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ORIGINAL PAPER

The T-box transcription factor brachyury behaves as a tumor suppressor in gliomas

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Abstract

The oncogene brachyury (*TBXT*) is a T-box transcription factor that is overexpressed in multiple solid tumors and is associated with tumor aggressiveness and poor patient prognosis. Gliomas comprise the most common and aggressive group of brain tumors, and at the present time the functional and clinical impact of brachyury expression has not been investigated previously in these neoplasms. Brachyury expression (mRNA and protein) was assessed in normal brain (n = 67), glioma tissues (n = 716) and cell lines (n = 42), and further *in silico* studies were undertaken using genomic databases totaling 3115 samples. Our glioma samples were analyzed for copy number (n = 372), promoter methylation status (n = 170), and mutation status (n = 1569 tissues and n = 52 cell lines) of the brachyury gene. The prognostic impact of brachyury expression was studied in 1524 glioma patient tumors. The functional impact of brachyury on glioma proliferation, viability, and cell death was evaluated both *in vitro* and *in vivo*.

Brachyury was expressed in the normal brain, and significantly downregulated in glioma tissues. Loss of brachyury was associated with tumor aggressiveness and poor survival in glioma patients. Downregulation of brachyury was not associated with gene deletion, promoter methylation, or inactivating point mutations. Brachyury re-expression in glioma cells was found to decrease glioma tumorigenesis by induction of autophagy. These data strongly suggest that brachyury behaves as a tumor suppressor gene in gliomas by modulating autophagy. It is important to note that brachyury constitutes an independent positive biomarker of patient prognosis. Our findings indicate that the role of brachyury in tumorigenesis may be tissue-dependent and demands additional investigation to guide rational interventions.

Keywords: autophagy; biomarker; brachyury; EMT; glioblastoma; gliomas; prognosis; *TBXT*; tumor suppressor

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No conflicts of interest were declared.

Introduction

Gliomas are the most common primary brain tumors and present high morbidity and mortality [1]. Histologically, the main glioma subtypes have an astrocytic and oligodendrocytic lineage and are categorized according to the World Health Organization (WHO) in four grades of malignancy (I–IV). Glioblastoma (WHO grade IV;

GBM) is the most aggressive and the most frequent form of glioma [2]. The newest glioma sub-classification encompasses four molecular subtypes based on its gene expression profile: proneural, neural, classical, and mesenchymal [3]. Nevertheless, only a few molecular markers such as mutations of the *TERT* promoter, *IDH1*, loss of 1p/19q, expression of ATRX, and *MGMT* methylation status are currently implemented in a routine setting [4–6]. Moreover, GBM patients' response to TMZ-based chemotherapy is modest (median of 15 months), and the 5-year overall survival rate is less than 5% [7,8]. The complexity and inability to predict patient outcomes together with glioma aggressiveness illustrate the urgent need for new GBM biomarkers.

The transcription factor brachyury (*TBXT*) is the prototype of the T-box transcription factor family, with a central role in notochord and mesoderm specification [9]. In development, brachyury misexpression is associated with several congenital defects, mainly neural-tube defects, and homozygous embryos die after a few days of gestation [10]. Its pivotal involvement in the pathogenesis of chordomas—a tumor derived from the notochord—first established a role of brachyury in cancer. More recently, brachyury was described to be upregulated in several tumors, including gastrointestinal stromal tumors (GIST) [11], breast [12], lung [13,14], colorectal [15], prostate [16], and testicular [17] cancer. It is notable that brachyury was reported as an independent biomarker of poor prognosis of these tumors [11–13,15,16]. Consequently, an anti-brachyury vaccine (GI-6301) was developed [18] and is being tested in a Phase II clinical trial (www.clinicaltrials.gov, 2015 – NCT02383498) in patients with chordoma. Recently, expression of *brachyury* (*TBXT*) was reported in approximately 30% of the gliomas from the TCGA database, but no associations with progression-free survival were observed [19].

In the present study, we performed an extensive analysis of brachyury's clinical impact and role in glioma tumorigenesis and surprisingly found brachyury to be an independent positive biomarker of prognosis in glioma patients.

Materials and methods

Tissue samples

A series of formalin-fixed paraffin-embedded (FFPE) glioma tissue samples, totaling 675 gliomas, including 103 pilocytic astrocytomas [WHO grade I, not otherwise specified (NOS)], 39 diffuse astrocytomas (WHO grade II, NOS), 39 anaplastic astrocytomas (WHO grade III, NOS), 436 glioblastomas (WHO grade IV, NOS), 58 anaplastic oligodendrogliomas (WHO grade III, NOS), and 67 non-tumoral tissues were used to assess brachyury expression profiles and clinical impact in gliomas. Overall, 196 cases were pediatric (0–20 years old), and 434 adult and follow-up were available for 413 glioma patients. The clinicopathological features of gliomas across different histological subtypes are presented in supplementary material, Supplementary materials and methods and Table S1. A series of 40 frozen gliomas (3 pilocytic astrocytomas, 4 diffuse astrocytomas, 5 anaplastic astrocytomas, and 28 glioblastomas: NOS) was used for mRNA expression analysis. Local Ethical Review Committees previously approved the present study, and all the samples enrolled in the present study were unlinked and unidentified from their donors.

Immunohistochemistry analysis

Histological slides with 4-µm-thick tissue sections were subjected to immunohistochemistry using a streptavidin-biotin peroxidase complex system, as described previously [11,16,17]. Brachyury immunostaining was validated using three different anti-brachyury antibodies (1:200 for sc-20109, Santa Cruz Biotechnology, Heidelberg, Germany; 1:500 for AF2085, R&D Systems, Minneapolis, MN, USA; 1:75 for ab57480, Abcam, Cambridge, UK) (supplementary material, Figure S1A). Testis tissues were used as a positive control for brachyury immunostaining, as described (supplementary material, Figure S1B) [17].

Sections were scored in a double-blinded fashion (GCA and MMM) for cytoplasm expression following a semi-quantitative criterion based on the intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong) as previously [11,16]. Samples with scores 0 and 1 were considered negative, and those with scores 2 and 3 were considered positive. Nuclear expression was also scored in positive cases (scores 2 and 3) where cases with ≥25% nuclear staining were considered positive, and cases with <25% of nuclear staining were considered negative.

