Tohoku J. Exp. Med., 2021, **255**, 247-256

Tac2-N Promotes Glioma Proliferation and Indicates Poor Clinical Outcomes

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As the most common tumor of central nervous system in adults, glioma is characterized with poor prognosis. Tac2-N (TC2N) is a newly discovered protein that play potential roles in lung cancer and breast cancer progression. Here we aimed to investigate the expression, clinical significance, and function of TC2N in glioma. The mRNA level of *TC2N* in glioma patients was extracted from TCGA datasets. Immunohistochemistry staining was conducted to test protein expression of TC2N in glioma tissues. Chi-square test was used to assess correlations between TC2N expression and patients' clinicopathological characteristics. Kaplan-Meier method was used to plot survival curves. The prognostic predictive role of TC2N was evaluated by univariate and multivariate analyses. Knockdown assays were performed in U87 and U251 cell lines, respectively. Cell proliferation, colony formation, and subcutaneous mice xenografts were used to reveal the tumor-related role of TC2N in glioma. Compared with normal brain tissues, the mRNA level of *TC2N* was significantly higher in glioma tissues, whose dysregulated higher mRNA level was correlated with poorer overall survival. Similarly, higher protein expression of TC2N was observed in cases with larger tumor size and advanced WHO grades. Univariate and multivariate analyses identified TC2N as a novel independent prognostic factor of gliomas. In vitro and in vivo data demonstrated that TC2N interference can remarkably prevent glioma cell proliferation and tumor growth. In conclusion, high TC2N expression is significantly correlated with poor overall survival of glioma patients via enhancing tumor growth.

Keywords: glioma; prognosis; proliferation; TC2N
Tohoku J. Exp. Med., 2021 November, **255** (3), 247-256.

Introduction

Occurring in the brain or glial tissues, glioma accounts for the most common tumor in central nervous system (Jung et al. 2019). Gliomas can be divided into low-grade gliomas (LGG) and glioblastomas (GBM). The LGG refers to those with WHO grade II and grade III, including astrocytomas, oligodendrogliomas and oligoastrocytomas (Louis et al. 2007). GBM includes those with WHO grade IV and represents the most aggressive type of gliomas (Ostrom et al. 2019). Surgical resection is the only curative treatment for glioma patients. Adjuvant therapeutic options include radiotherapy and chemotherapy (Weller et al. 2014, 2017). However, the prognosis of gliomas is far from satisfied. Although the post-operative survival of LGG differs among patients and ranges between 1-15 years (Ricard et al. 2012), the median survival time for GBM patients was only 15 months with a 5-year overall survival rate of 6.8% (Zhang et al. 2021). Hence, there is an urgent demand to identify novel prognostic prediction biomarkers for accurately predicting the prognosis and distinguishing patients for personalized therapeutic treatment.

Tac2-N (TC2N) is an atypical C-terminal-type (C-type) tandem C2 protein that localizes to nucleus, initially reported in 2001 (Fukuda and Mikoshiba 2001). As a novel protein, the physiological and pathological role of TC2N is poorly understood, which may participate in venous thrombosis and von Willebrand disease (Morange et al. 2011; Sanders et al. 2015). Interestingly, the oncogenic function of TC2N was recently discovered. According to the data by

Received July 8, 2021; revised and accepted August 9, 2021. Published online November 26, 2021; doi: 10.1620/tjem.255.247.
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Hao et al. (2019b), TC2N was remarkably upregulated in lung cancer compared with adjacent normal lung tissues, whose upregulation was significantly associated with poor outcome. The similar tumor-promoting role of TC2N was later reported in breast cancer (Hao et al. 2019a) and gastric cancer (Shen et al. 2020; Xu et al. 2021b). However, the expression pattern and tumor-relate function of TC2N in other malignancies remain unknown.

Here we firstly investigate the mRNA level of TC2N in glioma via online data mining, which demonstrated its increased transcription in glioma tissues and unfavorable prognostic associations. Secondly, we validated its protein expression profile in a retrospective cohort containing 102 glioma patients, thus confirming its independent prognostic predictive role in glioma patients. Finally, we conducted both in vitro and in vivo experiments to further illustrate the tumor-promoting effect of TC2N in glioma progression.

