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RESEARCH ARTICLE

Molecular and Morphologic Correlates of the Alternative Lengthening of Telomeres Phenotype in High-Grade Astrocytomas

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Abstract

Recent studies suggest that the telomere maintenance mechanism known as alternative lengthening of telomeres (ALT) is relatively more common in specific glioma subsets and strongly associated with *ATRX* mutations. We retrospectively examined 116 high-grade astrocytomas (32 pediatric glioblastomas, 65 adult glioblastomas, 19 anaplastic astrocytomas) with known ALT status using tissue microarrays to identify associations with molecular and phenotypic features. Immunohistochemistry was performed using antibodies against ATRX, DAXX, p53 and $IDH1^{R132H}$ mutant protein. *EGFR* amplification was evaluated by fluorescence *in situ* hybridization (FISH). Almost half of fibrillary and gemistocytic astrocytomas (44%) demonstrated ALT. Conversely all gliosarcomas (n = 4), epithelioid (n = 2), giant cell (n = 2) and adult small cell astrocytomas (n = 7) were ALT negative. The ALT phenotype was positively correlated with the presence of round cells ($P = 0.002$), microcysts ($P < 0.0002$), IDH1 mutant protein ($P < 0.0001$), ATRX protein loss ($P < 0.0001$), strong P53 immunostaining ($P < 0.0001$) and absence of *EGFR* amplification ($P = 0.004$). There was no significant correlation with DAXX expression. We conclude that ALT represents a specific phenotype in high-grade astrocytomas with distinctive pathologic and molecular features. Future studies are required to clarify the clinical and biological significance of ALT in high-grade astrocytomas.

INTRODUCTION

Telomere maintenance mechanisms are essential for long-term tumor growth. In 85% to 90% of human cancers, telomere length appears to be maintained, or increased, through up-regulation of the enzyme telomerase, a reverse transcriptase with the ability to synthesize new DNA using an internal RNA template (2, 11). However, specific cancer subsets exhibit the alternative lengthening of telomeres (ALT) phenotype, a telomerase-independent telomere maintenance mechanism (14). In ALT, the homologous recombination machinery is recruited to maintain telomeres. In a recent study, the ALT phenotype was relatively more prevalent in gliomas as compared to most other tumor types. ALT was present in 27% of high-grade astrocytomas, as compared to 3.7% of the 6110 overall cancer cases examined (7). A previous study showed longer patient survival in ALT-positive glioblastomas, as well as an association with IDH1 mutant protein expression, suggesting they represent a less-aggressive tumor subtype with a better prognosis (16).

In an exciting study this past year, the ALT phenotype showed a 100% concordance in gliomas, medulloblastomas and pancreatic neuroendocrine tumors with inactivating somatic mutations in the alpha thalassemia/mental retardation syndrome X-linked (ATRX) or death domain-associated protein (DAXX) genes (6). Mutations in genes encoding for these proteins were originally identified in pancreatic neuroendocrine tumors using unbiased sequencing studies (8). *ATRX* loss is also present in the majority of cell lines demonstrating ALT (14). Other studies have confirmed the increased frequency of *ATRX* mutations in glioblastoma (GBM) of young adults and children (21), grade II–III astrocytomas (9), as well as mutations in *DAXX* and *H3F3A* (encoding for the histone variant H3.3) in a subset (21). *H3F3A* mutations are particularly prevalent (78%) in diffuse intrinsic pontine gliomas (DIPG) of children (26), and are associated with a worse clinical outcome (10).

A clinical manifestation of germline *ATRX* mutations is a syndrome characterized by severe mental retardation (23). Multiple *in vivo* studies have confirmed ATRX functional importance in the nervous system (1). In the nucleus, ATRX cooperates with the molecular chaperone DAXX to incorporate H3.3 into heterochromatin at telomeres (12), providing a mechanistic link to the ALT phenotype. Loss of ATRX function leads to numerous cellular

aberrations, including abnormal methylation and gene expression patterns, as well as chromosome missegregation (19). In this study, we evaluated associations of the ALT phenotype with morphologic characteristics that on histopathology may be associated with more clinically favorable infiltrating gliomas subtypes, such as cellular monotony and microcysts. Given the strong association of the ALT phenotype with *ATRX/DAXX* mutations, and the important role they play in chromatin remodeling, we also evaluated chromatin quality by histology, as well as correlations with ATRX/DAXX expression and molecular alterations in high-grade astrocytomas.