Cell lines and transfection

One cell line derived from normal astrocytes (NHAi), 16 human glioma cell lines, 3 glioma stem cell lines, and 18 primary glioma cell lines were evaluated. SNB-19, U373, A172, GAMG, and U87 cell lines were transfected with full-length human brachyury cloned into the pcDNA4/T0 vector (Invitrogen), designated pcBrachyury, or with pcDNA4/T0 empty vector (4/T0); brachyury expression was knocked-down in NHAi cell lines, as described previously [16]. UCH1 and UCH2 chordoma cells were used as positive controls and kindly provided by the Chordoma Foundation (https://www.chordomafoundation.org). Detailed information about the cell lines used is presented in supplementary material, Supplementary material and methods.

Western blotting and immunofluorescence analyses

Immunodetection was achieved using antibodies for human brachyury (1:500, sc-20109, Santa Cruz Biotechnology; 1:400, AF2085, R&D Systems; and 1:250, ab57480, Abcam), p62 (1:200, clone pw9860, EnzoLife Sciences, Farmingdale, NY), LC3 (1:1000, clone 2775, Cell Signaling Technology, Leiden, The Netherlands), β-actin (1:1000, sc-1616, Santa Cruz Biotechnology), and GAPDH (1:1000, sc-69 778, Santa Cruz

Biotechnology). The analysis of apoptosis-related proteins was assessed using a human apoptosis array kit (Proteome Profiler Array ARY009; R&D Systems). Brachyury protein subcellular localization analysis in glioma cell lines was performed as described previously [16]. Brachyury specificity was validated by western blotting in brachyury-negative and brachyury-positive cells (supplementary material, Figure S1C,D).

Viability and proliferation functional assays

MTS and BrdU assays were used to evaluate the viability and proliferation capacity of cells over time, as described previously [16,20]. Soft agar anchorage-independent colony formation assays were performed as described previously [20,21].

Flow cytometry analysis

Cell death was evaluated using annexin V-FITC and propidium iodide (PI), according to the manufacturer's instructions (BD Biosciences, San José, CA, USA). Cell cycle analysis was carried out using the CycleTEST Plus DNA Reagent Kit (BD Biosciences). Both cell death and cell cycle analysis were analyzed by flow cytometry (LSRII, BD Biosciences).

Autophagy assays (p62, LC3-I/II and acridine orange staining)

Cells were plated on a 6-well plate 1 day before the start of the experiment to allow the cells to attach. On the next day, cells were serum-starved in DMEM (0% FBS) or in HBSS (Sigma-Aldrich, MO, USA), with or without the addition of Bafilomycin A1 (Sigma-Aldrich) to inhibit LC3 degradation (control). After 1 h of treatment, cells were harvested and collected for protein extraction. p62 and LC3I/II conversion was evaluated by western blotting (described above). Formation of acidic vesicular organelles, a morphological characteristic of autophagy, was quantitated by acridine orange staining at a final concentration of 1 µg/ml (A8097, Sigma-Aldrich) added to treated cells for 15 min at 37 °C. Subsequently, cells were analyzed by flow cytometry (BD FACSCanto II, BD Biosciences) using the software BD FACSDiva (BD Biosciences).

In vivo chicken chorioallantoic membrane (CAM) assay and mouse xenograft model

To assess *in vivo* tumorigenicity and angiogenesis, we used the CAM assay as described previously [20,21].

Eight-week-old athymic nude Foxn1nu male mice were obtained from Charles River Laboratories, maintained and housed at ICVS, in a pathogen-free environment under controlled conditions of light and humidity. Animal experiments were approved by institutional and national ethical committees (Direção Geral de Alimentação e Veterinária, Portugal) and carried out following European Union Directive 2010/63/EU. Single-cell suspensions of 2.7×10^6 SNB19 4/T0, SNB-19 pcBrachyury, U373 4/T0, or U373 pcBrachyury cells in 200 µl of phosphate-buffered saline (PBS) were injected subcutaneously in the right flank of the mice. Mice were weighed, and tumor volumes were calculated twice a week using caliper measurements ($V = \pi (d2 \times D)/6$), where d is the minor tumor axis and D is the major tumor axis). Mice were euthanized when one of the tumors reached the maximum volume of 4 cm^3. Tumors were weighed, divided, and fixed in paraformaldehyde (4% PFA) then paraffin-embedded for histological and immunohistochemical analysis, or snap frozen. Tumor histology was evaluated using 3 µm sections stained conventionally with hematoxylin and eosin (H&E).

In silico analysis (Oncomine, TCGA, REMBRANDT, Ivy, Cosmic, cBioPortal databases)

Glioma tumor gene expression, copy number, methylation, mutation, and clinical data (in a total of 3115 samples) were collected from Oncomine, TCGA, Rembrandt, Ivy, Cosmic, and cBioPortal databases. The prognostic value of brachyury was further evaluated in the datasets with information for overall survival ($n = 1076$). The categorization of patient samples was assigned into low (lowest 25%), moderate, and high (highest 25%) subgroups according to the levels of brachyury mRNA expression [12]. A detailed description of *in silico* analysis is described in supplementary material, Supplementary materials and methods.

Statistical analysis

Correlations between brachyury expression and clinicopathological data were assessed using the chi-square test (χ^2 test). Differences between survival rates were evaluated by univariate (log-rank test) and multivariate survival analysis (Cox proportional hazard model). Statistical analyses were performed using SPSS-v19.0 (IBM Corp, Armonk, NY, USA). Simple comparisons were analyzed using Student's *t*-test and two-way analysis of variance (ANOVA) (Bonferroni post test) for comparison of two conditions over time using Prism GraphPad-v5.0a (GraphPad Software, San Diego, CA, USA). The level of significance in the statistical analyses is indicated as * = $p < 0.05$, as ** = $p < 0.01$ or as *** = $p < 0.001$.

Results

Brachyury is expressed in normal brain, and its loss correlates with glioma aggressiveness

To investigate the role of brachyury in gliomas, we initially assessed its mRNA levels using RT-qPCR on a small series that included normal brain ($n = 10$, five fetal and five adult), glioma stem cells ($n = 4$), immortalized normal astrocyte cell line ($n = 1$), and established and primary glioma cell lines ($n = 34$). We found *TBXT* expression in normal brain samples, while absent or at