Materials and Methods

Online data mining

Transcription of TC2N in glioma tissues and normal brain tissues was obtained from the TCGA database via the UALCAN server (http://ualcan.path.uab.edu/analysis.html). The mRNA level of TC2N was presented as transcripts per million (TPM).

Patients' enrollment

A total of 102 paraffin-embedded samples from patients diagnosed with gliomas were selected from the Qingzhou People's Hospital and all diagnoses were validated by pathological examination. All patients underwent surgical treatment in our hospital. The clinicopathological information and survival time of enrolled cases were obtained during follow-up. This study was conducted in accordance with the World Medical Association Declaration of Helsinki and approved by the Ethics Committee of Qingzhou People's Hospital. All patients signed informed consent before data collection.

Immunohistochemistry staining (IHC)

The paraffin-embedded tissues were sliced into 5-μm sections, followed by deparaffinized in xylene, rehydrated in gradient ethanol, and boiled in 100 mM sodium citrate solution (pH 6.0) to block endogenous peroxidase activity. Then slides were exposed to 3% hydrogen peroxide for 10 minutes before incubation with primary TC2N antibody (1:500; Abcam, Cambridge, MA, USA) at 4°C overnight. After then, slides were incubated with secondary antibody at room temperature for 1 hour before visualized using 3,3'-diaminobenzidine, tetrahydrochloride (DAB) reagent.

IHC results were evaluated by two independent pathologists who were blinded to patients' characteristics. The staining intensity of IHC images were scored as followings: 0 (negative); 1 (slight staining); 2 (yellow staining); and 3 (dark staining). The proportion of positively stained cells was scored as followings: 0 (no positive cell); 1 (1%-25%); 2 (26%-50%); 3 (51%-75%) and 4 (76%-100%) (Liu et al. 2017). The product of intensity and proportion scores was regard as final IHC score, ranging 0-12. Accordingly, all the 102 patients were divided into negative expression of TC2N (n = 60, IHC score 0-5) or positive expression of TC2N (n = 42, IHC score 6-12).

Cell lines and knockdown

Human glioma cell lines U87 and U251 cells were purchased from Chinese Academy of Science. Both cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, CA, USA) in a humidified atmosphere with 5% CO_2 at 37°C. Knockdown was achieved by using specific shRNAs targeting TC2N using scrambled shRNA as control as described by others (Hao et al. 2019a). Both the TC2N-shRNAs and scrambled shRNA were inserted into GV248 vector and then transfected into cultured cells using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfected cells were selected using puromycin by limited diluted strategy.

Western blotting (WB)

Protein expression levels were tested via WB as we described before (Hou et al. 2017). Briefly, total proteins were extracted from cells by radioimmunoprecipitation assay lysis buffer. Protein concentrations were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Pittsburgh, PA, USA). Equal amount of proteins (20 μg) were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane. After blocking with 5% skim milk, membrane was incubated with primary antibodies at 4°C overnight. On the second day, the membranes were incubated with the horseradish peroxidase (HRP)-labeled secondary antibody and incubated with enhanced chemiluminescence solution. The immunoreactivity was tested by a ECL detection system. All experiments were carried out in triplicates.

Cell Counting Kit 8 (CCK-8) assay

Cell viability was evaluated using a CCK-8 kit (Dojindo, Kumamoto, Japan). In brief, cells were seeded in 96-well plates and cultured for different time points (8 h, 24 h, 48 h, 72 h, and 96 h). CCK-8 solution (10 μl) was then added into each well to incubate for another 90 min. The absorbance at 450 nm was finally tested by using a microplate reader. All experiments were carried out in triplicates (Zhao et al. 2021).

Colony formation assay

Transfected cells were seeded into 6 well plates at 1,000 cells per well and incubated for 10 days. After then, cells were stained with 0.5% crystal violet and cell clones were counted (Liu et al. 2021). All experiments were carried out in triplicates.

