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Original Article

TERT expression increases with tumor grade in a cohort of *IDH*-mutant gliomas

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Abstract: The molecular mechanisms underlying progression from astrocytoma to secondary glioblastoma are poorly understood. Telomerase reverse transcriptase (TERT), a gene encoding for the catalytic subunit of telomerase, is upregulated in various cancers. Upregulation of TERT is a likely mechanism by which malignant cells delay senescence and evade cell death. TERT activity is also the primary mechanism by which malignant cells replenish telomeres, with the other means of telomere replacement being the alternative lengthening of the telomeres (ALT) system. The ALT system is known to be upregulated in tumors harboring loss of function mutations in ATRX. This study analyzed aggregate data on TERT and ATRX expression in astrocytoma, anaplastic astrocytoma, and secondary glioblastoma and then supplemented the data with our findings. In data obtained from Oncomine, significantly higher TERT expression is seen in astrocytomas and secondary glioblastomas compared to normal brain tissue. Additionally, The Cancer Genome Atlas data shows that TERT expression is a significant predictor of overall survival in low-grade gliomas. However, studies comparing the expression of TERT across all grades of astrocytomas had not been performed to date. Using immunohistochemical staining, we showed that controlling for ATRX and IDH mutational status, TERT expression increased with tumor grade in a cohort of patient-derived astrocytoma, anaplastic astrocytoma, and secondary glioblastoma samples. These findings indicate that TERT expression increases as astrocytomas become more aggressive tumors, and probably plays a role in their progression.

Keywords: Glioma, TERT, telomerase, astrocytoma

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain malignancy in adults, producing 3.19 new cases per 100,000 annually. GBM is also the most lethal primary brain tumor, with a median survival of only 15 months [1, 2]. This is despite aggressive and multimodal treatment, including surgical resection, chemotherapy, radiation therapy, and immunotherapy [2-4]. Reflecting on the poor outcome and relatively ineffective treatments associated with GBM, it is clear that further research is vital to understand GBM better and achieve new insight into the mechanisms underlying their growth and development.

GBMs are stratified into the two broad categories of primary and secondary GBM, respectively. Primary GBMs arise de novo and are slightly more aggressive, while secondary GBMs arise from a lower-grade tumor such as a diffuse astrocytoma (WHO grade II) or an anaplastic astrocytoma (WHO grade III) [5-7]. The tumors are also, with few exceptions, able to be distinguished by the presence or absence of mutations in the isocitrate dehydrogenase enzyme (IDH). IDH is acitric acid cycle enzyme that catalyzes the conversion from isocitrate to α-ketoglutarate, thereby generating NAD(P)H [5, 8]. Mutations in IDH1 and IDH2 are frequently found in secondary GBM but are rare in primary tumors [9]. An overview of the various pathways through which GBMs may evolve in adults and their associated genetic mutations is shown in **Figure 1**.

As mentioned above, IDH mutations are highly prevalent in secondary GBMs [5, 9]. GBM patients with IDH mutations tend to be younger and experience better clinical outcome than those with wild-type IDH [5, 7, 9]. Beyond IDH,