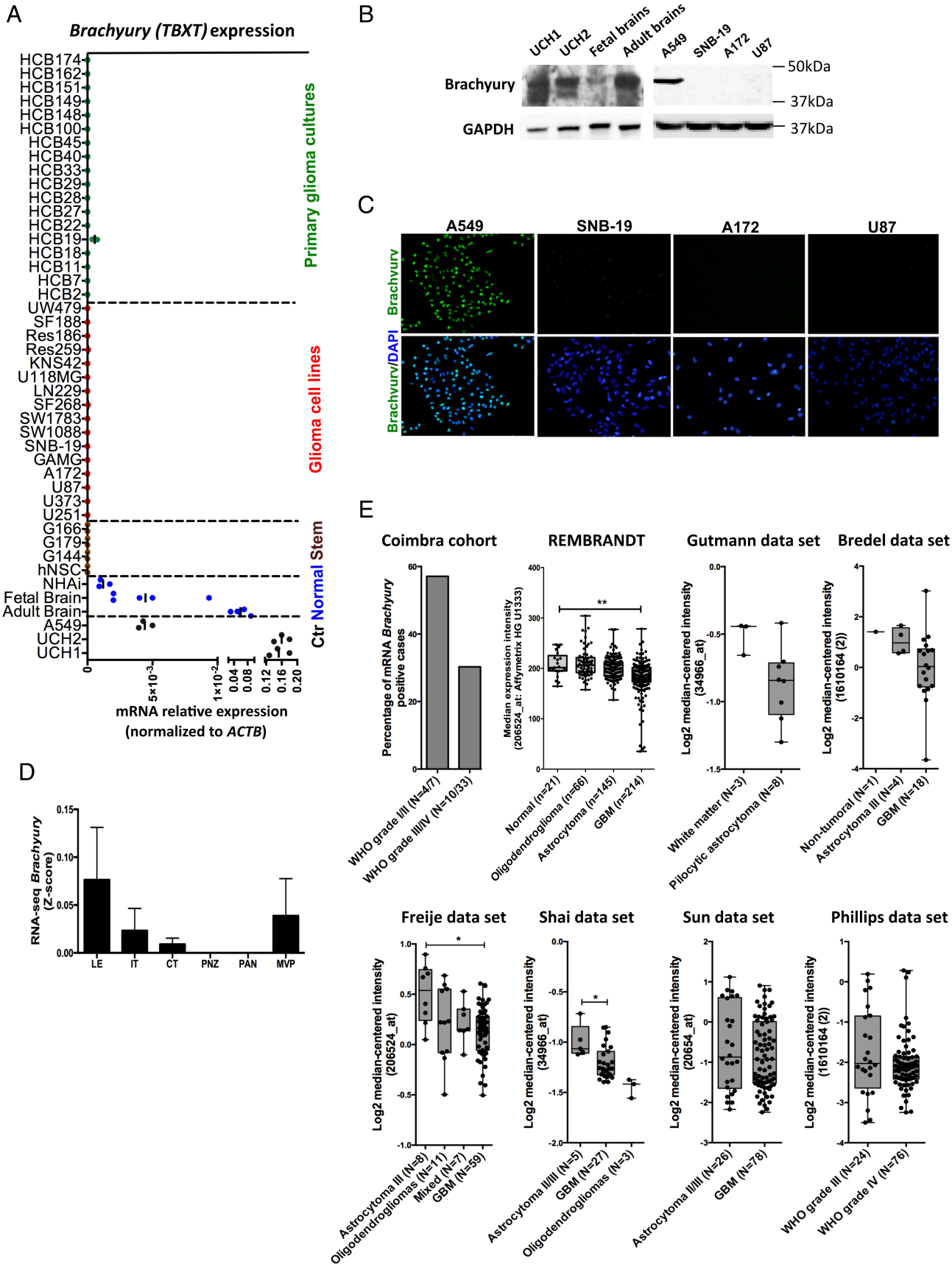

Figure 1. Legend on next page.

low levels in all glioma cell lines studied (Figure 1A). These data were corroborated at the protein level by western blot and immunofluorescence (Figure 1B,C).

To further investigate the low levels of brachyury in gliomas compared with normal brain tissue, we analyzed *TBXT* expression by RNA sequencing (Ivy database). We observed that, within the different anatomical structures of the tumor, *TBXT* is preferentially expressed in the leading edge (1–3 tumor cells/100 cells), while it was absent or weakly expressed in the cellular tumor (ratio of tumor to normal cells approximately 500/1) (Figure 1D). These data suggest that *TBXT* is preferentially expressed in regions with a higher concentration of normal cells. Then, we analyzed *TBXT* mRNA levels in eight independent data sets of glioma patients from the Rembrandt, Oncomine (Figure 1E) and TCGA (supplementary material, Figure S2) databases, in a total of 1307 glioma and 32 non-tumor samples. *TBXT* expression was validated in our cohort of 40 human fresh glioma samples (Coimbra cohort) (Figure 1E, supplementary material, Figure S2A). We found that *TBXT* mRNA levels were: (1) significantly lower in glioma samples when compared with non-tumoral tissue; (2) inversely associated with higher-grade gliomas (Figure 1E; supplementary material, Table S2); and (3) TBXT loss was significantly associated with the mesenchymal GBM subtype (supplementary material, Figure S2B).

Subsequently, we studied brachyury protein expression in a series of 675 gliomas of different malignant grades (supplementary material, Table S1) and 67 non-tumoral adjacent tissues by immunohistochemistry (Table 1, Figure 2A,B). We found that brachyury was expressed in the majority (56/67; 83.6%) of non-tumoral adjacent brain tissues, predominantly in the cytoplasm of neurons and in both cytoplasm and nucleus of other glial cells. In glioma tissues, brachyury presented a sole cytoplasm or cytoplasm/nuclear staining pattern (Figure 2A). Overall, we found that 49.0% (331/675) of glioma tissues were negative, 50.9% (344/675) had cytoplasm staining, and 16.0% (108/675) presented with cytoplasm/nuclear staining (Table 1). Isolated nuclear staining was never detected. As at the transcript level, we observed progressive reduction of brachyury immunostaining along with increased tumor grade (WHO I: 76.7%; WHO II: 61.5%; WHO III: 39.8%; WHO IV: 43.3%) (Figure 2B). Clinically, nuclear brachyury staining was statistically correlated with histology ($p < 0.001$), low-grade gliomas ($p < 0.001$), low KPS ($p = 0.005$), and absence of tumor recurrence ($p < 0.001$) (Table 1). No differences were observed within GBMs when subdivided into age groups, namely pediatric ($n = 108$) and adult ($n = 336$). The association between nuclear staining and clinical data is in agreement with the fact that brachyury plays a functional role as a transcription factor.

Low levels of brachyury predict poor prognosis in glioma patients

To investigate the impact of brachyury expression in patients' survival, we performed univariate and multivariate survival analysis in eight independent cohorts of glioma patients totaling 1554 glioma samples (mRNA: seven cohorts, $n = 1141$; and one for protein: $n = 413$) with available survival data (Figure 2C, Table 2 and supplementary material, Figure S2D).