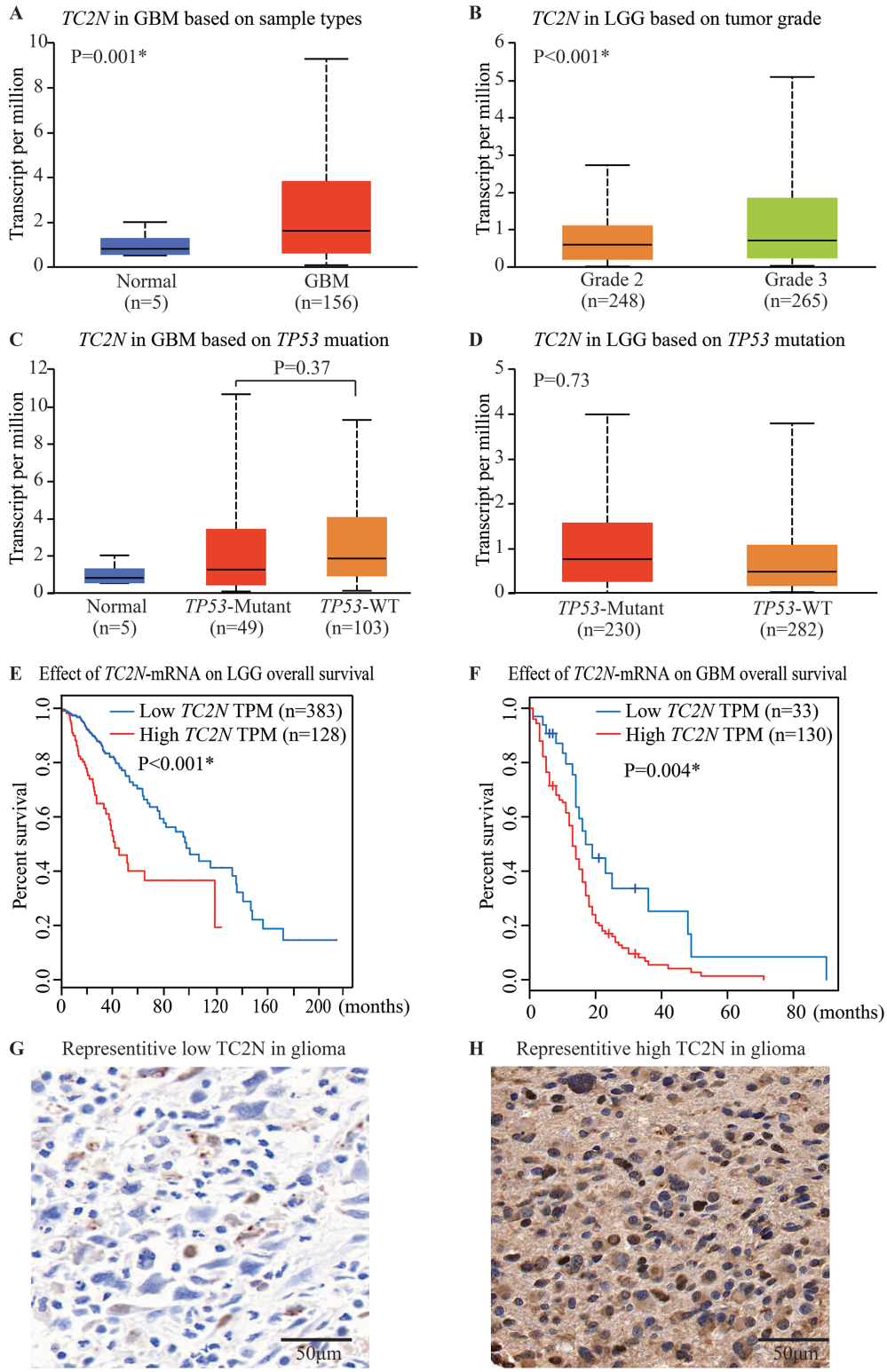

Fig. 1. *TC2N*-mRNA expression and its clinical relevance in gliomas from TCGA datasets.
(A) The mRNA level of *TC2N* was significantly higher in glioblastoma (GBM) tissues compared to that in normal brain tissues. The Y axis was labeled as transcript per million (TPM). (B) The mRNA level of *TC2N* was positively correlated with WHO grade in low-grade gliomas (LGG). Patients with WHO grade III showed significantly higher *TC2N*-mRNA level than those with WHO grade II. (C) *TC2N* showed no statistically significant difference in GBM tissues with or without *TP53* mutation. (D) *TC2N* showed no statistically significant difference in LGG tissues with or without *TP53* mutation. (E) Higher *TC2N* TPM is correlated with poorer overall survival of LGG patients. (F) Higher *TC2N* TPM is correlated with poorer overall survival of GBM patients. (G) Representative negative TC2N protein expression in glioma tissues. (H) Representative positive TC2N protein expression in glioma tissues.