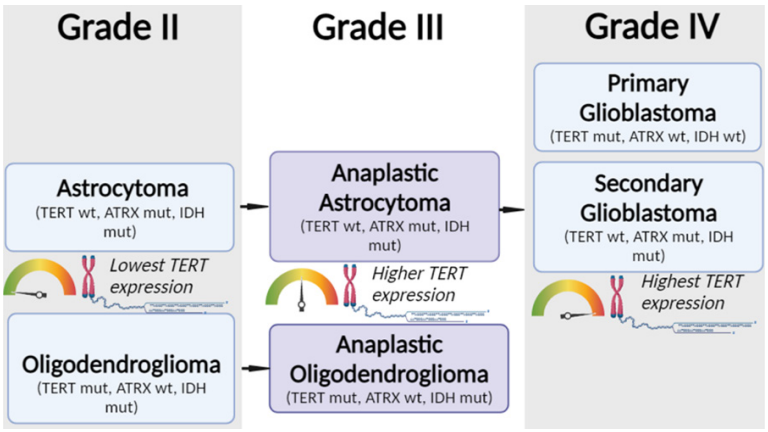

Figure 1. The lineages of progression for malignant gliomas in adults are shown. A molecular signature of astrocytomas, anaplastic astrocytomas, and secondary glioblastomas is that they are more likely to harbor ATRX mutations and less likely to have mutations in TERT as compared to GBM and oligodendroglioma. Additionally, both astrocytomas and oligodendrogliomas are characterized by mutations in IDH, a molecular feature not typically seen in primary GBM. In our study, we showed that TERT expression increases as a function of tumor grade as astrocytomas progress to more aggressive tumors.

mutations in two other genes have also been heavily investigated regarding GBM classification: TERT and ATRX (Alpha Thalassemia/Mental Retardation Syndrome X-Linked), both involved in telomere maintenance [10]. Although both TERT and ATRX mutations are involved in telomere maintenance, they achieve this by distinct mechanisms. TERT mutations have been shown to increase telomerase activity [10, 11], whereas ATRX loss is strongly correlated with the alternative lengthening of telomeres (ALT) system [10, 12-14]. ALT is a telomerase-independent mechanism of telomere lengthening, which departs from most tumor cells that depend on telomerase [15, 16]. However, the current understanding of ALT indicates that its mechanism of function is reliant on homologous DNA recombination [16-18]. A more specific understanding of how ALT functions has not yet been determined. The body of literature surrounding the use of telomere-maintenance-related genes (such as TERT and ATRX) as markers for GBM classification and predictors for clinical prognosis is growing rapidly. Several published papers associate TERT and ATRX mutations with specific glioma subgroups [19-21] and variable prognoses in a clinical setting [20, 22]. In this study, we aim to provide a clear narrative surrounding the aggregate data available on TERT and ATRX expression in astrocytoma, anaplastic astrocytoma, and secondary glioblastoma and augment this data with our results obtained from a cohort of patients with these tumors.

Methods

Immunohistochemistry

The human glioblastoma surgical biopsy specimens were obtained from Saint Francis Medical Center (Peoria, IL) and processed in accordance with the UICOMP Institutional Review Board-approved protocols (#85193). The paraffin-embedded sections were deparaffinized overnight at 65°C. The slides were processed as per the standard protocols. Briefly, the slides were then blocked in Carbo-Free blocking solution (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. The sections were then incubated with primary antibodies in the same blocking solution at 4°C for 24 hours. The primary antibody dilutions were 1:1000 mouse anti-TERT (Santa Cruz Biotechnology, Santa Cruz, CA) and 1:2000 rabbit anti-ATRX (Santa Cruz Biotechnology, Santa Cruz, CA). Following incubation with primary antibody, sections for TERT and ATRX were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in a 1:2000 dilution for 1 hour. The slides were washed, and nuclear staining was then performed with hematoxylin. The slides were mounted and incubated overnight at 60°C before imaging. The sections were viewed under light microscopy and scored for TERT immunoreactivity based on the intensity and frequency of positive cell staining. Slides were scored as strongly positive (++), weakly positive (+), or negative (-) for TERT. The mutational status for ATRX and IDH were determined using IHC immunostaining by the neuropathologist, Dr. Sarah E. Bach.

Data collection

The survival data presented were collected by The Cancer Genome Atlas (TCGA) and retrieved from UALCAN using the TCGA analysis function. In addition, expression data were obtain-