At the protein level, patients were divided into three groups regarding brachyury subcellular localization: (1) negative ($n = 219$), (2) cytoplasm ($n = 155$), and (3) nucleus ($n = 39$). We found that patients with brachyury nuclear-positive glioma exhibited a better prognosis (Figure 2C) both by univariate ($p < 0.001$) and multivariate analysis ($p = 0.044$) (Table 2) when compared with negative and cytoplasm subgroup patients. No differences were observed between the negative and cytoplasm subgroups, indicating that probably brachyury is not relevant in the cytosol. The prognostic impact of brachyury in glioma patients was validated in seven different cohorts at the transcript level (Figure 2C). In agreement with brachyury protein, patients with absence or low levels of *TBXT* expression had significantly shorter overall survival than patients whose tumors expressed high *TBXT* levels (Figure 2, Table 2). We also found that *TBXT* downregulation was associated with glioma recurrence, but not with chemotherapy (supplementary material, Figure S2D). It is important to note that in the multivariate analysis, the association between brachyury and overall survival was independent of other clinical prognostic variables

Figure 1. Brachyury expression in normal and gliomas samples. (A) Reverse-transcription quantitative PCR (RT-qPCR) analysis showing that *brachyury* (*TBXT*) is expressed in normal adult and fetal brain while it is negative or weakly expressed in establish and primary glioma cell lines and in glioma stem cells. Data for *TBXT* mRNA is presented as absolute values of three independent extractions in triplicate for the cell lines. Expression values for 10 different normal brain tissues (5 adult and 5 fetal) are also represented—blue dots. (B) Western blot for brachyury (AF2085) in one sample of normal brain tissues (one adult and one fetal) and in glioma cell lines. Chordoma cell lines UCH1, UCH2, and the lung cancer cell line A549 were used as positive controls. (C) Immunofluorescence demonstrating that brachyury (sc-20109) is negative in glioma cell lines. (D) RNAseq analysis for brachyury expression (extracted from Ivy Glioblastoma Atlas Project) in different anatomic structures of GBM isolated by laser microdissection. *Brachyury* is absent or low-expressed in the core of the tumor and necrotic zones and is highly expressed in the periphery of the tumor (LE) (containing between 1–3 tumor cells per 100 cells). Five structures are presented: LE, leading-edge; IT, infiltrating tumor; CT, cellular tumor; PNZ, perinecrotic zone; PAN, pseudopalisading cells around necrosis; and MVP, microvascular proliferation. (E) *TBXT* mRNA values in different glioma data sets. Data are present as box plots, where whiskers indicate the range of the data and the horizontal bar represents the median. The validation Coimbra cohort shows the percentage of cases positive for *TBXT* in human glioma samples. Conventional semi-quantitative RT-PCR was used to assess *TBXT* presence in Coimbra glioma fresh samples (supplementary material, Figure S2A). *, $p < 0.05$. All tumors were classified as not otherwise specified (NOS), following WHO 2016 guidelines [2].

Table 1. Clinical-pathological correlation with brachyury protein staining*

Clinical-pathological data	Brachyury IHC				
	n	Negative (%)	Cytoplasm (%)	Nucleus† (%)	*p* value
Gender					
Male	339	184 (54.3)	99 (29.2)	56 (16.5)	0.082
Female	270	122 (45.2)	96 (35.6)	52 (19.3)	
Cellular lineage					
Astrocytic	617	302 (48.9)	208 (33.7)	106 (17.3)	**0.007**
Oligoastrocytic	59	36 (61.0)	22 (37.3)	1 (1.7)	
Histology					
Pilocytic astrocytoma	103	24 (23.3)	2 (1.9)	77 (74.8)	**<0.001**
Diffuse astrocytoma	39	15 (38.5)	12 (30.8)	12 (30.8)	
Anaplastic astrocytoma	39	23 (59.0)	15 (38.5)	1 (2.5)	
Oligodendrogliomas	58	35 (60.3)	22 (37.9)	1 (1.7)	
Glioblastoma	434	240 (55.3)	177 (40.8)	17 (3.9)	
WHO grade					
Low (I, II)	142	39 (27.5)	14 (9.9)	89 (62.7)	**<0.001**
High (III, IV)	533	298 (55.9)	216 (40.5)	19 (3.6)	
KPS					
≤70	80	23 (27.5)	31 (38.8)	27 (33.8)	**0.005**
>70	93	39 (41.9)	16 (17.2)	38 (40.9)	
Recurrence					
No	65	17 (26.2)	3 (4.6)	45 (69.2)	**<0.001**
Yes	31	15 (48.4)	7 (22.6)	9 (29.0)	

*All tumors were classified as not otherwise specified (NOS), following WHO 2016 guidelines [2].
†Nuclear and cytoplasm staining. Bold numbers: statistically significant.

(age, gender, WHO grade malignancy, and chemotherapy) in all cohorts analyzed with a significant number of cases (both protein and mRNA) (Table 2). There was no statistical significance in three mRNA data sets due to the lower number of cases, which preclude the multivariate analysis (Table 2).

The present data show that loss of brachyury is an important biomarker of poor outcome in glioma and is strongly suggestive of the gene having a tumor suppressor role in this neoplasm.

Brachyury downregulation was not due to gene deletion, promoter methylation, or mutation status

To explore the underlying mechanisms of *TBXT* downregulation in gliomas, gene copy number aberrations, DNA methylation, and mutations were evaluated *in silico* using the TCGA, cBio Portal, and COSMIC databases (supplementary material, Figure S3, Tables S3 and S4).

We used copy number variant (CNV) analysis to investigate deletion within the *TBXT* region as a potential genomic rearrangement mechanism for loss of brachyury expression in gliomas. *TBXT* deletion was found in 14.0% (51/372) of GBM cases, 86.0% (320/372) of tumors revealed a normal diploid status, and no gene amplification was detected. No association with *TBXT* expression levels was found (supplementary material, Figure S3A). *TBXT* gene promoter hypermethylation was found in 32.9% (56/170) of GBM cases, yet was not significantly associated with *TBXT* transcript levels (supplementary material, Figure S3B). These results suggested that copy number deletions and hypermethylation are unlikely to be involved in the regulation of brachyury expression in gliomas. We also interrogated whether inactivating somatic point mutations could explain the lack of *TBXT* gene expression. We found only three different mutations: Two were considered missense (1141G > A and 563C > T) and one was silent (1119C > A) (supplementary material, Table S3). The lower frequency of mutations (0.2%, three cases in 1569 glioma samples) (supplementary material, Table S4) suggests that the lack of *TBXT* expression in gliomas is unlikely to be a result of the presence of inactivating point mutations in this gene.

