
P161	29	Female	119	4	Glioblastoma	Alive
P162	47	Female	26	4	Glioblastoma	Deceased
P163	76	Male	-	4	Glioblastoma	Unknown
P164	52	Male	9	4	Glioblastoma	Deceased

Table 2: Summary of Whole Exome Sequencing Statistics (n=57)

Case	Tumor/ Normal	Filtered Bases (G)	Mapped to Genome	Bases Mapped to target region (G)	Percent Mapped to target region	Targeted bases with at least 10 reads (M)	Targeted bases with at least 10 reads (%)	Total Coverage			Distinct Coverage		
								Average Raw Coverage	Average High Quality Coverage	Effective Coverage	Average Raw Coverage	Average High Quality Coverage	Effective Coverage
P37	Tumor	7.62	83%	2.04	27%	43.45	86%	40.5	37.9	93%	37.7	35.4	93%
P37	Normal	9.5	84%	2.5	26%	45.15	90%	49.6	48.2	95%	45.3	44.1	95%
P81	Tumor	10.6	88%	6.18	58%	47.14	94%	122.6	110.8	96%	95.9	88.2	95%
P81	Normal	8.55	88%	5.14	60%	46.87	93%	102.1	98.4	96%	89.1	86.1	96%
P82	Tumor	9.07	87%	5.39	60%	46.76	93%	107.1	96.6	95%	89.5	81.8	95%
P82	Normal	9.23	87%	5.43	59%	47.41	94%	107.8	103.5	96%	94.3	90.9	96%
P83	Tumor	10.45	87%	6.32	61%	47.14	94%	124.6	112.1	96%	97.7	89.5	95%
P83	Normal	8.31	86%	4.63	56%	46.63	93%	91.4	87.4	96%	79.5	76.4	95%
P84	Tumor	9.02	87%	5.45	60%	47.31	94%	107.3	96.1	96%	90	81.7	96%
P84	Normal	8.84	87%	5.27	60%	46.87	93%	103.8	99.7	96%	90.6	87.4	96%
P103	Tumor	6.73	86%	3.69	55%	45.86	91%	73.2	69.3	95%	59.4	56.6	94%
P103	Normal	5.86	87%	3.32	57%	46.24	92%	65.8	64	96%	56	54.7	95%
P104	Tumor	6.65	86%	3.62	54%	45.61	91%	71.8	68.4	95%	54.4	52.3	94%
P104	Normal	8.42	86%	4.65	55%	46.72	93%	92.3	90.5	96%	69.9	68.8	95%
P105	Tumor	7.23	87%	3.73	52%	45.91	91%	74.2	69.3	95%	57.7	54.6	94%
P105	Normal	7.59	87%	4.09	54%	45.89	91%	81.2	79.1	95%	60.5	59.3	94%
P106	Tumor	7.76	87%	3.76	48%	45.85	91%	74.7	70.1	95%	63.2	59.8	94%
P106	Normal	8.92	86%	4.54	51%	46.55	92%	90.2	87.9	96%	74.9	73.2	95%
P107	Tumor	10.13	87%	5.24	52%	46.9	93%	103.3	91.6	96%	90.7	81.3	95%
P107	Normal	13.6	88%	7.64	56%	47.45	94%	150.5	144.3	96%	115.9	112	96%

P108	Tumor	6.51	87%	3.59	55%	45.83	91%	71	63.7	94%	63.5	57.5	94%
P108	Normal	6.43	87%	3.58	56%	46.03	91%	70.8	67.7	95%	65.5	62.8	95%
P109	Tumor	8.25	88%	4.54	55%	46.38	92%	89.5	81.7	95%	80.3	73.9	95%
P109	Normal	8.66	88%	4.92	57%	46.99	93%	97	93.5	96%	85.3	82.5	96%
P110	Tumor	8.51	88%	4.68	55%	46.51	92%	92.5	84.3	95%	79.2	72.9	95%
P110	Normal	7.44	88%	4.32	58%	46.4	92%	85.3	82.3	95%	72.9	70.5	95%
P111	Tumor	8.49	88%	4.83	57%	46.48	92%	95.4	85.8	95%	84.1	76.4	95%
P111	Normal	8.17	88%	4.85	59%	46.64	93%	95.7	91.6	96%	77.5	74.7	95%
P112	Tumor	8.9	88%	4.78	54%	46.33	92%	94.5	84.7	95%	81.4	73.7	95%
P112	Normal	10.3	88%	5.73	56%	46.85	93%	113.1	108.2	96%	91.9	88.5	95%
P114	Tumor	8.72	87%	4.83	55%	46.74	93%	95.2	85.7	95%	82.4	75	95%
P114	Normal	9.03	88%	5	55%	46.64	93%	98.5	94.8	96%	85.6	82.7	95%
P115	Tumor	7.54	88%	4.38	58%	46.12	92%	86.3	78.6	95%	65.8	60.9	94%
P115	Normal	8.32	88%	4.59	55%	46.67	93%	90.6	87	96%	79.4	76.5	95%
P116	Tumor	11.61	88%	6.56	57%	47.25	94%	129.3	117.1	96%	107.5	98.6	96%
P116	Normal	11.51	89%	6.26	54%	47.51	94%	123.6	118.3	96%	106	102	96%
P117	Tumor	5.82	87%	3.44	59%	44.97	89%	68.2	64.1	93%	55.1	52.3	93%
P117	Normal	6.12	88%	3.6	59%	46.02	91%	71.4	69.5	95%	63.7	62.2	95%
P118	Tumor	6.91	87%	4.09	59%	45.87	91%	80.8	75.9	95%	66.9	63.4	94%
P118	Normal	6.92	88%	4.15	60%	46.45	92%	82	79.9	95%	69.5	67.9	95%
P119	Tumor	9.21	88%	5.4	59%	47	93%	106.4	95	96%	85.3	77.4	95%
P119	Normal	9.02	88%	5.19	58%	46.47	92%	102.4	97.9	95%	81.5	78.4	95%
P121	Tumor	8.6	88%	4.7	55%	46.43	92%	92.8	82.8	95%	81.1	73.2	95%
P121	Normal	9.19	89%	5.02	55%	46.86	93%	99.1	94.6	96%	86.3	82.7	96%
P122	Tumor	10.39	88%	5.87	57%	46.26	92%	116	103.4	95%	77.6	71.4	94%
P122	Normal	10.17	88%	5.46	54%	46.85	93%	107.8	102.7	96%	91.7	87.9	96%

P123	Tumor	8.34	90%	4.89	59%	47.63	95%	96.4	85.1	96%	86.3	76.9	96%
P123	Normal	8.32	88%	4.7	57%	46.79	93%	92.7	88.3	96%	80.5	77	96%
P124	Tumor	9.9	92%	4.73	48%	47.57	94%	93.4	83.2	96%	83.8	75.3	96%
P124	Normal	10.78	92%	6.35	59%	48.37	96%	124.9	119.2	97%	114.2	109.3	97%
P125	Tumor	8.25	91%	4.41	53%	47.08	94%	87	77.7	96%	79.2	71.3	96%
P125	Normal	8.32	92%	4.65	56%	47.87	95%	91.7	87.3	97%	84.1	80.3	97%
P126	Tumor	9.1	92%	4.9	54%	47.37	94%	96.8	86.1	96%	91.2	81.5	96%
P126	Normal	9.48	91%	5.32	56%	47.91	95%	104.8	99.6	97%	97.6	93	97%
P127	Tumor	8.47	91%	4.52	53%	47.49	94%	89.1	78.8	96%	83.5	74.3	96%
P127	Normal	10.15	91%	5.59	55%	48.09	96%	110.2	104.7	97%	103.5	98.6	97%
P128	Tumor	7.92	91%	4.41	56%	46.99	93%	87.2	76.6	95%	80.5	71.2	95%
P128	Normal	10.16	90%	5.85	58%	47.93	95%	115.5	109	97%	102.6	97.3	97%
P129	Tumor	8.56	91%	4.7	55%	47.21	94%	92.7	81.7	96%	85.5	75.9	96%
P129	Normal	10.13	91%	5.82	57%	48	95%	114.7	108	97%	105.8	99.9	97%
P130	Tumor	8.11	92%	4.33	53%	47.63	95%	85.6	72.6	96%	80.5	68.8	96%
P130	Normal	8.09	91%	4.73	59%	47.3	94%	93.4	87.6	96%	86.2	81.2	96%
P132	Tumor	10.18	91%	5.47	54%	47.68	95%	107.9	92.1	96%	100.8	86.7	96%
P132	Normal	6.7	91%	3.64	54%	47.29	94%	71.8	66.8	96%	66.7	62.2	96%
P133	Tumor	7.95	91%	4.43	56%	46.9	93%	87.4	77.4	96%	81.9	73	95%
P133	Normal	9.43	92%	5.3	56%	48.07	95%	104.4	98.6	97%	96.5	91.5	97%
P134	Tumor	11.02	91%	5.21	47%	47.67	95%	102.8	90.5	96%	97.4	86.2	96%
P134	Normal	7.94	91%	4.44	56%	47.71	95%	87.4	82.7	97%	82.2	77.9	97%
P141	Tumor	8.84	88%	4.79	54%	46.59	93%	94.4	81.1	95%	84.7	73.6	95%
P141	Normal	8.72	88%	4.78	55%	46.35	92%	94.5	87.6	95%	84.5	78.9	95%
P142	Tumor	10.35	88%	5.63	54%	46.93	93%	111	95.4	95%	97.1	84.7	95%
P142	Normal	8.64	88%	5.01	58%	46.91	93%	98.8	91.5	96%	82	76.6	95%

P143	Tumor	9.38	89%	5.01	53%	46.76	93%	99	88.8	95%	90	81.3	95%
P143	Normal	8.44	88%	4.52	54%	46.52	92%	89.2	85.3	95%	76.5	73.5	95%
P144	Tumor	7.98	88%	4.11	51%	46.58	93%	81.1	72.3	95%	75.2	67.5	95%
P144	Normal	9.45	88%	5.25	56%	47.03	93%	103.5	98.8	96%	90.3	86.6	96%
P145	Tumor	8.47	89%	4.64	55%	47.09	94%	91.5	80.2	96%	83.2	73.6	96%
P145	Normal	9.76	88%	5.23	54%	46.9	93%	103.2	98.2	96%	92.5	88.3	96%
P146	Tumor	9.61	88%	5.6	58%	47.58	95%	110.2	97.2	96%	94.2	84.2	96%
P146	Normal	9.39	89%	5.11	54%	47.71	95%	100.5	94.8	97%	91.7	86.8	97%
P147	Tumor	10.63	90%	6.21	58%	47.63	95%	122.3	108.6	96%	103.4	93.1	96%
P147	Normal	7.65	91%	4.61	60%	47.53	94%	90.8	86.3	96%	51.1	49.3	96%
P148	Tumor	10.44	90%	5.82	56%	47.47	94%	114.7	101.7	96%	105.5	94.2	96%
P148	Normal	8.58	91%	5.08	59%	47.95	95%	99.9	94.8	97%	92.8	88.2	97%
P149	Tumor	7.77	88%	4.14	53%	46.46	92%	82.1	77.3	95%	56.1	53.6	95%
P149	Normal	9.87	88%	5.26	53%	47.24	94%	104.3	101.8	96%	72	70.7	96%
P150	Tumor	9.58	88%	4.99	52%	46.29	92%	98.9	92.3	95%	72.2	68.4	95%
P150	Normal	9.1	88%	4.91	54%	47.48	94%	97.3	94.4	97%	73.5	71.7	96%
P151	Tumor	8.38	88%	4.34	52%	46.16	92%	86.1	80.1	95%	66.1	62.4	95%
P151	Normal	10.47	88%	5.3	51%	46.63	93%	105.1	102.5	96%	80.3	78.6	95%
P152	Tumor	7.65	88%	3.88	51%	45.49	90%	76.9	73	94%	54.3	52.2	94%
P152	Normal	9.28	87%	4.66	50%	46.5	92%	92.5	90.6	96%	72.1	70.9	95%
P153	Tumor	9.11	88%	4.61	51%	45.89	91%	91.5	83.9	94%	59.7	56.1	94%
P153	Normal	10.14	88%	5.07	50%	46.48	92%	100.7	97.5	95%	75.8	73.9	95%
P154	Tumor	9.88	88%	4.89	49%	46.32	92%	97	89.1	95%	70.1	65.7	95%
P154	Normal	9.34	88%	4.58	49%	46.18	92%	90.9	88.1	95%	70.7	68.8	95%
P155	Tumor	9.63	88%	5.56	58%	46.92	93%	109.5	92.2	95%	86.5	74.7	95%
P155	Normal	9.64	88%	5.55	58%	47.48	94%	109.4	100.1	96%	94	86.6	96%

P156	Tumor	9.52	88%	5.32	56%	46.52	92%	104.9	88.9	95%	89.9	77.4	95%
P156	Normal	8.62	87%	4.87	57%	47.06	93%	96	87.7	96%	75.9	70.2	96%
P157	Tumor	8.34	89%	4.74	57%	46.12	92%	93.5	77	94%	80.3	67.4	94%
P157	Normal	10.35	88%	5.68	55%	46.62	93%	112.1	102.7	95%	94.3	87.1	95%
P158	Tumor	8.31	88%	4.49	54%	45.63	91%	88.8	73.8	94%	77	65	93%
P158	Normal	11.9	88%	6.71	56%	47.01	93%	132.5	121.4	96%	110.6	102.3	95%
P159	Tumor	8.94	88%	4.93	55%	46.5	92%	97.2	82.3	95%	85.2	73.1	95%
P159	Normal	10.84	88%	5.99	55%	46.94	93%	118.1	109.7	96%	99.5	93	96%
P160	Tumor	9.3	88%	5.1	55%	46.62	93%	100.6	85.1	95%	87.9	75.3	95%
P160	Normal	7.91	88%	4.51	57%	46.52	92%	89.1	82.8	95%	77.8	72.7	95%
P161	Tumor	9.4	89%	5.15	55%	46.14	92%	101.7	86	94%	85.8	73.8	94%
P161	Normal	9.71	88%	5.35	55%	46.65	93%	105.7	97.5	95%	86.8	80.7	95%
P162	Tumor	8.18	87%	4.44	54%	45.74	91%	87.7	73.8	94%	74.8	63.9	94%
P162	Normal	9.9	88%	5.25	53%	46.41	92%	103.9	95.7	95%	88.2	81.8	95%
P163	Tumor	9.5	88%	5.08	54%	46.74	93%	100.2	82.8	95%	86.3	72.5	95%
P163	Normal	9.07	88%	4.65	51%	46.37	92%	91.7	83.7	95%	79	72.5	95%
P164	Tumor	8.35	87%	4.51	54%	46.38	92%	88.8	74.2	95%	77.7	65.9	95%
P164	Normal	10.44	88%	5.81	56%	47.33	94%	114.3	104.5	96%	96.3	88.8	96%

2.3.2 Genetic alterations identified in A2s

Exome sequencing of seven A2s revealed 93 somatic mutations. On average, A2s contained 13 somatic mutations, with 92% of targeted regions covered by 10x high quality reads or more. *IDH1*, *ATRX*, and *TP53* were co-mutated in 3/7 (43%) of our patient cohort, and no other gene was mutated in more than one grade II astrocytoma (Figure 1). We did not find any high copy number gain or deletion by our methodology in A2s.

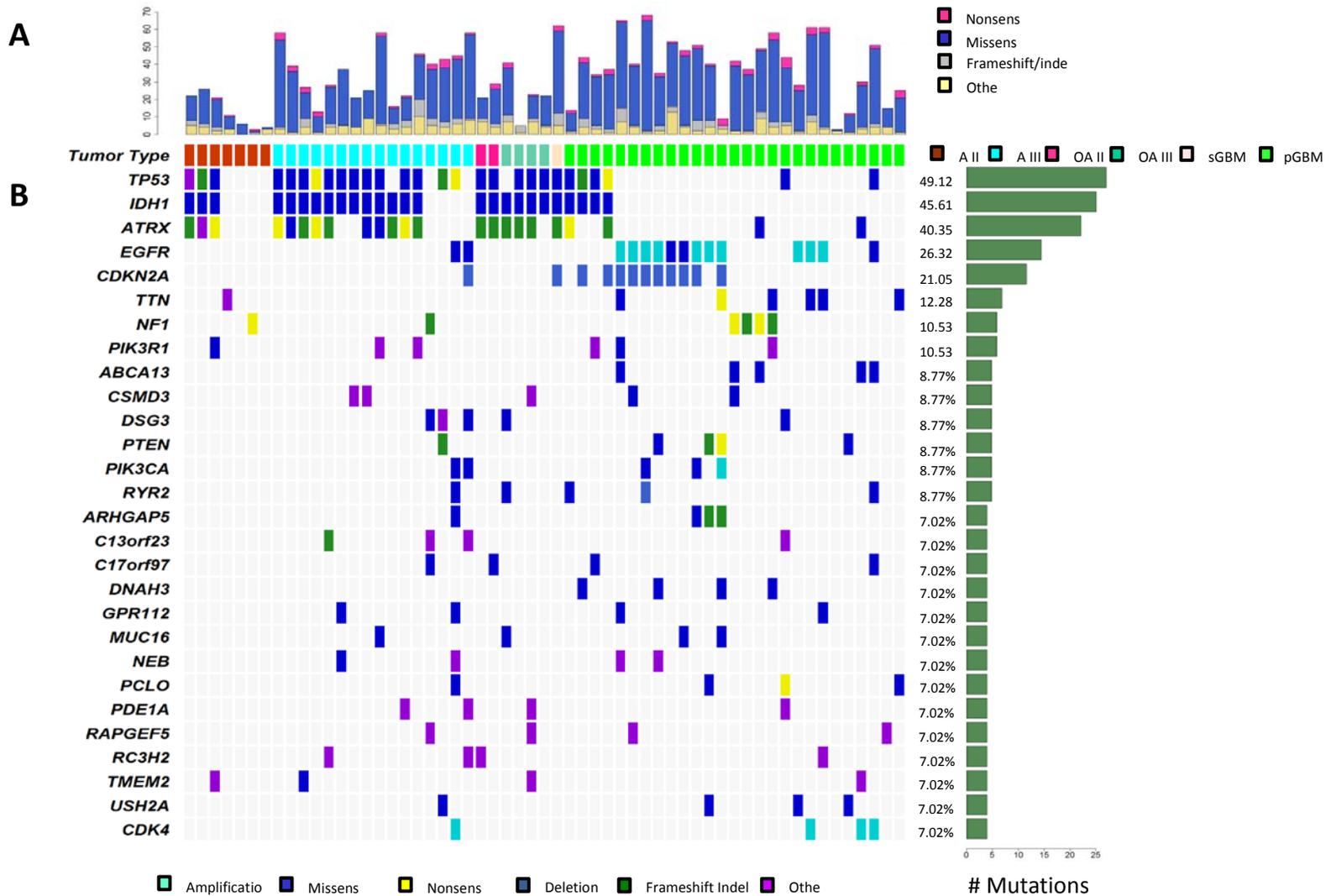


Figure 1: Genetic landscape of progressive astrocytomas.

Figure 1 (Continued): Genetic landscape of progressive astrocytomas.

Mutational analysis utilizing exome sequencing of matched tumor and normal pairs for 57 progressive astrocytomas, oligoastrocytomas, and GBMs. (A) Distribution of mutation types (Nonsense, Missense, Frameshift/Indel, Other) are reported for each tumor. (B) Depiction of Mutation Spectrum for each tumor is shown. Represented are genes which were mutated in four or more gliomas. The frequency (%) and number of gene alterations in the tumor cohort is represented on the right.

2.3.3 Genetic alterations identified in A3s

Exome sequencing of 16 A3s revealed 576 mutations. On average, A3s contained 36 somatic mutations with 92% of targeted regions covered by 10x high quality reads or more. Mutations in the genes *IDH1*, *ATRX*, and *TP53* were the most frequent events in A3s confirming previously published studies identifying them as critical astrocytoma derived mutations (Figure 1) (Yan, Parsons et al. 2009, Jiao, Killela et al. 2012, Kannan, Inagaki et al. 2012, Liu, Gerges et al. 2012). Copy number alterations were infrequent in A3s, only 3 of 16 tumors contained detectable alterations via exome analysis with a median of 22 genes targeted by copy number alterations (range: 2-47) in our cohort.

Notch signaling pathway disruption has been previously reported in low grade gliomas (Somasundaram, Reddy et al. 2005, Xu, Yu et al. 2009). Here, we report mutations in Notch pathway members in 5/16 (31%) A3s. *NOTCH1*, *NOTCH2*, *NOTCH4*, and *NOTCH2NL* were mutated in 2, 1, 1, and 1 cases, respectively. Notably, we observed a recurrent *NOTCH1* missense A465T mutation in 2 cases. This mutation resides within the extracellular epidermal growth factor-like (EGF) repeats of the Notch1 protein (Fiuza and Arias 2007, Gordon, Vardar-Ulu et al. 2007). We also observed two *PIK3CA* and two

PIK3R1 mutations in 16 A3 tumors. Sequencing also revealed a novel recurrent mutation in desmoglein 3 (*DSG3*), a calcium binding transmembrane glycoprotein present in desmosomes (Amagai, Ishii et al. 1995), in 3/16 (19%) of A3s, in 1/4 OA3s (25%), and in 1/28 (4%) GBMs. All five *DSG3* alterations occurred in *IDH1* wild type tumors.

Of great interest to the brain tumor community is the evolution of genetic mutations as astrocytomas progress to higher grade lesions. To this end we have performed exome sequencing on a pair of astrocytomas that progressed from A2 (Tumor P110) to an A3 (Tumor P112). While both tumors harbored the same number of mutations, the mutational spectrum was quite distinct. Two genes, *IDH1* and *ADRBK1*, contained mutations at the exact same residue in both tumors. Furthermore, both P110 and P112 contained *TP53* mutations. However, the mutation was located at a different residue within *TP53*, R273C in P110 and P177R in P112. These results suggest that mutations in *IDH1* are an early gene mutation, and that progressive tumors result from independent clonal expansions from a common *IDH1*-mutated population of cells.

2.3.4 Genetic alterations identified in OAs

OAs present a great diagnostic challenge to neuropathologists as they show histological properties of both astrocytomas and oligodendrogliomas (Louis, Ohgaki et al. 2007). Recently, studies have suggested that at least genetically, the majority of these “mixed histology” tumors contain genetic events representative of either astrocytomas, namely mutations in *IDH1*, *ATRX*, and *TP53*, or oligodendrogliomas, namely chr 1p/19q

LOH and mutations in *CIC* and/or *FUBP1* (Bettegowda, Agrawal et al. 2011, Jiao, Killela et al. 2012, Liu, Gerges et al. 2012, Yip, Butterfield et al. 2012). We assessed exomes of two OA2s and four OA3s, revealing 157 somatic mutations. On average OAs contained 26 somatic mutations (range: 21-41), and 94% of targeted regions were covered by 10x or more high quality reads. *IDH1* (100%, 6/6), *ATRX* (83%, 5/6), and *TP53* (83%, 5/6) were the most commonly mutated genes in this cohort (Figure 1).

2.3.5 Genetic alterations identified in GBMs

To compare the genetic landscape of A2, A3, OA2, OA3 and secondary GBMs to primary GBMs, we next sequenced the exome of 28 GBMs. Exome sequencing identified 1,177 somatic mutations. Primary GBMs on average contained 42 somatic mutations with 93% of targeted regions covered by 10x high quality reads or more. Copy number alterations were frequent in primary GBMs, averaging 32 events per tumor (range: 1-132).

Confirming previous studies, the EGFR/PTEN/PI(3)K pathway is the most frequently affected pathway in GBMs (Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008, Duncan, Killela et al. 2010, Verhaak, Hoadley et al. 2010, Brennan, Verhaak et al. 2013). We found frequent genetic alterations of *EGFR* in 13/28 (46%), *PTEN* mutations in 4/28 (14%), *PIK3CA* mutations in 3/28 (10%), and *PIK3R1* mutations in 3/28 (10%) of the GBMs (Figure 1). However, *EGFR*, *PIK3CA* and *PIK3R1* were also mutated in lower grade tumors (Figure 1). We found two *EGFR* (13%), two *PIK3CA*

(13%), two *PIK3R1* (13%), and one *PTEN* (7%) mutation from A3s, and one *PIK3R1* mutation from A2s. *PTEN*, *PIK3CA* and *PIK3R1* mutations were mutually exclusive. Furthermore, *PIK3CA* mutations were found exclusively in *IDH1*-wildtype tumors (P=.05, Fisher's exact test, two-tailed) whereas four of six *PIK3R1* mutations were found in the *IDH1* mutant subgroup (P=0.39, Fisher's exact test, two-tailed). Within our GBM cohort were five GBMs harboring mutations in *IDH1* and/ or *ATRX*, mutations typically associated with progressive astrocytomas.

2.4 Discussion

Despite decades of research, the prognosis for patients with malignant gliomas remains dismal. Recently, significant progress has been made in elucidating the genetic aberrations in GBMs (Phillips, Kharbanda et al. 2006, Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008, Verhaak, Hoadley et al. 2010, Sturm, Witt et al. 2012, Brennan, Verhaak et al. 2013, Frattini, Trifonov et al. 2013). We sought to determine the genetic landscape of A3s and compare the mutation spectrum to other subtypes of gliomas including A2s, OAs, and GBMs. Our results represent the largest scale of exomic sequencing of progressive astrocytomas to date. Here, we report that mutations in *IDH1*, *ATRX*, and *TP53* are particularly prevalent in A3s; whereas *EGFR* and *CDKN2A* were the most frequently altered genes in GBMs, a finding that corroborates previous studies (Wen and Kesari 2008, Ohgaki and Kleihues 2009, Jiao, Killela et al. 2012, Kannan, Inagaki et al. 2012). These findings will aid in improving the classification

of brain tumors, and the selection of patients with genetically homogeneous tumors for clinical trials.