MATERIALS AND METHODS

Patient population and tissue microarray

We studied 116 high-grade astrocytomas (32 pediatric GBM, 65 adult GBM, 19 anaplastic astrocytomas) from 112 patients using tissue microarray (TMA) sections. Patient demographics are summarized in Table 1. Among the GBM group, 14 were classified as secondary GBM based on progression from a documented grade II (n = 4) or grade III (n = 10) infiltrating glioma precursors. No diffuse intrinsic pontine gliomas were studied. Four 0.6 mm cores were included per tumor. Patient demographics and outcome data were abstracted from retrospective chart review. All studies were approved by the Johns Hopkins Institutional review board.

ALT assessment

We studied high-grade astrocytomas (WHO grade III and IV) that were recently part of a large survey of ALT in cancer (7). Telomere-specific fluorescence *in situ* hybridization (FISH) was performed as previously described (7, 9). Briefly, ALT-positive cases were identified by large, very bright intranuclear foci of telomere FISH signals marking ALT-associated telomeric DNA in interphase nuclei of fixed tissue specimens. Cases were classified as ALT positive if they met the following criteria: first, the presence of ultra-bright intranuclear foci of telomere FISH signals (ALT-associated telomeric foci), with integrated total signal intensities for individual foci being >10-fold that of the per cell mean integrated signal intensities for all telomeric signals in individual benign stromal cells within the same case; second, ≥1% of tumor cells displaying these ALT-associated telomeric foci. Cases lacking ALT-associated telomeric foci in which at least 500 cells were assessed were considered ALT negative. Areas exhibiting necrosis were excluded from consideration.

Histologic evaluation

Histologic evaluation was performed in whole Hematoxylin and Eosin sections in all cases with available slides (n = 92). Tumors were evaluated by two observers (FJR, DN). All tumors were placed in one general histologic subtype (fibrillary, gemistocytic, small cell, gliosarcoma, giant cell and epithelioid). Tumors were also evaluated for chromatin quality (fine vs. coarse), as well as the presence or absence of microcysts, and round cells (Figure 1). Tumors were interpreted as having round cells when focal monotonous features/halos were identified in the tumor (at least one high-power field) in the presence of a largely pleomorphic neoplasm with astrocytic cytology. Tumors with a convincing oligodendroglial component were not included. Small cell astrocytoma, characterized by uniform oval (rather than round) cells, with pronounced mitotic activity, and lacking microcysts as previously described (18), was also excluded from the group of tumors composed of round cells.

Immunohistochemical studies

Immunohistochemistry was performed using antibodies against ATRX (rabbit polyclonal, Sigma-Aldrich, St. Louis, MO, USA, 1:600), DAXX (rabbit polyclonal, Sigma-Aldrich, 1:100), p53

Table 1. Demographics and IDH1(R132H) status.

	Anaplastic astrocytoma	Pediatric GBM	Adult GBM	All groups
Number (% total)	19 (16)	32 (28)	65 (56)	116
Median age (range)	37 (11–62)	14 (<1–17)	55 (22–86)	40 (<1–86)
Sex				
Male	10 (53)	15 (56)	34 (53)	59 (54)
Female	9 (47)	12 (44)	30 (47)	51 (46)
Anatomic location				
Left	7 (39)	6 (33)	29 (45)	42 (42)
Right	11 (61)	12 (67)	35 (55)	58 (58)
Frontal	13 (68)	6 (25)	22 (34)	41 (38)
Frontotemporal	1 (5)	0	5 (8)	6 (6)
Frontoparietal	1 (5)	5 (21)	7 (11)	13 (12)
Parietal	1 (5)	2 (8)	8 (13)	11 (10)
Parietoccipital	0	1 (4)	5 (8)	6 (6)
Temporal	3 (16)	3 (13)	13 (20)	19 (18)
Temporoparietal	0	0	3 (5)	3 (3)
Hemispheric	0	1 (4)	1 (2)	2 (2)
Cerebellum	0	4 (17)	0	4 (4)
Spinal Cord	0	2 (8)	0	2 (2)
IDH1(R132H)	14 (74)	5 (16)	4 (6)	23 (20)

