

TRIM28 as an independent prognostic marker plays critical roles in glioma progression

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Abstract Tripartite motif (TRIM) proteins are involved in tumorigenesis. Here, we examined the expression, biological function, and clinical significance of tripartite motif containing 28 (TRIM28) in glioma, a locally aggressive brain tumor. First, TRIM28 expression was significantly higher in glioma ($n = 138$) than in non-glioma controls ($n = 6$). TRIM28 expression was positively correlated with tumor malignancy, and associated with poor overall survival (OS) and progression-free survival (PFS). Notably, TRIM28 expression was negatively correlated with p21 expression in patients with glioblastoma multiforme (GBM). A multivariate analysis that included relevant measures indicated that high TRIM28 expression is an independent prognostic factor for poor OS and PFS in GBM patients. In experiments with cultured glioma cells, down-regulating TRIM28 with shRNA increased p21

expression, and induced cell cycle arrest at the G1 phase. In a xenograft model, down-regulating TRIM28 suppressed tumor growth. These results indicate that over-expression of TRIM28 is associated with poor outcome in glioma patients.

Keywords TRIM28 · Gliomas · Proliferation · Prognosis · Malignancy

Abbreviations

TRIM	Tripartite motif
TRIM28	Tripartite motif containing 28
OS	Overall survival
PFS	Progression-free survival
GBM	Glioblastoma multiforme
TMA	Tissue microarray
IHC	Immunohistochemistry
qRT-PCR	Quantitative real-time polymerase chain reaction
TCGA	The Cancer Genome Atlas
MIB-1	Mind bomb E3 ubiquitin protein ligase 1
KPS	Karnofsky performance status
MGMT	O-6-methylguanine-DNA methyltransferase
STR	Subtotal resection
GTR	Gross total resection

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Introduction

Gliomas, and glioblastoma multiforme (GBM) in particular, are locally invasive brain tumors with poor prognosis, even after treatment with temozolomide and/or radiation [1, 2].

E3 ligase, a member of the ubiquitin–proteasome system, has been implicated in tumorigenesis on the basis of

regulation of oncoproteins and tumor inhibitors [3–6]. Tripartite motif (TRIM) family of proteins function as E3 ligases via their RING-finger domain [7]. More than 70 members of the TRIM family have been identified so far. Several of these factors have been associated with oncogenic processes, such as apoptosis, cell proliferation and transcriptional regulation [8]. Some of the TRIM gene family members (such as TRIM19 and TRIM24) are transported to other genes and play important roles in tumorigenesis and cancer progression [9, 10]. Other TRIM proteins, including TRIM25 and TRIM68, regulate nuclear receptor activation [8]. TRIM28 expression is elevated in a variety of tumors, including breast cancer, gastric cancer and lung cancer [11–16]. In a clinical study in patients with early-stage lung tumor, TRIM28 expression was correlated with overall survival (OS) [17]. In a mouse model for aging, TRIM28 knockdown could reverse senescence [18].

In the current study, we compared TRIM28 gene expression in glioma tissues acquired from surgical resection versus non-glioma tissues. Functional significance of TRIM28 was examined in cultured glioma cells and a xenograft model using shRNA gene silencing.

Materials and methods

Cell lines and culture

Four representative glioma cell lines (SHG44, GL261, U87 and U251: from the Chinese Academy of Sciences Shanghai Branch Bank of Cells) were cultured in DMEM medium containing 10 % fetal bovine serum (GIBCO; Carlsbad, CA, USA) as previously described [19].

Patients and follow-up

A total of 138 glioma tissue microarray (TMA) samples were obtained from patients who underwent curative resection between 2002 and 2009 in the Department of Neurosurgery, Huashan Hospital, Fudan University. The patient characteristics are shown in Table S1. Of them, 70 cases were GBM (Table S2). Six control brain specimens were randomly chosen from patients undergoing trauma/epilepsy surgery. Ethical approval was provided by the Ethics Committee of Huashan Hospital. Written informed consent was obtained from each patient.

Construction of the TMAs and immunohistochemistry (IHC)

Detailed description of the construction of the TMA and IHC are available in the Supplementary Materials and Methods.

Transfection, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting

Detailed description of the transfection experiments with shRNA for TRIM28, qRT-PCR and Western blotting analysis are available in the Supplementary Materials and Methods.