Several genes not previously linked to gliomas were identified in this study. We found *DSG3* mutated in 19% of A3s, all of which do not harbor the *IDH1* mutation. *DSG3* has been reported to be expressed at high levels in head and neck squamous cell carcinoma, and has been implicated as a potential biomarker for detection of this cancer's lymph node metastases (Patel, Martin et al. 2013). Furthermore, within A3s we identified frequent mutations (5/16, 31%) in members of the Notch pathway (*NOTCH1*, *NOTCH2*, *NOTCH4*, *NOTCH2NL*). Notch family members have been reported as differentially expressed in astrocytomas and have been implicated in gliomagenesis (Purow, Haque et al. 2005, Xu, Yu et al. 2009). We observed a recurrent missense mutation among two astrocytomas converting amino acid 465, alanine to threonine. *NOTCH1*-A465T is located within an EGF like repeat domain, where additions of O-fucose to Ser/Thr is predicted and resides near a critical GlcNAc'ylation site (Shao and Haltiwanger 2003, Takeuchi and Haltiwanger 2010). This exact residue is also reported mutated in one colon adenocarcinoma in the COSMIC database (Sample ID COS1863429), suggesting this may be a hotspot mutation that may play a role in other cancer types. The spectra and frequency of Notch mutations we observed in astrocytomas further supports the notion of Notch pathway aberrations as a critical player in astrocytoma transformation.

Our exome sequencing of primary GBMs confirmed previous findings, highlighting frequent mutations in *EGFR* (46%), deletions of *CDKN2A* (39%), *TP53* mutations (25%), *NF1* mutations (15%), and *PTEN* mutations (15%) in primary GBMs (Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008, Brennan, Verhaak et al. 2013). Recent reports have identified *TET2*, a gene encoding the enzyme which catalyzes 5-methylcytosine to 5-hydroxymethylcytosine, to be frequently mutated in AML, and *TET* mutations are mutually exclusive with *IDH1/2* mutations in AML (Tahiliani, Koh et al. 2009, Figueroa, Abdel-Wahab et al. 2010, Ito, D'Alessio et al. 2010). Mutations in *IDH1/2* or *TET* have resulted in epigenetic alterations including a hypermethylated phenotype in gliomas and AML, respectively (Figueroa, Abdel-Wahab et al. 2010, Turcan, Rohle et al. 2012). It is of interest to note that we observed two *TET2* mutations in our GBM subset and that one primary GBM, P134, harbors a *TET2* mutation and an *IDH1* R132 mutation. Additional investigation of the epigenetic features of this tumor is necessary to make further conclusions about the potential synergy between these two epigenetic modifiers. Overall, the data contained here represents the largest exome sequencing study of progressive gliomas to date. We have elucidated the genetic landscape of progressive gliomas encompassing A2s, OA2s, A3s, OA3s, secondary GBMs, and primary GBMs, uncovering genes not previously linked to progressive astrocytomas. Furthermore, this study highlights the vast genetic differences between

progressive astrocytomas and primary GBMs, providing further evidence of two uniquely distinct disease entities.

3. Frequent *ATRX*, *CIC*, *FUBP1*, and *IDH1* mutations refine the classification of malignant gliomas

3.1 Introduction

Recent research has begun to characterize the molecular landscape of gliomas to better understand their molecular pathogenesis and to aid in their classification for clinical management. High-throughput molecular studies by The Cancer Genome Atlas and other groups have focused on the more common and lethal grade IV GBMs and identified molecular subgroups of those tumors (Phillips, Kharbanda et al. 2006, Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008, Noushmehr, Weisenberger et al. 2010, Verhaak, Hoadley et al. 2010). Major findings include frequent activating mutations in isocitrate dehydrogenase genes, *IDH1* and *IDH2* (“IDH mutation” refers to a mutation in either gene), in secondary GBMs (>50%) and rarely in primary GBMs (<5%) (Parsons, Jones et al. 2008, Ichimura, Pearson et al. 2009, Yan, Parsons et al. 2009, Hartmann, Hentschel et al. 2010, Reitman and Yan 2010). Additionally, inactivating alterations in Alpha Thalassemia/Mental Retardation Syndrome X-linked (*ATRX*) were recently identified in 7% of adult GBMs and in 14-31% of pediatric GBMs (Heaphy, de Wilde et al. 2011, Schwartzentruber, Korshunov et al. 2012). *ATRX* is critical for normal telomere homeostasis by regulating incorporation of histone variant H3.3 into telomeric chromatin (Lewis, Elsaesser et al. 2010, Eustermann, Yang et al. 2011, Iwase, Xiang et al. 2011), and *ATRX* alterations are associated with an alternative lengthening of telomeres (ALT) phenotype among GBMs (Heaphy, de Wilde et al. 2011, Schwartzentruber,

Korshunov et al. 2012). Recent GBM exome sequencing studies also confirmed frequent mutations in the tumor suppressor gene *TP53* among secondary GBMs (65%), and less frequently in primary GBMs (28%) (Parsons, Jones et al. 2008, Watanabe, Nobusawa et al. 2009, Verhaak, Hoadley et al. 2010, Schwartzentruber, Korshunov et al. 2012).

The molecular properties of grade II-III gliomas are less well-characterized than grade IV GBMs. Lower-grade gliomas share molecular features with secondary GBMs and with a subset of primary GBMs, including frequent IDH mutations (>70%) (Hartmann, Meyer et al. 2009, Ichimura, Pearson et al. 2009, Yan, Parsons et al. 2009), a proneural gene expression signature (Phillips, Kharbanda et al. 2006, Ducray, Idbaih et al. 2008, Verhaak, Hoadley et al. 2010), and a glioma CpG island hypermethylator phenotype (G-CIMP) (Noushmehr, Weisenberger et al. 2010). Grade II-III astrocytomas frequently harbor *TP53* mutations, which are uncommon in oligodendrogliomas (Ohgaki and Kleihues 2009, Yan, Parsons et al. 2009). ALT occurs frequently in grade II-III astrocytomas (63%) but less commonly in oligodendrogliomas (20%) (Hakin-Smith, Jellinek et al. 2003, Chen, Hakin-Smith et al. 2006, McDonald, McDonnell et al. 2010, Heaphy, Subhawong et al. 2011). However, whether *ATRX* mutations occur in adult grade II-III astrocytomas, and whether they may associate with ALT in those tumors, remains unknown.

Co-deletion of chromosomal arms 1p and 19q is a well-known prognostic marker found frequently (>60%) in grade II-III oligodendrogliomas (Smith, Perry et al. 2000,

Jenkins, Blair et al. 2006, Cairncross and Jenkins 2008). Recent exomic sequencing of oligodendrogliomas revealed inactivating mutations in two tumor suppressor genes: homolog of *Drosophila capicua* (*CIC*) and far-upstream binding protein 1 (*FUBP1*) in 53% and 15% of oligodendrogliomas, respectively (Bettegowda, Agrawal et al. 2011, Sahm, Koelsche et al. 2012, Yip, Butterfield et al. 2012). Since *CIC* is located on chromosomal arm 19q and *FUBP1* is located on chromosomal arm 1p, 1p/19q loss is thought to be a mechanism to inactivate *CIC* and *FUBP1* (Bettegowda, Agrawal et al. 2011). Among grade II-III gliomas, *CIC* and *FUBP1* mutational status has only been investigated in oligodendrogliomas, oligoastrocytomas and preselected astrocytomas with 19q loss (Sahm, Koelsche et al. 2012).

Given the difficulty in classifying gliomas, in particular grade II-III gliomas, we sought to characterize the landscape of genetic alterations in numerous common subtypes of brain tumors. We focused on both alterations that have been discovered recently in at least one glioma subtype by high-throughput molecular analyses (*ATRX*, *CIC*, *FUBP1*) and on classic alterations (*IDH*, *TP53*, 1p/19q) that could be routinely detected in a molecular genetics laboratory. This analysis uncovered frequent alterations in *ATRX*, *CIC*, and *FUBP1* in new glioma subtypes and identified an association between *ATRX* alterations and ALT in grade II-III gliomas. The mutational pattern identified here also revealed mutually exclusive, highly recurrent, multi-gene mutational signatures that correlated well with the clinical features of the patients.

3.2 Methods

3.2.1 Patient Samples

Snap-frozen tumor tissue and matched blood was obtained from patients from The Preston Robert Tisch Brain Tumor Center Biorepository at Duke University Medical Center (n=291), Johns Hopkins Medical Institutes (n=27), and the University of Sao Paulo, Brazil (n=45) with informed consent from the patients under protocols approved by the Institutional Review Boards of the respective institutions. Tissue sections were centrally reviewed by board-certified neuropathologists to ensure that $\geq 95\%$ of the section consisted of tumor cells and to confirm histopathological diagnosis. DNA was extracted from snap-frozen tissue obtained from primary brain tumors or from xenografts that were serially passaged in mice that were derived from primary brain tumors. All samples were obtained in accordance with the Health Insurance Portability and Accountability Act (HIPAA). 9 grade I pilocytic astrocytomas, 15 grade II astrocytomas, 44 grade III astrocytomas, 15 grade IV secondary GBMs, 94 grade IV primary GBMs, 25 grade IV pediatric GBMs, 21 grade II oligodendrogliomas, 29 grade III oligodendrogliomas, 18 grade II oligoastrocytomas, 22 grade III oligoastrocytomas, 65 medulloblastomas, 2 pleomorphic xanthoastrocytoma, and 5 ependymomas were sequenced. Secondary GBM designates a GBM which was resected > 1 year after a prior diagnosis of a lower grade glioma (grade I-III). Pediatric GBM samples were defined if between the ages of 0-20 and adult GBM was classified as ages 21+. One grade IV GBM was not included in mutation frequency analyses as described below. Pilocytic

astrocytomas, pleomorphic xanthoastrocytomas, ependymomas, and medulloblastomas were not considered for clinical analyses of grade II-IV gliomas. Median and mean ages reported in Table 1 was based on patients with available clinical information. Patients with survival of 1 month or less, likely due to surgical complications, were excluded from the survival analysis.

3.2.2 Sequencing

DNA was isolated from frozen tissue and the entire coding sequences of *ATRX*, *CIC*, and *FUBP1* were analyzed by Sanger sequencing (Sjoblom, Jones et al. 2006) for 363 tumors. 1p and 19q copy number was determined by microsatellite marker analysis and mutational status of *TP53*, *IDH1*, and *IDH2* was determined, as described previously (Yan, Parsons et al. 2009). All coding exons of *ATRX*, *CIC*, and *FUBP1* were examined in 363 tumors for this study. Death domain associated protein (*DAXX*) was sequenced in all grade II-III astrocytomas and oligodendrogliomas because it is mutated in pancreatic neuroepithelial tumors and pediatric GBMs (Jiao, Shi et al. 2011, Schwartzenuber, Korshunov et al. 2012) and because it functions in the same telomere-regulating pathway as *ATRX* (Lewis, Elsaesser et al. 2010). No *DAXX* mutations were found in those gliomas. *ATRX* mutational status (but not *CIC* or *FUBP1* mutational status) for a subset of GBMs, medulloblastomas, and 13 oligodendrogliomas were reported in a previous resequencing study (Heaphy, de Wilde et al. 2011). No grade II-III gliomas aside from those 13 oligodendrogliomas were examined for *ATRX* in that analysis. *CIC*

and *FUBP1* mutational status for 7 oligodendrogliomas were reported in a previous whole-exome sequencing study (Bettegowda, Agrawal et al. 2011). *CIC* and *FUBP1* status from other oligodendrogliomas, and for non-oligodendroglioma brain tumors reported here were not included in that analysis. Sequencing was performed by PCR amplification followed by Sanger sequencing as described previously (Sjoblom, Jones et al. 2006).

For *IDH1*, *IDH2*, *TP53*, and 1p/19q status, sequencing efforts on an overlapping panel of 445 brain tumors for those alterations (Yan, Parsons et al. 2009) were supplemented by analyzing these markers in all remaining tumors for which additional tumor DNA or tissue was available. *IDH1* and *IDH2* status were based on the presence or absence of either R132 or R172 mutations, respectively, from exon 4 sequencing of either gene. *TP53* mutational status, as well as 1p and 19q chromosomal status based on microsatellite markers, were determined as described previously (Yan, Parsons et al. 2009). All mutations were validated with Sanger sequencing in tumors and matched normal controls. One sample pGBM IV-2, harbors a somatic deletion adjacent to the intron exon edge of *ATRX* and is likely to affect functionality; this sample was considered to have an *ATRX* mutation for the purposes of this study. Another sample, GBM-13, had a “hypermutator” phenotype and harbored an extreme number of mutations that were likely to be passenger mutations, as discussed in a previous exomic

sequencing analysis (Parsons, Jones et al. 2008). This case was excluded from analyses of mutation frequency, mutation co-occurrence, and patient characteristics.

3.2.3 Telomere FISH, and Immunolabeling

Telomere length was assessed by FISH in 71 samples with available formalin-fixed, paraffin-embedded tissue, and p53 labeling was performed by immunofluorescence (Meeker, Gage et al. 2002, Koorstra, Hong et al. 2009, Heaphy, de Wilde et al. 2011, Heaphy, Subhawong et al. 2011, Jiao, Shi et al. 2011). Loss of *ATR*X nuclear immunolabeling indicates that *ATR*X is inactivated by genetic or epigenetic changes not identifiable by sequencing of *ATR*X exons (Heaphy, de Wilde et al. 2011, Molenaar, Koster et al. 2012), therefore tumors with *ATR*X mutation and/or loss of *ATR*X nuclear immunolabeling in tumor cells but not normal cells as assessed by immunohistochemistry (Heaphy, de Wilde et al. 2011) were considered to have an *ATR*X alteration. Fisher's exact test was used to determine whether two aberrations were associated among a group of patients.

Deparaffinized slides were hydrated, steamed for 20 minutes in citrate buffer (catalog# H-3300; Vector Laboratories), dehydrated and hybridized with a Cy3- labeled peptide nucleic acid (PNA) probe complementary to the mammalian telomere repeat sequence ([N-terminus to C-terminus] CCCTAACCCCTAACCCCTAA). Following post-hybridization washes, the desired primary antibody was applied (anti-*ATR*X, as described above), followed by application of species-appropriate Alexa 488 fluorescent

secondary antibody (Molecular Probes Cat.# A-11034 or A-11001) and nuclear counterstaining with DAPI . Slides were imaged with a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc., Ontario, CA) and appropriate fluorescence excitation/emission filters. Grayscale images were captured using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital camera, pseudo-colored and merged.

The FISH and immune-labeled slides were assessed and scored independently (C. Heaphy and A.K. Meeker). Large, ultra-bright telomere repeat DNA aggregates are unique to ALT-positive cell populations and are significantly larger and brighter than the FISH signals emanating from individual telomeres in the same cell population (for further details, see detailed discussion of these foci as markers for the ALT phenotype in Supplementary Online Material of (Heaphy, de Wilde et al. 2011)). In this study, gliomas were classified as ALT-positive if they met the following criteria: (i) the presence of ultra-bright, intra-nuclear foci of telomere FISH signals, with signal intensities and areas for individual foci being significantly larger than any telomeric signals in individual benign cells within the same case and (ii) $\geq 1\%$ of neoplastic cells displaying ALT-associated telomeric DNA foci. Tumor samples lacking ALT-associated telomeric foci in which at least 5000 cells were assessed were considered ALT-negative. In all cases, areas exhibiting necrosis were excluded from consideration.

3.2.4 ATRX Immunohistochemistry

Heat-induced antigen retrieval was performed in a steamer using citrate buffer (catalog# H-3300, Vector Laboratories) for 30 minutes. Endogenous peroxidase was blocked (catalog# S2003, Dako) and serial sections were then incubated with primary antibody; anti-*ATRX* (1:400 dilution; catalog# HPA001906, Sigma-Aldrich, lot R00473) for 1 hour at room temperature. The primary antibodies were detected by 30 minute incubation with HRP-labeled secondary antibody (catalog# PV6119, Leica Microsystems) followed by detection with 3,3'-Diaminobenzidine (Sigma-Aldrich), counterstaining with Harris hematoxylin, rehydration and mounting. Only nuclear labeling of either protein was evaluated. The immune-labeled glioma slides were assessed and scored (R.F. DeWilde, F. Rodriguez, and A.K. Meeker); internal controls included endothelial cells (including within intratumoral vessels), and benign neurons, which demonstrated strong nuclear immunolabeling for *ATRX*. 29 tumors were analyzed for *ATRX* nuclear staining, 14 of which were also analyzed by sequence analysis. Tumors were considered to have an *ATRX* alteration if either a mutation was present when analyzed by sequencing, or if *ATRX* nuclear expression was lost when tumor tissue was analyzed by immunostaining, as done previously (Heaphy, de Wilde et al. 2011). p53 nuclear protein immunohistochemistry was performed as described previously (Koorstra, Hong et al. 2009). Of the 93 tumors that were considered to have *ATRX* alterations, eight tumors

without a detectable *ATRX* mutation demonstrated loss of *ATRX* nuclear staining in tumor cells but not normal cells.

3.2.5 Survival Analysis

Survival was analyzed for adult patients (>20 years old at diagnosis) with grade II-IV gliomas with available survival and age information. 156 patients from Duke (Cohort A) and 43 patients from Brazil (Cohort B) met these criteria. A Mantel-Cox log-rank test was used to determine whether survival was significantly different between two or more groups of patients. A two-tailed Student's t-test and single factor ANOVAs were used to determine whether mean age was significantly different between two or three groups of patients, respectively.

3.3 Results

3.3.1 *ATRX* inactivation is linked to mutations in TP53 and IDH, and to altered telomeres

ATRX alterations were found in 93 out of 363 brain tumors, in ten tumor subtypes (Table 3). *ATRX* alterations occurred frequently in grade II astrocytomas (67%, n=15), grade III astrocytomas (73%, n=44), secondary GBMs (57%, n=14), and in tumors of mixed astrocytic and oligodendrocytic lineage (68%, n=40). In contrast, *ATRX* alterations were rare in primary GBMs (4%, n=94) and uncommon in pediatric GBMs (20%, n=25) and pure oligodendroglioma tumors (14%, n=50). Surprisingly, while all of the *ATRX* mutations in pediatric GBMs were found near the carboxy-terminal Helicase

domain as observed previously (Jiao, Shi et al. 2011, Schwartzenuber, Korshunov et al. 2012), mutations in adult gliomas instead distributed evenly (Figure 2).

Table 3: Frequency of mutations in 13 tumor types

Tumor Classification	No. of tumors analyzed	Mean age (yrs.)	Median age (yrs.)	Male Sex (%)	IDH 1/2		ATR X		TP53		CIC		FUBP1	
					Mut No.	%	Mut No.	%						
Astrocytic tumors	203	37	26	54%	71	35%	59	29%	91	45%	1	0.50%	2	1%
<i>Pilocytic Astrocytoma (grade I)</i>	9	7	4	56%	0	0%	0	0%	NA	NA	0	0%	0	0%
<i>Pleomorphic xanthoastrocytoma (grade II)</i>	2	11	11	0%	0	0%	0	0%	2	100%	0	0%	0	0%
<i>Astrocytoma (grade II)</i>	15	25	30	60%	10	67%	10	67%	9	60%	0	0%	0	0%
<i>Anaplastic Astrocytoma (grade III)</i>	44	36	35	61%	37	84%	32	73%	36	82%	0	0%	0	0%
<i>Secondary Adult glioblastoma (grade IV)</i>	14	37	36	64%	8	57%	8	57%	11	79%	0	0%	0	0%
<i>Adult glioblastoma (grade IV)</i>	94	56	57	60%	13	14%	4	4%	22	23%	1	1%	2	2%
<i>Pediatric glioblastoma (grade IV)</i>	25	12	12	79%	3	12%	5	20%	11	44%	0	0%	0	0%
Oligodendroglial	50	40	41	55%	46	92%	7	14%	7	14%	23	46%	12	24%
<i>Oligodendroglioma (grade II)</i>	21	36	35	48%	18	86%	5	24%	5	24%	8	38%	3	14%
<i>Anaplastic Oligodendroglioma (grade III)</i>	29	43	46	62%	28	96%	2	7%	2	7%	15	52%	9	31%
Oligoastrocytic (Mixed)	40	41	38	58%	39	98%	27	68%	27	68%	3	8%	2	5%
<i>Oligoastrocytoma (grade II)</i>	18	42	39	61%	18	100%	10	56%	11	61%	1	6%	1	6%
<i>Anaplastic Oligoastrocytoma (grade III)</i>	22	40	37	55%	21	95%	17	77%	16	73%	2	9%	1	5%
Ependymoma (grade II)	5	NA	NA	NA	0	0%	0	0%	NA	NA	0	0%	0	0%
Medulloblastoma (grade IV)	65	9	7	54%	0	0%	1	2%	1	NA	2	3%	0	0%

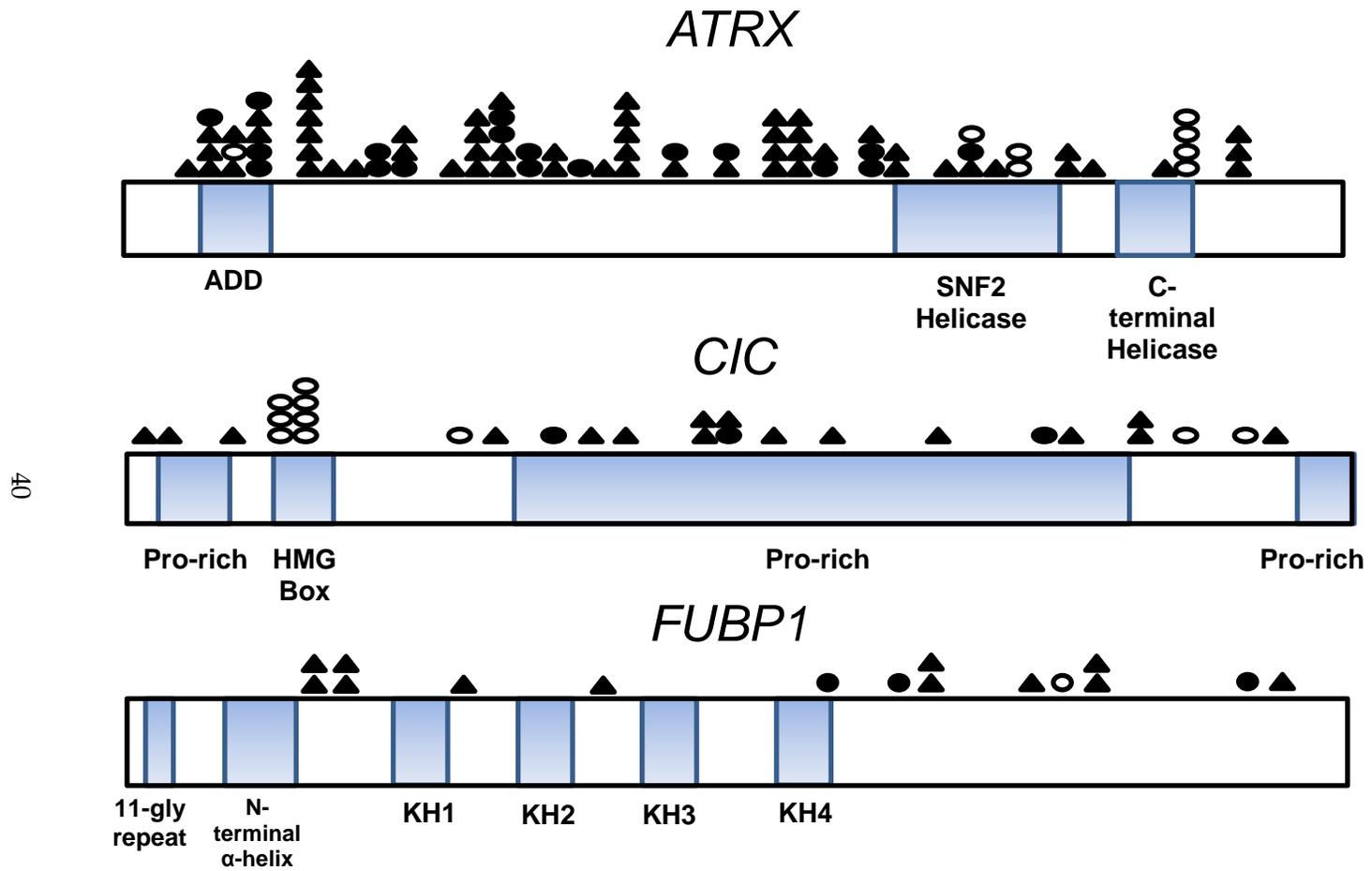


Figure 2: Distribution of ATRX, CIC, and FUBP1 mutations in gliomas.

Figure 2 (Continued): Distribution of ATRX, CIC, and FUBP1 mutations in gliomas.

Schematics of the coding sequences of ATRX, CIC, and FUBP1 are shown. Mutations were predominantly truncating mutations such as frameshifts (shaded triangles) and nonsense mutations (shaded circles). Empty circles correspond to missense mutations. ATRX contains a DNA-binding ATRX-DNMT3-DNMT3L (ADD) domain and an ATPase Helicase Domain. ATRX alterations occurred in 20% of pediatric GBMs (n=25) and all of those mutations clustered in the Helicase domain of ATRX as described previously (Schwartzentruber, Korshunov et al. 2012). ATRX mutations in adult gliomas instead distributed evenly. CIC contains four highly conserved domains, the HMG (high mobility group) box responsible for DNA binding and proline-rich (pro-rich) domains. Among CIC mutations (n=29), a predilection for missense mutations occurred in the HMG box domain (24%). Mutations in FUBP1 (n=16) occurred 3' of the N-terminal α -helix domain, which controls binding of FUBP1 to the FBP-interacting repressor (FIR). Gene representations are to scale.

The association between *ATRX* alterations and other frequent molecular alterations was analyzed among the grade II-IV gliomas (Figure 3 and Figure 5). *IDH* mutations were observed in 87 of 88 adult gliomas with an *ATRX* alteration (99%). We termed tumors with this genetic signature "I-A gliomas", denoting that these tumors had alterations in either *IDH1* or *IDH2* and in *ATRX*. Most I-A adult gliomas also had mutations in *TP53* (94%, n=87), and the majority of adult gliomas with *TP53* mutations had the I-A signature (72%, n=113). The I-A signature was tightly associated with ALT among grade II-III gliomas (n=71): 100% (49/49) of gliomas with ALT were I-A, and 98% (49/50) of gliomas with I-A were ALT positive (P<0.001, Figure 4). *ATRX* alterations and 1p/19q loss rarely co-occurred (only one case, P<0.001).