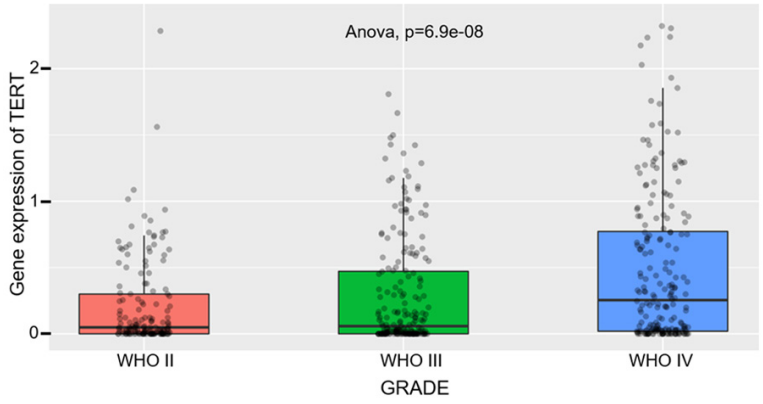

Figure 2. TERT expression is significantly correlated with tumor grade (ANOVA, P=6.9e-08), with the highest levels seen in WHO grade IV tumors.

ed using the Oncomine database and the Chinese Glioma Genome Atlas.

Data analysis

TERT expression data across WHO tumor grades obtained from Oncomine and the Chinese Glioma Genome Atlas was analyzed using analysis of variance (ANOVA). Data comparing TERT expression between normal brain tissue, anaplastic astrocytoma, secondary GBM, astrocytoma, and normal brain tissue were analyzed using an unpaired t-test. Patient survival data obtained from TCGA to show the effect of TERT expression on patient survival and the effect of TERT expression level and tumor grade on patient survival were analyzed using ANOVA.

Results

TERT expression increases with tumor grade in a general sample of gliomas

An extensive data analysis was performed at the study onset to determine existing aggregate data on TERT expression in glioma. Data from the Chinese Glioma Genome Atlas had shown that TERT expression significantly correlates with tumor grade in a pooled sample of gliomas (**Figure 2**). It is worth emphasizing, however, that the currently available data here do not stratify by histologic or molecular classification of the gliomas.

TERT expression is increased in astrocytoma and secondary glioblastoma compared to normal brain tissue

To determine the extent to which prior studies have delineated the expression of TERT in astrocytic tumors, datamining of the Oncomine database was conducted. Findings from Beroukhim et al. [23] had shown that secondary glioblastoma showed significantly higher TERT expression compared to both normal brain tissue (P<0.01) and anaplastic astrocytoma (P<0.01) (**Figure 3A**). Additionally, in a cohort of astrocytomas (n=43), significantly higher TERT expression was seen compared to normal brain tissue (P<0.01) (**Figure 3B**). Notably, data directly comparing astrocytoma, anaplastic astrocytoma, and secondary glioblastoma for TERT expression was lacking.

TERT expression is correlated with lower survival rates in grade II and grade III gliomas

Next, to validate the prognostic significance of TERT expression in malignant brain tumors, aggregate data were obtained from TCGA. Patients whose tumors had high TERT expression exhibited significantly decreased survival rate (P<0.0041) compared to patients harboring tumors with medium/low TERT expression (**Figure 4A**). This was the case for both grade II and grade III gliomas, although tumor grade remained a more significant prognostic factor than TERT expression. This is evidenced in **Figure 4B**, where grade III tumors with low/medium TERT expression show significantly improved survival over grade III gliomas with high TERT expression but still exhibit worse outcomes compared to grade II gliomas with high TERT expression.

Aggregate data combining both grade II and grade III tumors (**Figure 4B**) confirmed that TERT was significantly associated with decreased survival (P<0.001) in low-grade gliomas. These findings are in line with prior meta-analyses [24, 25].