The prognostic value of *TBXT* copy number loss and methylation was also investigated in 364 and 125 GBM patients, respectively. No association was found between copy number loss, *TBXT* promoter hypermethylation, and glioma patient overall survival (supplementary material, Figure S2D). Nevertheless, patients with hypermethylation of *brachyury* promoter present poor prognosis when compared with hypomethylated patients (supplementary material, Figure S2D).

Brachyury inhibits glioma cell viability

To understand the biological role of brachyury in human gliomas, four brachyury-negative glioma cell lines were manipulated to express brachyury (described from now on as pcBrachyury) (Figure 3A, supplementary material, Figure S1D, and Figure S4A,B). The normal astrocytic cell line (NHAi), which endogenously expresses brachyury, was used to assess the role of brachyury silencing (shBrachy) (supplementary material, Figure S4A). We initially assessed the effect of brachyury in cell-line viability (MTS) and tumorigenesis (clonogenic assays). We

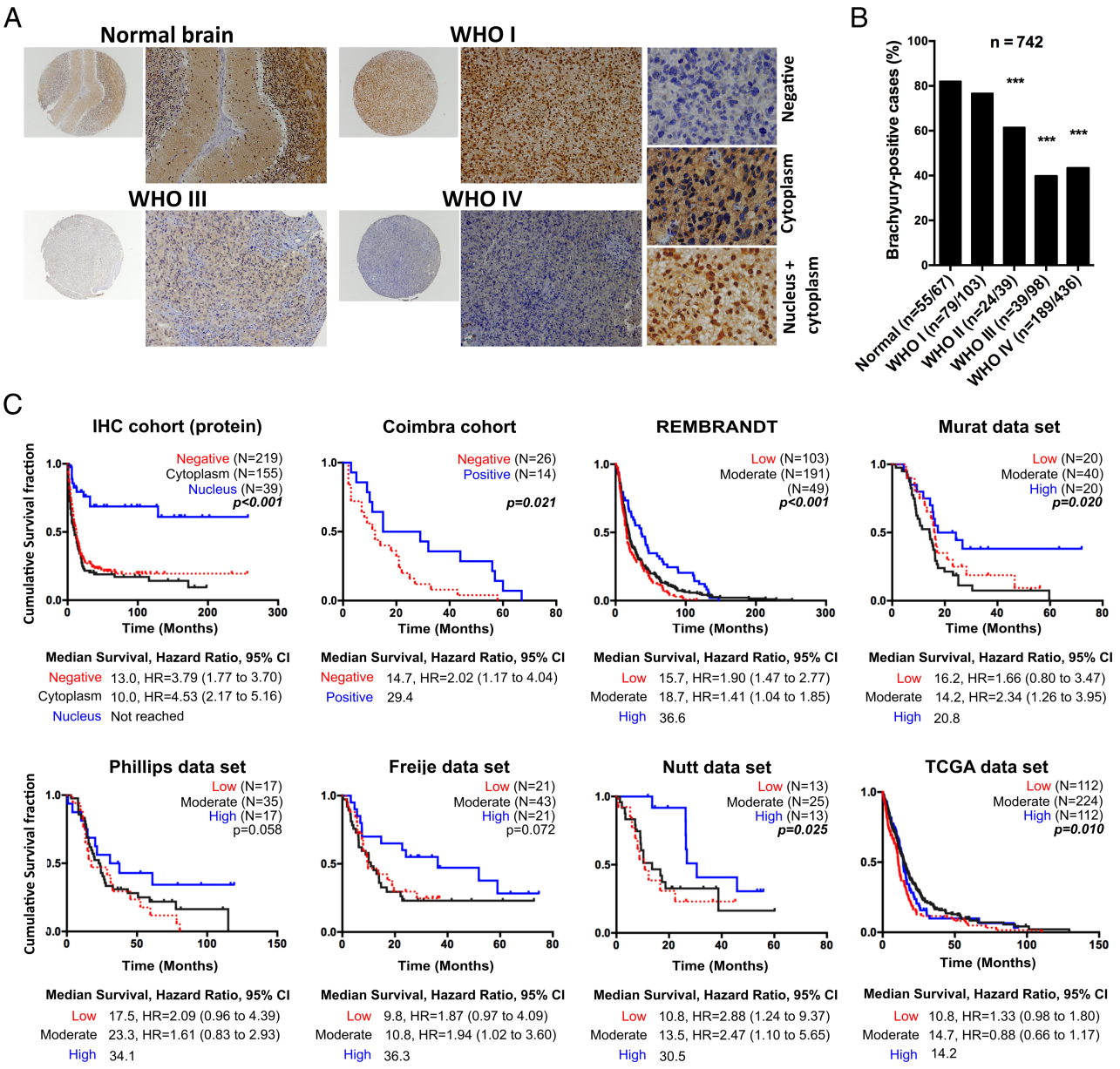

Figure 2. Loss of brachyury expression is correlated with tumor progression and glioma patient survival. (A) Immunohistochemistry analysis of brachyury (sc-20109) was performed in 67 normal brain tissues and in 742 glioma samples. Brachyury was found highly expressed in the normal brain and was lost progressively with glioma aggressiveness. Cytoplasm only and nuclear/cytoplasm brachyury staining was found in glioma tissues. (B) Graphical representation of all samples analyzed by immunohistochemistry. Data are presented as the percentage of positive cases for brachyury staining. (C) Kaplan–Meier analysis between brachyury protein staining (IHC cohort) or *TBXT* (*brachyury*) mRNA levels (Coimbra, REMBRANDT, Murat, Phillips, Freije, Nutt, TCGA) and glioma patient survival. The categorization of patients' samples was assigned into low (lowest 25%), moderate, and high (highest 25%) subgroups according to the levels of *TBXT* mRNA expression. Hazard ratios (HR) of the low- and median-score groups with 95% confidence intervals are shown at the bottom. ***, $p < 0.001$. All tumors were classified as not otherwise specified (NOS), following WHO 2016 guidelines [2].

found that brachyury overexpression was able to significantly decrease cell viability in all glioma cell lines (Figure 3B, supplementary material, Figure S4C), whereas its downregulation increases cell viability in the NHAi cells (supplementary material, Figure S4C). By tumorigenic colony formation assays, we found that brachyury leads to a lower number of colonies formed, both in an anchorage-dependent and anchorage-independent manner (Figure 3C, supplementary material, Figure S5A), corroborating the previous findings of decreased viability of brachyury-positive cells. We next investigated whether the decrease in viability was associated with cell proliferation and/or metabolic alterations. Brachyury did not affect cell proliferation as assessed by BrdU or Ki67 staining (Figure 3D, supplementary material, Figure S4D,E), or affect glioma cell cycle distribution (Figure 3E, supplementary material, Figure S4G,H), glucose consumption or lactate