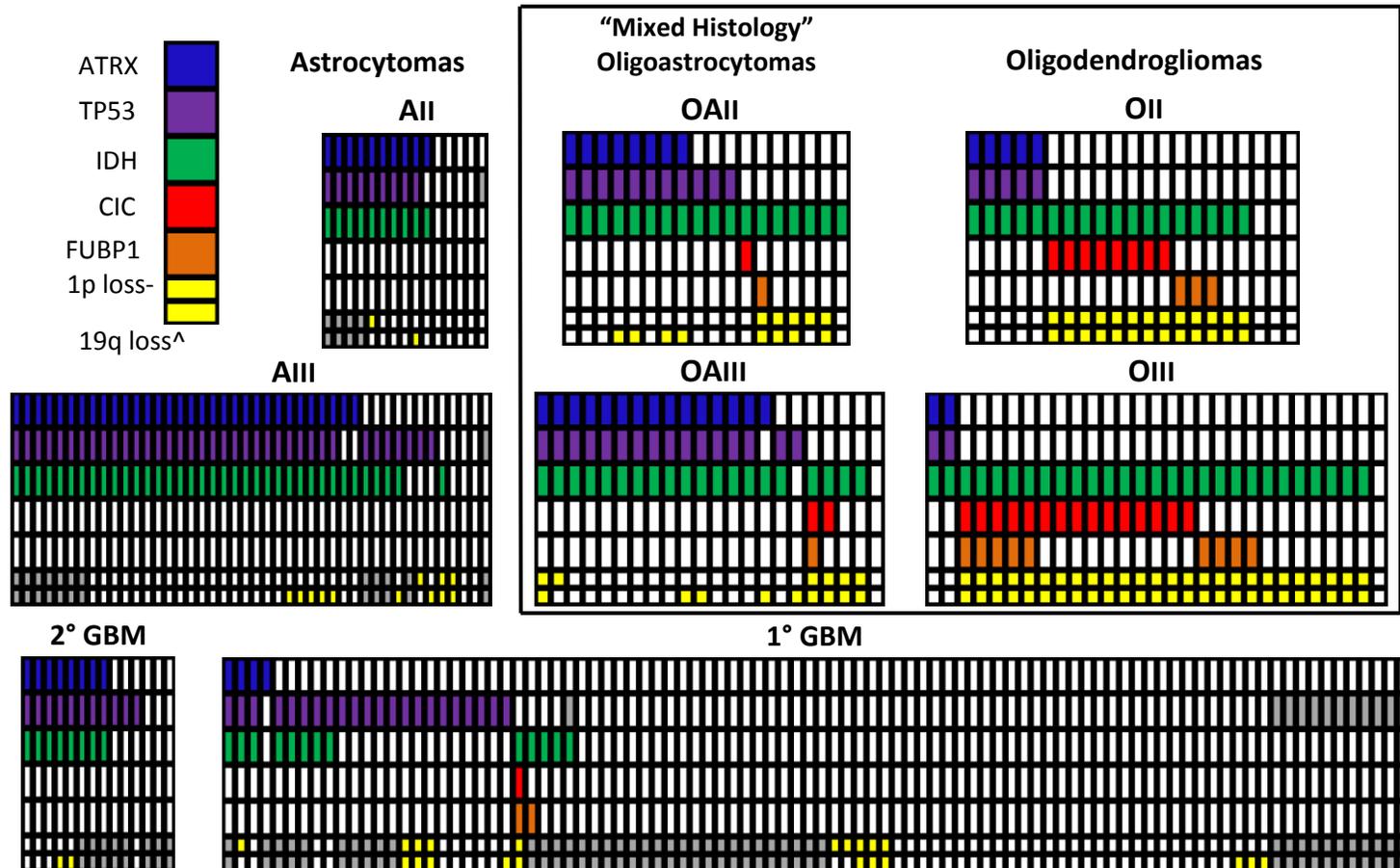


Figure 3: Distribution of ATRX, TP53, IDH, CIC, and FUBP1 mutations, and of chromosomes 1p and 19q loss, in grade II-IV gliomas.

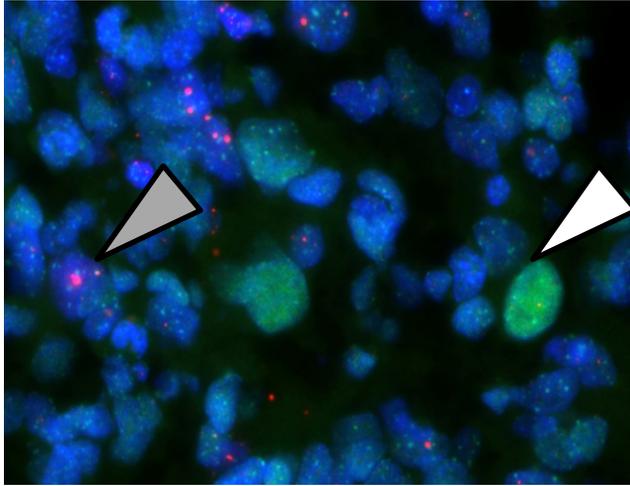
Figure 3 (Continued): Distribution of ATRX, TP53, IDH, CIC, and FUBP1 mutations, and of chromosomes 1p and 19q loss, in grade II-IV gliomas.

Data are from 15 grade II astrocytomas (AII), 44 grade III astrocytomas (AIII), 21 grade II oligodendrogliomas (OII), 29 grade III oligodendrogliomas (OIII), 18 grade II oligoastrocytomas (OAII), 22 grade III oligoastrocytomas (OAIII), 14 secondary GBMs (2° GBM), 94 primary GBMs (1° GBM), and 25 pediatric GBMs. chromosome 1p status is indicated in the top of the lower row, and chromosome 19q status is indicated in the bottom of this cell, with yellow coloring indicating loss of the indicated chromosomal arm. Gray cells denote analyses which were not informative or for which additional genetic material was not available for analysis.

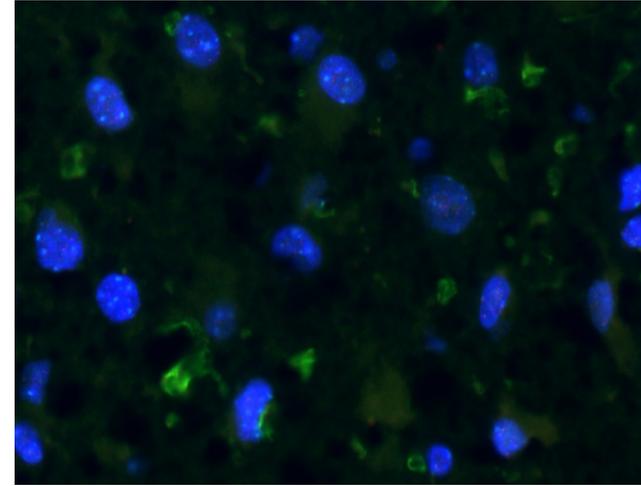
3.3.2 CIC and FUBP1 mutations are linked to IDH mutations and 1p/19q loss

Sequencing analysis of *CIC* and *FUBP1* genes in the same brain tumors revealed 29 and 16 somatic mutations, respectively (Figure 2 and Table 3). The highest frequency of *CIC* and *FUBP1* mutations occurred in grade II (38% and 14%, n=21) and grade III oligodendrogliomas (52% and 31%, n=29). In contrast, *CIC* and *FUBP1* mutations were rare in primary GBMs (1% and 2%, n=94) and completely absent (0%, n=59) in grade II-III pure astrocytic tumors. Seven tumors had concurrent *CIC* and *FUBP1* mutations. Every glioma with a *CIC* or an *FUBP1* mutation exhibited an *IDH* gene mutation (Figure 3 and Figure 5). Almost every glioma with a *CIC* or an *FUBP1* mutation exhibited loss of both chromosomes 1p and 19q (98%, n=36). Therefore, we termed tumors that had mutations in *IDH1* or *IDH2* combined with either 1p/19q loss, *CIC* mutation, or *FUBP1* mutations as “I-CF gliomas.” *CIC* and *FUBP1* mutations were mutually exclusive with *ATRX* or *TP53* mutations ($P < 0.001$ for all four comparisons).

ALT-positive



ALT-negative



	No ATRX alteration	ATRX alteration
ALT-negative	21	1
ALT-positive	0	49

Tumor type	No. with ALT/ Total analyzed (%)
Grade II astrocytoma	3/3 (100)
Grade III astrocytoma	22/29 (76)
Grade II oligodendroglioma	1/3 (33)
Grade III oligodendroglioma	0/4 (0)
Grade II oligoastrocytoma	8/13 (62)
Grade III oligoastrocytoma	15/19 (79)

Figure 4: ALT associates with the ATRX alterations and the I-A signature.

Figure 4 (Continued): ALT associates with the ATRX alterations and the I-A signature.

(A) Representative grade III astrocytoma section that was positive for ALT is shown on the left. A nucleus demonstrating large, ultra-bright telomeres (pink) is indicated with the shaded arrow. A p53 positive nucleus (green), which reflects p53 dysfunction, is indicated with the empty arrow. A representative image of a grade III astrocytoma section that was negative for both ALT and for p53 nuclear staining is shown on the right. The number of tumors with ALT and/or ATRX alterations is shown, along with the percentage of each tumor type with ALT. All tumors with ATRX alterations were I-A, that is, they also had an IDH mutation (n=50); the remaining tumors were IDH wild-type and did not have an ATRX alteration (n=21).

For the purposes of this study, other adult gliomas that did not fall into the I-A or I-CF signatures were categorized as I-X gliomas (i.e. not I-A or I-CF). Nearly half of adult gliomas included in this study (47%, n=257) were classified as I-X gliomas. Seventy-four percent of the I-X gliomas were diagnosed as grade IV primary GBMs and 95% of primary GBMs were classified as I-X gliomas. Most, but not all I-X gliomas (87%) were *IDH* wild type. Mutant *TP53* was found in 26% of these tumors, and most of the I-X gliomas with *IDH* mutations (69%) also had *TP53* mutations. Seven percent of adult gliomas were *ATRX* mutant or showed loss of 1p and 19q and were considered I-X gliomas because they lacked *IDH* mutations.

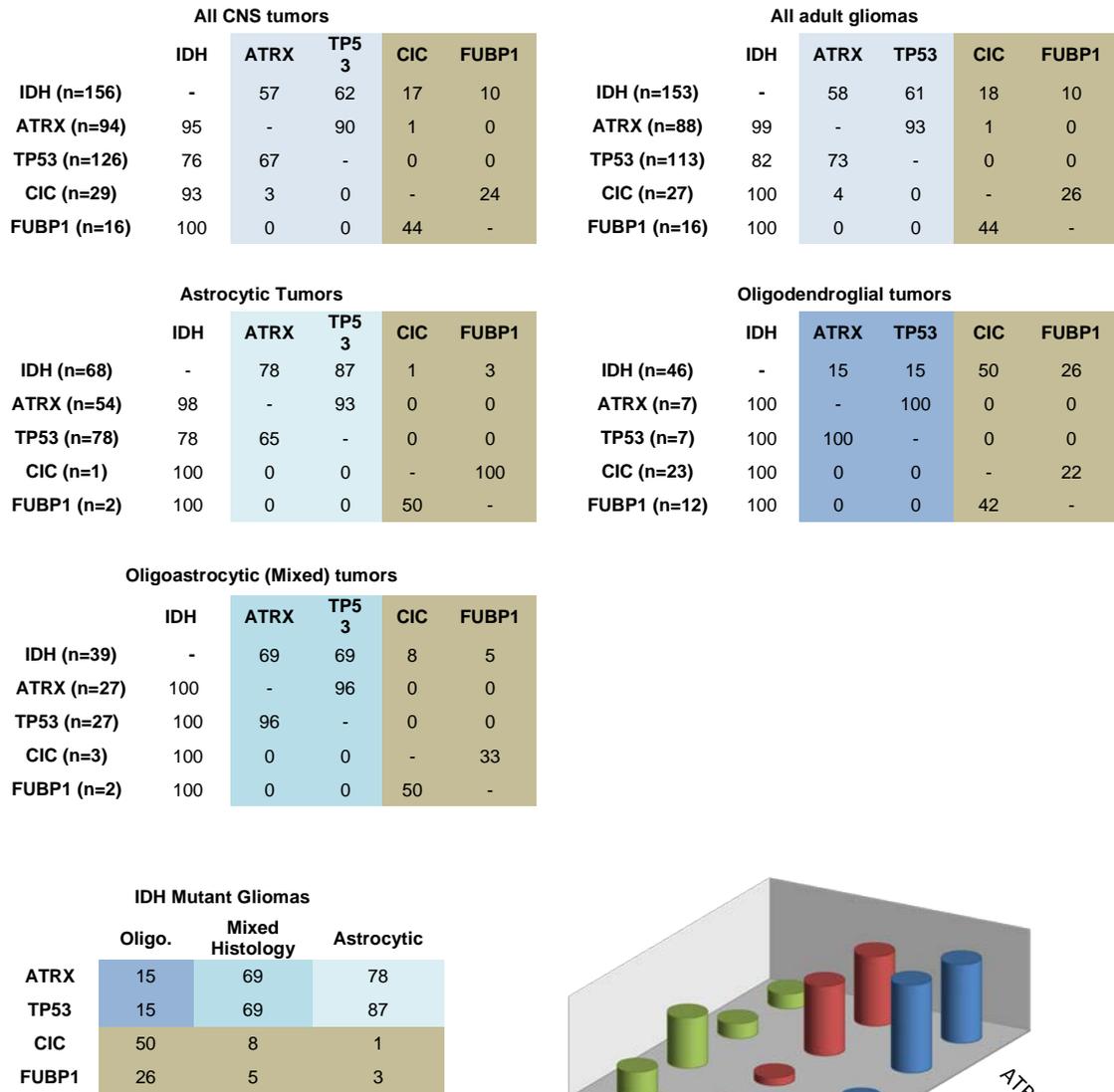


Figure 5: Co-occurrence of IDH, ATRX, CIC, and FUBP1 mutations in 363 brain tumors.

Figure 5 (Continued): Co-occurrence of IDH, ATRX, CIC, and FUBP1 mutations in 363 brain tumors.

Groups include all CNS tumors (n=363), adult grade II-IV gliomas (n=257), oligodendroglial tumors (n=50), astrocytic tumors (n=167), mixed histology tumors (oligoastrocytomas, n=40), and all IDH-mutated adult gliomas (n=153). The graph displays mutation frequencies of in IDH-mutated astrocytic, oligodendroglial and mixed histology tumors based on histopathological diagnosis (n=153). Astrocytic tumors were defined in this figure as grade II astrocytomas (AII), grade III astrocytomas (AIII), secondary GBMs (2° GBM), and primary GBMs (1° GBM). Percentage of co-occurrence is shown.

3.3.3 Pathologic and clinical characteristics of I-A, I-CF, and I-X gliomas

On the basis of the mutational patterns described above, gliomas could be classified as either I-A, I-CF, or I-X. We sought to determine whether these glioma genetic signatures were associated with any differences between the clinical characteristics of the patients who bore the tumors. Survival, age at diagnosis, and histopathological diagnosis data were collected for 199 grade II-IV adult glioma patients, including a cohort of glioma patients from the United States (n=156 patients) and a cohort of glioma patients from Brazil (n=43 patients). Age, median survival, and histopathological diagnosis of patients with I-A, I-CF, and I-X tumors were similar in the two cohorts (Table 4). Distribution of genetic signatures among the combined cohort of 199 patients is shown in Table 5 and was as follows: gliomas classified as astrocytic were primarily I-A (32%) or I-X (66%) and rarely carried the I-CF (2%) genetic signature.

Oligodendroglial tumors were primarily I-CF (77%) but a significant proportion harbored the I-A (16%) or I-X (7%) genetic signatures.

Table 4: Clinical characteristics from glioma patients in two cohorts.

	Cohort A*	Cohort B*
Total No. patients	156	43
No. grade II (%)	27 (17)	4 (9)
No. grade III (%)	61 (39)	8 (19)
No. grade IV (%)	68 (44)	31 (72)
No. oligodendroglial (%)	7 (5)	0 (0)
No. mixed histology (%)	44 (28)	0 (0)
No. astrocytic (%)	105 (67)	43 (100)
No. I-A (%)	47 (30)	12 (28)
Mean age at diagnosis \pm S.D., years	35 \pm 8	30 \pm 5
Median survival, months	59	43
No. I-X (%)	70 (45)	30 (70)
Mean age at diagnosis \pm S.D., years	56 \pm 14	50 \pm 14
Median survival, months	17	11
No. I-CF (%)	39 (25)	1 (2)
Mean age at diagnosis \pm S.D., years	43 \pm 13	53
Median survival, months	94	N.R.

*Cohort A was from Duke University Medical Center, United States. Cohort B was from the University of Sao Paulo, Brazil.

Table 5: Characteristics of patients with I-A, I-CF, and I-X genetic signatures

	I-A	I-CF	I-X	p Value*
Age (mean ± S.D., years)	34 ± 7	44 ± 11	54 ± 15	<0.001
(median, years)	33	46	56	
Gender (% male)	56	54	65	0.33
Survival (median, months)	51	96	13	<0.001
All Cases (n=199)	59 (29.6)	39 (19.6)	101 (50.8)	
Astrocytomas (n=148)	48 (32.4)	3 (2.0)	97 (65.6)	
Oligodendrogliomas (n=44)	7 (15.9)	34 (77.3)	3 (6.8)	
Mixed Histology (n=7)	4 (57.1)	2 (28.6)	1 (14.3)	
Grade II (n=31)	15 (48.4)	12 (38.7)	4 (12.9)	
Grade III (n=69)	34 (49.3)	25 (36.2)	10 (14.5)	
Grade IV (n=99)	10 (10.1)	2 (2.0)	87 (87.9)	
Secondary GBM (n=12)	7 (58.3)	0 (0.0)	5 (41.7)	
IDH Mutant Tumors (n=111)	59 (53.2)	39 (35.1)	13 (11.7)	

The 199 patients analyzed above are the subset of all adult (>20 yr) grade II-IV glioma patients who had age and survival information

*One-way ANOVA for difference in mean age distribution, Chi-squared test for difference in gender proportions, and log-rank test for difference in survival

Age at diagnosis and survival parameters were first analyzed among the group of 199 grade II-IV glioma patients, and then among subgroups of patients with specific tumor types. At the time of diagnosis, patients with I-A gliomas were younger (mean age of 34) than patients with I-CF tumors (mean age of 44, $P<0.001$) and patients with I-X tumors (mean age of 54, $P<0.001$). This stratification in age was independent of tumor grade, as patients with I-A tumors were younger than patients with the other two signatures among the group of grade III patients (34, 46, and 46 years old, $P<0.001$) and the group of grade IV patients (29, 53, and 55 years old, $P<0.001$).

Among all grade II-IV glioma patients, those whose tumors bore the I-A and I-CF signatures survived longer than those with I-X tumors (median 51, 96, and 13 months, $P<0.001$) (Figure 6). Even when stratified by grade, patients whose tumors bore the I-A and I-CF signature survived longer than patients with the I-X signature among the subgroup of grade III gliomas (median 51, 127, and 22 months, $P<0.001$, Figure 6). In contrast, there was not a significant difference in survival between groups of grade III gliomas that were stratified by standard histopathological diagnostic criteria ($P=0.37$, log-rank test, Figure 6). I-A patients also survived longer than I-X patients among GBMs (median 31 vs. 13 months, $P=0.016$,) and among all astrocytomas (grade II-IV, median 51 vs. 13 months, $P<0.001$) (Figure 7). Significant differences in median survival between I-A, I-CF, and I-X signatures were not observed among the grade II glioma patients or grade II-III oligodendrogliomas, or between *CIC* and *FUBP1* mutated patients (Figure 7).

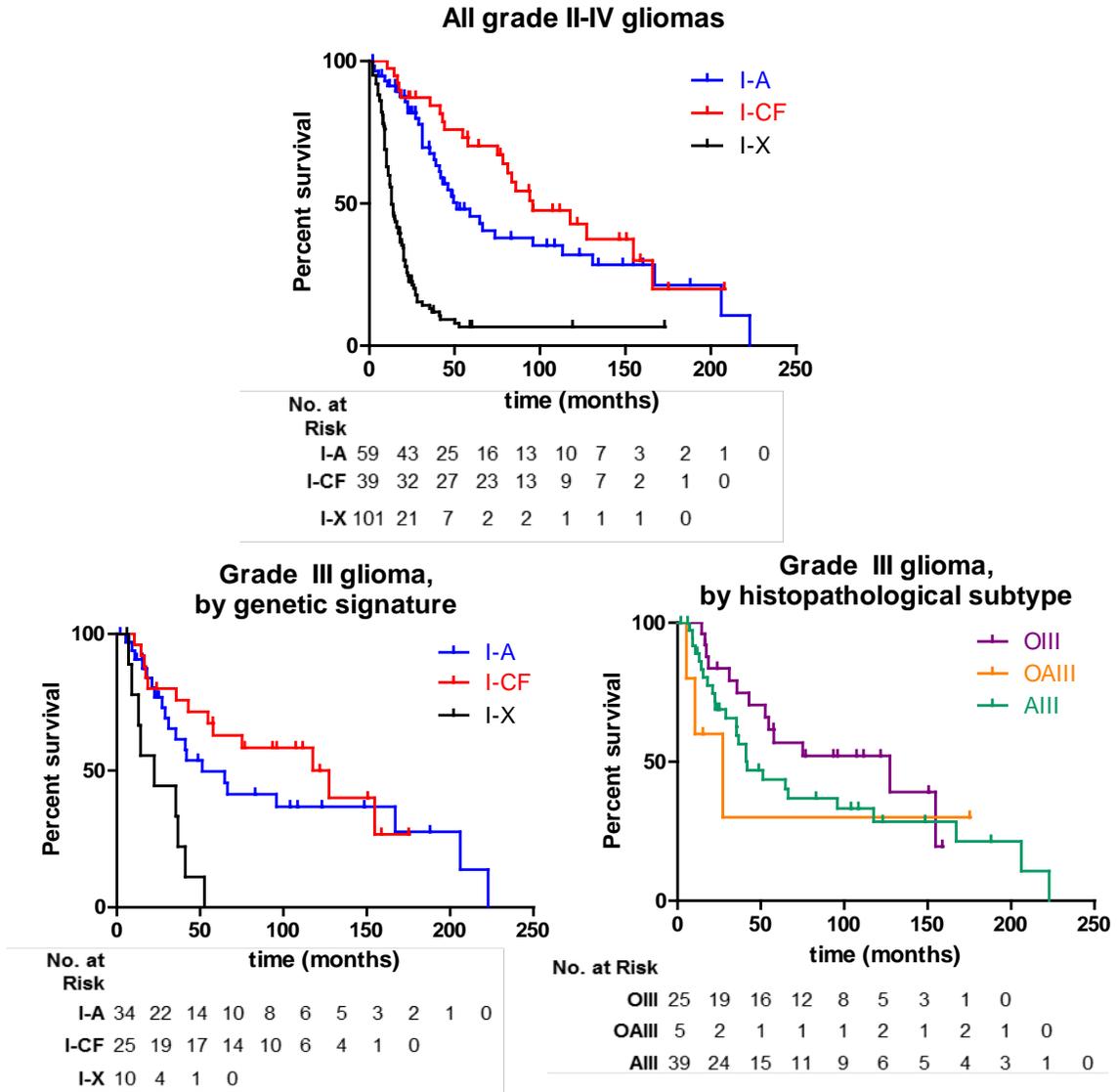


Figure 6: Survival of glioma patients with I-A, I-CF, and I-X genetic signatures.

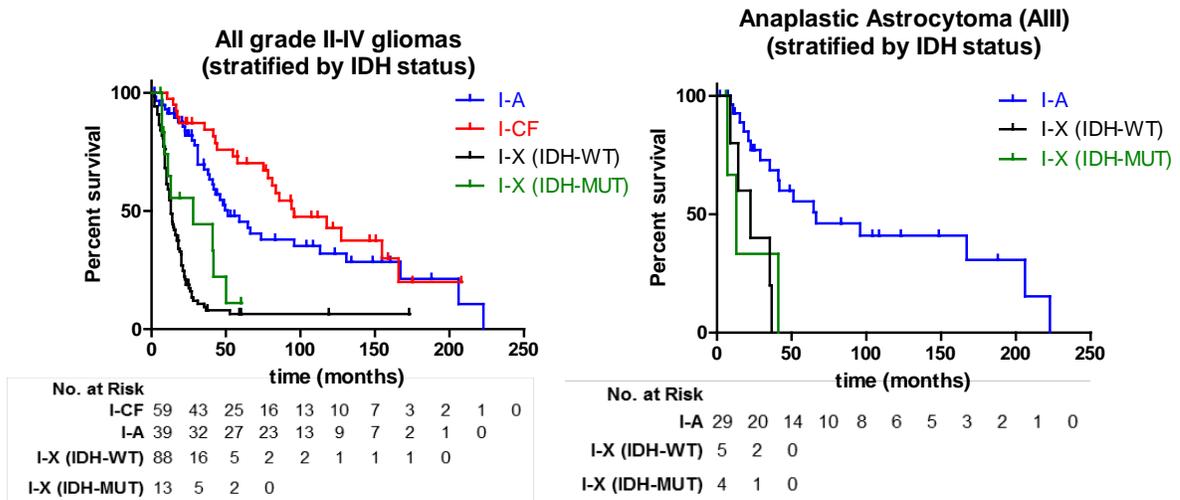


Figure 6 (Continued): Survival of glioma patients with I-A, I-CF, and I-X genetic signatures.