Cell proliferation, colony formation, flow cytometry and xenograft experiments

Detailed description of the cell proliferation assay, colony formation assay, flow cytometry assay of the cell cycle and xenograft experiments are available in the Supplementary Materials and Methods.

Statistical analysis

Statistical analysis was performed with the SPSS 19.0 software program (SPSS, Chicago, IL, USA). Data are presented as the mean \pm standard deviation. Student's t-test and Fisher's exact probability were used for comparisons between groups. OS and progression-free survival (PFS) were evaluated using the Kaplan–Meier method for univariate analysis, and differences were assessed using the log-rank test. For the multivariate analysis, Cox proportional hazards regression model was used. Values of $p < 0.05$ were considered statistically significant.

Results

Bioinformatics analysis of TRIM family expression

We searched the Cancer Genome Atlas (TCGA) for differential expression of TRIM family proteins in GBM tissue ($n = 483$) vs. normal brain tissue ($n = 10$) (Fig. 1a). The analysis using a nanobody-based reverse proteomics approach [20] revealed high TRIM28 expression in GBM stem-like cells [20]. Such a result was confirmed by an analysis of the Murat database (Fig. 1b).

TRIM28 is overexpressed in glioma and correlates with poor prognosis

Examination of TRIM28 expression in 11 cases of glioma ($n = 4, 3,$ and 4 for grade II, III and IV) and 3 control tissues by qRT-PCR and Western blotting revealed significantly higher TRIM28 mRNA ($p = 0.009$) and protein ($p = 0.034$) in glioma (Fig. 2a, b). TRIM28 IHC and counterstaining with hematoxylin and eosin (H&E) revealed localization of TRIM28 in cell nuclei (Fig. 2c). Upon IHC analysis, the TRIM28 protein was substantially higher in glioma ($n = 138$) than in control tissues ($n = 6$) ($p = 0.002$,

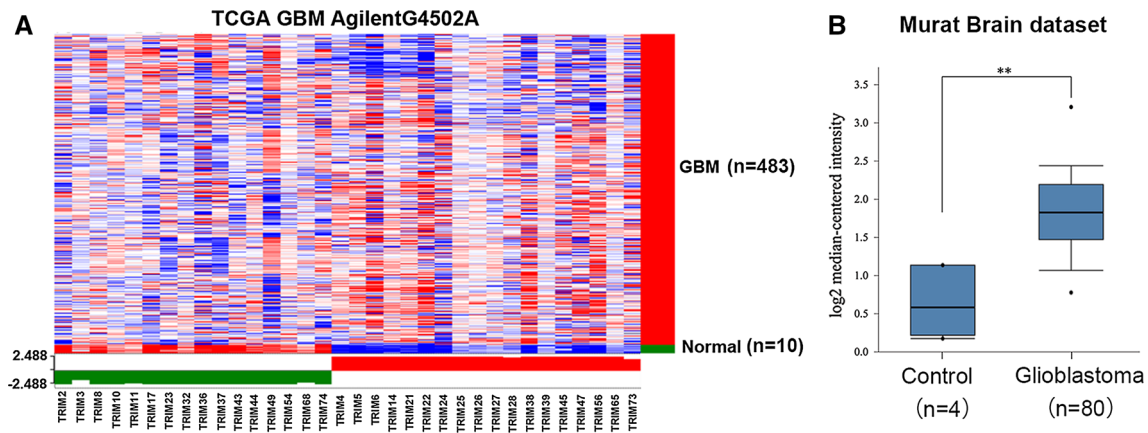


Fig. 1 Expression of TRIM28 in glioma tissues. **a** Heat maps of the differentially expressed TRIM family members in normal brain tissue (n = 10) and in GBM samples from the TCGA database (n = 483). **b** The mRNA expression of TRIM28 in the control brain and GBM in the Murat database. (**p < 0.01)