Table 2. Univariate and multivariate analyses of brachyury expression and overall survival in glioma patients

Source	Cohort (months)	Brachyury	n	Overall Survival		
				Median (95% CI)	p value	
					Log-Rank	Cox*
mRNA	Coimbra	Negative	26	14.7 ± 2.7	**0.021**	**0.007**
		Positive	14	29.4 ± 6.2		
	Rembrandt	Low	103	15.7 ± 1.5	**0.002**	**0.021**
		Moderate	191	18.7 ± 1.6		
		High	49	36.6 ± 9.6		
	Murat	Low	20	16.2 ± 0.8	**0.020**	**0.042**
		Moderate	40	14.2 ± 3.1		
		High	20	20.8 ± 8.5		
	Phillips	Low	17	17.5 ± 8.4	0.058	0.362
		Low	35	23.3 ± 2.9		
		High	17	30.8 ± 10.0		
	Freije	Low	21	9.8 ± 5.7	0.072	0.523
		Moderate	43	10.8 ± 2.6		
		High	21	36.3 ± 12.8		
	Nutt	Low	37	10.8 ± 2.5	**0.025**	0.098
		Moderate	25	13.5 ± 4.2		
		High	13	30.5 ± 3.1		
	TCGA	Low	112	10.8 ± 0.6	**0.010**	**0.014**
		Moderate	225	14.7 ± 0.8		
		High	112	14.2 ± 1.3		
Protein		Negative	219	13.0 ± 1.2	**<0.001**	**0.045**
		Cytoplasm	155	10.0 ± 1.6		
		Nuclear	39	NR		

Bold numbers are statistically significant.
NR, not reached.
*Adjusted for patient age, gender and WHO grade.

production (supplementary material, Figure S4F). However, by cytometry analysis, we found a significant increase in the sub-G0 phase in pcBrachyury cells compared with 4/T0 controls (Figure 3E,F and supplementary material, Figure S4G,I), suggesting higher rates of cell death in brachyury-expressing cells.

Brachyury promotes cell death through the induction of autophagy and alterations in the apoptotic pathway

To explore the cell death mechanisms in brachyury-positive cells, we performed Annexin/PI staining; however, no significant alterations were found (Figure 3E, Figure S4J). We did observe an increased expression of pro-apoptotic proteins (BAX, HIF-1α, p-P53, and FADD) and decreased levels of anti-apoptotic protein (cIAP, HSP27, HSP70, and XIAP), yet no differences were observed in cleaved-PARP and caspase 3 (Figure 3G). We further investigated whether cell death associated with brachyury overexpression could be due to autophagy. Using orange acridine staining (FACS) and conventional p62 and LC3I/II conversion assays (western blotting), we observed that brachyury expression strongly increased autophagy in glioma cells, both at the basal state and after autophagy stimulation (Figure 3H, I and supplementary material, Figure S4K). Taken together, our results indicate that brachyury sensitizes cells to apoptosis, possibly by altering the levels of several apoptotic proteins, and enhances autophagy in glioma cells, ultimately resulting in a decrease of cell viability and increased cell death.

In vivo tumor growth effect of Brachyury

To better understand whether the *in vitro* effects are reflected in an *in vivo* context, we assessed its tumorigenic role using both the chick embryo chorioallantoic membrane (CAM) model and subcutaneous nude mouse xenograft model. In the CAM assay, we observed that the number of tumors formed by brachyury-positive cells was significantly less than by brachyury-negative cells (supplementary material, Figure S5B, C). Despite the observed decreased tumor size using brachyury-positive cells compared with brachyury-negative cells, no statistical significance was attained in both models (Figure 4A–C and supplementary material, Figure S5B–D). Corroborating the *in vitro* findings, no differences were observed in the number of proliferative cells as assessed by Ki67 staining (Figure 4D, and supplementary material, Figure S5E). We also observed that brachyury did not influence the number of vessels formed (supplementary material, Figure S5B). Brachyury expression both at protein and mRNA levels was confirmed in tumors formed in CAM and mice xenografts (Figure 4E, and supplementary material, Figure S5F,G). Although brachyury overexpression reduces tumor growth *in vivo,* our results suggest that its effect may be tissue/microenvironment-dependent to trigger apoptotic or autophagic pathways.