Kaplan-Meier estimates of survival are shown for adult glioma patients, with the number of patients at risk at each time point for each group shown below the respective plot. Among all adult patients with grade II-IV gliomas ($n=199$), those with I-A tumors and with I-CF tumors survived significantly longer (median 51 and 96 months) than patients with I-X tumors (13 months; $P<0.001$ for both comparisons). Among grade III glioma patients with tumors of all histologies ($n=69$), there was a significant difference in survival between groups of patients with I-A or I-CF tumors and patients with I-X tumors (I-A and I-X, $P=0.002$; I-CF and I-X, $P<0.001$). There was not a significant difference in survival when the same grade III glioma patients were stratified by the histopathological diagnosis of their tumors ($P=0.37$). When all 199 grade II-IV gliomas were additionally stratified by *IDH* status, patients with I-A tumors and patients with I-CF tumors still survived significantly longer (median 51 and 96 months) than patients with *IDH*-mutated I-X tumors, and also longer than patients with *IDH*-WT I-X tumors (median 28 and 13 months, respectively; $P<0.001$ for all four comparisons). When grade III astrocytoma patients ($n=39$) were stratified by genetic signature and *IDH* status of their tumors, patients with I-A tumors survived longer (66 months) than grade III astrocytoma patients with either *IDH*-mutated I-X tumors (22 months, $P=0.005$) and patients with *IDH*-WT I-X tumors (13 months, $P=0.002$).

IDH mutations are associated with improved survival in gliomas (Nobusawa, Watanabe et al. 2009, Yan, Parsons et al. 2009, Hartmann, Hentschel et al. 2010). Among all gliomas with *IDH* mutations, patients with *IDH* mutations in the I-X group had a shorter median survival (28 months) than patients with I-A tumors (51 months, $P=0.007$) or I-CF tumors (96 months, $P<0.001$) (Figure 6). In the grade III astrocytoma group, patients with I-A tumors survived much longer (66 months) than patients with *IDH*-mutated I-X tumors (13 months, $P=0.002$) and *IDH* wild-type I-X tumors (22 months, $P=0.005$) (Figure 6). These results suggest that the I-A and I-CF signatures may provide more prognostic information than *IDH* mutational status alone.

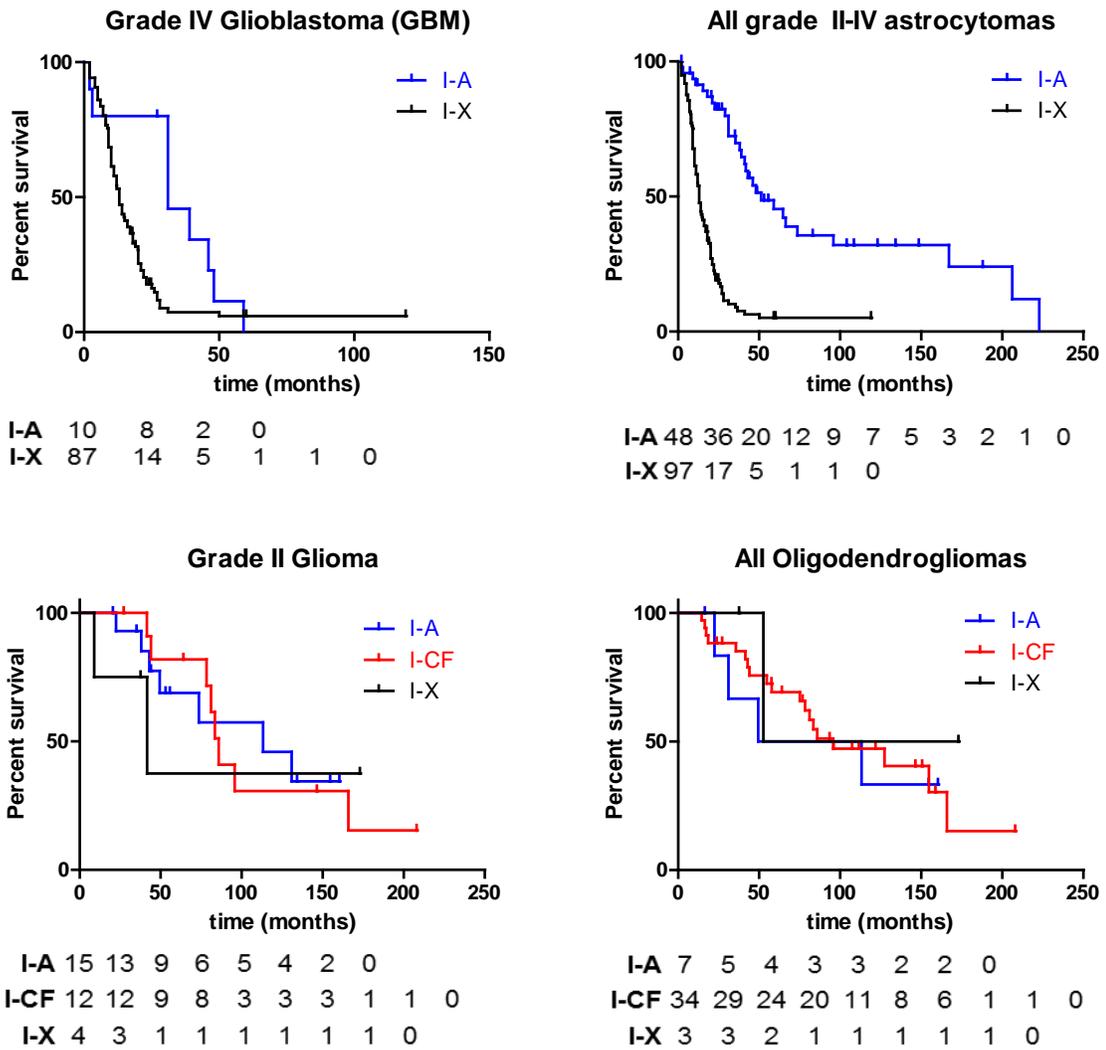


Figure 7: Survival of patients with I-A and I-CF genetic signatures among different glioma subgroups.

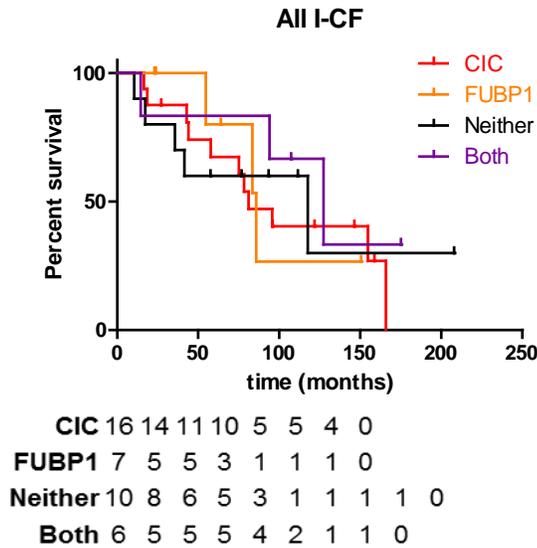


Figure 7 (Continued): Survival of patients with I-A and I-CF genetic signatures among different glioma subgroups.

Kaplan-Meier estimates of overall survival are shown for subgroups of 199 patients with grade II-IV gliomas, with the number of patients at risk at each time shown below each respective plot. Survival was significantly different between grade IV glioblastoma patients (n=97) with I-A and I-X signatures (P=0.02, log-rank test). There was a significant difference in survival between astrocytoma patients (grade II-IV, n=145) whose tumors had I-A or I-X signatures (P<0.001, log-rank test). A significant difference in survival between patients with I-A, I-CF, and I-X tumors was not observed among all grade II glioma patients (n=31, P=0.79, log-rank test) or among oligodendroglioma patients (grade II-III, n=44; P=0.76, log-rank test). A significant difference in survival between patients with FUBP1 mutation, CIC mutation, both mutations, or neither mutation among all grade II-IV glioma patients with the I-CF signature (n=39) was not observed (P=0.43, log-rank test).

3.3.4 I-A and I-CF signatures can discriminate gliomas with mixed histopathology

40 gliomas with mixed oligodendrocytic and astrocytic histopathology were examined to determine their I-A, I-CF, and I-X status (Table 6). 33 of these cases showed incomplete loss at loci on 1p36, 1p32, and 19q in prior clinical FISH testing and exhibited light microscopic appearances that were considered to be equivocal between oligodendroglioma or astrocytoma based on nuclear morphology and cytologic appearance. Greater than half of all mixed histology gliomas were I-A (65%), although I-CF (23%) and I-X (10%) genetic signatures were also found in this subgroup. In one case, the tumor had a “mixed” genetic signature: one grade III oligoastrocytoma demonstrated both an *ATRX* alteration and 1p/19q loss. When grouped by genetic signature, the age of oligoastrocytoma patients showed the same pattern observed for other glioma types: mean age at diagnosis was 35 for I-A patients, 49 for I-CF patients, and 59 for I-X patients ($P=0.001$). Survival statistics were not tabulated because this cohort was only recently collected and median follow-up was insufficient.

Table 6: Characteristics of mixed histology tumors analyzed in this study.

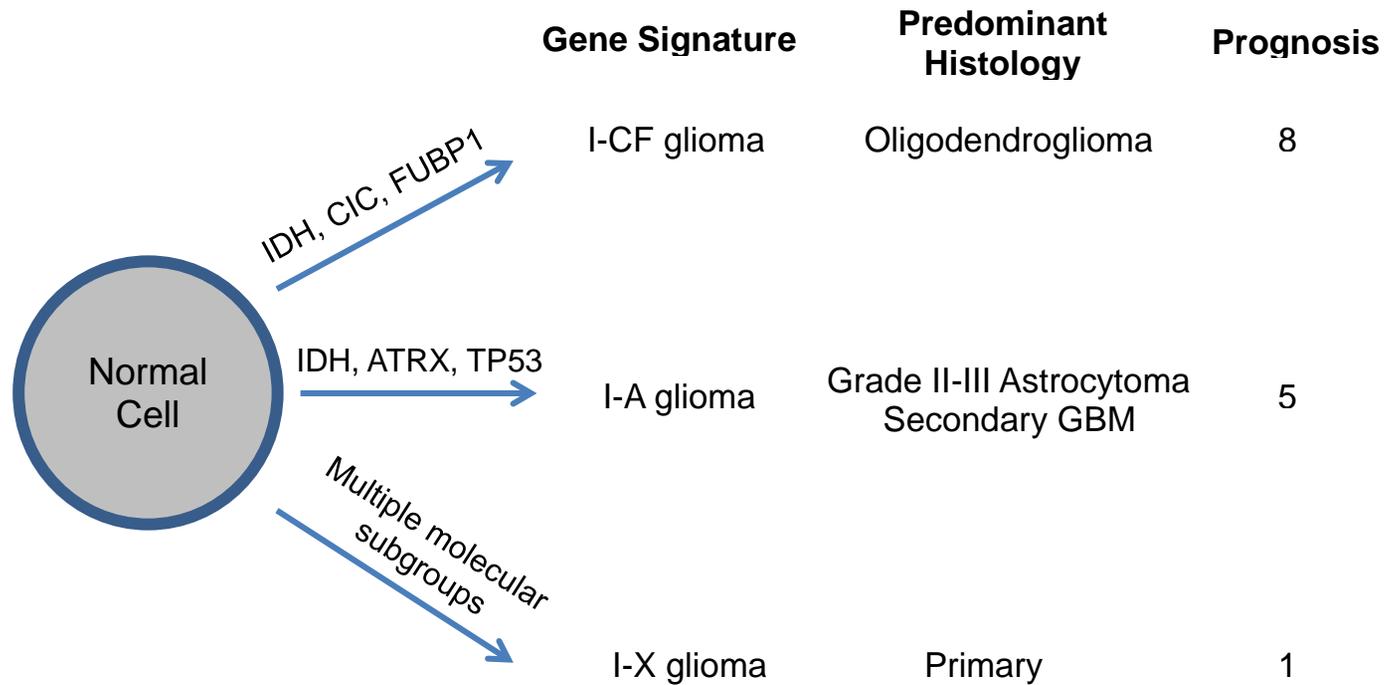
Sample ID	Diagnosis	Age	Sex	ATRX	ATRX IHC	ALT Staining	IDH1	IDH2	TP53	CIC	FUBP1	1p and 19q LOH	I-A, I-CF or I-X
1	OAI	38	F	MUT	NA	NA	MUT	no	MUT	no	no	no	I-A
2	OAI	80	M	no	Positive	Negative	MUT	no	no	no	no	no	I-X
3	OAI	45	M	no	NA	NA	MUT	no	no	no	no	Yes	I-CF
4	OAI	52	F	no	Positive	Negative	MUT	no	no	no	no	Yes	I-CF
5	OAI	21	F	no	Negative	Positive	MUT	no	MUT	no	no	no	I-A
6	OAI	52	F	MUT	NA	NA	MUT	no	MUT	no	no	no	I-A
7	OAI	38	M	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
8	OAI	51	M	no	Positive	Negative	MUT	no	no	no	no	no	I-X
9	OAI	35	F	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
10	OAI	61	M	no	NA	NA	MUT	no	MUT	no	no	no	I-X
11	OAI	52	M	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
12	OAI	25	F	no	Positive	Negative	MUT	no	no	MUT	no	no	I-CF
13	OAI	30	M	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
14	OAI	32	M	no	Negative	Positive	MUT	no	MUT	no	no	no	I-A
15	OAI	32	M	no	Positive	Negative	no	MUT	no	no	no	Yes	I-CF
16	OAI	34	M	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
17	OAI	39	F	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
18	OAI	46	M	no	NA	NA	MUT	no	no	no	MUT	Yes	I-CF
19	OAI	65	M	MUT	Negative	Positive	MUT	no	no	no	no	no	I-A
20	OAI	38	F	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
21	OAI	44	F	MUT	NA	NA	MUT	no	MUT	no	no	no	I-A
22	OAI	49	M	no	Positive	Negative	MUT	no	no	no	no	Yes	I-CF
23	OAI	31	M	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
24	OAI	34	F	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
25	OAI	25	M	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
26	OAI	32	M	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
27	OAI	32	M	no	Negative	Positive	MUT	no	MUT	no	no	no	I-A
28	OAI	26	F	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
29	OAI	49	F	MUT	NA	NA	MUT	no	MUT	no	no	no	I-A
30	OAI	30	M	MUT	Negative	Positive	MUT	no	MUT	no	no	no	I-A
31	OAI	55	M	MUT	NA	NA	MUT	no	MUT	no	no	no	I-A
32	OAI	48	M	no	Positive	Negative	MUT	no	no	MUT	no	Yes	I-CF
33	OAI	22	F	no	Negative	Positive	MUT	MUT	MUT	no	no	no	I-A
34	OAI	44	F	no	NA	NA	no	no	no	no	no	no	I-X

35	OAIII	2	F	MUT	NA	Positive	MUT	no	MUT	no	no	no	I-A
36	OAIII	71	M	no	NA	NA	MUT	no	no	no	no	Yes	I-CF
37	OAIII	42	M	no	Negative	Positive	MUT	no	MUT	no	no	Yes	Mixed I-A and I-CF
38	OAIII	30	M	no	Negative	Positive	MUT	no	MUT	no	no	no	I-A
39	OAIII	45	F	no	NA	NA	MUT	no	no	MUT	MUT	Yes	I-CF
40	OAIII	35	F	MUT	NA	Positive	MUT	no	MUT	no	no	no	I-A

*OAII, grade II oligoastrocytoma; OAIII, grade III oligoastrocytoma. Age is age at diagnosis.

3.4 Discussion

The association between *ATRX* mutations with *IDH* mutations, and the association between *CIC/FUBP1* mutations and *IDH* mutations as well as 1p/19q loss defined I-A and I-CF genetic signatures. These signatures are especially common among the grade II-III gliomas and remarkably homogeneous in terms of genetic alterations and clinical characteristics. I-A tumors were defined by alterations in *ATRX* and in *IDH*; they almost always had *ALT* and *TP53* mutations, typically had an astrocytic histological component, and were often diagnosed in the fourth decade of life. I-CF tumors were defined by *IDH* mutations and by alterations in either *CIC*, *FUBP1*, and/or 1p/19q, rarely displayed *ALT*, typically had an oligodendroglial component and were often diagnosed in the fifth decade of life. This molecular classification system distinguishes the prognosis of gliomas with I-A and I-CF genetic signatures from each other and from the poorly-performing, genetically heterogeneous I-X group (Figure 8).



I-CF – tumors mutant in IDH, CIC and/or FUBP1 with 1p/19q

I-A – tumors mutant in IDH and ATRX with altered telomeres (ALT)

I-X – tumors without I-A or I-CF mutations; represents multiple molecular subgroups, similar to primary GBM

Figure 8: Model for molecular classification of gliomas.

Figure 8 (Continued): Model for molecular classification of gliomas.

Alterations in IDH and ATRX comprise the I-A signature, and alterations in IDH as well as one or more of CIC, FUBP1, or combined 1p/19q loss comprise the I-CF signature. I-X gliomas do not have either signature and likely represent multiple molecular disease subgroups. The I-A and I-CF signatures arise early in the pathogenesis of a glioma from a normal precursor cell. I-CF gliomas are typically grade II-III oligodendrogliomas and patients with these tumors survive for about 8 years. I-A gliomas are typically grade II-III astrocytomas or grade IV secondary GBMs and patients with these tumors survive for about 5 years. I-X gliomas are typically grade IV primary GBMs and patients with these tumors survive for about 1 year.

Our mutational analysis expands on recent reports that *ATRX*, *CIC*, and *FUBP1* mutations are frequent in specific nervous system tumor types (Bettegowda, Agrawal et al. 2011, Jiao, Shi et al. 2011, Cheung, Zhang et al. 2012, Sahm, Koelsche et al. 2012, Schwartzenuber, Korshunov et al. 2012, Yip, Butterfield et al. 2012). *ATRX* mutations were recently found to define a clinically distinct population of teenaged and young adult neuroblastoma patients, who often had a protracted course with death occurring many years after diagnosis (Cheung, Zhang et al. 2012). Our results reveal striking parallels to the *ATRX*-mutated neuroblastoma patients, since *ATRX*-mutated glioma patients are also relatively young adults and demonstrate a protracted but ultimately fatal course (median overall survival 51 months). In contrast to a previous report that *FUBP1* mutations always co-occurred with a *CIC* mutation among a cohort of 18 oligodendrogliomas and 42 oligoastrocytomas reported by Sahm and colleagues (Sahm, Koelsche et al. 2012), we did not observe any significant associations between *CIC* and *FUBP1* mutations among 50 oligodendrogliomas and 40 oligoastrocytomas (Figure 2).

However, we did observe a case with *CIC* mutation that occurred in the absence of 1p/19q loss, which is a situation that was observed in one oligodendroglial tumor by Sahm and colleagues (Sahm, Koelsche et al. 2012). In addition to expanding on previous preliminary reports of the frequency of *ATRX*, *CIC*, and *FUBP1* mutations in selected tumor types, our study for the first time identifies associations between these mutations and age at diagnosis and clinical outcome among glioma patients.

Mutational analysis of *ATRX*, *CIC*, and *FUBP1* refined the prognostic information provided by the known prognostic markers *IDH*, *TP53*, and 1p/19q (McLendon, Herndon et al. 2005, Louis, Ohgaki et al. 2007, Wen and Kesari 2008, Nobusawa, Watanabe et al. 2009, Hartmann, Hentschel et al. 2010). This refinement is shown by comparing tumors with isolated *IDH* mutations to patients who had these mutations in the context of a full I-A or I-CF genetic signature (Figure 6). 1p/19q status alone also may not be sufficient to determine whether a tumor contains alterations in *CIC* or *FUBP1*, since *CIC* mutation occurred in the absence of 1p/19q alteration in one of the cases studied. Also, the presence of an oligoastrocytoma with “mixed” I-A and I-CF signatures raises the possibility that a subset of histopathologically mixed tumors are truly heterogeneous in a manner that may not be detected by routine 1p/19q analysis alone. *IDH*-mutated gliomas are a subset of the gliomas with the proneural gene expression signature (Verhaak, Hoadley et al. 2010), and *IDH* mutations tightly overlap with the G-CIMP hypermethylator phenotype among gliomas (Noussmehr,

Weisenberger et al. 2010, Turcan, Rohle et al. 2012). Therefore, the I-A and I-CF genetic signatures provide a molecular platform to further subclassify the broader proneural and G-CIMP molecular subgroups of gliomas.

In contrast to the I-A and I-CF genetic signatures, which represent homogeneous genetic disease entities, I-X tumors are a genetically heterogeneous group. Based on their association with poor patient survival and advanced patient age, and on their lack of *IDH* or *ATRX* mutations, I-X tumors are similar to primary GBMs (Ohgaki and Kleihues 2009, Yan, Parsons et al. 2009, Heaphy, de Wilde et al. 2011) and to *IDH* wild-type low-grade gliomas (Figarella-Branger, de Paula et al. 2011, Kim, Lachuer et al. 2011). Patients with these tumors can be further classified into prognostic subgroups on the basis of other mutational, copy number, gene expression, and DNA methylation alterations such as *EGFR* amplification, *PTEN* deletion, gene expression signatures, and other previously-described markers (Phillips, Kharbanda et al. 2006, Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008, Ohgaki and Kleihues 2009, Noushmehr, Weisenberger et al. 2010, Verhaak, Hoadley et al. 2010). Our genetic classification scheme provides a prognostic tool to identify uncommon I-A and I-CF tumors among the grade IV gliomas and to classify the vast majority of grade II-III gliomas.

Many tumors classified as one glioma subtype by histopathological criteria bore hallmark genetic and clinical features of another tumor type in the present study. 16% (7/39) of oligodendrogliomas and 3% (3/87) of primary GBMs bore the I-A genotype,

suggesting that a subset of tumors diagnosed as oligodendrogliomas or primary GBMs are genetically more similar to grade II-III astrocytomas. 11% (15/132) of grade II-III gliomas and GBMs lacked I-A or I-CF gene signatures altogether and had a poor survival (I-X tumors) and are likely genetically more similar to primary GBMs than to other grade II-III gliomas. Moreover, 98% (39/40) of gliomas containing histopathological features of both oligodendrogliomas and astrocytomas could be unambiguously assigned to a genetic signature: 26 were I-A, 9 were I-CF, and 4 were I-X.

Currently, there are no significant differences in the treatment of gliomas based on astrocytic or oligodendrocytic differentiation, or based on any molecular prognostic markers. Treatment decisions are based on WHO tumor grade (Wen and Kesari 2008, Holdhoff and Grossman 2011) despite the fact that outcomes vary significantly between different glioma subgroups (Smith, Perry et al. 2000, Phillips, Kharbanda et al. 2006, Louis, Ohgaki et al. 2007, Cairncross and Jenkins 2008). With the development of personalized therapies for glioma patients, a refined classification that incorporates genetic signatures may become critical for their management. As sequencing technology declines in cost, genotypic analysis is expected to become increasingly accessible to patients, not only as part of clinical trials, but also as part of standard clinical care. In the interim, these data suggest that new therapeutic trials for patients with gliomas should incorporate genetic signatures, as well as histopathologic signatures, to help to classify patients for eligibility for a specific treatment and to determine their outcomes.

4. TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal

4.1 Introduction

Telomeres are nucleoprotein complexes at the ends of eukaryotic chromosomes that are required for chromosomal integrity. Several hundred nucleotides of telomere repeats cap each chromosomal end, and in the absence of telomerase activity, telomeres shorten with each cell division (Aubert and Lansdorp 2008). Eventually, uncapped telomeres trigger cell death or senescence. Cancer cells seem to divide *ad infinitum* and therefore, require some telomere maintenance mechanism to avoid this fate. Because telomerase activity is generally higher in cancer cells than normal cells, it was originally believed that telomerase was somehow activated in cancer cells (Kim, Piatyszek et al. 1994, Shay and Bacchetti 1997, Li, Zhao et al. 1998, Kang, Kwon et al. 1999, Akiyama, Hideshima et al. 2002). However, it was subsequently realized that telomerase was only inactive in terminally differentiated cells and that normal stem cells in self-renewing tissues retained telomerase activity (Morrison, Prowse et al. 1996, Forsyth, Wright et al. 2002, Hiyama and Hiyama 2007, Aubert and Lansdorp 2008). Because normal stem cells must replicate throughout the long lifetimes of mammals (which can be more than a century in humans), it is clear that such cells must also retain telomerase activity. Because normal stem cells are thought to be the progenitors of cancers, there would be no need to specifically activate telomerase in cancer cells; the enzyme was already active

in the precursors, just as were the hundreds of other enzymes and proteins normally required for cell proliferation.

This view was challenged by the discovery of another mechanism for maintaining telomere length [i.e., alternative lengthening of telomeres (ALT)] (Cesare and Reddel 2010, Heaphy, de Wilde et al. 2011, Jiao, Shi et al. 2011). ALT occurs in the absence of telomerase activity and seems to be dependent on homologous recombination. It occurs in a particularly high fraction of certain tumor types, such as sarcomas, pancreatic neuroendocrine tumors, and brain tumors, but rarely in most common tumor types, such as those tumor types of the colon, breast, lung, prostate, or pancreas (Heaphy, Subhawong et al. 2011). Why would cancer cells need ALT if telomerase activity was already constitutively active in their precursors? This question was highlighted by the discovery that many ALT cancers harbor mutations in alpha thalassemia/mental retardation syndrome X-linked (*ATRX*) or death-domain associated protein (*DAXX*), genes encoding proteins that interact with each other at telomeres (Heaphy, de Wilde et al. 2011, Jiao, Shi et al. 2011). Presumably, the absence of functional *ATRX/DAXX* complexes permits the homologous recombination resulting in ALT. At minimum, these data were compatible with the ideas that there could be a selective advantage for genetic alterations that results in telomere maintenance and that telomerase is not indefinitely activated in all normal stem cell precursors of cancers.

Another challenge to the idea that genetic alterations were not required for telomerase activation in cancer was raised by the finding that mutations of the telomerase reverse transcriptase (*TERT*) promoter occurred in ~70% of melanomas and in a small number of tumor cell lines derived from various tissue types (Horn, Figl et al. 2013, Huang, Hodis et al. 2013). Importantly, only 5 of 110 cell lines derived from lung, stomach, ovary, uterus, or prostate cancers harbored *TERT* promoter mutations, whereas 19 mutations were found among 37 cell lines derived from various other tumor types. This situation is analogous to the situation for ALT, which is infrequently observed in common epithelial cancers but is observed more regularly in tumors derived from nonepithelial cells, particularly sarcomas and brain tumors (Heaphy, Subhawong et al. 2011).