TERT expression increases as a function of tumor grade and is highest in secondary glioblastoma

While there are extensive data in the literature measuring TERT expression across tumor grade in glioma samples, previous studies typically

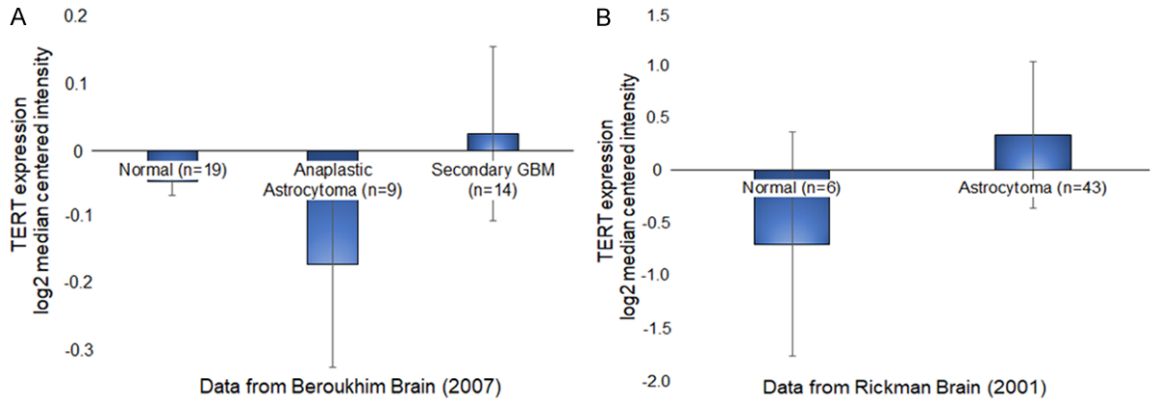

Figure 3. A. TERT expression is significantly higher in secondary glioblastoma (n=14) compared to both normal brain tissue (n=19) and anaplastic astrocytoma (n=9) (P<0.01) by unpaired t-test. B. TERT expression is significantly greater in astrocytoma (n=45) compared to normal brain tissue (n=6) (P<0.01) by unpaired t-test.

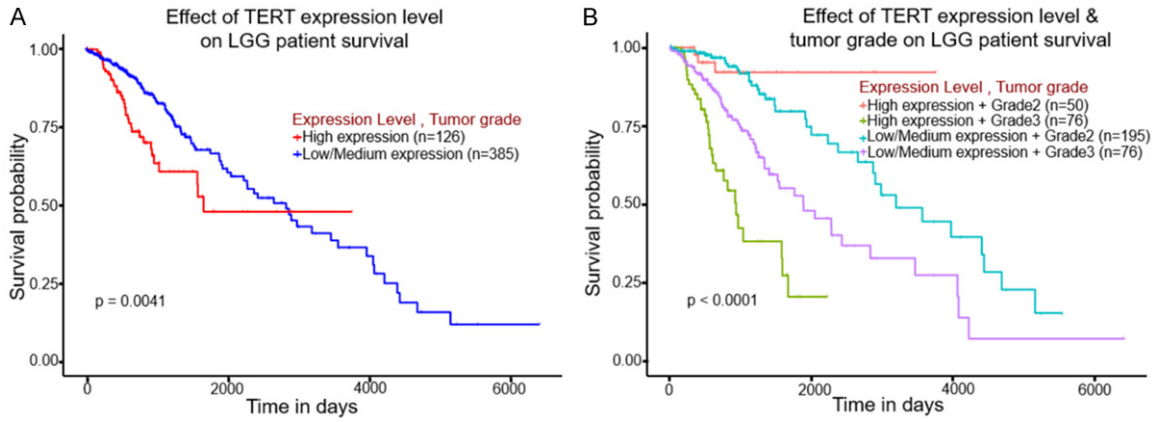

Figure 4. A. High TERT expression (n=126) in low-grade gliomas is associated with decreased survival (P=0.0041) compared to gliomas with low TERT expression (n=385) by unpaired t-test. B. Patients with grade 3 gliomas (n=76) and grade 2 gliomas (n=50) with high TERT expression exhibit worse survival (P<0.0001) as compared to patients with grade 2 (n=195) and grade 3 (n=76) gliomas exhibiting low TERT expression by ANOVA.