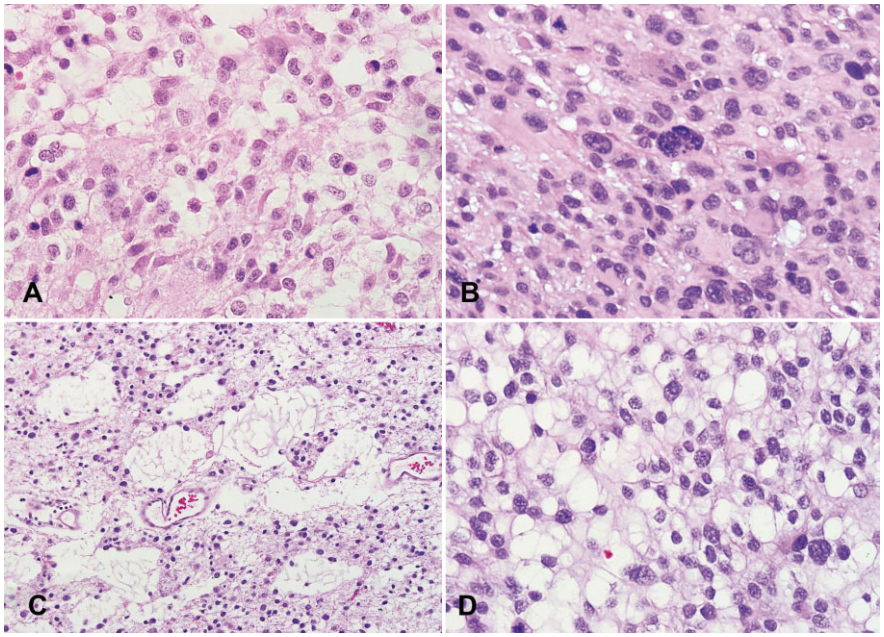

Figure 1. Histologic evaluation to identify morphologic correlates with the ALT phenotype. Histologic evaluation of the different tumors included chromatin quality as either fine (**A**) or coarse (**B**), as well as the presence of microcysts (**C**) and round cells (**D**).

(clone BP53-11, Ventana, Tuczon, AZ, USA; prediluted) and $IDH1^{R132H}$ (clone H09, Dianova, Hamburg, Germany; 1:50) mutant protein on TMA tissue sections. $IDH1^{R132H}$ was scored as positive or negative. P53, ATRX and DAXX were scored using a four tiered scale: 3+ = strong immunoreactivity in 50%–100% cells, 2+ = 10%–50% of tumor cells or strong reactivity in 1%–10% of cells; 1+ = weak reactivity in 1%–10% of tumor cells, 0 = absent immunoreactivity. The median value of several evaluable cores was used to arrive at a final score in each tumor.

Fluorescence *in situ* hybridization

EGFR amplification was evaluated by FISH using commercially available probes targeting *EGFR* with the corresponding centromere 7 (Abbott Molecular, Des Plaines, IL, USA). Amplification was defined as target to control probe ratio >2 in more than 5% of cells.

Statistical analysis

Correlation between categorical variables was performed using the chi square or Fisher's exact tests, and overall survival by log-rank or Wilcoxon tests. Continuous variables were evaluated with the Wilcoxon test. *P*-values of <0.05 were considered to be statistically significant. Statistical analyses were performed using JMP version 10 software (SAS Institute, Inc., Cary, NC, USA).

RESULTS

ALT is associated with grade and morphologic subtype in high-grade astrocytoma

ALT was identified in 40 cases (34%), including 17 (89%) grade III astrocytomas, and 23 (24%) grade IV astrocytomas, as previously reported (7), as well as in eight (57%) secondary vs. 15 (18%) primary GBM, a difference that was statistically significant ($P = 0.004$; Fisher's exact test). A total of 23 (20%) tumors expressed $IDH1^{R132H}$ (Table 1). Lower grade precursors for the secondary GBM were not available for testing. Patients with ALT-positive tumors had a median age of 33 years (range 5–58) at diagnosis vs. 50 years (range <1–86) for ALT-negative tumors. ALT status was concordant in tumor specimens obtained from the same patients. There were no significant associations with gender or anatomic location.

When focusing on histologic subtypes of high-grade astrocytoma, almost half of fibrillary and gemistocytic astrocytomas (41%) were ALT positive. Conversely all gliosarcomas (n = 4), epithelioid (n = 2), giant cell (n = 2) and adult small cell astrocytomas (n = 7) were ALT negative. The ALT phenotype was positively correlated with the presence of round cells ($P = 0.002$) and microcysts ($P < 0.0003$), but not with chromatin quality or the presence of giant cells. When looking at specific subgroups, the positive association of ALT with the presence of microcysts maintained significance in the GBM group ($P = 0.02$), while the presence of rare giant cells was significantly associated with ALT in GBM ($P = 0.03$). Associations between ALT and morphologic features are summarized in Table 2.