Table 1. Characteristics of glioma patients and their correlations with TC2N protein level.

Variables	Cases (n = 102)	TC2N protein expression		P value
		Negative (n = 60)	Positive (n = 42)	
Sex				
Female	38	20	18	0.328
Male	64	40	24	
Age (years)				
≤ 48	50	31	19	0.523
> 48	52	29	23	
Tumor size				
≤ 5.0 cm	61	43	18	0.003*
> 5.0 cm	41	17	24	
WHO grade				
II	23	17	6	0.019*
III	35	24	11	
IV	44	19	25	
KPS score				
≤ 90	66	38	28	0.729
> 90	36	22	14	
Resection pattern				
GTR	54	37	17	0.091
STR	23	12	11	
PR	25	11	14	

*P < 0.05 by Chi-square test.
GTR, gross total resection; STR, subtotal resection; PR, partial resection, or biopsy.

Xenografts

Subcutaneous xenografts were conducted in nude mice model for in vivo assays. Briefly, 2×10^5 transfected U87 or U251 cells were subcutaneously injected into BALB/c nude mice. Then the size of xenografts was measured by every 5 days for one month. The xenograft volume was calculated as: volume = (π× length × width^2) / 6. After one month, all xenografts were isolated and weighted (Xu et al. 2021a).

Statistical analyses

Statistical analyses were carried out using SPSS 18.0 software. The different mRNA level of TC2N in samples from TCGA database was compared by Mann-Whitney test. Kaplan-Meier curves of overall survival were plotted and compared by log-rank test. Multivariable Cox regression analysis was used to test the independent prognostic effect of variables. Student's t-test was used to compare the differences between groups in cellular and xenografts experiments. $P < 0.05$ was considered statistically significant.

Results

Patients' information

This retrospective study contains 102 glioma patients that underwent surgical treatment in our hospital. Up to 62.7% (64/102) cases were males while the other 38 cases were females. The median age at disease diagnosis of enrolled patients was 48 years old. The Karnofsky Performance Scale (KPS) score was also evaluated for each patient. Accordingly, 36 cases were scored as larger than 90, while the other 66 cases with KPS score ≤ 90. Among them, 54 cases underwent gross total resection (GTR), 23 cases underwent subtotal resection (STR), and the other 25 cases accepted partial resection (PR). After surgical resection, the tumor size and WHO grade were exanimated by the Department of Pathology. 41 cases showed tumor size larger than 5.0 cm in diameter, and the other 61 cases with tumor diameter ≤ 5.0 cm. As for the WHO grade, 23 cases were diagnosed with WHO grade II, 35 cases with WHO grade III, and the other 44 cases with WHO grade IV. The median follow-up time was 34.5 months, ranging from 5 months to 93 months. By the end of follow up, there were 74 cases dead and 28 cases alive. Kaplan-Meier survival curve indicated a 5-year overall survival rate as 37.1%.

The mRNA and protein expression of TC2N in gliomas

We firstly evaluated the mRNA level of *TC2N* in GBM and LGG from TCGA datasets, respectively. As shown in Fig. 1A, GBM tissues showed significantly higher *TC2N* transcripts than that in normal brain tissues (P = 0.001). Similarly, *TC2N* exhibited higher mRNA level in grade III LGG samples than that in grade II LGG samples (P <

Table 2. Overall survival of glioma patients by univariate analyses.