These findings prompted us to formulate a hypothesis about the mechanisms responsible for telomerase activity in cancers. We suggest that there are two ways to maintain telomere lengths as cells divide: (i) through epigenetic regulation of telomerase activity, which occurs in stem cells of tissues that are rapidly renewing, and (ii) through somatic mutations that maintain telomere lengths, such as mutations in the *TERT* promoter or mutations in *DAXX* or *ATRX*. Those cancers that originate in tissues that are constantly self-renewing, such as cancers of the epithelia of the gastrointestinal tract and skin or bone marrow, would be unlikely to harbor telomere-maintaining mutations, because telomerase is already epigenetically activated in their precursor cells. In

contrast, tumors arising from cells that are not constantly self-renewing, such as neurons, glial cells, fibroblasts, hepatocytes, islet cells, and pancreatic ductal epithelial cells, might frequently harbor such mutations. A corollary of this hypothesis is that tumor types exhibiting high frequencies of ALT would also exhibit high frequencies of *TERT* mutations, and these mutations would be distributed in a mutually exclusive fashion. To test these hypotheses as well as answer other questions related to the role of *TERT* promoter mutations in various cancer types, we determined the prevalence of *TERT* promoter mutations in a large number of tumors.

4.2 Methods

All clinical information and tissue were obtained with consent and Institutional Review Board approval from the various institutions donating material to this study, and they were obtained in accordance with the Health Insurance Portability and Accountability Act. Tissue sections were reviewed by board-certified pathologists to ensure that $\geq 50\%$ of the cells used for DNA purification were neoplastic and confirm histopathological diagnosis. Oligonucleotides with the sequences 5'-M13-GGCCGATTCGACCTCTCT-3' and 5'-AGCACCTCGCGGTAGTGG-3', where M13 is a universal sequencing priming site with sequence 5'-tgtaaacgacgccagt-3', were used to PCR-amplify the proximal *TERT* promoter containing C228 and C250 (chr5: 1,295,228; chr5: 1,295,250, respectively; hg19) for Sanger sequencing using standard methods (Sjoblom, Jones et al. 2006). Primary GBM copy number data as well as ALT status were

derived from the data published in refs. (Jiao, Killela et al. 2012), (Duncan, Killela et al. 2010), and (Yan, Parsons et al. 2009), and *OTX2* copy number expression was derived from the data published in ref. (Adamson, Shi et al. 2010). Brain tumor patients were treated at the Tisch Brain Tumor Center at Duke. For the purposes of this study, secondary GBM designates a GBM that was resected >1 y after a prior diagnosis of a lower-grade glioma (grades I–III), and all other GBMs were considered to be primary GBMs. Pediatric GBM samples were defined as those samples occurring before 21 y of age.

4.3 Results

4.3.1 TERT Promoter Analysis Results

We attempted to evaluate at least 20 individual specimens of common tumor types and fewer specimens of rare tumor types, depending on availability of specimens in our laboratories. In those tumor types in which our pilot studies showed a significant number of mutations, additional tumors were evaluated. Melanomas and tumors of the lung, stomach, and esophagus were excluded, because they had already been adequately evaluated in the seminal papers cited (Horn, Figl et al. 2013, Huang, Hodis et al. 2013). When primary tumors rather than cell lines were used, we ensured that the fraction of neoplastic cells was >50% through histopathologic examination of frozen sections of the tissue blocks used for DNA purification. In those cases in which the neoplastic content was <50%, we microdissected the lesions to enrich the neoplastic

content to >50%. Primers were designed to amplify the region containing the two *TERT* mutations that were previously described—C228T and C250T—corresponding to the positions 124 and 146 bp, respectively, upstream of the *TERT* ATG start site (Horn, Figl et al. 2013, Huang, Hodis et al. 2013). The PCR fragments were then purified and analyzed by conventional Sanger sequencing.

In all, we evaluated *TERT* promoter mutations in 1,230 tumor specimens and identified 231 mutations (18.8%) (Table 7). C228T and C250T mutations accounted for 77.5% and 20.8% of the alterations, respectively. Additionally, we detected four mutations that had not been observed previously: three C228A mutations and one C229A mutation. All four of these mutations as well as a representative subset of the C228T and C250T mutations (n = 59) were somatic, as evidenced by their absence in normal tissues of the patients containing the mutations in their tumors.

Table 7: Frequency of TERT mutations in human cancers.

Tumor type*	No. tumors	No. tumors mutated (%)
Chondrosarcoma	2	1 (50)
Dysembryoplastic neuroepithelial tumor	3	1 (33.3)
Endometrial cancer	19	2 (10.5)
Ependymoma	36	1 (2.7)
Fibrosarcoma	3	1 (33.3)
Glioma†	223	114 (51.1)
Hepatocellular carcinoma	61	27 (44.2)
Medulloblastoma	91	19 (20.8)
Myxofibrosarcoma	10	1 (10.0)
Myxoid liposarcoma	24	19 (79.1)
Neuroblastoma	22	2 (9)
Osteosarcoma	23	1 (4.3)

Ovarian, clear cell carcinoma	12	2 (16.6)
Ovarian, low grade serous	8	1 (12.5)
Solitary fibrous tumor (SFT)	10	2 (20.0)
Squamous cell carcinoma of head and neck	68	11 (16.8)
Squamous cell carcinoma of the cervix	22	1 (4.5)
Squamous cell carcinoma of the skin	5	1 (20)
Urothelial carcinoma of bladder	21	14 (66.6)
Urothelial carcinoma of upper urinary epithelium	19	9 (47.3)

*No mutations were found in acute myeloid leukemia (n=48), alveolar rhabdomyosarcoma (n=7), atypical lipomatous tumor (n=10), breast carcinoma (n=88), cholangiosarcoma (n=28), central/conventional chondrosarcoma (n=9), chronic lymphoid leukemia (n=15), chronic myeloid leukemia (n=6), colorectal adenocarcinoma (n=22), embryonal rhabdomyosarcoma (n=8), esthesioneuroblastoma (n=11), extraskeletal myxoid chondrosarcoma (n=3), fibrolammellar carcinoma of the liver (n=12), gall bladder carcinoma (n=10), gastrointestinal stromal tumor (n=9), hepatoblastoma (n=3), leiomyosarcoma (n=3), conventional lipoma (n=8), low grade fibromyxoid sarcoma (n=9), malignant peripheral nerve sheath tumor (n=3), medullary thyroid carcinoma (n=24), meningioma (n=20), mesothelioma (n=4), pancreatic acinar carcinoma (n=25), pancreatic ductal adenocarcinoma (n=24), pancreatic neuroendocrine tumor (n=68), prostate carcinoma (n=34), spinal ependymoma (n=9), synovial sarcoma (n=16), or undifferentiated pleomorphic soft tissue sarcoma (n=10) samples.

†Glioma comprises 11 subtypes; see Table 10

The 1,230 tumors represented 60 tumor types. In 26 of these tumor types, at least 15 individual tumors were evaluated (comprising a total of 1,043 individual tumors) (Figure. 9). In the remaining tumor types, only a small number of samples (2–12) was available, in part because these tumor types are generally uncommon in Western populations (Table 7). Among the tumor types in which at least 15 individual tumors were available for study, a clear distinction could be made. Eighteen of these tumor types had only occasional *TERT* promoter mutations (zero to three mutations, comprising 0–15% of the tumors of each type) (Figure. 9). We classified these tumor

types as TERT-low (TERT-L), because they had a low frequency of *TERT* promoter mutations. Eight other tumor types were classified as TERT-high (TERT-H) because of their relatively high prevalence of *TERT* promoter mutations (16–83% of the tumors of each type).

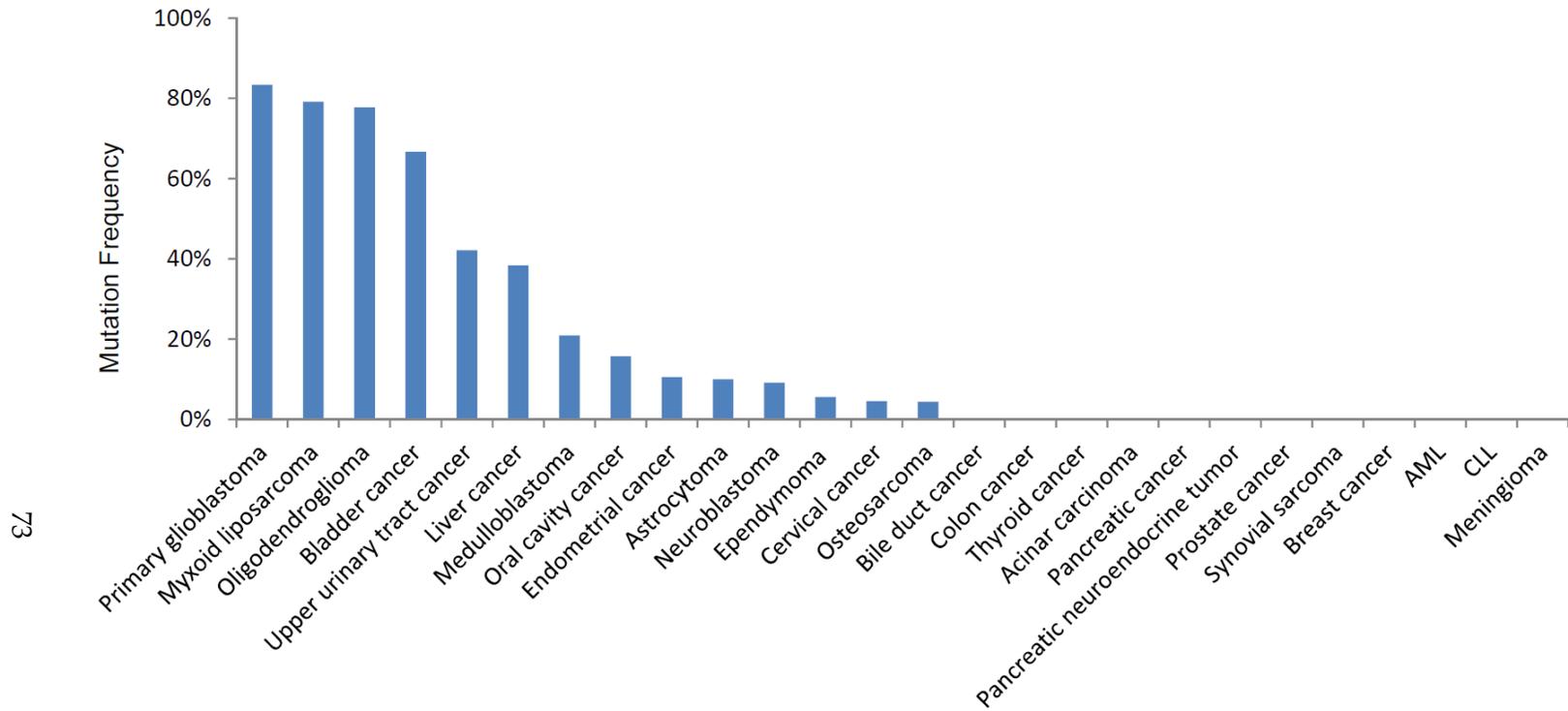


Figure 9: Frequency of TERT promoter mutations

15 or more tumors were analyzed in 26 tumor types. Gliomas are divided into primary GBM, astrocytoma (including astrocytoma grades II and III, as well as secondary GBM), and oligodendroglioma.

The TERT-L tumor types included some of the most prevalent cancers, including epithelial tumors of the breast, prostate, thyroid, pancreas, gall bladder, uterus, and colon (as well as tumors of the lung, stomach, and esophagus based on prior studies) (Horn, Figl et al. 2013, Huang, Hodis et al. 2013) and leukemias. In fact, no *TERT* mutations were identified in any specimen of 30 tumor types that we studied, comprising a total of 546 tumors (Table 7). Some nonepithelial cancers, such as synovial sarcomas, chordomas, neuroblastomas, osteosarcomas, and ependymomas, were also TERT-L.

Eight TERT-H tumor types were identified (in addition to the previously described melanomas) (Horn, Figl et al. 2013, Huang, Hodis et al. 2013). These tumors included tumors of the CNS, transitional cell carcinomas of the urinary tract, hepatocellular carcinomas, myxoid liposarcomas, and oral cavity carcinomas. Although only a small number of TERT-H tumors (other than melanomas) were examined in previous studies (Huang, Hodis et al. 2013), mutations in gliomas, hepatocellular, and oral cavity carcinomas were detected, which would be expected on the basis of the high frequency of mutation in these tumors types (Table 7).

4.3.2 Clinical and Molecular Correlations in TERT-H Tumors

4.3.2.1 Sarcomas

One of the highest frequencies of *TERT* promoter mutation was found in myxoid liposarcoma (19 of 24 tumors, 79% with mutation). Myxoid liposarcomas account for

more than one-third of all liposarcomas and ~10% of all adult soft tissue sarcomas (Conyers, Young et al. 2011). Patients are relatively young, with a peak age range between 30 and 50 y. At the genetic level, the most characteristic change is a t(12;16)(q13;p11) chromosomal translocation that results in the fusion of the *FUS* and *DDIT3* genes (Goransson, Andersson et al. 2009, Conyers, Young et al. 2011). The cellular origin of these tumors is unknown, but preadipocytic progenitor cells and mesenchymal stem cells have been implicated (Charytonowicz, Terry et al. 2012); after embryogenesis, the mitotic activity of these cells is thought to be low. Other sarcomas, also thought to originate from mesenchymal cells that do not self-renew in the absence of damage, were not TERT-H (Table 7). These sarcomas included synovial sarcomas (0% of 16 tumors) and osteosarcomas (4.3% of 23 tumors). Of note, myxoid liposarcomas have been previously shown to have a relatively high prevalence of ALT (24% of 38 tumors) (Costa, Daidone et al. 2006, Heaphy, Subhawong et al. 2011). The data, in aggregate, are compatible with the idea that myxoid liposarcomas almost always genetically activate telomere maintenance genes through either *TERT* promoter mutations or ALT.

4.3.2.2 Hepatocellular Carcinomas

Hepatocellular carcinomas (HCCs) are the third leading cause of cancer mortality worldwide, and their incidence is increasing in the United States (Altekruse, McGlynn et al. 2009). Most HCCs in the United States are associated with Hepatitis B or C Virus infection, whereas others are associated with alcoholic cirrhosis; 44% of HCC samples

that we evaluated harbored *TERT* promoter mutations (27/61). This finding makes *TERT* the most commonly mutated gene yet observed in this tumor type (Li, Zhao et al. 2011, Guichard, Amaddeo et al. 2012). The mutations seemed to occur relatively early in tumorigenesis, because they were observed in 39% of stage I well-differentiated HCCs (Table 8). *TERT* mutations were observed in virally associated tumors as well as cases without any underlying liver disease at similar frequencies (Table 8). There was also no difference in the prevalence of *TERT* promoter mutations with respect to sex, age, or ethnicity (Table 8). ALT has been observed in 7% of 121 HCCs studied previously (Heaphy, Subhawong et al. 2011).

Table 8: Hepatocellular carcinoma patient data

Sample ID	Age (y)	Race	Sex	Stage	Differentiation	Focality	Underlying liver disease	Largest tumor size (cm)	Mutations in known drivers	<i>TERT</i> status
RK66	77	W	F	T2	Moderate	Single	HCV	4.5	CTNNB1	C228T
RK67	57	W	M	T1	Moderate	Single	HCV	3.5		WT
RK113	71	B	F	T2	Well	Multifocal	HCV	2.8	CTNNB1, ARID2	C228T
RK142	67	W	M	T2	Poor	Single	HCV	7.2	TP53	C228T
RK148	66	B	M	T1	Well	Single	HCV	4.5	CTNNB1, ARID2	C228T
RK153	58	W	F	T2	Well	Multifocal	HCV	2.8	CTNNB1, ARID2	C228T
RK168	62	B	F	T1	Well	Single	HCV	1.8		WT
RK179	62	W	M	T2	Poor	Multifocal	HCV	5.5	CTNNB1	C228T
RK183	65	B	F	T3b	Moderate	Multifocal	HCV	7.5	TP53	C228T
RK190	66	W	M	T1	Well	Single	HCV	1.6	TP53	WT
RK191	61	B	M	T2	Moderate	Multifocal	HCV	3.5	TP53	C228T
6700T	79	B	M	T1	Moderate	Single	HCV	4	ARID2	C228T
HCC 41 PT	67	UNK	M	UNK	Moderate	UNK	HBV	3.9		WT
HCC 42 PT	55	UNK	M	UNK	Moderate	UNK	HBV	1.7		WT
HCC 43 PT	64	UNK	M	UNK	Moderate	UNK	HBV	8		WT
HCC 45 PT	49	UNK	F	UNK	Poor	UNK	HCV	4		WT
HCC 46 PT	54	UNK	M	UNK	UNK	UNK	HBV	11	TP53	C228T
HCC 47 PT	53	UNK	M	UNK	UNK	UNK	HCV	1.3	ARID2	C228T
HCC 48 PT	48	UNK	M	UNK	Poor	UNK	HBV and ETOH	9		C228T
HCC 196	55	B	F	T1	Moderate	Single	HCV	1.8		WT
HCC 193	77	W	F	T2	Poor	Single	None	18		WT

HCC 192	60	W	F	T2	Moderate	Single	Cryptotenic liver disease	10	CTNNB1, ARID2	C228T
HCC 334	63	Asian	M	T1	Moderate	Single	HBV	3.4		WT
HCC 395	80	W	M	T1	Well	Single	None	6		C228T
HCC 712	61	W	M	T1	Moderate	Single	HCV	1.6		WT
RK3	59	W	M	T1	Well	Single	None	11	CTNNB1	C228T
RK5	70	W	M	T1	Moderate	Single	None	6.5	CTNNB1	C228T
RK9	40	W	F	T2	Moderate	Single	None	3.7		WT
RK15	59	Asian	F	T2	Moderate	Two	HBV	2.5		WT
RK63	63	B	M	T2	Moderate	Two	ETOH	5		C228T
RK65	55	W	M	T1	Well	Single	None	17		WT
RK69	71	W	M	T2	Moderate	Single	None	4.4		WT
RK73	65	B	M	T2	Moderate	Single	HBV	3.5		C228T
RK110	62	W	M	T1	Moderate	Single	ETOH	2		C228T
RK111	33	W	F	T1	Well	Single	Hepatic adenoma	1		WT
RK112	50	B	F	T2	Well	Single	None	9	TP53	WT
RK117	47	W	F	T2	Moderate	Single	None	15		WT
RK124	19	B	F	T3A	Moderate	Diffuse	None	Diffuse		C228T
RK129	45	W	F	t1	Well	Single	None	5		C228T
RK132	76	Asian	M	T1	Well	Single	HBV	4	TP53	WT
RK134	69	Asian	M	T1	Moderate	Single	None	3.2		C228T
RK139	74	W	M	T1	Moderate	Single	None	4.5		C228T
RK150	37	B	M	T2	Poor	Two	HBV	7		WT
RK165	46	Asian	M	T2	Poor	Single	HBV	5	TP53	C228T
RK166	65	Asian	F	T1	Moderate	Single	HBV	3	TP53	WT
RK174	60	W	M	T1	Moderate	Single	None	12	CTNNB1	WT
RK176	64	W	F	T1	Moderate	Single	None	8		WT
RK177	52	Asian	M	T1	Moderate	Single	HBV	1.2		WT

RK184	74	W	F	T2	Well	Two	None	5.3		C228T
RK193	73	Asian	M	T2	Moderate	Single	HBV	5.4		WT
RK194	76	W	M	T1	Well	Single	HBV	2.5	CTNNB1	WT

Only hepatocellular carcinoma (HCC) patients for which clinical information is known are tabulated. ETOH, alcohol; F, female; HBV, hepatitis B virus; HCV, hepatitis C virus; M, male; UNK, unknown; W, white; B, black.

4.3.2.3 Urinary Tract Cancers

Urothelial carcinoma of the bladder is the fourth most common type of cancer in American males. In 2013, over 73,000 patients will be diagnosed with bladder cancer leading to approximately 15,000 deaths in the US alone (Siegel, Naishadham et al. 2013). Two-thirds of the 21 urothelial carcinomas of the bladder that we studied harbored *TERT* promoter mutations. We were also able to evaluate 19 urothelial carcinomas of the upper urinary tract, a much less common anatomic site for this histopathologic subtype of tumor. Nine of nineteen upper urinary tract urothelial carcinomas harbored *TERT* mutations. *TERT* mutations are, therefore, the most frequently mutated genes yet identified in urothelial carcinoma of either the bladder or upper urinary tract (Gui, Guo et al. 2011). The prevalence of ALT in bladder cancers is very low (1% of 188 cancers) (Heaphy, Subhawong et al. 2011).

4.3.2.4 Head and neck cancers

Head and neck cancers are almost always squamous cell carcinomas and can occur throughout the oral cavity lining (mucous membranes of the cheek, hard and soft palate, tongue, supraglottis, etc.). It is the sixth most common cancer in the world, and 50,000 cases occurred in the United States in 2012. We identified *TERT* promoter mutations in 17% of 70 oral cavity cancers that we evaluated. However, the anatomic distribution of the cases with *TERT* promoter mutations was striking: 11 of 12 cancers

with *TERT* promoter mutations were in the oral tongue, although only 23 of 70 total cases originated in the oral tongue ($P < 0.0001$, Fisher exact probability test, two-tailed) (Table 9). The basis for this extraordinary selectivity is curious given the shared characteristics of the squamous epithelium lining the tongue and other parts of the head and neck, including the oral cavity. Moreover, we evaluated 22 squamous cell carcinomas of another site (the cervix) and found only one *TERT* mutation (4.5%) (Table 7). Most cervical squamous cell carcinomas and a subset of head and neck squamous cell carcinomas are caused by human papillomavirus, which can activate telomerase by expressing E6 and E7 viral oncogenes (Liu, Dakic et al. 2009). These findings raise the possibility that human papillomavirus infection and *TERT* mutation may be alternative mechanisms to activate telomerase among squamous cell carcinomas. We were unable to test correlations between *TERT* promoter mutations and HPV status or other clinical parameters because of the small number of patients with available data (Table 9). There have been no ALT cases identified among 70 head and neck cancers, including 41 oral cavity cancers (Heaphy, Subhawong et al. 2011).

Table 9: Oral cavity cancer patient data

Sample ID	Age (y)	Sex	Site/subsite	Histology	HPV status	Tobacco use	Mutations in known drivers	TERT status
HN 103 PT	54	M	Floor of mouth	SCC	N/A	Y		WT
HN 104 PT	50	F	Mandible	SCC	N/A	Y	TP53	WT
HN 108 PT	49	M	Tongue	SCC	N/A	N		WT
HN 111 PT	45	M	Hard palate	SCC	N/A	Y		WT
HN 112 PT	40	F	Tongue	SCC	N/A	N	FBXW7	WT
HN 115 PT	54	F	Floor of mouth	SCC	N/A	Y	NOTCH1, TP53	WT
HN 116 PT	56	M	Floor of mouth	SCC	N/A	Y		WT
HN 120 PT	46	M	Floor of mouth	SCC	N/A	Y		WT
HN 124 PT	53	M	Tongue	SCC	N/A	Y	CDKN2A	C228T
HN 125 PT	UNK	M	Larynx	SCC	UNK	UNK	TP53	WT
HN 127 PT	52	M	Larynx	SCC	N/A	Y		WT
HN 129 PT	59	M	Supraglottis	SCC	N/A	Y	TP53	WT
HN 133 PT	50	M	Glottis	SCC	N/A	Y	CDKN2A, TP53	WT
HN 134 PT	66	M	Tonsil	SCC	Y	UNK		WT
HN 137 PT	65	M	Tonsil	SCC	Y	Y	FBXW7	WT
HN 138 PT	58	M	Oropharynx/ hypopharynx	SCC	UNK	Y	TP53	WT
HN 139 PT	57	F	Tonsil	SCC	UNK	Y	TP53, NOTCH1	WT
HN 143 PT	49	M	Tonsil	SCC	Y	N		WT
HN 145 PT	58	F	Base of tongue	SCC	Y	UNK		WT
HN 146 PT	44	M	Oropharynx	SCC	UNK	Y		WT
HN 147 PT	72	M	Base of tongue	SCC	UNK	Y		WT
HN 148 PT	46	M	Tonsil	SCC	UNK	Y	NOTCH1	WT

HN 151 PT	54	M	Tonsil	SCC	Y	Y		WT
HN 152 PT	69	F	Tongue	SCC	N/A	Y		C228T
HN 305 PT	50	M	Floor of mouth	SCC	N/A	Y		WT
HN 306 PT1	59	M	Floor of mouth	SCC	N/A	Y		WT
HN01 PT	44	F	Tongue	SCC	N/A	N	TP53	WT
HN02PT-2	64	M	Tongue	SCC	N/A	UNK		C228T
HN03 PT	46	F	Tongue	SCC	N/A	Y		WT
HN04 PT	42	M	Base of tongue	SCC	UNK	N		WT
HN05 PT2	64	F	Alveolar ridge	SCC	N/A	Y		C250T
HN06 PT	72	F	Tongue	SCC	N/A	N		WT
HN07 PT	66	F	Floor of mouth	SCC	N/A	Y		WT
HN08 PT	43	M	Tongue	SCC	N/A	Y		C250T
HN09PT	34	M	Tongue	SCC	N/A	Y	TP53	C228T
HN10PT	82	M	Tongue	SCC	N/A	Y		WT
HN11PT	63	F	Tongue	SCC	N/A	Y	TP53, CDKN2A, HRAS, PIK3CA	WT
HN13PT	71	F	Tongue	SCC	N/A	Y		WT
HN14PT	37	F	Tongue	SCC	N/A	N	TP53, NOTCH1	C228T
HN15PT	67	F	Hard palate	SCC	N/A	Y		WT
HN16PT	63	M	Tongue	SCC	N/A	Y	TP53	WT
HN17PT DNA	65	F	Tonsil	SCC	Y	UNK		WT
HN18PT DNA	59	M	Tonsil	SCC	Y	N		WT
HN19PT DNA	42	M	Tonsil	SCC	Y	N		WT
HN20PT DNA	63	M	Tonsil	SCC	Y	Y		WT
HN21PT DNA	59	M	Tonsil	SCC	N	Y		WT
HN22PT DNA	52	F	Supraglottis	SCC	N/A	Y	TP53, CDKN2A	WT
HN24PT DNA	71	M	Hypopharynx	SCC	N/A	Y	TP53	WT
HN25PT DNA	50	M	Tonsil	SCC	UNK	Y		WT

HN26PT DNA	59	M	Tonsil	SCC	UNK	Y		WT
HN27PT DNA	32	F	Tongue	SCC	N/A	Y	TP53	C228T
HN28PT DNA	70	M	Tonsil	SCC	UNK	N		WT
HN30PT DNA	56	M	Supraglottis	SCC	N/A	N		WT
HN31PT DNA	49	M	Tonsil	SCC	N	N		WT
HN32PT tumor	58	M	Supraglottis	SCC	N/A	Y	TP53	WT
HN33PT tumor	83	M	Floor of mouth	SCC	N/A	Y	TP53, FBXW7	WT
HN34PT tumor	49	M	Base of tongue	SCC	UNK	Y		WT
HN35PT tumor	68	M	Tongue	SCC	N/A	UNK	TP53	WT
HN39PT tumor	44	M	Tonsil	SCC	UNK	Y		WT
HN41PT tumor	42	M	Tonsil	SCC	Y	Y	PIK3CA	WT
HN42PT tumor	56	M	Base of tongue	SCC	Y	N		WT
HN43PT tumor	59	M	Tonsil	SCC	UNK	Y		WT
TUNG 1 PT	35	F	Tongue	SCC	N	N		WT
TUNG 2 PT	51	M	Tongue	SCC	N	N	NOTCH3	WT
TUNG 3 PT	43	F	Tongue	SCC	N	N	NOTCH1	C228T
TUNG 4 PT	45	M	Tongue	SCC	N	N	NOTCH2NL	WT
TUNG 6 PT	45	M	Tongue	SCC	N	N	TP53, NOTCH1, NOTCH2	C228T
TUNG 7 PT	34	F	Tongue	SCC	N	N		C228T

F, female; HPV, human papillomavirus; M, male; N, no; N/A, not applicable; SCC, squamous cell carcinoma; UNK, unknown; Y, yes.