ALT is associated with ATRX protein loss

The presence of ALT was associated with increasing extent of ATRX loss as an ordinal variable ($P < 0.0001$). Complete ATRX protein loss was present in nine (of 39; 23%) ALT-positive cases, compared to four (of 72; 6%) ALT-negative cases ($P < 0.01$; Figure 2). In contrast, there was no significant correlation with DAXX expression ($P = 0.26$), and complete DAXX protein loss was not identified in any case. ATRX and DAXX immunostaining scores in association with ALT are summarized in Table 3.

Table 2. ALT associations with histologic subtypes and morphologic features in high-grade astrocytomas.

N (% of ALT status)	ALT+	ALT–	*P*-value
Fibrillary astrocytoma	29 (85)	36 (62)	NA
Gemistocytic astrocytoma	3 (9)	4 (7)	NA
Small astrocytoma	2 (6)	10 (17)	NA
Gliosarcoma	0	4 (7)	NA
Giant cell astrocytoma	0	2 (3)	NA
Epithelioid	0	2 (3)	NA
Microcysts	12 (35)	3 (5)	0.0002
Rare giant cells	15 (44)	18 (31)	0.25
Round cells	22 (65)	18 (32)	0.002
Coarse chromatin	23 (67)	46 (79)	0.21
Microcalcifications	5 (15)	8 (14)	1.00

NA = not applicable

Table 3. Immunohistochemical scoring of ATRX and DAXX.

	ATRX score (n %)				DAXX score (n %)			
	0	1	2	3	0	1	2	3
ALT+	9 (69)	17 (65)	8 (47)	6 (11)	0	0	4 (25)	36 (39)
ALT–	4 (31)	9 (35)	9 (53)	50 (89)	0	3 (100)	12 (75)	57 (61)

ALT is associated with distinct molecular and phenotypic features in high-grade astrocytoma

To evaluate the relationship of the ALT phenotype with other common molecular aberrations in high-grade glioma, we examined the presence of IDH^{R132H}, p53 immunoreactivity and EGFR amplification in our cohort. The presence of ALT was strongly associated with IDH^{R132H} mutant protein expression in the group as a whole ($P < 0.0001$) as well as in every astrocytoma subcategory. In fact, every IDH^{R132H}-positive tumor demonstrated ALT, although 17 (of 93) (18%) IDH^{R132H}-negative tumors also demonstrated ALT. ALT was also positively correlated with p53 nuclear labeling ($P < 0.0001$), with 24 (of 38; 63%) ALT-positive cases demonstrating strong (3+) nuclear immunolabeling, compared to 12 (of 75; 16%) ALT-negative cases. Conversely, there was an inverse association with *EGFR* amplification and the presence of ALT, with 30 (of 32; 94%) cases in which the receptor was amplified lacking ALT ($P = 0.0001$). Interestingly, the two anaplastic astrocytomas lacking ALT were the only anaplastic astrocytomas demonstrating *EGFR* amplification. These tumors also showed strong ATRX (3+) staining, weak (1+) p53 nuclear labeling and lacked $IDH1^{R132H}$ expression. Molecular results are illustrated in Figure 3. Immunophenotypic and molecular features are summarized by tumor subtype in Table 4.

Survival analysis

To determine the clinical significance of the ALT phenotype in high-grade astrocytoma, we evaluated ALT-positive and ALT-negative tumors with respect to survival. When analyzing all high-grade astrocytomas, ALT was associated with better overall survival ($P = 0.0007$), and there was a nonstatistical trend for better overall survival in tumors with complete ATRX loss ($P = 0.15$; Figure 4). However, there were no significant differences in outcome associated with ALT or ATRX loss in adult or pediatric GBM, or this combined group. Interestingly, the two ALT-negative anaplastic astrocytomas also demonstrated an adverse overall survival in this subgroup, 11 and 13 months after diagnosis, respectively.