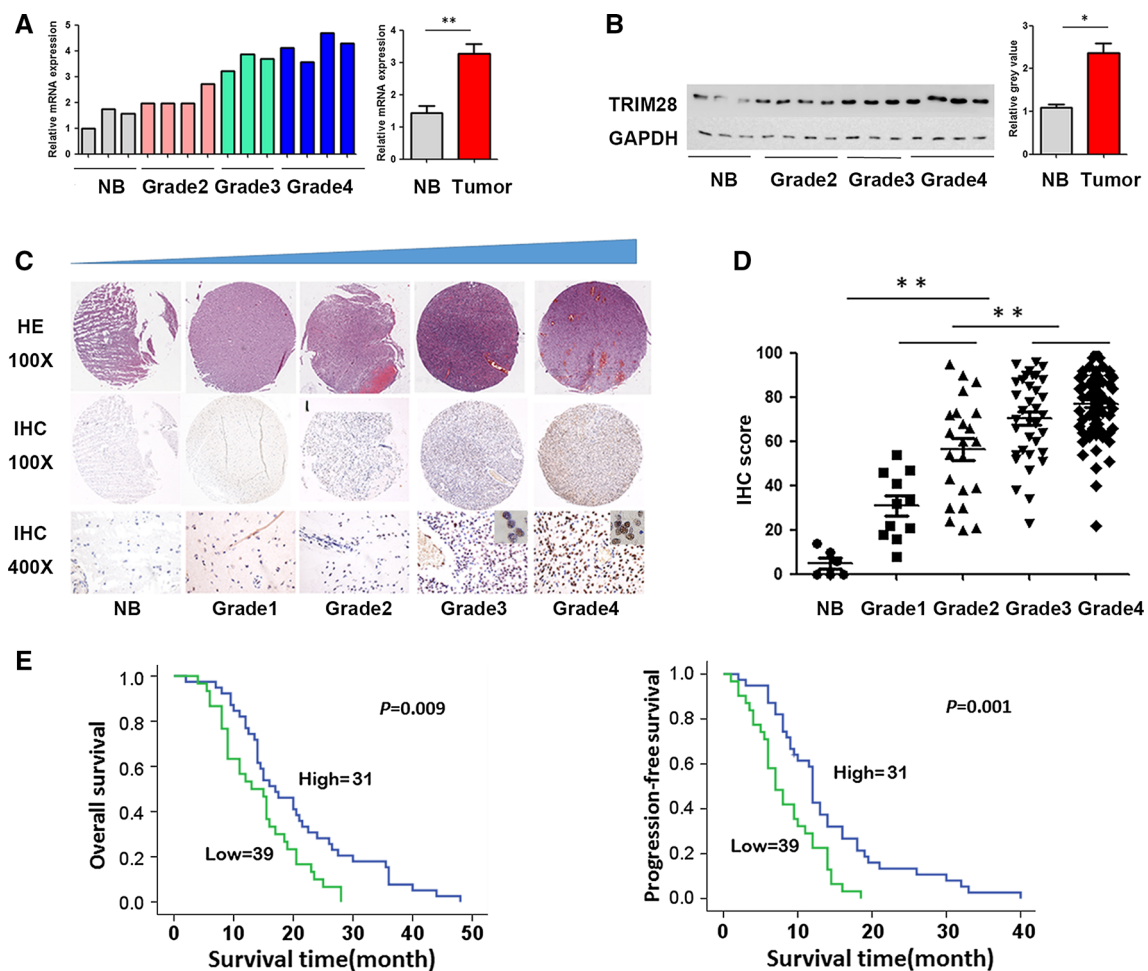


Fig. 2 TRIM28 is overexpressed in glioma tissues and associated with poor prognosis. **a, b** A total of 11 cases of glioma (including 4 grade II, 3 grade III and 4 grade IV) and 3 control tissues evaluated by qRT-PCR (p = 0.009) and immunoblotting (p = 0.034). **c** Representative photomicrographs of the tumor sections following IHC analysis for TRIM28. **d** Elevated TRIM28 expression is correlated with the histological malignancy. Expression of TRIM28 was higher in glioma tissues than in control tissue (p = 0.002), and in high-grade glioma compared with low-grade glioma (p = 0.009). **e** Kaplan–Meier survival curves indicating the cumulative survival as a function of time for patients with high TRIM28 expression versus those with low expression (OS p = 0.009, PFS p = 0.001). The patients with high TRIM28 expression experience significantly worse outcomes. (*p < 0.05, **p < 0.01)

Table 1 Correlation of TRIM28 expression with the clinical and molecular characteristics in archival GBM patients