4.3.2.5 Medulloblastomas

Medulloblastoma is the most common malignant brain tumor of childhood (Gilbertson and Ellison 2008). *TERT* mutations occurred in 21% of 91 medulloblastomas that we evaluated. As with the oral cavity cancers, *TERT* mutations were not distributed randomly among the medulloblastoma patients. Although medulloblastomas are usually diagnosed at a young age, those medulloblastomas with *TERT* mutations were diagnosed at a considerably older age (median = 6 vs. 16 y, $P = 0.0012$, t test assuming unequal variances, two-tailed) (Figure 10A). This observation has important implications for understanding the basis for the selectivity of the tumor types harboring *TERT* promoter mutations (Discussion); 45 of 90 patients had been assessed previously for orthodenticle homeobox 2 (*OTX2*) gene amplification and expression, and alterations in this transcription factor are known to correlate with clinically distinct molecular subtypes of medulloblastomas (Adamson, Shi et al. 2010). *OTX2* expression was >100-fold higher in medulloblastomas patients without *TERT* promoter mutations than in those patients with *TERT* promoter mutations (note the log scale in Figure 10B). The high levels of *OTX2* expression were usually the result of *OTX2* gene amplification (Figure 10C). The association of *TERT* promoter mutations with an older age at diagnosis and a lack of *OTX2* overexpression raises the possibility that *TERT* mutations occur in a specific clinical and molecular subtype of medulloblastoma. The most likely molecular subtype of medulloblastoma that may be enriched for *TERT* mutations is the

noninfant sonic hedgehog subtype, which is characterized by an older age at diagnosis and lower expression of *OTX2* (Kool, Koster et al. 2008, Northcott, Korshunov et al. 2011). Larger studies will be needed to make this association more definitive. ALT has been observed in 7% of 55 medulloblastomas studied previously (Heaphy, Subhawong et al. 2011).

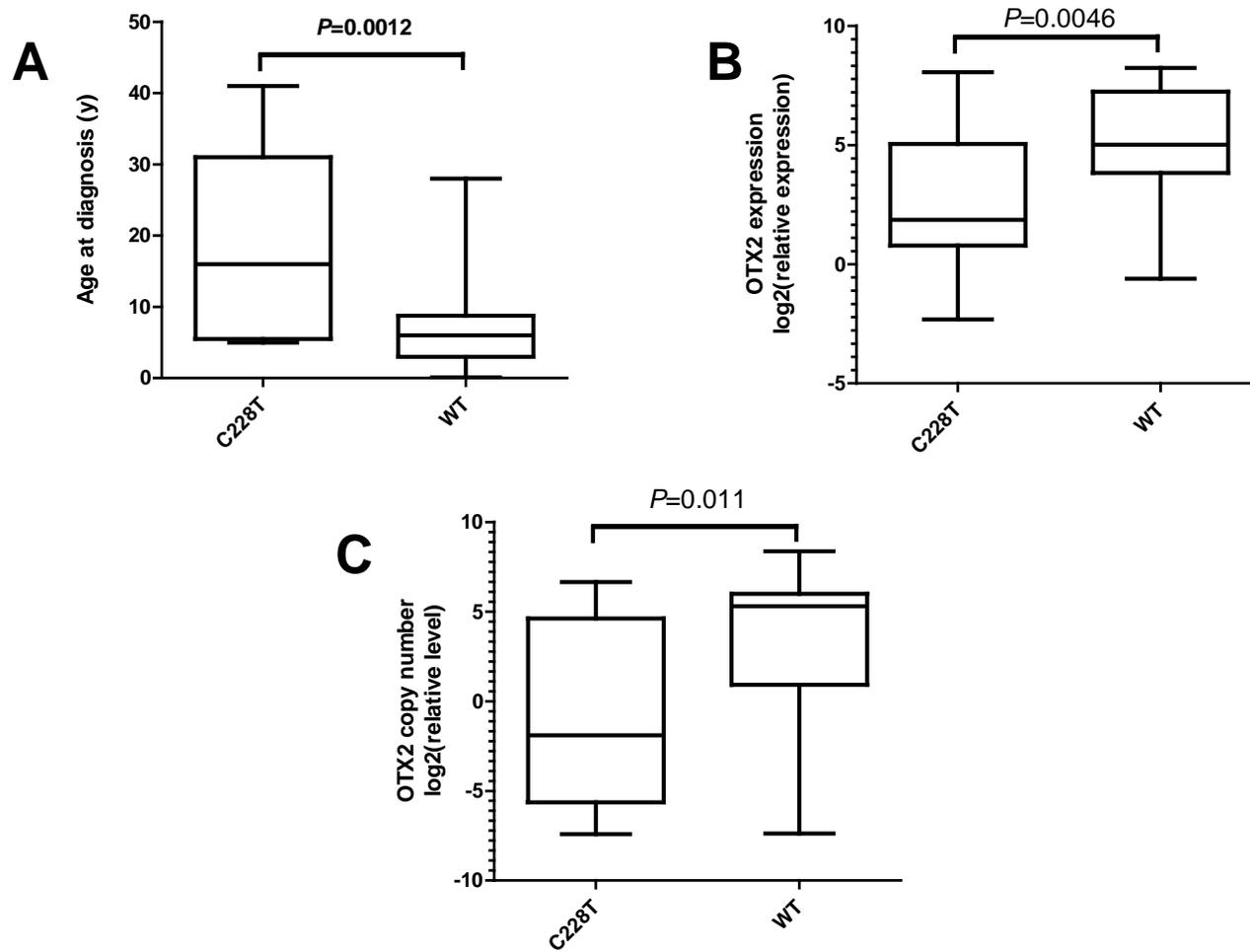


Figure 10: Telomerase reverse transcriptase (TERT) promoter mutations and clinical characteristics of medulloblastomas.

Figure 10 (Continued): Telomerase reverse transcriptase (TERT) promoter mutations and clinical characteristics of medulloblastomas.

(A) Age at diagnosis of medulloblastomas patients with and without tumoral TERT promoter mutations. (B) Relative tumoral OTX2 expression, which was assessed by quantitative PCR, among patients with TERT-mutated and non-TERT-mutated tumors. (C) Relative tumoral OTX2 copy number, which was assessed by quantitative PCR, among patients with TERT-mutated and non-TERT-mutated tumors.

4.3.2.6 Gliomas

Gliomas are the most common CNS tumor type and accounted for >14,000 deaths in the United States last year (Jansen, Yip et al. 2010). Histopathological and clinical criteria established by the World Health Organization are used to characterize these tumors into several subtypes (Jansen, Yip et al. 2010). We considered the four main subtypes individually (Table 10).

Table 10: TERT mutations in glioma subtypes

Glioma subtype	WHO grade	No. of tumors studied	No. of tumors with TERT promoter mutation	Tumors with TERT mutation (%)
Primary GBM, adult	IV	78	65	83
Primary GBM, pediatric	IV	19	2	11
Astrocytoma	II	8	0	0
Astrocytoma	III	27	4	15
Astrocytoma	IV	5	0	0
Oligodendroglioma	II	19	12	63
Oligodendroglioma	III	26	23	88
Oligoastrocytoma	II	9	2	22
Oligoastrocytoma	III	15	4	27

4.3.2.7 Primary glioblastomas

These primary glioblastomas (GBMs) are the most common malignant brain tumors in adults, accounting for ~17% of all intracranial tumors, and they confer the worst survival (median of ~15 mo) (Dolecek, Propp et al. 2012). These high-grade (grade IV) tumors have no detectable precursor lesions and have been referred to as de novo tumors. The prevalence of *TERT* promoter mutations was remarkably high in GBMs of adults (83% of 78 tumors) (Table 10). This prevalence is higher than the prevalence of any other genetic mutation in this tumor type (Parsons, Jones et al. 2008). These findings provide a molecular mechanism responsible for the high levels of *TERT* mRNA and telomerase activity observed in GBMs (Boldrini, Pistoiesi et al. 2006). For 51 of 78 primary GBM tumors, data on other common genetic alterations as well as clinical data were available (Figure. 11A). Interestingly, *EGFR* amplification, a classic molecular feature of primary GBM, exclusively occurred in tumors with *TERT* mutations ($P = 0.0006$, Fisher exact probability test, two-tailed). Conversely, no association was identified between *TERT* mutation and either *TP53* mutation or *CDKN2A* deletion. Importantly, the frequency of *TERT* promoter mutations was considerably less in primary GBMs of pediatric patients (11% of 19 tumors) than adult patients (Discussion) (Table 10). ALT was observed in 11% of 105 adult GBM and 44% of pediatric GBM (i.e., the reverse of the pattern observed for *TERT* promoter mutations) (Heaphy, Subhawong et al. 2011). Primary GBM patients without *TERT* mutations survived considerably

longer, on average, than patients with such mutations (median = 27 vs. 14 mo, $P = 0.01$ by the log rank test) (Figure. 12).

Figure 11(Continued): Mutations of selected genes in glioma subtypes.

(A) Distribution of TERT mutations and other genetic events in 51 primary GBMs. (B) Distribution of TERT mutations and other genetic events among 40 astrocytomas, including grades II–III astrocytomas and grade IV secondary GBMs. (C) Distribution of TERT mutations and other genetic events among 45 oligodendrogliomas. (D) Distribution of TERT mutations and other genetic events among 24 oligoastrocytomas. World Health Organization tumor grade is indicated under each column. Light gray cells denote WT status in tumors.

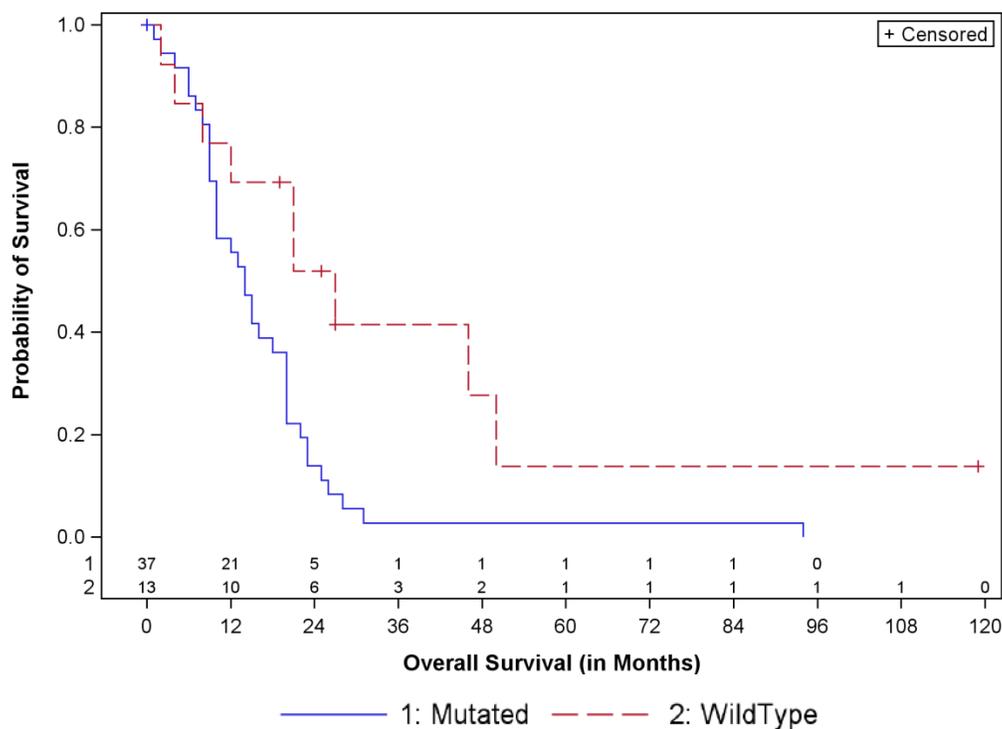


Figure 12: Survival of primary GBM patients with TERT promoter-mutated tumors.

Kaplan–Meier analysis of 50 primary GBM patients stratified by TERT promoter mutational status. Patients with TERT promoter WT tumors (n = 13) survived longer than patients with TERT promoter-mutated tumors (n = 37); median survival was 27 mo among the patients with TERT promoter WT tumors compared with 14 mo among patients with TERT promoter-mutated tumors. The estimated hazard ratio was 0.38 (95% confidence interval = 0.18, 0.81; P = 0.01, log rank test).

4.3.2.8 Astrocytomas

Infiltrative astrocytic tumors frequently progress, with recurrent lesions often of higher grade than the original lesions excised at surgery. They are most often grade II or III but can progress to grade IV (at which point they are often termed secondary GBMs). Astrocytomas of any stage rarely contained *TERT* promoter mutations (10% of 40 total samples) (Table 10). Instead, they more frequently contained isocitrate dehydrogenase 1 (*IDH1*) or isocitrate dehydrogenase 2 (*IDH2*) mutations (75% of 40 tumors), *ATRX* mutations (70% of 40 tumors), and *TP53* mutations (73% of 40 tumors) (Figure. 11B). ALT has been observed in 63% of 57 astrocytomas, consistent with the high prevalence of *ATRX* mutations (Heaphy, Subhawong et al. 2011). The lack of activating *TERT* mutations in *IDH1* mutant tumors is also corroborated by the lack of *TERT* mRNA and telomerase activity observed in these lesions (Boldrini, Pistolesi et al. 2006).

4.3.2.9 Oligodendrogliomas

Like astrocytomas, oligodendrogliomas often progress, and they frequently contain *TERT* promoter mutations (78% of 45 tumor samples) (Table 10). Oligodendroglioma was the only tumor type studied (of all types, including non-CNS tumors) in which C250T mutations were nearly as frequent as C228T mutations. In oligodendrogliomas, 43% of tumors with *TERT* mutations contained C250T substitutions, whereas in other gliomas, only 10% did ($P < 0.001$, Fisher exact probability test, two-tailed). Interestingly, 91% of 45 oligodendrogliomas that were evaluated for

ATRX and *TERT* sequence alterations contained either an *ATRX* coding or a *TERT* promoter mutation, suggesting that genetic alterations resulting in telomere maintenance are required for tumorigenesis of this subtype. Oligodendrogliomas have long been known to contain characteristic losses of chromosome arms 1p and 19q, and these losses reflect inactivation of the *CIC* gene on chromosome 19q and in some cases, inactivation of the *FUBP1* gene on chromosome 1p (Maintz, Fiedler et al. 1997, Bromberg and van den Bent 2009, Bettegowda, Agrawal et al. 2011). Accordingly, 78% of 45 oligodendrogliomas contained chromosome arm 1p or 19q losses of heterozygosity (Figure 11C) (34–36). Moreover, nearly all of them contained *IDH1* or *IDH2* mutations (93%).

4.3.2.10 Oligoastrocytomas

As their name implies, these tumors are mixed, with histologic features of both oligodendrogliomas and astrocytomas. This mixture, in part, reflects the difficulties in distinguishing the various glioma subtypes from one another on the basis of histopathologic or clinical criteria (Jiao, Killela et al. 2012). The genetic features of this tumor subtype reflect this mixture: the prevalence of *TERT* promoter mutations (25% of 24 tumors) was intermediate between oligodendrogliomas and astrocytomas, as were the frequencies of chromosome (Chr) 1p/19q losses and *IDH1/2*, *TP53*, and *ATRX* mutations (Figure. 11D).

4.3.3 ALT Vs. TERT

ALT has been observed in tumors of the CNS (particularly gliomas) more frequently than tumors of any other tissue type. Given that *TERT* promoter mutations are also common in gliomas, the relationship between these two features could be determined with high confidence. The tumors depicted in Figure 11 had previously been evaluated for alterations in *ATR*X, which is a nearly perfect surrogate for the ALT phenotype (Heaphy, de Wilde et al. 2011, Jiao, Killela et al. 2012). Our data show that there were 50 gliomas with *ATR*X mutations and 83 gliomas with *TERT* mutations; 0 of 83 tumors with *TERT* mutations contained *ATR*X mutations ($P < 0.0001$, Fisher exact probability test, two-tailed).

4.4 Discussion

The results described above, as well as the results published in refs. (Horn, Figl et al. 2013) and (Huang, Hodis et al. 2013), provide evidence that supports one of the hypotheses raised in the Introduction and refutes others. The first of these hypotheses was that *TERT* mutations would only be observed in tumors derived from tissues that are not constantly self-renewing under normal circumstances. This hypothesis was supported in part: the vast majority of *TERT* promoter mutations occurred in tumors derived from tissues that do not continually self-renew. The TERT-H tumor types include only melanomas, certain subtypes of glioma, medulloblastomas, squamous cell cancers of the tongue, liposarcomas, HCCs, and urinary tract cancers. The normal

transitional cells of the urinary tract have very low proliferative indices ($0.64\% \pm 0.52\%$), much lower than indices of gastrointestinal tract, bone marrow, or skin (King, Matteson et al. 1996). Normal hepatocytes also do not turnover often (Aikata, Takaishi et al. 2000), and glial cells are thought to have limited capacity for self-renewal (Spalding, Bhardwaj et al. 2005).

Two other observations also support the hypothesis. Pediatric primary GBMs rarely contained *TERT* mutations (11%), whereas adult primary GBMs frequently did (83%). Pediatric GBMs are presumably derived from cells that are still dividing at the time of tumor initiation, and therefore, there is no selective advantage conferred by activating telomerase through a genetic mutation. Adult GBMs, in contrast, are presumably derived from postmitotic cells, and they should require telomerase activation. Similarly, medulloblastomas are embryonal tumors that typically arise from precursor cells with high self-renewal rates that do not usually persist in adults. This finding is consistent with our observation that the mean age of medulloblastoma patients with *TERT* mutations was considerably older than the mean age of medulloblastoma patients without *TERT* mutations (Figure. 10A).

There are, however, exceptions that belie the hypothesis that *TERT* mutations occur only in non-self-renewing tissues. The epithelium that lines the tongue constantly self-renews, but many squamous carcinomas of the tongue harbored *TERT* mutations (Table 9). Additionally, the squamous epithelia of the tongue certainly would not be

expected to self-renew less than other squamous epithelia of the oral cavity, but the latter rarely harbored *TERT* mutations (Table 9). This finding may suggest that squamous carcinomas of the tongue originate from a different cell of origin than other oral cavity squamous carcinomas. Conversely, only a subset of the tumor types derived from non-self-renewing tissues was TERT-H. For example, the TERT-H tumors included myxoid liposarcomas but not synovial sarcomas. Moreover, cells of the pancreas (the islets of Langerhans and the ductal epithelial cells) rarely renew, but pancreatic tumors of all types (pancreatic neuroendocrine tumors, acinar carcinomas, and pancreatic ductal adenocarcinomas) were all TERT-L. The most that we can conclude at present is that non-self-renewing cell types are the major sources of TERT-H tumors but that non-self-renewal is only one of the factors that determines whether tumor cells with *TERT* promoter mutations will have a selective growth advantage over adjoining cells.

The first corollary to the hypothesis raised in the Introduction was that tumor types that displayed ALT would be those types that harbored *TERT* promoter mutations. This corollary is soundly refuted by these data, at least in general terms. Although tumor types of the CNS and liposarcomas had high frequencies of ALT as well as high frequencies of *TERT* promoter mutations, these tumor types were the exceptions rather than the rule. For example, pancreatic neuroendocrine tumors have very high frequencies of ALT but no evidence of *TERT* mutations. Conversely, bladder cancers frequently have *TERT* mutations but never have ALT (Heaphy, Subhawong et al. 2011).

Additionally, even among gliomas, pediatric GBMs have high frequencies of ALT and low frequencies of *TERT* mutations, whereas adult GBMs have the reverse pattern.

The second corollary was that the selective advantage afforded by *TERT* mutation would be equivalent to the advantage afforded by *ATRX* mutation (conferring ALT). This hypothesis was most effectively tested in gliomas, in which both *ATRX* coding and *TERT* promoter mutations were common. There was a striking mutual exclusivity with respect to *ATRX* and *TERT* mutations ($P < 0.0001$), lending strong support to this idea.

These results also raise many unanswered questions. In some tumor types, such as gliomas, we can imagine that all tumors have genetically activated telomere maintenance programs through mutations in either *TERT* or *ATRX*. However, in other tumor types with frequent *ATRX* mutations, such as pancreatic neuroendocrine tumors, what is responsible for activating telomerase in the fraction of cases not exhibiting ALT if it is not a mutation in the *TERT* promoter? Similarly, what is responsible for activating telomerase in those tumors derived from non-self-renewing cell types in which neither ALT nor *TERT* mutations is frequently observed, such as synovial sarcomas or osteosarcomas? Also, there are occasional individual tumors among the TERT-L types that have *TERT* promoter mutations (e.g., cervical cancers, ovarian cancers, and in (Huang, Hodis et al. 2013), lung cancers). What distinguishes these occasional cancers from others of the same histopathologic subtype? Whole-genome sequencing studies,

rather than those studies limited to the exome, might provide answers to these questions.

The results recorded here have practical as well as basic scientific implications. Two-thirds of bladder cancers had *TERT* promoter mutations, making it the most commonly mutated gene yet identified in invasive urothelial carcinoma of the bladder. Given the persistently high mortality rate despite multimodality treatment in this group of patients, these mutations represent ideal urinary biomarkers to detect bladder cancers at an early stage and to follow patients for evidence of progression or recurrence once they have been diagnosed (Cheng, Zhang et al. 2011). Similarly, the high prevalence of *TERT* promoter mutations in HCCs and glioma subtypes provides excellent candidate biomarkers for early detection (HCC) or monitoring (HCC in the plasma and gliomas in the cerebrospinal fluid) (Baraniskin, Kuhnhen et al. 2012, Singal, Nehra et al. 2012).

Another practical implication involves diagnostics. We conjecture that tumors with *TERT* promoter or *ATRX* mutations are derived from different precursor cells and that either type of precursor cell is different from those types that are the precursors of tumors without such mutations. This distinction could aid classification of the tumors in clinically meaningful ways. For example, Figure 11 and Figure 13 outline the major genetic alterations occurring in the three most common types of gliomas. On the basis of the data in Figure 11 A–C, we speculate that oligodendrogliomas that lack *TERT* mutations but contain *ATRX* mutations may behave more like astrocytomas than

oligodendrogliomas and vice versa. Similarly, the primary GBMs without *TERT* mutations (15% of the total) may behave more like advanced progressive astrocytomas, which generally lack *TERT* mutations. This possibility is supported by the observation that those primary GBM patients without *TERT* mutations had a longer survival, on average, than other primary GBM patients (Figure. 12).

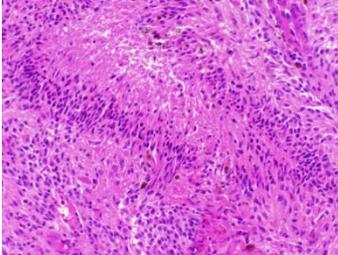
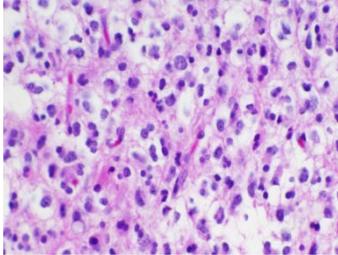
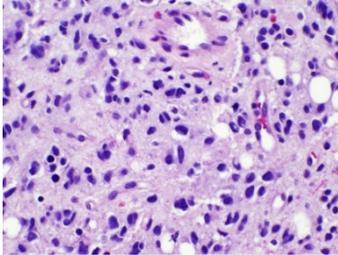
	Primary Glioblastoma	Oligodendroglioma	Progressive Astrocytoma
			
WHO Grade	IV	II-III	II-IV
<i>TERT & IDH status</i>	<i>TERT</i> mutant, <i>IDH1/2</i> wild type	<i>TERT</i> mutant, <i>IDH1/2</i> mutant	<i>TERT</i> wild type, <i>IDH1/2</i> mutant
Telomere Maintenance Mechanism	<i>TERT</i> Mutation	<i>TERT</i> Mutation	Alternative lengthening of telomeres
<i>Other Frequent Molecular Alterations</i>	<i>EGFR</i> amplification <i>CDKN2A/CDKN2B</i> deletion	<i>1p/19q</i> loss <i>CIC</i> mutation <i>FUBP1</i> mutation	<i>TP53</i> mutation <i>ATRX</i> mutation

Figure 13: Isocitrate dehydrogenase 1 (IDH1) and TERT mutations delineate oligodendrogliomas, primary glioblastomas (GBMs), and progressive astrocytomas.