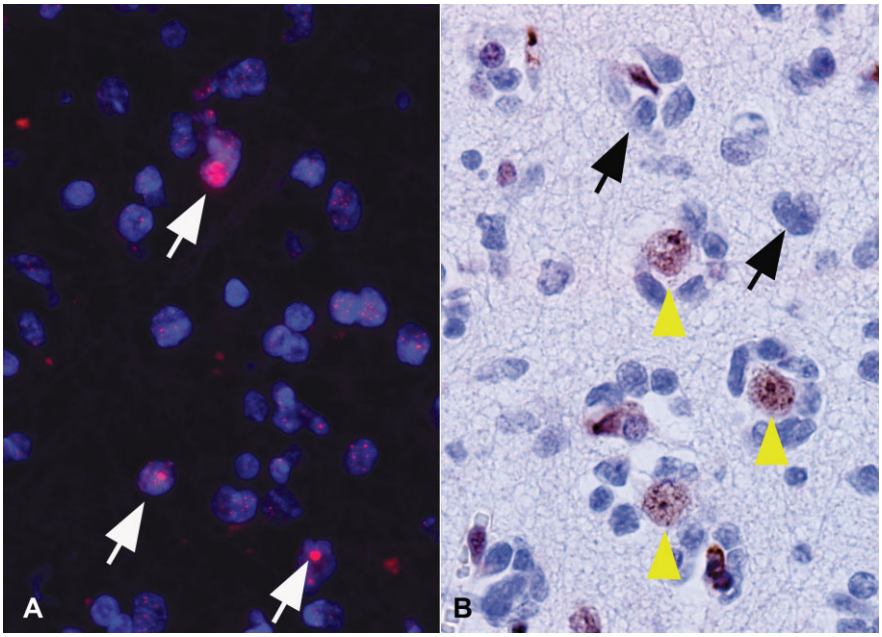

Figure 2. ALT phenotype and ATRX loss in high-grade astrocytomas. The ALT phenotype is characterized in tissue sections by ultra-bright signals using telomere-specific FISH (white arrows, **A**). Loss of nuclear ATRX protein expression by immunohistochemistry in tumor cells (black arrows). Preserved immunoreactivity in neurons (yellow arrowheads) serve as an internal control (**B**).