Variables	Cases (n = 102)	Overall survival (months)		P value
		Mean ± SD	3-year OS rate	
Sex				0.967
Female	38	45.0 ± 4.5	55.30%	
Male	64	43.3 ± 4.3	44.50%	
Age (years)				0.378
≤ 48	50	46.5 ± 4.5	48.70%	
> 48	52	40.9 ± 4.4	48.10%	
Tumor size				0.004*
≤ 5.0 cm	61	50.9 ± 3.9	60.40%	
> 5.0 cm	41	32.4 ± 4.8	30.70%	
WHO grade				< 0.001*
II	23	72.4 ± 5.2	87.00%	
III	35	45.2 ± 5.2	56.40%	
IV	44	26.9 ± 3.5	21.90%	
KPS score				0.873
≤ 90	66	44.0 ± 4.0	47.70%	
> 90	36	42.5 ± 4.9	50.00%	
Surgery				0.118
GTR	54	49.2 ± 4.4	55.60%	
STR	23	41.6 ± 5.8	47.10%	
PR	25	34.5 ± 6.4	34.70%	
TC2N protein expression				< 0.001*
Negative	60	56.1 ± 4.0	71.30%	
Positive	42	25.5 ± 3.3	19.00%	

*P < 0.05 by log rank test.
GTR, gross total resection; STR, subtotal resection; PR, partial resection, or biopsy.

0.001) (Fig. 1B). The relationship between *TC2N* mRNA level and *TP53* mutation was also evaluated in both GBM and LGG, respectively (Fig. 1C, D). According to the TCGA datasets, no statistically significant correlation was observed between *TP53* mutation and *TC2N* mRNA expression. Of note, patients with higher *TC2N* mRNA level exhibited poorer overall survival of both LGG and GBM patients (Fig. 1E, F).

To further explore the protein expression of TC2N, we next conducted IHC experiments in glioma samples from our retrospective cohort. Accordingly, TC2N exhibited distinct staining intensities in different glioma tissues (Fig. 1G, H). Therefore, we divided our cohort into negative-TC2N expression group (n = 60) and positive-TC2N expression group (n = 42). Chi-square test revealed a positive correlation between TC2N protein expression and the WHO grade of gliomas (P = 0.019, Table 1). In addition, gliomas with larger tumor size exhibited significantly higher TC2N protein expression level (P = 0.003, Table 1). In contrast, no statistically significance was observed between TC2N expression with patients' sex, age, KPS score, nor surgical pattern. The results by Chi-square tests implied that TC2N may be closely correlated with the progression of gliomas.

Prognostic predictors of glioma patients

The prognostic predictive role of each enrolled variable was evaluated by univariate test, respectively (Table 2). Based on the overall survival curves and log-rank tests (Fig. 2), a significant role of TC2N on help predicant glioma patients' survival was demonstrated. In detail, patients with positive TC2N showed significantly lower 5-year overall survival rate (19.0%) compared to those with negative TC2N expression (71.3%). The median survival time of negative-TC2N group was 56.1 ± 4.0 months (mean ± SD), while it decreased to 25.5 ± 3.3 months of the positive-TC2N group (P < 0.001). Meanwhile, patients with larger glioma size also showed poorer overall survival compared to those with smaller ones (median survival time 32.4 ± 4.8 months vs. 50.9 ± 3.9 months, P = 0.004). As a well-recognized prognostic factor with wide clinical application, WHO grade also showed predicative significance on the overall survival of gliomas (P < 0.001).

To identify the independent effect of each variable on patients' survival, we next conducted multivariate analysis using a Cox regression model. Accordingly, WHO grade instead of tumor size exhibited independent prognostic significance (Table 3). Importantly, positive TC2N protein