Clinical characteristics	High No.	Low No.	p	Molecular characteristics	High No.	Low No.	p
Age (No.)			0.934	MIB-1			0.325
≥55	17	21		High	5	10	
<55	14	18		Low	26	29	
Sex (No.)			0.724	P53			0.650
Male	18	21		High	16	18	
Female	13	18		Low	15	21	
Location			0.518	MGMT			0.677
Frontal	11	10		High	11	12	
Parietal	3	3		Low	20	27	
Temporal	7	8					
Other site	1	6					
Combined	9	12					
Extent of resection (No.)			0.881				
GTR	25	32					
STR	6	7					

Fisher's exact probability test was used to analyze for the significance of the location group, and Pearson's Chi squared test was used to analyze for the significance of the other variables ($p < 0.05$)

No. number, *KPS* Karnofsky performance score, *GTR* gross total resection, *STR* subtotal resection, *MIB-1* Mind bomb E3 ubiquitin protein ligase 1, *MGMT* O-6-methylguanine-DNA methyltransferase

Table 2 Univariate and multivariate cox regression analyses of OS and PFS in archival GBM patients

Variables		OS			PFS		
		Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis	
			p	HR		95 % CI	p
Gender	Female versus male	0.883	NS		0.959	NS	
Age, year	≤55 versus >55	0.562	NS		0.952	NS	
KPS	<70 versus ≥70	0.006	1.811	1.016–3.227	0.044	0.035	1.492 0.841–2.648 0.171
Extent of resection	GTR versus STR	0.268	NS		0.106	NS	
MIB-1	High versus low	0.911	NS		0.987	NS	
TP53	High versus low	0.125	NS		0.498	NS	
MGMT	High versus low	0.686	NS		0.924	NS	
Trim28	High versus low	0.009	1.776	1.069–2.950	0.026	0.002	2.120 1.263–2.350 0.004

OS overall survival, PFS progression-free survival, KPS Karnofsky performance status, HR hazard ratio, 95 % CI 95 % confidence interval, MIB-1 Mind bomb E3 ubiquitin protein ligase 1, NS not significant

Fig. 2d). Higher TRIM28 expression was also evident in high-grade versus low-grade glioma ($p = 0.009$, Fig. 2d).

In the 70 GBM cases, high and low TRIM28 expression was noted in 32 and 38 cases, respectively. Both the OS ($p = 0.009$) and PFS ($p = 0.001$) differed between gliomas that expression high versus low levels of TRIM28. High TRIM28 expression was associated with poor prognosis (Fig. 2e). The 1- and 2-year OS was 76.3 and 56.3 %, respectively for GBM with low

TRIM 28 expression, and 26.3 and 12.5 %, respectively for GBM with high TRIM28 expression. The 1- and 2-year PFS was 42.1 and 25.0 %, respectively for GBM with low TRIM28 expression, and 13.2 and 3.1 %, respectively for GBM with high TRIM28 expression.

The prognosis did not correlate with gender, sex, extent of resection, tumor location, or expression of Mind bomb E3 ubiquitin protein ligase 1 (MIB-1) and TP53 (Table 1).

A univariate analysis indicated better prognosis in patients with a Karnofsky performance status (KPS) at 70 or above (OS: $p = 0.006$; PFS: $p = 0.035$) (Table 2). In addition, reduced TRIM28 expression was associated with longer OS ($p = 0.009$) and PFS ($p = 0.002$) (Table 2). A multivariate analysis using Cox proportional hazard regression model confirmed poor OS ($p = 0.026$, HR = 1.776, 95 % CI 1.069–2.950) and PFS ($p = 0.004$, HR = 2.120, 95 % CI 1.263–2.350) in glioma with high TRIM28 expression. KPS at above 70 was also a prognostic factor for OS ($p = 0.044$, HR = 1.811 95 % CI 1.016–3.227) but not for PFS ($p = 0.171$).

TRIM28 knockdown decreases cell proliferation and induces G1 arrest

Examination of TRIM28 expression in 4 representative glioma cell lines by qRT-PCR and Western blot revealed more robust increase in TRIM28 expression in U251 and U87 cells versus in SHG44 and GL261 cells versus normal brain tissues (Fig. 3a).