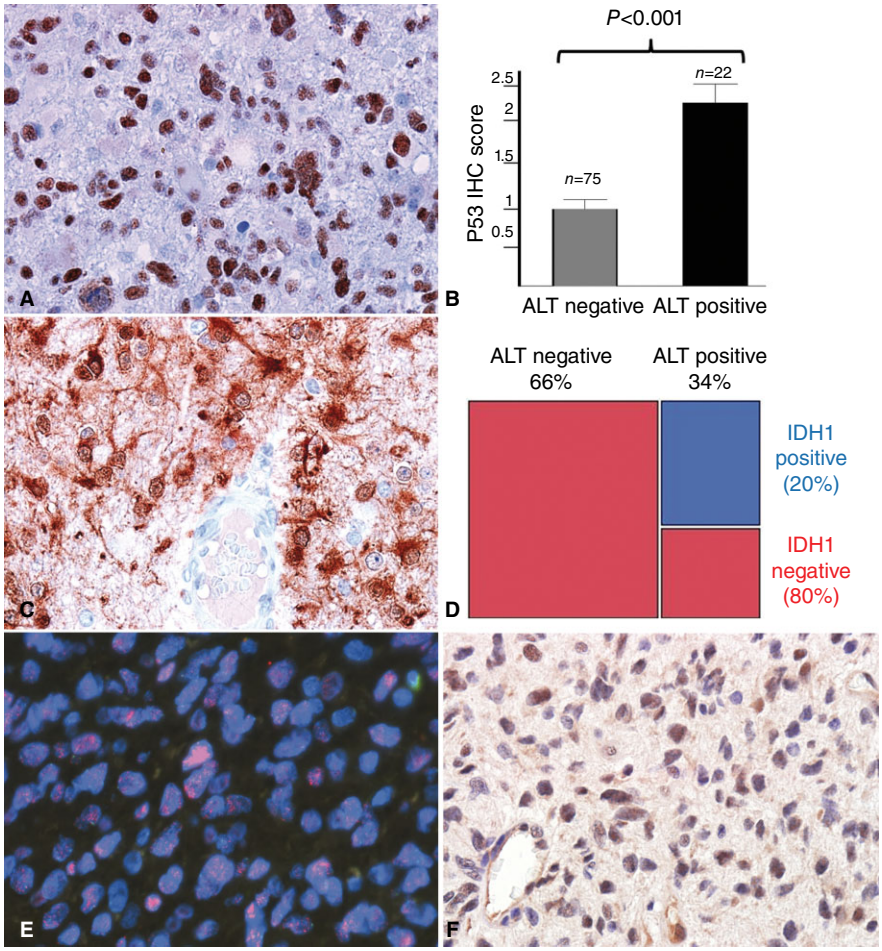

Figure 3. Immunohistochemical and molecular alterations associated with ALT in high-grade astrocytomas (n = 116). Strong p53 nuclear labeling in an infiltrating astrocytoma (**A**). Increased p53 immunoreactivity was associated with the presence of ALT (Wilcoxon test; **B**). Cytoplasmic protein expression of IDH1^{R132H} (**C**) was strongly associated with the presence of ALT (**D**). *EGFR* amplification in an anaplastic astrocytoma with intact ATRX protein expression (**F**).

DISCUSSION

Our progressive understanding of the molecular basis of high-grade gliomas has been the product of the recognition of particular phenotypic subsets as a result of recurrent somatic mutational events. The association of the ALT phenotype, which occurs at a relatively high frequency in anaplastic astrocytomas and glioblastomas compared to other tumor types, and concurrent *ATRX* mutations has provided key insights into the role of chromatin remodeling proteins in brain cancer, particularly in the pediatric population.

Here we provide additional clinical, phenotypic and genetic correlations of the ALT phenotype in high-grade astrocytomas. Our morphologic analysis revealed an increased frequency of ALT in fibrillary and gemistocytic astrocytoma subtypes, as well as an association with microcysts and the presence of round cells. These findings suggest that the ALT phenotype is overrepresented in tumors with a more favorable histology, and absent in subgroups

Table 4. Molecular and immunohistochemical findings by tumor subtype and ALT associations.

n/total	AA	Peds GBM	Adult GBM	Secondary GBM	Primary GBM	All GBM
Complete ATRX protein loss (IHC)	3/19	4/31*	6/62*	1/14	9/79*	10/93*
Complete DAXX protein loss (IHC)	0	0	0	0	0	0
IDH1 mutant protein	14/19*	5/32*	4/65*	6/14*	3/83*	9/97*
Absent *EGFR* amplification†	17/19*	26/29	27/55*	8/10	46/74*	53/84*
Strong P53 IHC	9/18	15/31*	12/64*	8/14	19/81*	27/95*
Presence of ALT	17/19 (89%)	14/32 (44%)	9/65 (14%)	8/14*	15/83	23/97 (24%)

*Associated with the presence of ALT ($P < 0.05$).

†None of the EGFR amplified adult GBMs were ALT+, while 2 (of 3) peds GBMs with *EGFR* amplification were ALT+.

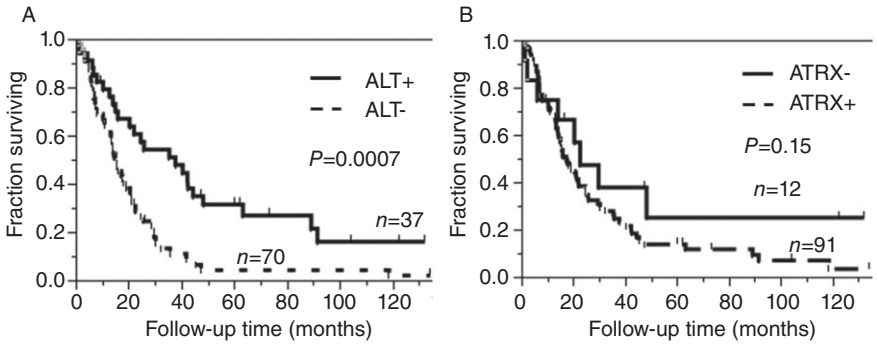

Figure 4. ALT is associated with longer survival in high-grade astrocytoma. Univariate analysis demonstrating increased survival associated with ALT-positive tumors compared with ALT-negative tumors (**A**). Nonsignificant trend for increased overall survival in tumors with ATRX loss (**B**).

that are almost exclusively of the primary (*de novo*) glioblastoma subtype, for example, gliosarcoma, adult small cell astrocytoma and giant cell glioblastoma (13). However, these findings must be interpreted with caution as the histologic subtypes other than fibrillary astrocytomas were comparatively small to draw firm conclusions.