Figure 13 (Continued): Isocitrate dehydrogenase 1 (IDH1) and TERT mutations delineate oligodendrogliomas, primary glioblastomas (GBMs), and progressive astrocytomas.

TERT promoter combined with IDH1 mutational status allow for refinement of the classification of the three most common types of gliomas. World Health Organization (WHO) grade, typical TERT promoter and IDH1 mutational status, telomere maintenance mechanism, and other frequent molecular alterations are shown for each group of tumors.

5. Mutations in IDH1, IDH2, and in the TERT promoter define clinically distinct subgroups of adult malignant gliomas

5.1 Introduction

Gliomas are the most common primary malignant tumor of the central nervous system and account for 24% of brain tumors (Dolecek, Propp et al. 2012). Tumor grades range from grade I to grade IV and are based on histopathological and clinical criteria established by the World Health Organization (WHO) (Louis, Ohgaki et al. 2007, Dolecek, Propp et al. 2012). The progression between grades along with the potential for mixed histology presents neuropathologists with diagnostic challenges that often rely on subjective measures. Consequently, diagnoses among different pathologists and institutions have weak correlations that may result in variable treatment and management of each tumor grade (Louis, Ohgaki et al. 2007, Wen and Kesari 2008). The subjective nature of these analyses stresses the importance of an accurate, unbiased, and objective means of diagnosis. This is crucial for stratification of patients with biologically similar tumors in clinical trials, and could aid in the selection of targeted therapeutic regimens. The discovery of biomarkers that objectively identify each tumor's unique molecular signature is a necessary next-step in managing patient outcomes more effectively. Genetic signatures performed on pathologically relevant tissues will be a potentially useful supplement to clinicians in refining and clarifying patient stratification.

Characterization of the genetic landscape of gliomas has been at the forefront of cancer research in order to better aid prognostication and classification of clinical outcomes (Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008). High-throughput screens have paid particular attention to understanding the genomic variability between each subgroup of glioma. The Cancer Genome Atlas and other groups, including ours, have begun to identify the molecular subgroups of these tumors and delineate which tumor types harbor which mutations (Phillips, Kharbanda et al. 2006, Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008, Yan, Parsons et al. 2009, Verhaak, Hoadley et al. 2010, Jiao, Killela et al. 2012, Brennan, Verhaak et al. 2013, Johnson, Mazar et al. 2013). For example, *IDH1/2* mutations that occur frequently in secondary GBMs (>50%) are infrequent in primary GBMs (<5%) (Yan, Parsons et al. 2009, Jiao, Killela et al. 2012).

Recent findings have established frequent mutations in the promoter region of telomerase reverse transcriptase (*TERT*) in a multitude of cancers, including melanomas, liposarcomas, bladder cancer, urinary tract cancers, and gliomas (Griewank, Murali et al. 2013, Horn, Figl et al. 2013, Huang, Hodis et al. 2013, Killela, Reitman et al. 2013, Koelsche, Sahm et al. 2013, Liu, Wu et al. 2013, Vinagre, Almeida et al. 2013). *TERT* is a subunit of the telomerase enzyme that, when expressed, allows cells to avoid senescence. This is especially noted as *TERT* is mutated in high frequencies in cells with low rates of self-renewal, such as melanocytes, urothelial cells, and glial cells (Kim,

Piatyszek et al. 1994, Shay and Bacchetti 1997, Horn, Figl et al. 2013, Huang, Hodis et al. 2013, Killela, Reitman et al. 2013). Of interest to glioma genomics, *TERT* promoter mutations occur in 70-80% of primary GBMs and >70% of oligodendrogliomas, but occur less frequently in both lower grade astrocytomas and most oligoastrocytomas (Arita, Narita et al. 2013, Killela, Reitman et al. 2013, Koelsche, Sahm et al. 2013).

The discovery of *TERT* promoter mutations in these subsets of gliomas creates an opportunity for genomics to supplement histopathological analysis, especially when combined with *IDH1/2* mutation status. Here, we have assessed the characteristic variance between *IDH1/2* and *TERT* promoter mutations among several glioma subtypes that help refine the diagnosis of gliomas. The assay, based upon three polymerase chain reactions (PCR), provides pathologists with a manageable and reliable diagnostic supplement in the form of a simple, yet robust genetic signature unique to each tumor type.

5.2 Methods

5.2.1 Sample Collection, Processing, and Sequencing

Adult glioma (18 ≥ years old) and corresponding clinical information were obtained with consent and Institutional Review Board approval from the Preston Robert Tisch Brain Tumor Center BioRepository at Duke University in accordance with the Health Insurance Portability and Accountability Act. Newly diagnosed versus recurrent glioma status and vital status were determined by clinical chart review. Fresh frozen

tissue sections (first and last sections from the block, stained with hematoxyline and eosin) were reviewed by a board-certified neuropathologist (REM) to confirm original clinical histopathologic diagnosis and to ensure intervening studied sections contain \geq 80% tumor cells. DNA was extracted from 240 grade IV GBMs, 88 grade II and grade III astrocytomas, 58 grade II and grade III oligoastrocytomas, and 87 grade II and grade III oligodendrogliomas. Of the 473 tumors, 160 gliomas had been analyzed in our previous studies of the *TERT* promoter (Killela, Reitman et al. 2013). Isolated DNAs were PCR amplified for the *TERT* promoter, exon 4 of *IDH1*, and analyzed via Sanger sequencing for 473 tumors as described previously (Sjoblom, Jones et al. 2006, Yan, Parsons et al. 2009, Killela, Reitman et al. 2013). Additionally, on those cases that did not harbor mutations in *IDH1* we amplified exon 4 of *IDH2* and analyzed them via Sanger sequencing. 1p and 19q copy number was evaluated by microsatellite marker analysis and via 1p and 19q FISH testing in a certified clinical laboratory as described previously (Reifenberger, Reifenberger et al. 1994, Bigner, Matthews et al. 1999, Yan, Parsons et al. 2009).

5.2.2 Statistical Methods

Clinical and demographic characteristics at the time of diagnosis were summarized for all patients and stratified by histologic tumor type. Means and standard deviations were used to describe interval variables, whereas frequency distributions were used to describe categorical variables. Unpaired t-tests were used to compare the

mean age of patients with and without *TERT* promoter mutations. The Kaplan-Meier estimator was used to describe OS. OS was defined from time of surgery to death or last follow-up. Multivariable Cox models were used to assess the effect of *TERT* promoter and *IDH1/2* mutations on OS adjusting for baseline tumor characteristics. The generalized R2 statistic was used to assess the strength of association between covariates. Associations between categorical variables were analyzed using Fisher exact tests.

5.3 Results

5.3.1 *TERT* promoter mutations are frequent in primary GBMs and oligodendrogliomas but uncommon in lower grade astrocytoma

To assess the prevalence and prognostic impact of *TERT* promoter mutations we sequenced the proximal *TERT* promoter hotspot mutations (C228T and C250T) in 473 adult gliomas. We identified *TERT* promoter mutations in 281 (59.4%) tumors (Figure 14). In agreement with previous studies (Killela, Reitman et al. 2013, Liu, Wu et al. 2013, Nonoguchi, Ohta et al. 2013), we identified *TERT* promoter mutations in 74.2% of grade IV GBMs (178/240). *TERT* promoter mutations were also common in oligodendrogliomas (79.3%); however, *TERT* promoter mutations were less frequently identified in grade II-III astrocytomas (18.2%, 16/88). Furthermore, we observed a moderate frequency of *TERT* promoter mutations in oligoastrocytomas (31.0%, 18/58). As expected, GBMs were diagnosed in older patients when compared to other histologic

subtypes studied here (Table 11). Within each tumor type, *TERT* promoter mutations were associated with an older age at diagnosis (Table 12).

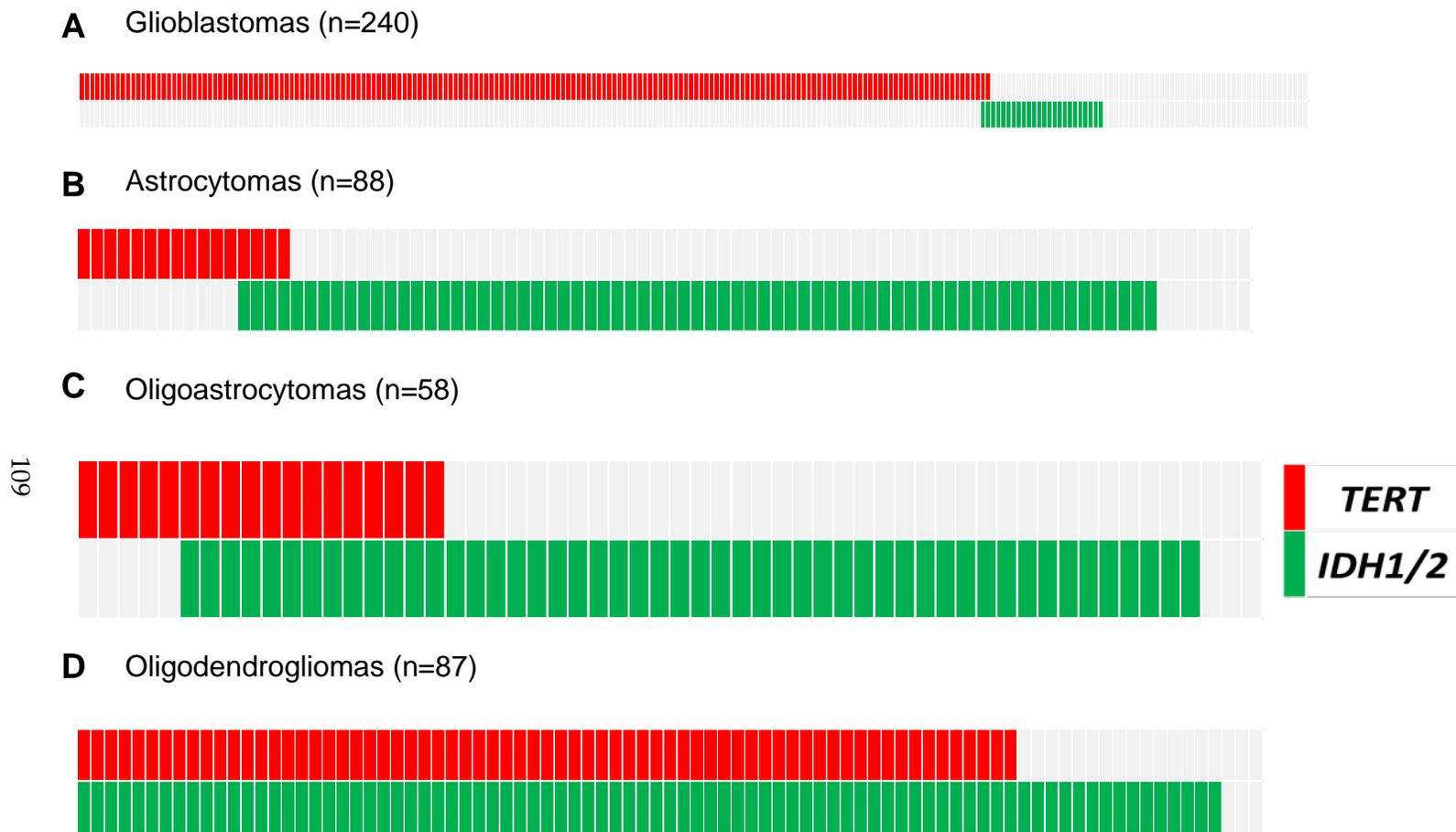


Figure 14: Distribution of TERT promoter and IDH1/2 mutations in a panel of 473 adult gliomas.

Figure 14 (Continued): Distribution of TERT promoter and IDH1/2 mutations in a panel of 473 adult gliomas.

Mutational analysis of 473 adult gliomas for TERT promoter and IDH1/2 mutations. Data are from 240 Grade IV GBM (A), 88 Grade II-III astrocytomas (B), 58 Grade II-III oligoastrocytomas (C), and, 87 Grade II-III oligodendrogliomas (D). Mutation status is indicated by color shading, with gray coloring indicating wild type sequence, red indicating mutations in the TERT promoter, and green indicating mutations in IDH1/2.

Table 11: Clinical Characteristics of Cohort

	GBM (N=240)		A (N=88)		OA (N=58)		O (N=87)	
Age (mean, SD)	54.9	13.4	39.0	10.4	42.0	13.2	41.2	11.2
Gender								
Male	146	60.8%	54	61.4%	36	62.1%	60	69.0%
Female	94	39.1%	34	38.6%	22	37.9%	37	31.0%
Grade								
II	0	0.0%	40	45.4%	28	48.3%	44	50.6%
III	0	0.0%	48	54.6%	30	51.7%	43	49.4%
IV	240	100.0%	0	0.0%	0	0.0%	0	0.0%
Diagnosis Status								
Newly Diagnosed	132	55.0%	50	56.8%	30	51.7%	42	48.3%
Recurrent	65	27.1%	35	39.8%	24	41.4%	31	35.6%
Not Available	43	17.9%	3	3.4%	4	6.9%	14	16.1%
TERT Status								
Mutant	178	74.2%	16	18.2%	18	31.0%	69	79.3%
Wildtype	62	25.8%	72	81.9%	40	69.0%	18	20.7%
IDH1/2 Status								
Mutant	24	10.0%	69	78.4%	50	86.2%	84	96.5%
Wildtype	216	90.0%	19	21.6%	8	13.8%	3	3.5%
TERT-IDH1/2 Status								
TERT wt / IDH wt	40	16.7%	7	7.9%	3	5.2%	3	3.5%
TERT wt / IDH mut	22	9.2%	65	73.9%	37	63.8%	15	17.2%
TERT mut / IDH wt	176	73.3%	12	13.6%	5	8.6%	0	0.0%
TERT mut / IDH mut	2	0.8%	4	4.6%	13	22.4%	69	79.3%
1p/19q Status								
Wildtype/Wildtype	0	0.0%	21	23.9%	26	44.8%	9	10.4%
Wildtype/19q	0	0.0%	7	7.9%	11	19.0%	0	0.0%
1p/Wildtype	0	0.0%	0	0.0%	2	3.5%	0	0.0%
1p/19q	0	0.0%	2	2.3%	12	20.7%	47	54.0%
Not Available	240	100.0%	58	65.9%	7	12.1%	31	33.6%

Table 12: Age at diagnosis in gliomas as determined by TERT promoter genotype

		<i>TERT</i> Mutant	<i>TERT</i> WT	p-value
GBM	Age (mean ± SD)	57 ± 12	49 ± 16	0.0003
	median yrs	57	52	
A	Age (mean ± SD)	44 ± 10	38 ± 10	0.0379
	median yrs	43	36	
OA	Age (mean ± SD)	50 ± 12	38 ± 12	0.0008
	median yrs	50	35	
O	Age (mean ± SD)	42 ± 10	37 ± 14	0.0933
	median yrs	40	33	

5.3.2 Co-occurring mutations in TERT promoter and IDH1/2

IDH1/2 mutations are a well-established molecular feature of gliomas (Yan, Parsons et al. 2009). To define the co-occurrence of IDH1/2 mutations and the presence of TERT promoter mutations, we determined the status of *IDH1* and *IDH2* mutations in the same cohort of 473 gliomas and identified mutations in 47.9% (227/473) of tumors (Figure 14 and Table 11). *IDH1/2* mutations were much less prevalent among GBMs (10%), and much more common in grade II-III astrocytomas (78.4%), oligoastrocytomas (86.2%) and oligodendrogliomas (96.5%). *TERT* mutations occurred in the absence of *IDH1/2* mutations in GBMs (73.3%, 176/240). However, in oligodendrogliomas, the *TERT*

promoter mutation always occurred in the setting of the *IDH1/2* mutation, which is frequent in both oligodendrogliomas and astrocytomas (Figure 14) (Yan, Parsons et al. 2009). The cross-tabulation of *TERT* promoter and *IDH1/2* mutations aligned with three of the four histologic subtypes. GBMs were characterized as primarily *TERT* promoter mutant/*IDH* wildtype (73.3%), grade II-III astrocytomas were predominantly *TERT* promoter wildtype/*IDH* mutant (73.9%), and the majority of oligodendrogliomas mainly harbored mutations in both the *TERT* promoter and *IDH1/2* (79.3%). A majority of oligoastrocytomas (63.8%) exhibited the *IDH* mutation in the absence of *TERT* promoter mutations, much like grade II-III astrocytomas; however, a fraction (22.4%) of oligoastrocytomas presented with both *TERT* promoter and *IDH1/2* mutations, similar to oligodendrogliomas (Figure 14).

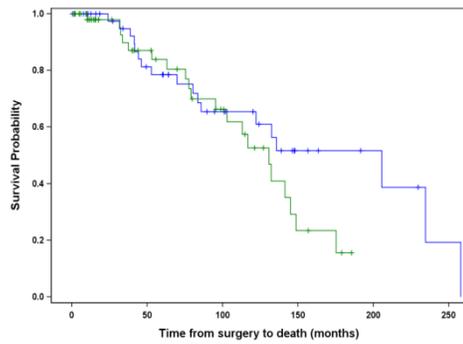
5.3.3 *TERT* promoter and *IDH1/2* mutations have distinct tumor distributions and are associated with OS.

We next sought to determine whether the combination of *TERT* promoter and *IDH1/2* mutations are associated with OS. Clinical information (survival, age at diagnosis, and histopathological diagnosis) was available for our cohort of 473 adult gliomas in both treated and untreated patients (Table 11). As grade is a well-known prognostic factor in glioma patients, we first investigated whether distinct tumor subgroups could be distinguished using only *TERT* promoter and *IDH1/2* mutation status within each grade (Figure 15, Table 13). Among the 112 Grade II gliomas, 103 were characterized by either mutations in both *TERT* and *IDH* or *IDH* alone. The median

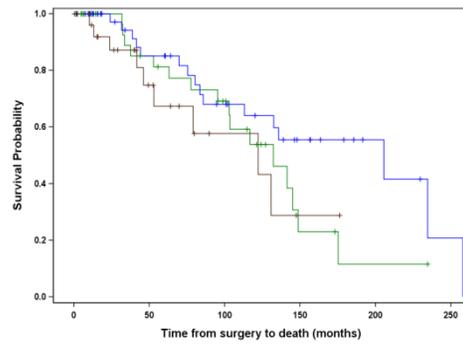
OS of those tumors harboring mutations in both *TERT* promoter and *IDH1/2*, the predominant genetic signature in oligodendrogliomas, was longer than those tumors with an *IDH1/2* mutation only, typically seen in grade II-III astrocytomas (206 months vs. 131 months), but this difference was not statistically significant (log-rank $p=0.1754$) (Figure 15A). When stratified by histologic diagnosis, oligodendrogliomas had the best median OS among grade II astrocytomas, oligodendrogliomas, and oligoastrocytomas, as expected (median OS 205 months).

Among the 121 grade III tumors, 60 (50%) had *IDH1/2* mutations alone and 40 (34%) had mutations in both the *TERT* promoter and *IDH1/2*. Those with mutations in both the *TERT* promoter and *IDH1/2* had the largest median OS (127 months), followed by those with an *IDH1/2* mutation only (median OS 64 months), and those with neither mutation (median OS 32 months). Tumors with mutations in the *TERT* promoter alone, which was the predominant signature present in primary GBMs had the poorest OS (median OS 19 months). Four distinct subgroups of grade III gliomas were identified when stratified by the combination of the *TERT* promoter and *IDH1/2* mutation status (log-rank $p=0.0008$) (Figure 15B). Oligodendrogliomas again had the best median OS when grade III tumors were stratified by histologic subtypes (median OS 125 months), but OS did not significantly differ among the three histologic subtypes, which were astrocytomas, oligodendrogliomas, and oligoastrocytomas (log-rank $p=0.1626$).

a) Grade II

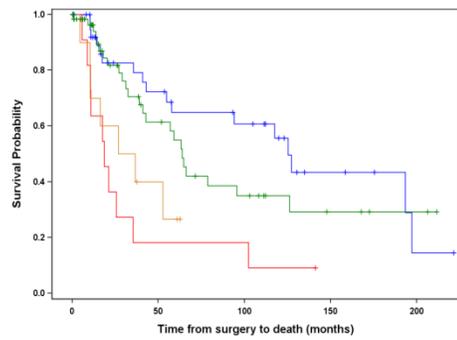


— *TERT* mut / *IDH* mut
 — *TERT* wt / *IDH* mut

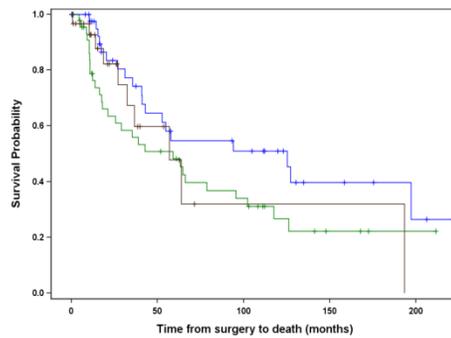


— O — A — OA

b) Grade III

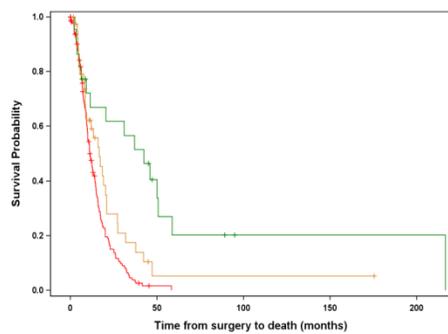


— *TERT* mut / *IDH* — *TERT* mut / *IDH* wt
 — *TERT* wt / *IDH* mut — *TERT* wt / *IDH* wt

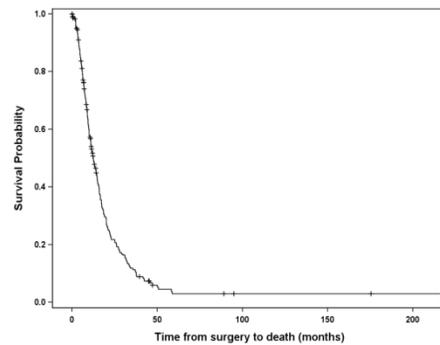


— O — A — OA

c) Grade IV



— *TERT* mut / *IDH* wt — *TERT* wt / *IDH* mut
 — *TERT* wt / *IDH* wt



— GBM

Figure 15: Overall Survival stratified by *TERT* promoter and *IDH1/2* mutational status and histology within each tumor grade.

Figure 15 (Continued): Overall Survival stratified by TERT promoter and IDH1/2 mutational status and histology within each tumor grade.

Overall survival was represented by Kaplan Meier plots for individual WHO tumor grade: a) Grade II (n=103), b) Grade III (n=121), c) Grade IV (n=218). Only subgroups with at least 10 patients were included in the analyses. Tumors were represented by mutations status on the left (TERT promoter status / IDH1/2 status) and histology on the right (A represents Astrocytomas, O represents Oligodendrogliomas, and OA represents Oligoastrocytomas).

Table 13: Summary of OS Stratified by TERT promoter and IDH1/2 Mutational Status by Grade

	<i>TERT/IDH</i> status	Total	# failed	OS in months (95% CI)	1 year OS (95% CI)	2 year OS (95% CI)	5 year OS (95% CI)	10 year OS (95% CI)
Grade II	<i>TERT</i> WT / <i>IDH</i> MUT	57	20	130.7 (95.1, 145.0)	98% (86.4%, 99.7%)	98% (86.4%, 99.7%)	83.8% (67.3%, 92.5%)	52.7% (32.3%, 69.5%)
	<i>TERT</i> MUT/ <i>IDH</i> MUT	46	18	205.5 (85.8, 257.9)	100%	97.4% (83.2%, 99.6%)	78.5% (61.5%, 88.6%)	65.4% (46.9%, 78.8%)
Grade III	<i>TERT</i> MUT/ <i>IDH</i> WT	11	10	18.6 (8.7, 35.3)	63.6% (29.7%, 84.5%)	36.4% (11.2%, 62.7%)	18.2% (2.9%, 44.2%)	9.1% (0.5%, 33.3%)
	<i>TERT</i> WT / <i>IDH</i> WT	10	7	31.8 (4.4, -)	70.0% (32.9%, 89.2%)	60.0% (25.3%, 82.7%)	26.7% (4.8%, 56.3%)	NE*
	<i>TERT</i> WT / <i>IDH</i> MUT	60	24	63.8 (40.9, 126.2)	96.3% (85.8%, 99.1%)	81.9% (66.8%, 90.6%)	55.0% (37.3%, 69.6%)	35.1% (19.3%, 51.3%)
	<i>TERT</i> MUT / <i>IDH</i> MUT	40	16	127.3 (57.7, 197.2)	94.4% (79.6%, 98.6%)	84.9% (67.3%, 93.4%)	66.6% (46.5%, 80.6%)	57.3% (36.2%, 73.6%)
Grade IV	<i>TERT</i> MUT / <i>IDH</i> WT	176	158	11.3 (10.0, 13.1)	47.6% (39.8%, 54.9%)	15.0% (9.9%, 21.1%)	0.0%	0.0%
	<i>TERT</i> WT / <i>IDH</i> WT	40	30	16.6 (8.6, 21.0)	59.1% (41.5%, 71.1%)	27.9% (13.6%, 44.2%)	5.2% (0.5%, 19.5%)	5.2% (0.5%, 19.5%)
	<i>TERT</i> WT / <i>IDH</i> MUT	22	16	42.3 (9.1, 50.6)	67.0% (42.7%, 82.8%)	61.8% (37.7%, 78.9%)	20.3% (5.4%, 41.8%)	20.3% (5.4%, 41.8%)

*NE=Not Estimable

** Genotypes with frequencies less than 10 were not included

A majority of the GBMs were characterized by mutations in the *TERT* promoter alone (73%), and this genetic signature also had the worst prognosis (median OS 11.3 months) (Figure 15, Table 13). Those without mutation in either marker had only a slightly better outcome (median OS 17 months), while those with an *IDH1/2* mutation alone, the signature characteristic of grade II-III astrocytomas and grade IV secondary GBMs, had the best outcome among the grade IV tumors (median OS 42 months). Within the primary and secondary GBMs, using the *TERT* promoter and *IDH1/2* alone, we were able to distinguish three significantly different subgroups (log-rank $p < 0.0001$), and these associations remained when adjusting for the factors of age and diagnosis (Table 14). The *TERT* promoter mutation is associated with poorer OS in GBMs, and as shown in the multivariable model, this association was also evident among tumors without an *IDH1/2* mutation (HR: 1.9, 95% CI: 1.2-2.9).