To functional significance of TRIM28 expression, we knocked down the expression of TRIM28 in U87 and U251 cells via transfection with pGMLV-GFP-vshRNA-TRIM28 (Fig. 3b). TRIM28 knockdown decreased cell proliferation

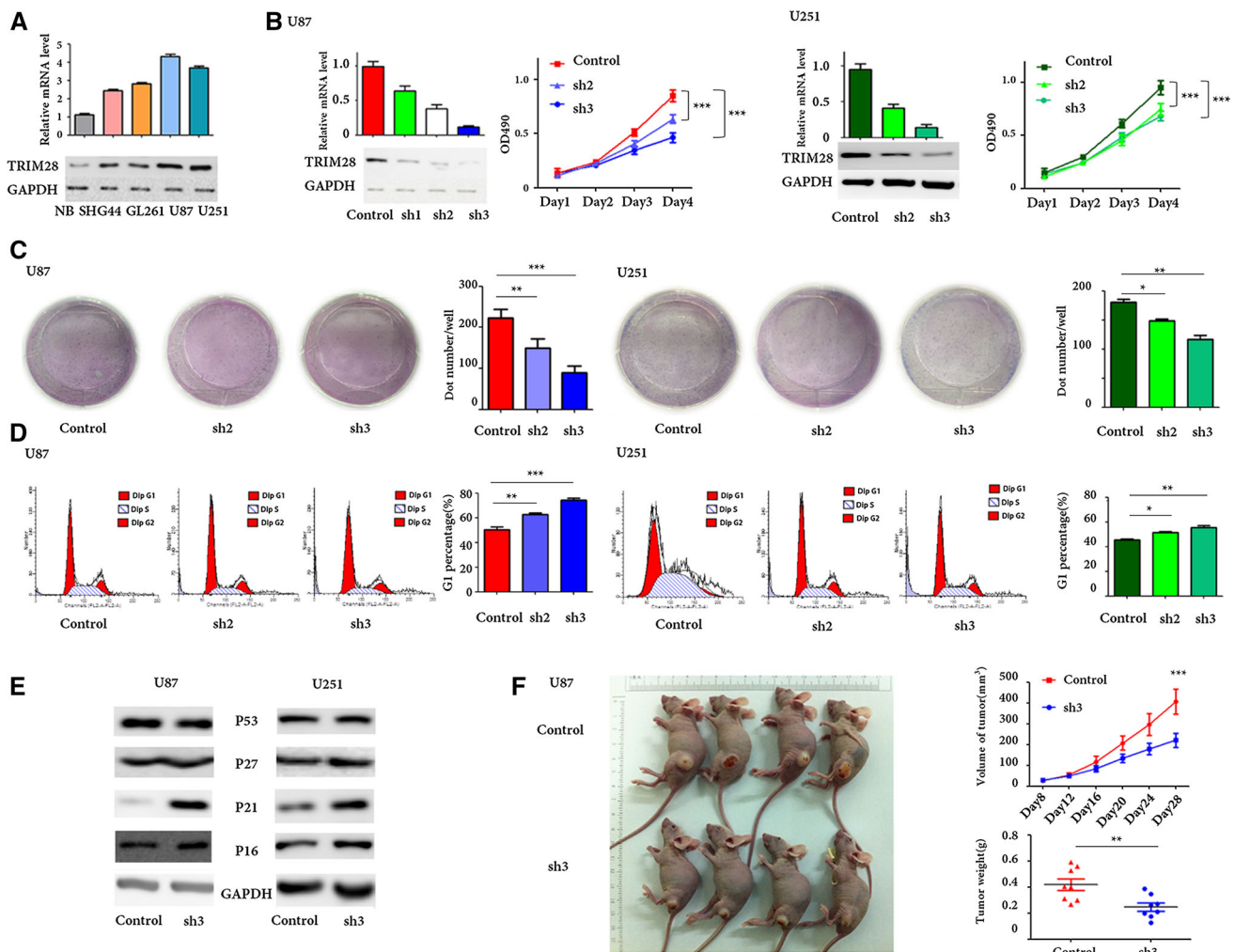


Fig. 3 TRIM28 down-regulation inhibited glioma cell proliferation. **a** qRT-PCR and immunoblotting analyses of the expression of TRIM28 in 4 glioma cell lines and control brain tissue. **b** Stably transfected glioma cells with decreased TRIM28 expression were established. Cell proliferation was assessed by an MTT assay. **c** The clone formation ability was impaired after TRIM28 interference.