In addition, the presence of microcysts and round cells may represent relatively more favorable histologic features supporting the association with ALT. Our interpretation of a minor component of round cells is unlikely to be related to an oligodendroglial component of a mixed glioma as ALT appears to be relatively less frequent in oligodendrogliomas compared to infiltrating astrocytomas (7, 9). p53 alterations are rare in oligodendrogliomas, and mutually exclusive with 1p19q deletion status (24), but were strongly associated with ALT further arguing against the inclusion of oligodendrogliomas in our cohort.

Our results support the concept that high-grade astrocytomas with ALT and ATRX loss represent a distinct molecular subset, characterized by frequent IDH1^{R132H}, lack of *EGFR* amplification and the presence of p53 alterations. By univariate analysis, we also identified an association with better overall survival and ALT in high-grade astrocytomas, as well as a trend of better overall survival with complete ATRX protein loss. However, it was not possible to separate this effect from grade as almost all anaplastic astrocytomas were ALT positive, and we could not detect a statistical significant difference in the glioblastoma group even after adjusting for age. This certainly could be related to sample size as a larger study of ALT in 573 GBM demonstrated a survival difference with better survival in patients with ALT-positive tumors (16). Although numbers of anaplastic astrocytomas were insufficient to draw any firm conclusions about correlation with survival, we noted that the only anaplastic astrocytomas lacking ALT in our study had *EGFR* amplification and an overall survival close to a year, similar to glioblastoma. Besides supporting the prognostic value of ALT in high-grade astrocytoma, this finding suggests that a subset of anaplastic astrocytomas, despite lacking histologic criteria of glioblastoma, that is, necrosis or microvascular proliferation, may represent in fact primary glioblastomas early on their evolution at the molecular level, as has been described for example in small-cell astrocytomas of adults (18).

The relationship with IDH^{R132H} is particularly intriguing. Mutations in *IDH1* or *IDH2* were identified initially by a comprehensive sequencing study of GBM (17), and since then, other studies have confirmed these alterations to be present in the majority of infiltrating gliomas, other than primary glioblastoma (5, 25, 27). IDH^{R132H} is the most frequent mutation present in I*DH1* in infiltrating gliomas, and its mutant protein product is recognized by a specific antibody with great sensitivity and specificity in tissue sections (3, 4). Of interest, recent studies have demonstrated profound epigenetic changes as a consequence of *IDH1* mutation, including induction of the CpG island methylator (CIMP) phenotype (22) and interference with histone demethylation resulting in global changes in gene transcription (15). The relationship between the CIMP phenotype and the ALT phenotype has not been fully explored but warrants further study. They are likely distinct in some settings because pediatric high-grade gliomas often show ALT and rarely have IDH1/2 mutations.

The interplay between ALT, *ATRX* loss and IDH^{R132H} suggests a unique molecular subgroup of infiltrating astrocytomas characterized by aberrant chromatin structure. Of note, every tumor in our cohort with IDH^{R132H} also demonstrated ALT. However, the presence of inactivating *ATRX* and *DAXX* mutations in association with ALT in tumors lacking *IDH1* mutations suggests that ATRX/DAXX alterations are more closely associated with the ALT phenotype than IDH1/2 alterations. Furthermore, ALT was relatively more frequent in pediatric glioblastoma, where IDH^{R132H} mutations are less prevalent. Rather, mutations in *H3F3A* are relatively more frequent in the pediatric age group. Collectively, these findings suggest that profound chromatin alterations resulting from multiple mutational events are essential molecular mechanisms responsible for an important subgroup of high-grade glioma.

It is also important to note that the association between ATRX protein loss and ALT was not perfect, unlike the association with *ATRX* mutations. *ATRX* mutations appear to be inactivating and result in protein loss (6), but interpretation of immunohistochemical staining in infiltrating gliomas is affected by preservation of the antigen in underlying non-neoplastic elements, which are not always possible to unambiguously separate from tumor cells. We have encountered similar problems before with other molecular/immunohistochemical correlative studies, for example, when interpreting MGMT protein loss in tumor sections (20).

In summary, our study demonstrates important associations between the ALT phenotype and the morphologic and molecular properties in high-grade astrocytomas. Further studies will continue to characterize the clinical and biological significance of these findings, and clarify specific mechanisms operating in these

tumors, as well as suggest specific therapies for an important category of human malignancy.

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