Table 14: Cox Model Predicting Median Overall Survival in GBMs

Parameter		DF	Hazard Ratio	95% Lower Confidence Limit	95% Upper Confidence Limit
<i>TERT</i>	Mutant vs. Wildtype	1	1.901	1.244	2.904
<i>IDH1</i>	Mutant vs. Wildtype	1	0.496	0.251	0.983
Tumor Status	Newly Diagnosed vs. Recurrent	1	0.481	0.340	0.679
Age		1	1.019	1.004	1.033

*198 tumors with all covariates available are included in the model

Given that both grade III and grade IV gliomas were successfully stratified into distinct subgroups based on *TERT* promoter and *IDH1/2* mutational status, and that each signature was associated with a similar median OS within grade, the effect of histology and genetic signature on OS was also examined across the grade III and IV gliomas together (Figure 16, Table 15). When grade III and IV gliomas were examined based on histology, GBMs predictably had by far the worst prognosis, and oligodendrogliomas experienced the best survival outcome; however, OS among the grade III astrocytomas and oligoastrocytomas was similar and difficult to distinguish (Figure 16A and Table 15). Nevertheless, when genetic signatures were applied to the same cohort of tumors, four distinct clinical subgroups emerged (Figure 16B). As observed, within grade III and IV gliomas separately, tumors with mutations in both *TERT* and *IDH1/2* had the best median OS (oligodendroglioma signature), followed by those with an *IDH1/2* mutation only (grade II-III astrocytoma and secondary GBM signature). Both tumors without mutation in either marker and those tumors with a *TERT* promoter mutation alone had a poorer prognosis, with the latter signature having the worst median OS. The strength of the association between OS and *TERT/IDH1/2* mutational status is similar to that of OS and histology (Generalized R²: 0.3132 and 0.2704, respectively).

Table 15: Summary of OS Stratified by Histology in Grades III and IV

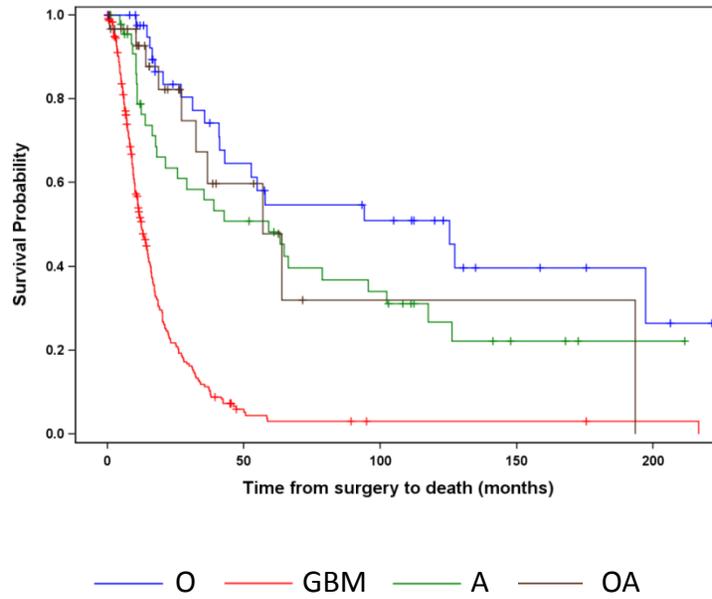
Histology	Total	# failed	OS in months (95% CI)	1 year OS (95% CI)	2 year OS (95% CI)	5 year OS (95% CI)	10 year OS (95% CI)
GBM	240	206	12.4 (10.9, 14.6)	51.2% (44.5%, 57.5%)	21.7% (16.4%, 27.6%)	3.0% (1.1%, 6.6%)	3.0% (1.1%, 6.6%)
A	48	29	59.0 (18.0, 95.6)	78.8% (63.2%, 88.4%)	63.5% (46.9%, 76.2%)	48.2% (32.1%, 62.5%)	26.7% (13.2%, 42.3%)
OA	30	9	56.9 (27.1, 193.3)	96.6% (77.9%, 99.5%)	85.8% (61.1%, 95.3%)	49.9% (19.4%, 74.4%)	33.3% (6.6%, 64.1%)
O	43	19	125.2 (40.9, -)	97.5% (83.5%, 99.6%)	83.5% (66.8%, 92.3%)	54.7% (36.1%, 69.9%)	51.0% (32.6%, 66.8%)

120

Table 16: Summary of OS Stratified by TERT promoter and IDH1/2 Mutational Status in Grades III and IV

<i>TERT/IDH status</i>	Total	# failed	OS in months (95% CI)	1 year OS (95% CI)	2 year OS (95% CI)	5 year OS (95% CI)	10 year OS (95% CI)
<i>TERT MUT / IDH WT</i>	187	168	11.5 (10.0, 14.0)	48.5% (41.0%, 55.7%)	16.4% (11.3%, 22.4%)	1.9% (0.5%, 5.5%)	1.0% (0.1%, 4.4%)
<i>TERT WT / IDH WT</i>	50	37	17.2 (10.5, 26.2)	61.4% (45.9%, 73.7%)	35.9% (21.8%, 50.2%)	10.4% (3.0%, 23.2%)	10.4% (3.0%, 23.2%)
<i>TERT WT / IDH MUT</i>	82	40	56.9 (38.8, 66.2)	87.8% (77.8%, 93.5%)	76.2% (63.9%, 84.8%)	44.3% (30.6%, 57.1%)	30.3% (18.0%, 43.6%)
<i>TERT MUT / IDH MUT</i>	42	18	125.2 (54.7, 197.2)	92.2% (77.8%, 97.4%)	83.2% (66.2%, 92.1%)	62.7% (43.4%, 77.1%)	53.9% (33.9%, 70.2%)

a)



b)

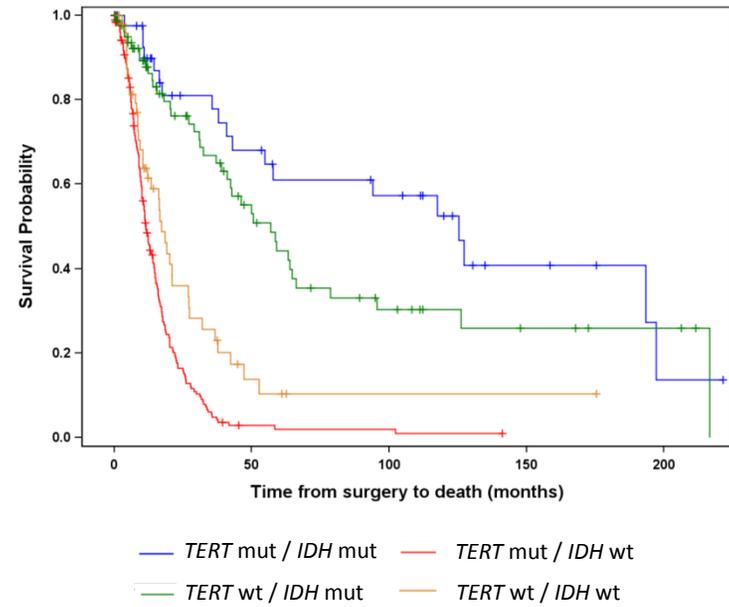


Figure 16: Overall Survival stratified by TERT promoter and IDH1/2 mutational status and histology among Grade III and IV patients.

Figure 16 (Continued): Overall Survival stratified by TERT promoter and IDH1/2 mutational status and histology among Grade III and IV patients.

Overall survival was represented by Kaplan Meier plots stratified by a) histology (A represents Astrocytomas, O represents Oligodendrogliomas, OA represents Oligoastrocytomas, and GBM represents Glioblastoma) and b) TERT promoter / IDH1/2 mutation status for all Grade III and Grade IV gliomas analyzed in this study.

5.4 Discussion

Our analysis of this tumor cohort expands upon previous reports identifying frequent *TERT* promoter mutations in gliomas (Arita, Narita et al. 2013, Killela, Reitman et al. 2013, Koelsche, Sahm et al. 2013, Liu, Wu et al. 2013, Nonoguchi, Ohta et al. 2013), examines the association between *TERT* promoter and *IDH1/2* mutations in glioma, and assesses their joint influence on OS. Utilizing a combined analysis of *IDH1/2* and *TERT* promoter mutations in adult glioma, we have derived a greatly expedited and simplified genetic signature of three common glioma subtypes, namely Grade II-III astrocytomas, oligodendrogliomas, and GBMs. Additionally, we show that oligoastrocytomas can be further classified.

Among patients with GBMs, we showed that the largest fraction of GBMs present with *TERT* promoter mutations. *IDH1/2* mutations are infrequent in these tumors and cluster within secondary GBMs. Three distinct subgroups were defined by the presence or absence of *TERT* promoter and *IDH1/2* mutations. Where patients harboring tumors with *TERT* promoter mutations alone had the poorest OS (median 11.3 months), patients with tumors bearing no mutations in either *TERT* or *IDH1/2* had a slightly better survival (median 16.6 months), and GBMs with *IDH1/2* mutation alone resulted in the best survival (median 42.3 months). Furthermore, these associations remained after adjustment for factors such as age. *TERT* promoter mutations predicted

poorer OS outcome in a multivariate model even in GBMs without *IDH1/2* mutations. This finding is in contrast with previous studies that did not report a significant difference in OS between *TERT* promoter-mutated and *TERT* promoter-wildtype non *IDH* mutated GBMs (Nonoguchi, Ohta et al. 2013). This finding will be of particular interest to clinicians as it may provide a tool to stratify non *IDH1/2* mutant GBMs and suggests that combined *IDH1/2* and *TERT* promoter genotyping will be useful for patient management. Because of variable treatment among these histological brain tumor groups, further analyses must include large cohorts of standardized treatment arms and measurements of other genetic features such as *MGMT* status, *EGFR* wildtype amplification, and the presence of *EGFRvIII* to confirm the validity of our findings. At a minimum, our current findings warrant further investigation and confirmation by other investigators. Also, genetic alterations of the *TERT* promoter may be particularly relevant given the development of therapeutics targeted against telomerase. Telomerase inhibitors have shown promise for treating GBM in preclinical models and are currently under investigation in clinical trials for several types of cancer (Zhang, Zou et al. 2006, Marian, Cho et al. 2010, Xia, Wang et al. 2012, Ruden and Puri 2013).

Conversely, *IDH1/2* mutations in grade II-III astrocytomas are frequent while *TERT* promoter mutations are uncommon. grade II-III oligodendrogliomas have frequent co-occurring mutations in the *TERT* promoter and *IDH1/2*. We provide evidence that over 86% of oligoastrocytomas in this cohort contain genetic signatures

representative of either astrocytoma (*IDH1/2* mutations alone) or oligodendroglioma (*TERT* promoter/ *IDH1/2*), signatures that we show are associated with OS.

Reproducibility of oligoastrocytoma diagnosis by histology alone displays variable diagnoses between neuropathologists within and among different institutions (Louis, Ohgaki et al. 2007, Wen and Kesari 2008, Ohgaki and Kleihues 2009). The presence of the *TERT* promoter and *IDH1/2* mutational status may be particularly useful to refine the classification of “mixed” oligoastrocytomas.

In addition to demonstrating the robust nature of these mutational patterns, we have further established that these genetic signatures are reliable when compared to the OS of patients derived from conventional histopathological diagnosis. As shown in Figures 15 and 16, mutations in the *TERT* promoter and *IDH1/2* effectively stratify patients into reproducible subgroups based on survival. This phenomenon was independent of grade among high grade astrocytomas as grade III and grade IV tumors mimicked this relationship when analyzed independently (Figure 15). Furthermore, the strength of these genetic signatures and their association with OS is illustrated by a slightly higher R2 (0.3132 vs. 0.2704) than by histology alone.

Two clinical subgroups exist among grade II tumors in the current cohort, as the power of the survival analysis was limited due to the smaller number of low grade gliomas. The grade II tumors exhibited genetic signatures with mutations in *IDH1/2* alone, and tumors with mutations in the *TERT* promoter and *IDH1/2*. Both subgroups

had a more favorable prognosis, with a median OS of 130.7 months in tumors with IDH1/2 mutations alone, and median OS of 205.5 months among patients whose tumors harbored *TERT* promoter and *IDH1/2* mutations. No grade II tumors exhibited *TERT* promoter mutations alone.

Within grade III-IV gliomas, those patients with the *TERT* promoter mutations alone had the poorest prognosis (median 11.5 months), while tumors bearing the events typically representative of astrocytomas (*IDH1/2* mutation) had a more favorable prognosis (median 56.9 months). Tumors harboring mutations typically seen in oligodendroglioma (both *TERT* promoter and *IDH1/2* mutation) had a more favorable prognosis (median 125.2 months). Tumors that did not harbor mutations in either the *TERT* promoter or *IDH1/2* comprised a unique clinical group with a short OS (median OS 17.2 months) that was distinct from *TERT* promoter mutated gliomas (median OS 11.5 months) (Table 16). As these gliomas, wildtype for both *TERT* promoter and *IDH1/2* mutations, represented a clinically distinct unit (Figure 15B and 15C) further investigation is required to delineate critical driver mutations in this subset of gliomas.

It is of interest to note that within each tumor type, a minority of tumors bore the genetic signature typically associated with other histological subtypes. In particular, 13.6% (12/88) of grade II-III astrocytomas bore *TERT* promoter mutations alone and occasional grade II-III astrocytomas harbored both *TERT* promoter and *IDH1/2* mutations (4/88, 4.6%). This suggests that at least genetically, these tumors may be more

similar to GBM and oligodendroglioma, respectively. Oligodendrogliomas were almost exclusively *TERT* promoter and *IDH1/2* mutated (79.3%, 69/87), but a fraction, 17.2% (15/87) harbored mutations in *IDH1/2* alone. In our cohort, no oligodendroglioma cases harbored *TERT* promoter mutations alone. A minor fraction of GBMs (0.8%, 2/240) contained mutations in both the *TERT* promoter and *IDH1/2* suggesting they were treated oligodendrogliomas that were diagnosed as small cell GBMs.

Loss of chromosomal arms 1p and 19q is a well-known genetic event associated with oligodendrogliomas that many neuropathologists use as a reliable test for diagnosing oligodendroglioma, a tumor generally associated with favorable prognosis and response to chemotherapy (Bigner, Matthews et al. 1999, Smith, Perry et al. 2000, Jenkins, Blair et al. 2006, Cairncross and Jenkins 2008). As a secondary analysis, the 69 oligodendrogliomas with 1p/19q status available were analyzed for an association with *TERT* promoter/*IDH1/2* mutational status. All 44 oligodendrogliomas with *TERT* promoter and *IDH1/2* mutations also had the 1p/19q allelic deletions and all but 3 of the 47 tumors with 1p/19q allelic losses also contained both *TERT* promoter and *IDH1/2* mutations, indicating that *IDH1/2* and *TERT* promoter mutational analysis may be comparable prognostic markers to 1p and 19q in oligodendrogliomas (Fisher exact $p < 0.0001$).

This study supports genotyping of *TERT* promoter and *IDH1/2* in gliomas as a rapid economical test requiring little tumoral DNA that could help inform clinicians as

to the predicted OS of these tumors that may differ from their predicted outcomes based on conventional histology alone. The *TERT* promoter mutations analyzed in this study lay only 22 base pairs apart, allowing for PCR amplification in a single amplicon. Additionally, the most frequent mutations in *IDH1* and *IDH2* occur in hotspot residues located at residues R132 and R172, respectively. Combined together, these three PCR amplicons allow for expedient turnaround, objective interpretation, and vast economic advantages to glioma patients.

The *TERT* promoter/*IDH1/2* mutational profiles of each tumor type can be used in several aspects of the clinical process including stratification of patients, examination of therapeutic response, and selection of treatment, among others. Given the background genes previously discovered in glioma, we hypothesize *TERT* promoter and *IDH1/2* mutations as the major driver genes that are consistently found in low-grade and high-grade adult gliomas. These gene mutation assays will support and expedite the diagnosis of brain tumors while supplementing histopathological evaluation. Measurement of these biomarkers could further increase the fidelity of glioma diagnosis in a rapid and cost-effective manner. Furthermore, the simplicity and affordability of these tests underscore their importance as a tool to aid neuropathologists in glioma diagnosis. Notably, these signatures can be applied to cases that present atypical morphologic features in standard histopathological analysis. Taken together these findings simplify the genetic classification of glioma. The ability of these genetic

signatures to stratify patients will refine and clarify the diagnostic accuracy of pathologists by supplementing standard histopathological criteria with genetic mutational analysis.

6. Summary and Future Directions

6.1 Summary and Future Directions

The biological diversity of malignant gliomas has confounded histopathological classification, and therapeutic strategies for decades. The recent focus on the molecular characterization of these tumors has provided insight and opportunities for more refined and informed patient diagnosis and management. While great strides have been achieved in elucidating fundamental genetic alteration in gliomas, the great depth and complexity of the glioma genome has been revealed in the process. Often, each histological subtype of glioma presents a unique genetic profile, and while many common alterations and themes have emerged, it is also evident that these signaling pathways are frequently targeted through multiple genetic alterations in each tumor, thereby making them difficult to manage clinically. Here, we describe a series of genomic investigations aimed at elucidating the fundamental and reproducible genetic alterations that can be used as glioma biomarkers to refine the classification of malignant gliomas.

First, we describe a genomic profiling analysis to characterize the genetic landscape of grade III anaplastic astrocytoma. In efforts to elucidated the key genetic alterations these tumors we performed whole exome mutational and copy number analysis on a cohort of 57 tumors including WHO grade II astrocytomas, grade III anaplastic astrocytoma, grade II oligoastrocytomas, grade III oligoastrocytomas, and a

panel of grade IV glioblastomas. In addition to confirming several findings from previous studies, we identified recurrent, frequently mutated genes and pathways including *IDH1*, *TP53*, *ATRX* in astrocytomas, and *EGFR* and *CDKN2A* in glioblastomas. We also identified genes previously unassociated with these tumors and frequent mutations in the *NOTCH* family including a recurrent *NOTCH1* mutation.

Additionally, we describe a series of experiments aimed at establishing the specificity and frequency of commonly altered genes, namely, *IDH1*, *IDH2*, *ATRX*, *CIC*, and, *FUBP1*, and their association with the clinical features of patients in numerous subtypes of malignant gliomas. We defined two highly recurrent mutational signatures in our glioma cohort: tumors with mutations in *IDH1/2* and *ATRX*, referred to as “I-A” tumors and tumors with mutations in *IDH1/2*, *CIC*, *FUBP1*, and/or loss of chromosomal arms 1p and 19q referred to as “I-CF” tumors. Additionally, we report that gliomas with mutations in *ATRX* are associated with an alternative lengthening of telomeres phenotype. These mutation signatures were also associated with the clinical features of patients, capable of predicting overall survival, providing a reproducible genetic signature for neuropathologists that may aid in diagnosis, prognosis, and treatment selection.

Next, we examined the relationship of mutations in the *TERT* promoter across a panel of cancer tissue, including gliomas. We assessed the status of the *TERT* promoter in a panel of over 1200 tumors, including gliomas. We found that tumors could be

divided into types with low (<15%, TERT-L) and high (\geq 15%, TERT-H) frequencies of *TERT* promoter mutations. The nine *TERT*-high tumor types almost always originated in tissues with relatively low rates of self renewal, including melanomas, liposarcomas, hepatocellular carcinomas, urothelial carcinomas, squamous cell carcinomas of the tongue, medulloblastomas, and subtypes of gliomas (including 83% of primary glioblastoma, the most common brain tumor type). Furthermore, Mutations in the *TERT* promoter were also frequent in oligodendroglioma (78%), and infrequent in astrocytomas (10%). As *ATRX* mutations have been implicated in the alternative lengthening of telomeres phenotype, particularly in gliomas, the mutually exclusive nature of *TERT* promoter and *ATRX* mutations provides insight into telomere maintenance mechanisms in gliomas. However, a subset of glioma patients did not harbor mutations in either the *TERT* promoter or *ATRX*. It is currently unclear as to what is responsible for telomere maintenance in these gliomas. We hypothesize that epigenetic alterations and mutations in telomere associated genes may be responsible for telomere maintenance in these gliomas, contributing to their malignant progression. Further investigation into this genetic subclass with studies encompassing the epigenome may address this question as methylation of the *TERT* promoter has been implicated in pediatric gliomas (Castelo-Branco, Choufani et al. 2013).

The discovery of *TERT* promoter mutation is a potential paradigm shift in cancer genomics, *TERT* mutations are located outside of the coding sequence of the genome,

and this area of the genome has largely been understudied. Current theories regarding the mechanisms of these mutations point to their generation of *de novo* transcription factor binding sites, which may be responsible for differences in gene expression. It is unlikely that *TERT* promoter mutations are the only genetic alteration in promoters of oncogenes and tumor suppressors, and future studies assessing the whole genome of these patients, not just those limited to the exome, are essential for understanding oncogenic mutations in these tumors. In addition to their implications for understanding the relationship between telomeres and tumorigenesis, *TERT* mutations may aid in the classification and prognostication of brain tumors.

Frequent mutations in isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) and the promoter of telomerase reverse transcriptase (*TERT*) represent two significant discoveries in glioma genomics. To further understand the relationship between these two mutations and investigate their potential as biomarkers for the classification and prognostication of brain tumors we analyzed the status of *IDH1*, *IDH2*, and the *TERT* promoter in a panel of 473 adult gliomas. We show that a simple, tripartite genetic signature involving *IDH1*, *IDH2*, and the *TERT* promoter can be used to distinguish several types of gliomas and is able to clinically predict the median overall survival of patients. Patients whose grade III-IV gliomas exhibit *TERT* promoter mutations alone predominately have primary GBMs associated with poor median OS (11.5 months). Patients whose grade III-IV gliomas exhibit *IDH1/2* mutations alone predominately have

astrocytic morphologies and exhibit a median OS of 57 months while patients whose tumors exhibit both *TERT* promoter and *IDH1/2* mutations predominately exhibit oligodendroglial morphologies and exhibit median OS of 125 months. A fourth clinical subtype involve tumors which are wild type for both alterations, and while prognosis of these patients remains poor (median OS 17.2 months), they have a distinct clinical response differing from tumors harboring *TERT* promoter mutant alone, tumors which are typically primary GBMs. Tumors wild type for both *IDH1/2* and *TERT* promoter mutations present an opportunity for further biological investigation and we hypothesize that wild type tumors will harbor genetic events more representative of the primary GBM genome, namely alterations targeting the *RTK/RAS/PI3K*, *TP53*, and *RB* pathways. Studies analyzing the whole genome of these patients are necessary next steps in deciphering the biological properties of this unique subgroup that are responsible for a slightly better prognosis than primary GBMs but ultimately fatal disease course.

While our previously established genetic signatures utilizing genes such as *ATRX*, *CIC*, and *FUBP1*, are successful in classifying subgroups of gliomas and correspond with clinical features of these patients, testing for these markers remains difficult and time consuming. *ATRX*, is a large, x- chromosome gene with 36 exons, resulting in expensive and laborious testing which can be difficult to interpret; *CIC* and *FUBP1* also present similar challenges. We show that on the basis of three simple

hotspot mutation PCR tests, *IDH1*, *IDH2*, and the *TERT* promoter, we can stratify gliomas into four distinct clinical subgroups capable of predicting median overall survival. The accessibility and simplicity of these tests will expedite, refine, and clarify the classification of gliomas moving forward and we provide further evidence supporting the roles of *TERT* promoter mutations and *IDH1/2* mutations as fundamental genetic alterations in gliomas. While the results of these tests are promising, future studies representing multi institution tumor cohorts and the standardization of clinical treatment arms are paramount for the establishment of *TERT* promoter and *IDH1/2* mutational analysis as a standard diagnostic tool in brain tumor clinics worldwide.

The lack of objective and reproducible glioma biomarkers has been a major challenge to the greater neuro-oncology community for years. Here we present evidence of a series of genomic alterations that occur in high frequency in gliomas, pointing to their importance in glioma tumorigenesis. Further, we provide evidence of their applicability as biomarkers that can be used clinically to stratify patients and predict overall survival. While the advent of these studies has resulted in novel genetic classification schemes, future studies determining the specific oncogenic mechanisms and dysregulation of signaling pathways associated with these mutations are paramount to our understanding of the malignant characteristics of gliomas.

Appendix A

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Biography

Patrick James Killela was born on November 13, 1984 in Freehold, NJ, to John and Christine Killela. Patrick attended North Carolina State University and graduated magna cum laude in the College of Agriculture and Life Sciences Honors Program in May of 2007 with a Bachelors of Science in Biological Sciences and a minor in Genetics. After his undergraduate degree, Patrick wanted to further his scientific training and joined the laboratory of Dr. Hai Yan in the Duke University Preston Robert Tisch Brain Tumor Center in July of 2007 as a Research Technician, a position he held for two years. Patrick began a journey investigating the diverse genetic aberrations that underlie the malignant transformation of brain tumors with a passion for the identification of clinically accessible genetic alterations and entered the Graduate School at Duke University in the Department of Pathology in September of 2009. Patrick has presented four abstracts and delivered a platform presentation at national scientific meetings and is an Associate Member of the American Association for Cancer Research.

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