d TRIM28 down-regulation induces G1 arrest. **e** P53, p27, p21 and p16 were assessed by immunoblotting after the down-regulation of TRIM28 in glioma cells. **f** The level of TRIM28 expression affected the growth abilities of glioma cells in the xenograft model of nude mice, as determined by the tumor size. The error bars represent the s.d. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

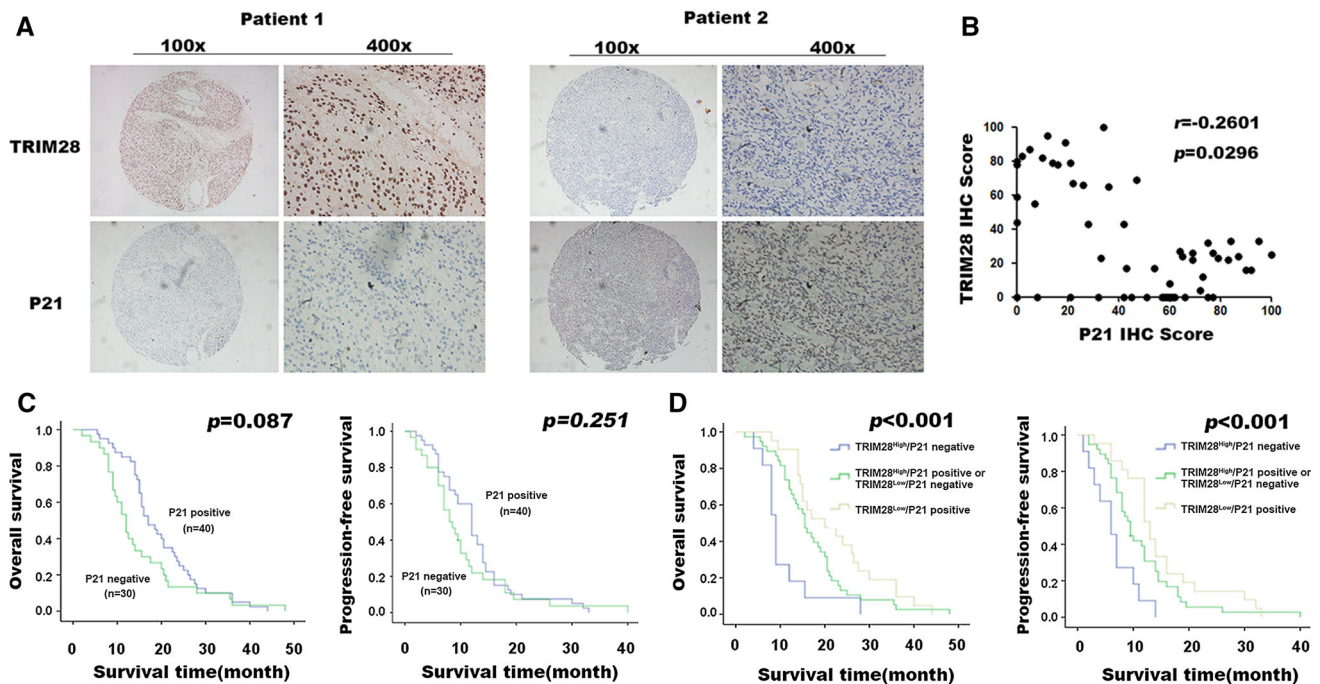


Fig. 4 Combination of TRIM28 overexpression and p21 down-regulation is associated with poor survival in glioma patients. **a** Representative immunostaining images of TRIM28 and p21. **b** The relationship between the protein levels of TRIM28 and those of p21 in glioma tissues. **c** Kaplan–Meier survival curves indicating no significance for survival time between patients with positive p21

expression versus those with negative expression (OS $p = 0.087$, PFS $p = 0.251$). **d** Kaplan–Meier survival curves indicating the cumulative survival as a function of time for patients with TRIM28 high/p21 negative expression versus those with TRIM28 low/p21 positive expression (OS $p < 0.001$, PFS $p < 0.001$). Glioma patients co-expressing TRIM28 high/p21 negative had the worst prognosis

(Fig. 3b) and clone formation (Fig. 3c) in U87 and U251 cells, and led to G1 arrest in glioma cells (Fig. 3d). TRIM28 knockdown significantly increased the expression of p21, slightly altered p53 and p16, but did not affect p27 expression (Fig. 3e). In a xenograft model using U87 cells, a decrease in TRIM28 expression inhibited tumor growth (Fig. 3f).