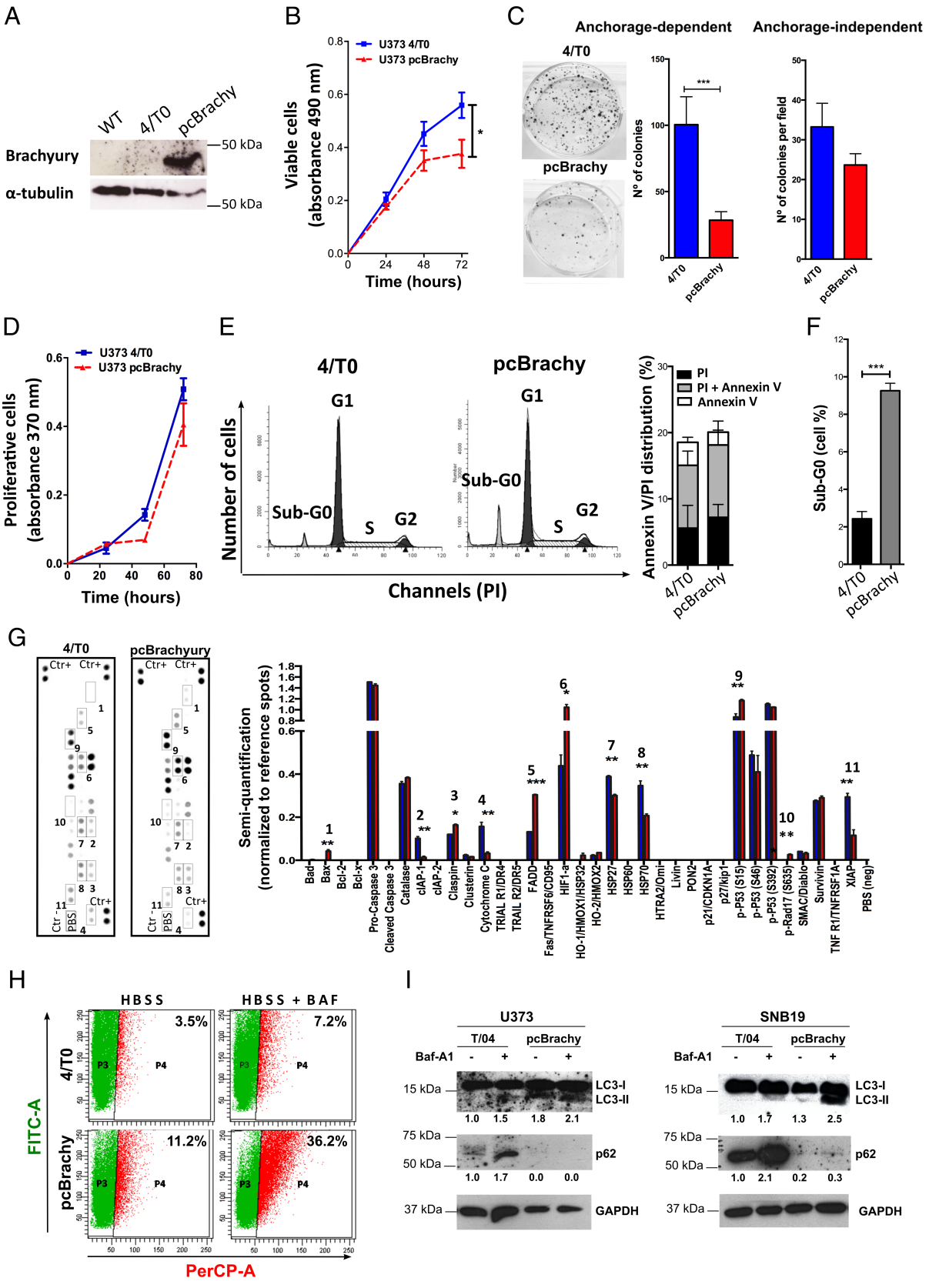

Figure 3. Brachyury expression decreases the tumorigenic capacity of glioma cells via the induction of cell death pathways and autophagy. (A) Immunoblot showing brachyury (AF2085) overexpression in a glioma cell line (U373) transfected with empty vector 4/T0 or with pcBrachyury plasmid. (B,C) Brachyury ectopic expression decreases glioma cell viability (MTS assay) and the number of colonies anchorage-dependent and anchorage-independent. (D,E) Brachyury has a minimal effect on BrdU incorporation (proliferation), cell cycle distribution, and Annexin/PI rates. (F) Brachyury-expressing cells present higher cell death (sub-G0). (G) Human apoptosis array showing that brachyury statistically increases the expression of pro-apoptotic proteins and decreases the anti-apoptotic proteins. (H,I) Brachyury is able to induce autophagy in glioma cells demonstrated by orange acridine staining, p62, and LC3 cleavage. Values are means ± standard error of the mean (SEM) of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

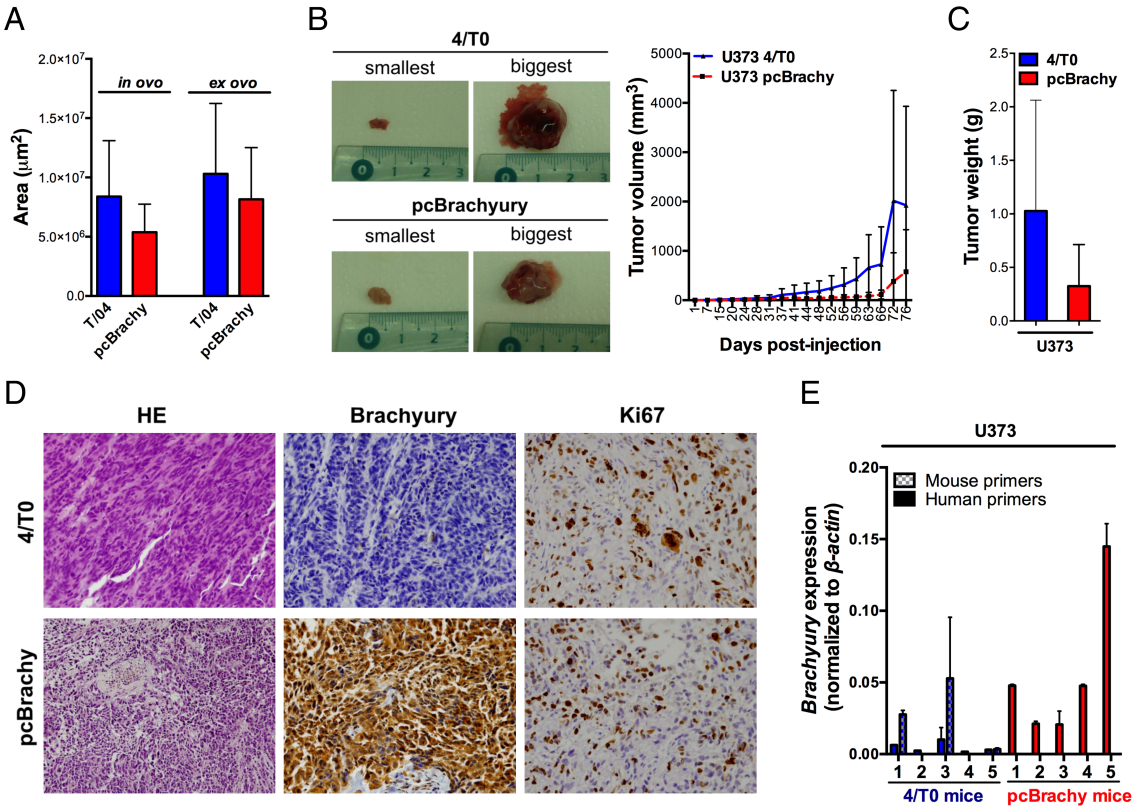

Figure 4. Brachyury overexpression does not significantly affect tumor growth *in vivo*. (A) *In vivo* CAM assay: A decrease in tumor area was observed in brachyury-positive tumors yet not statistically significant. (B) Representative images of *in vivo* subcutaneous tumor information in nude mice. (C) Graphical representation of tumor growth over time. Tumor volumes and weights using pcBrachyury cells are less than negative tumors, however, with a huge variance in size. (D) Histological images of tumors formed, brachyury expression (sc-20109 Ab) confirmation, and Ki67 staining. Histologically, tumors do not present any differences in Ki67 staining. (E) *TBXT* expression confirmation by RT-qPCR in subcutaneous tumors used in the present work.