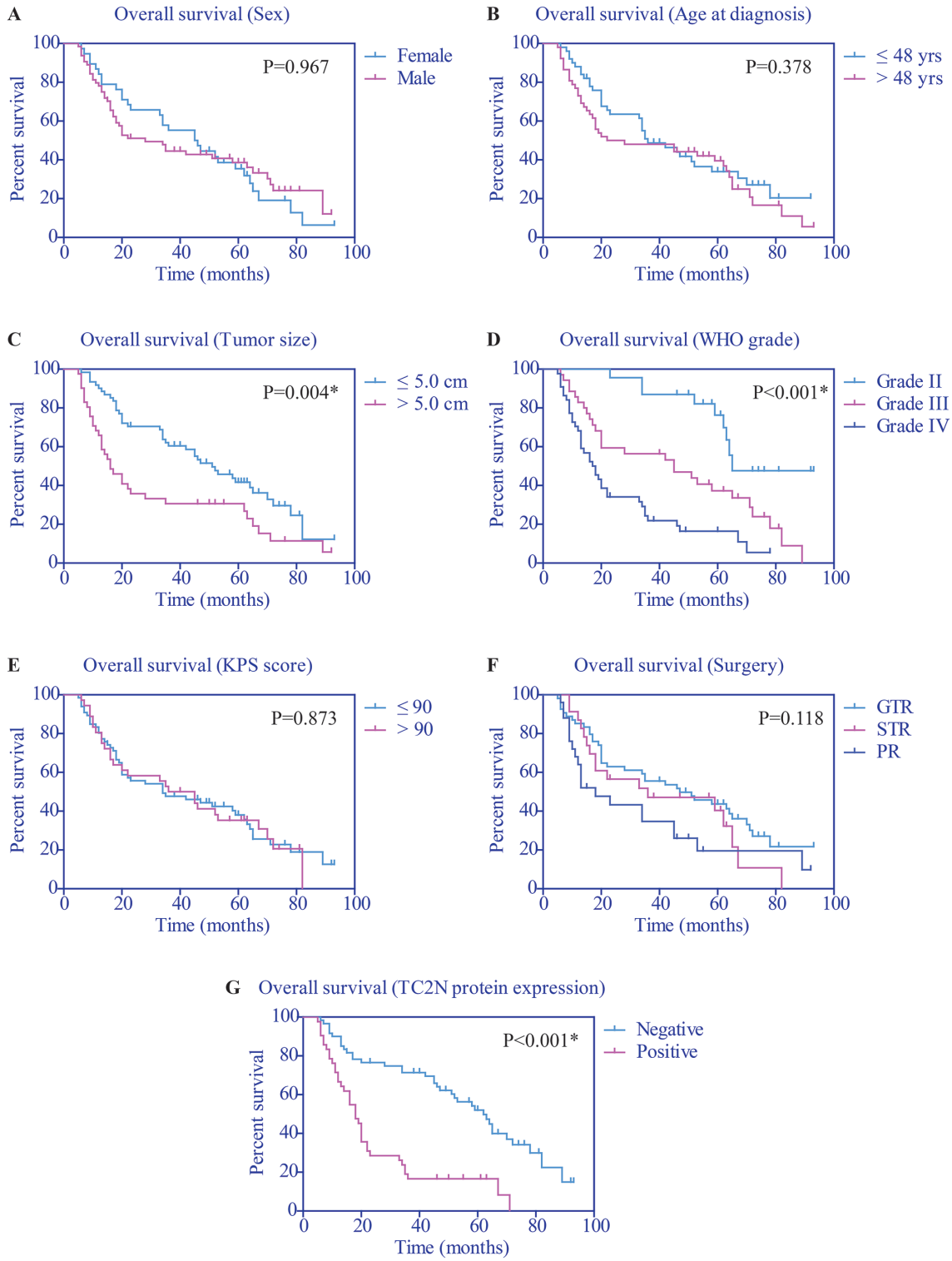

Fig. 2. Overall survival curves of 104 glioma patients by Kaplan-Meier assays.
The overall survival curves were plotted according to patients' sex (A), age (B), tumor size (C), WHO grade (D), Karnofsky Performance Scale (KPS) score (E), surgical pattern (F), and TC2N protein expression level (G). (F) GTR, gross total resection; STR, subtotal resection; PR, partial resection, or biopsy. *P < 0.05 by log-rank test.

expression in glioma tissues was also identified as a novel independent prognostic biomarker with the hazard ratio as 2.307 [95% confidence interval (CI) 1.320-4.033, P = 0.003].

TC2N interference suppressed glioma progression both in vitro and in vivo

Since clinical data revealed a potential involvement of TC2N in glioma progression and prognosis, we were

Table 3. Multivariate analysis of the OS of glioma patients.