TRIM28 and p21 expression levels are independent prognostic markers in glioma patients

To examine the relationship between TRIM28 and p21, we examined the expression of TRIM28 and p21 in 70 GBM tissue samples (representative staining in Fig. 4a). The results indicated a negative correlation between the expression of TRIM28 and p21 ($r = -0.2601$, $p = 0.0296$, Fig. 4b). Kaplan–Meier survival curves failed to show significant difference in survival time between patients with positive versus negative p21 expression (Fig. 4c), but did reveal poor prognosis in patients with high level of TRIM28 and negative p21 expression (Fig. 4d).

Discussion

TRIM proteins could affect (either promote or inhibit) oncogenesis and tumor progression by affecting cellular physiological processes, such as DNA repair, cell proliferation and apoptosis. The biological functions of TRIM family members as oncogenes or tumor suppressor genes have been extensively studied. In the present study, we analyzed TCGA data and found that 18 TRIM genes were up-regulated and 16 were down-regulated in 483 GBM tissue samples compared with 10 control brain tissues. Among the 18 genes with elevated expression, we focused on TRIM28, a molecule previously known to play important roles in the oncogenesis of several tumors [11, 13].

The clinical part of the current study showed that TRIM28 expression is positively associated with malignancy of glioma and poor patient prognosis. Next, we selected 4 representative glioma cell lines with a range of TRIM28 expression levels to investigate the role played by TRIM28. Inhibiting TRIM28 expression in the 2 cell lines with high TRIM28 expression (e.g., U87 and U251 cells)

with lentivirus-mediated pGMLV-GFP-shRNAs decreased cell proliferation and colony formation, and arrested cell cycle at the G1 phase. These results suggest that down-regulation of the TRIM28 protein plays an inhibitory role in the development of glioblastoma and may be a key regulator of the G1-S transition in glioblastoma cells. Extrapolation of the findings from cultured cells and xenograft models back into in vivo situations, however, must be attempted with caution.

TRIM28 is a member of the TRIM family of E3 ligases, and could activate or suppress transcription via various mechanisms under different contexts [21–24]. TRIM28 is known to be associated with the histone deacetylase complex NuRD and the histone methyltransferase SETDB1 and leads to the silencing of specific genes [25, 26]. Via other mechanisms, TRIM28 causes transcriptional activation through its recruitment to certain response elements, such as the Nur response element (NuRE) [23]. Santos et al. showed that in human embryonic lung fibroblast cell line IMR90, TRIM28 expression inhibit p16 induction without affecting p53 or p21 expression [27]. However, in our study, TRIM28 regulated the expression of p21 in glioma cells; such a finding was consistent with the negative correlation of TRIM28 with p21 in GBM tissues. P21 is a tumor suppressor gene and a cell cycle inhibitor. The down-regulation of p21 plays a vital role in the development of many cancers. The relationship between TRIM28 and p21 observed in the current study may partly explain the biological functions of TRIM28. Lee et al. demonstrated that sumoylated TRIM28 decreased H3-K9 and H3-K14 acetylation and augments H3-K9 methylation at the p21 promoter [28]. Also a result, the downregulation of p21 may also be related to the sumoylation of TRIM28 via a chromatin-silencing process.

In our experiments, TRIM28 was detected in cell nuclei but not in cytoplasm. E3 ligases, including TRIM28, catalyzes ubiquitination of the proteins destined for degradation, and by doing so, serves as an important post-translational mechanism to regulate various cell functions, such as transcriptional regulation, protein quality control, DNA repair and cell cycle regulation. Protein degradation indeed occurs in cytoplasm, but ubiquitination may occur in cell nuclei. [29].

In conclusion, the results from the current study highlighted the biological and clinical significance of TRIM28 expression in glioma. Importantly, the combination of negative p21 expression and high TRIM28 expression could predict a poor prognosis in GBM. Decreasing TRIM28 expression could inhibit the growth of glioma cells both in vitro and in vivo, suggesting that targeting TRIM28 could be a promising therapeutic approach to manage gliomas.

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Compliance with ethical standards

Conflict of interest None.

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