did not distinguish between different grade II and grade III gliomas in their analysis. This represents an important confound due to the distinct molecular signatures of different tumor lineages and grades (**Figure 1**). Additionally, literature evaluating TERT expression in secondary glioblastoma is exceedingly scarce, likely due to the relatively low incidence of these tumors compared to primary GBM [26]. Therefore, we sought to compare TERT expression across a cohort consisting of secondary GBMs and the precursor lesions to these tumors, namely, grade II astrocytomas and grade III anaplastic astrocytomas [27]. In this cohort (n=15), TERT immunoreactivity was found to increase as a function of tumor grade, with the highest expression seen in secondary GBMs (n=3). Representative images are shown in **Figure 5**. **Figure 5** reveals that TERT immunoreactivity in this cohort is significantly increased in secondary GBMs, particularly when compared to grade II astrocytoma samples. A more detailed description of the samples in this cohort, including the tumor grade, IDH, and ATRX mutational status, and TERT immunoreactivity, is shown in **Table 1**. All of the grade III and grade IV gliomas in the cohort were IDH and ATRX mutants. TERT immunoreactivity was markedly disparate as a function of tumor grade, particularly when solely comparing the IDH and ATRX mutant samples from each grade. Additionally, within the grade II tumors, TERT immunoreactivity was higher in the samples without ATRX mutations. However, addi-

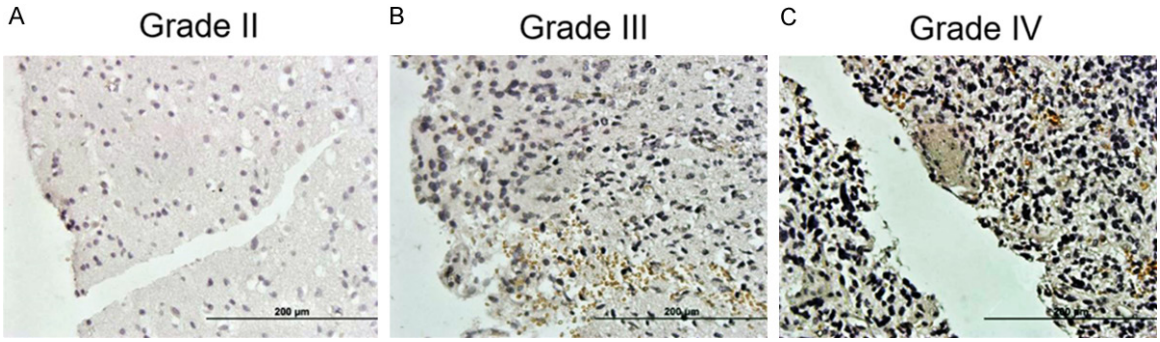

Figure 5. Representative images showing TERT expression in (A) astrocytoma, (B) anaplastic astrocytoma, and (C) secondary glioblastoma. The expression of TERT increased with tumor grade.

Table 1. Differential expression of TERT across a cohort of *IDH*-mutant gliomas

Sample ID	Tumor Type	Tumor Grade	IDH Status	ATRX Status	TERT Expression (IHC)	Average Staining Score
A	Astrocytoma	2	mutant	mutant	+	
B	Astrocytoma	2	mutant	mutant	-	
C	Astrocytoma	2	mutant	mutant	-	
D	Astrocytoma	2	mutant	Intact	+	0.67
E	Astrocytoma	2	mutant	intact	-	
F	Astrocytoma	2	mutant	mutant	++	
G	Anaplastic astrocytoma	3	mutant	mutant	++	
H	Anaplastic astrocytoma	3	mutant	mutant	++	
I	Anaplastic astrocytoma	3	mutant	mutant	+	1.5
J	Anaplastic astrocytoma	3	mutant	mutant	++	
K	Anaplastic astrocytoma	3	mutant	mutant	-	
L	Anaplastic astrocytoma	3	mutant	mutant	++	
M	Secondary Glioblastoma	4	mutant	mutant	++	
N	Secondary Glioblastoma	4	mutant	mutant	++	
O	Secondary Glioblastoma	4	mutant	mutant	++	

tional studies are needed to validate this association.