Variables	Hazard ratio	95% Confidence Interval	P value
Tumor size			
≤ 5.0 cm	Reference		
> 5.0 cm	1.511	0.912-2.504	0.109
WHO grade			
II	Reference		
III	3.094	1.447-6.618	0.004*
IV	5.133	2.397-10.991	< 0.001*
TC2N protein expression			
Negative	Reference		
Positive	2.307	1.320-4.033	0.003*

*P < 0.05 by Cox regression test.

encouraged to further test its detailed functions in glioma cell lines. Knockdown assays were achieved by using specific shRNAs targeting TC2N in both U87 and U251 cell lines (Fig. 3A, B). CCK-8 experiments were next conducted to compare the viabilities of transfected cells. Comparing with control shRNA, TC2N-knockdown significantly impaired the cell proliferation in both U87 and U251 cell lines (Fig. 3C, D). Similarly, colony formation data demonstrated a significant suppression effect of TC2N-shRNAs on the numbers of colonies (Fig. 3E, F).

Consistent with in vitro cellular results, mice xenografts experiments showed that silencing TC2N can significantly attenuate the xenograft growth (Fig. 4A, B). Consistently, the tumor size and tumor weight were remarkably smaller in xenografts generated by TC2N-knockdown cells compared to those generated by control cells (Fig. 4C-F).

Discussions

Unlike most malignancies, the major unfavorable characteristic of glioma is diffuse growth instead of metastasis. Identifying proliferation-related molecular biomarkers will be invaluable for both prognosis prediction and therapy development. For the first time, here we demonstrated the novel role of TC2N on enhancing cell viability of glioma cell lines. According to our data, silencing TC2N results in significantly inhibition on the proliferation and colony formation of U87 and U251 cells. Our findings are consistent with a recent study by Hao et al. (2019b), which reported the effect of TC2N on accelerating lung cancer proliferation via suppressing p53 signaling pathways. In lung cancer cells, TC2N promotes cell cycle by inhibiting Cdk5-induced phosphorylation of p53 via inducing Cdk5 degradation or disrupting the interaction between Cdk5 and p53 (Hao et al. 2019b). However, in our study, we did not find any significant correlation between TC2N mRNA levels and TP53 mutation status in TCGA samples. Another critical downstream signaling molecule of TC2N is AKT in breast cancer. As reported recently, TC2N can block AKT signaling in both PI3K dependent and independent way through weakening the interaction between ALK and p55γ or inhibiting the binding of EBP1 and AKT (Hao et al. 2019a), thus promoting breast cancer cell proliferation. Besides the cellular effects of TC2N in glioma cell lines, our study also provided in vivo evidence regarding to the tumor-suppressing role of TC2N-shRNA, thus highlighting its potential as a novel drug target. Indeed, knockdown of TC2N was reported to improve the sensitivity of gastric cancer cells to cisplatin, paclitaxel and 5-fluorouracil, while its overexpression exerted opposite effects (Shen et al. 2020). Besides proliferation, TC2N seems to also participate in tumor migration and invasion in lung cancer, breast cancer, and gastric cancer. Nevertheless, our data did not find any significant effect of TC2N-shRNA on affecting the migration nor invasion capacity of glioma cells (data not shown). Therefore, the detailed functional mechanisms of TC2N may be distinct among different tumor types, which deserve further investigation.

Consistent with its cellular effects, our data revealed the clinical relevance of TC2N in glioma. Firstly, TC2N exhibited significantly higher expression in both GBM and LGG than that in normal brain tissues, which was positively correlated with WHO grade and tumor size. Secondly, both the mRNA and protein expression levels of TC2N represented an unfavorable prognostic predictor of glioma patients according to TCGA datasets as well as our retrospective cohort. Moreover, multivariate analysis revealed that TC2N provided an independent effect on poorer overall survival of glioma patients, emphasizing its clinical significance. Therefore, testing the TC2N level in glioma tissues after surgical resection would be a possible strategy to help predict patients' survival.

Inevitably, our study has several limitations. Firstly, our retrospective cohort was collected from a single medical center with limited case numbers and the results may contains regional bias. Therefore, we tried to make our major conclusion more convincible by retrieving the mRNA level of TC2N and testing its prognostic predictive role in TCGA dataset. However, prospective testing in clinical trials would be invaluable for validating its significance and