Discussion

The present study describes for the first time that brachyury has tumor-suppressor functions in gliomas in addition to its established oncogenic properties in several other solid tumors. We also provide additional evidence that brachyury loss is associated with glioma aggressiveness, which is an independent biomarker of poor prognosis in several unique cohorts of patients with gliomas.

Brachyury was described described by us and others to be upregulated in several tumors types compared with normal tissues and to be linked directly to tumorigenesis and poor prognosis [11–17,22]. In contrast, in this study, we describe that brachyury is highly expressed in normal brain tissues (transcript and protein) but at low levels in glioma patients, and primary and established glioma cell lines. Indeed, a study from Jezkova *et al* in normal cells has described that the brain is the second normal tissue with higher *TBXT* expression levels [23]. To better understand the potential mechanisms that could underlie the loss of brachyury in gliomas, we have explored copy number deletion, promoter methylation status, and gene mutations; however, we were not able to find any correlation with brachyury expression. In addition to the association between TGF-β and brachyury expression [24], the exact mechanism by which brachyury is regulated in cancer is still unknown, and this warrants further investigation.

The results obtained in this work point to a novel and specific function of brachyury as a putative tumor suppressor in gliomas. A growing list of studies has reported deregulated levels of several T-box factors (TBX1, TBX2, TBX3, TBX4, TBX5, and TBX21) in different types of cancer, presenting both tumor-promoting and tumor-suppressor functions [25]. For instance, TBX3 seems to be a tumor suppressor in GBMs [26], whereas it acts as a tumor promoter in melanoma and head and neck cancers [27,28]. Although controversial, brachyury was also pointed to have dual functions in cancer, with some studies pointing to brachyury promoting lung cancer [13,14], whereas others are suggesting a tumor suppressor role also in lung cancer [29]. Unlike our work, Shah and colleagues suggest that brachyury can regulate *in vitro* glioma aggressiveness, but no association with progression-free survival was observed [19]. We analyzed eight independent cohorts with survival information, comprising a total of 1529 glioma patients, and using the same approach as the authors, we also did not find associations with progression-free survival. However, patients with absent or low levels of brachyury expression had a significant and independent shorter overall survival than patients whose tumors expressed

high brachyury levels, a result observed in all data sets. Shah *et al* also showed *in vitro* that brachyury inhibition can decrease proliferation and aggressiveness in glioma cultures via a concomitant expression of brachyury-YAP axis signaling [19]. Nevertheless, in GBM, TCGA data indicate that *TBXT* and *YAP* expression have a significant mutually exclusive pattern (supplementary material, Figure S6), indicating the distinct behavior of brachyury in gliomas. Contrasting with the results of Shah *et al*, we showed in five cell models that the presence of brachyury decreases *in vitro* cell viability without affecting the proliferation rates. Indeed, the association of brachyury in proliferation is still controversial: In colorectal cancer cells, Jezkova *et al* demonstrated that brachyury promotes proliferation via a p27kip1-dependent manner [30], whereas Huang *et al* [31] showed that brachyury blocks cell cycle progression in colorectal and lung cancer cells. Moreover, we found that brachyury was able to increase the expression of several pro-apoptotic proteins and autophagy that could explain the decrease in the *in vitro* cell viability and, consequently, in the tumor growth observed *in vivo*. Although future studies are necessary, we hypothesize that the involvement of cofactors, as well as the influence of molecules (microenvironment), define brachyury's role in sensitizing cells to death/autophagy. Some support for this hypothesis may be observed during embryo development, where brachyury function is influenced by the cofactor SMAD1 (in the presence of BMP4) or SMAD2/3 (in the presence of activin/Nodal) in the induction of mesoderm or endoderm gene expression, respectively [32]. Together, it is plausible that brachyury could also have different functions in cancer (glioma versus other tumor types) depending on the cofactors and the surrounding microenvironment.

Our results can have important implications regarding the ongoing Phase II clinical trial with the yeast–brachyury vaccine GI-6301 to treat patients with advanced carcinomas and chordomas (www.clinicaltrials.gov, 2015). The anti-brachyury vaccine induces the production of brachyury-specific T cells that are only able to recognize tumors cells brachyury-positive in an antigen-specific MHC class I-restricted manner (HLA-A2 positive) [14,33]. It has generally presumed that the central nervous system (CNS) is immune-privileged and that MHC-I is not expressed, which makes the use of this vaccine safe. Moreover, the presence of the blood–brain barrier might act to prevent brachyury-targeted molecules from entering and adversely affecting the brain. However, the presence of brachyury in normal brain tissues and its potential tumor suppression activity in gliomas indicates that the role of brachyury in the pathogenesis of several cancer types may be context-dependent and demands additional investigation to guide rational interventions.

In conclusion, the present data show that loss of brachyury is an important biomarker of poor outcome in gliomas and is strongly suggestive of a tumor suppressor role.

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Author contributions statement

FP and RMR conceived and designed the experiments. FP, AMC, SC, VS, and VMG performed the experiments. FP, AMC, GCA, MMM, SC, VS, VMG, CML, CC, APB, LN, GNMH, IWC, CJ, RPA, and RMR analyzed the data. FP, AMC, GCA, MMM, SC, VS, VMG, CML, CC, APB, LN, GNMH, IWC, CJ, RPA, and RMR contributed reagents/materials/analysis tools. FP and RMR wrote the initial manuscript. FP, AMC, GCA, MMM, SC, VS, VMG, CML, CC, APB, LN, GNMH, IWC, CJ, RPA, and RMR revised the manuscript. RPA and RMR supervised the study.

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*Cited only in supplementary material.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. Antibody validation in human tissues, brachyury-negative, and brachyury-positive glioma cells, and in genetically modified glioma cells

Figure S2. *Brachyury* (*TBXT*) expression, methylation, and gene copy number alterations

Figure S3. Correlation between copy number alterations, promoter methylation, and *brachyury* (*TBXT*) mRNA levels in gliomas

Figure S4. Brachyury decreases glioma viability and induces autophagy in different glioma cell lines.

Figure S5. *In vivo* studies: brachyury expression and tumorigenic role in CAM and xenografts models

Figure S6. *Brachyury* (*TBXT*) and *YAP1* expression are mutually exclusive in GBM patients

Table S1. Clinical-pathological features of glioma samples

Table S2. Clinical-pathological correlation with *brachyury* (*TBXT*) mRNA levels*

Table S3. Type of *brachyury* (*TBXT*) mutations in glioma samples

Table S4. Frequency of *brachyury* (*TBXT*) mutation in gliomas samples and glioma cell lines in the COSMIC and cBIOPortal databases