Discussion

In recent years, primary and secondary GBMs have been extensively distinguished from each other with regard to their genotype [27, 28]. As discussed previously, primary GBMs are far more likely to be IDH-wild type, while secondary GBMs and the precursor tumors astrocytoma and anaplastic astrocytoma harbor IDH mutations [29]. This defect in metabolism is thought to be at least partially responsible for the less aggressive nature of secondary GBMs compared to primary tumors [9]. With regards to TERT, several studies have found that primary GBMs are more likely to harbor activating mutations in the gene than secondary GBMs [27, 30]. In an aggregate study of 1206 adult gliomas, 77% of primary GBMs (n=309) were found to harbor mutations in TERT, as compared to a mutational frequency of 18% in secondary GBM (n=51) and 22% in astrocytoma (n=555) [30]. The frequency of TERT mutations also appears to vary greatly between oligodendroglioma and astrocytoma as well, with 96% of IDH mutant, 1p/19q co-deleted gliomas found to harbor TERT mutations. TERT mutations were also found to be highly prevalent in IDH wild-type gliomas, as well. In IDH mutant astrocytomas, however, the frequency of TERT mutations was found to be just 4% [31]. These activating mutations were thought to be a negative prognostic factor for primary GBMs. However, recent multivariate analyses

have concluded that TERT mutations are not an independent prognostic factor, and rather the findings of decreased survival in TERT mutant tumors are due to an inverse correlation with IDH mutations [27, 28, 31].

From these data, it follows that TERT expression, rather than mutational status, may be of greater pathologic significance in glioma progression [32]. A study with a sample of 22 primary GBMs and 20 secondary GBMs, found significantly higher levels of telomerase activity and TERT expression in secondary GBMs as compared to primary GBMs [31]. Using immunohistochemistry, Masui et al. detected elevated levels of TERT expression in both TERT wild type and TERT mutant gliomas. Additionally, they had found that overall TERT expression did not correlate with the mutational status of the glioma [33]. This indicates that several mechanisms may be responsible for regulating TERT expression, including epigenetic modifications.

With regard to telomere maintenance, TERT is just one of the two main variables, with ATRX status being critical as well [34]. It is well known that ATRX loss aberrantly induces the alternative lengthening of the telomeres (ALT) system, leading to uncontrolled cell proliferation and extensive telomere elongation [15]. Unlike TERT mutations, ATRX loss is thought to be pathologically significant [29]. A study by Ceccarelli et al. involved sequencing of 1122 gliomas and showed that ATRX, but not TERT promoter mutations were associated with increased telomere length [29]. In fact, some tumors with TERT mutations actually showed decreased telomere length compared to TERT wild-type tumors, regardless of IDH status [29]. The coincidence of TERT and ATRX mutations is very rare [31]. In a sample of 93 TERT mutant GBMs, only 6 samples also showed loss of ATRX ($P= 0.0022$) [35]. As such, predominantly TERT wild-type and ATRX mutant tumors such as secondary GBMs may display a greater efficiency in telomere maintenance and senescence compared to primary GBMs [32].

In this study, we examined TERT expression in a sample of 15 IDH-mutant gliomas. This included both secondary glioblastomas as well as their precursor lesions, grade II astrocytoma and grade III anaplastic astrocytoma. In our cohort, 80% of these samples also showed ATRX loss, comparable to the 89% coincidence of IDH and ATRX mutations reported in prior studies [36]. Through immunostaining, we identified a correlation between TERT immunoreactivity and tumor grade in this cohort. Given that ATRX loss is already present in 86% of IDH mutant, low-grade gliomas, these findings indicate that upregulations in TERT expression may be chiefly responsible for the greater senescence and telomere length of secondary GBMs as compared to lower grade gliomas [31, 33]. However, as IDH-mutant tumors are largely TERT wild-type, the mechanism by which TERT expression increases in these tumors remains unclear [33]. Further large-scale, quantitative analyses will be necessary to fully elucidate the significance of TERT expression in the evolution of astrocytomas toward grade IV secondary GBM.

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Disclosure of conflict of interest

None.

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