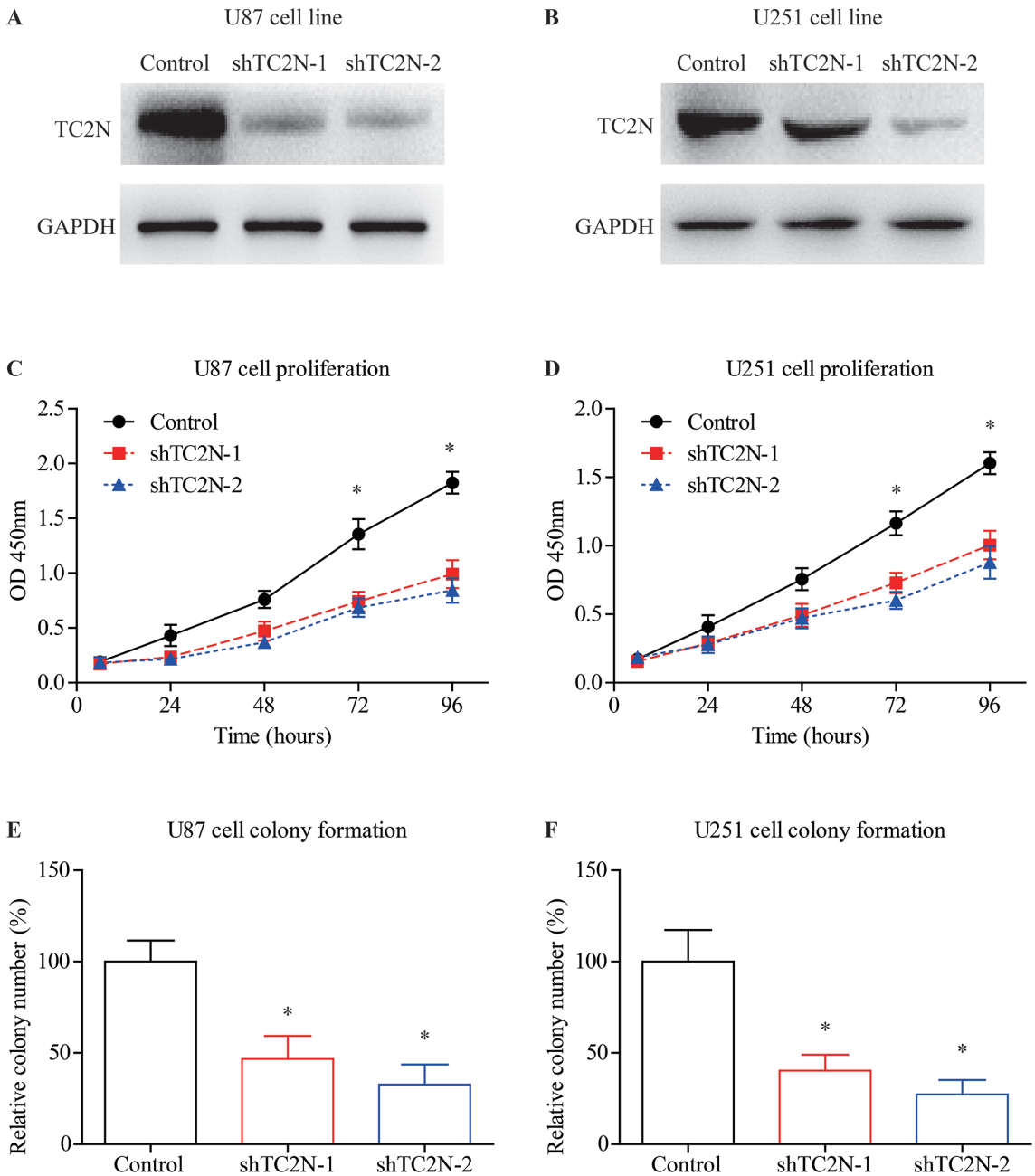

Fig. 3. Effect of TC2N-knockdown on inhibiting glioma proliferation.
(A, B) The knockdown efficiencies of TC2N-shRNAs (shTC2N-1 and shTC2N-2) in both U87 and U251 cells were tested via western blotting analysis, respectively. (C, D) Cell viability was tested via CCK-8 method, which revealed that silencing TC2N remarkably inhibited proliferation capacities of both U87 and U251 cells. (E, F) Colony formation assays were conducted to further explore the effect of TC2N on glioma proliferation. Accordingly, TC2N interference significantly impaired the colony formation process of both U87 and U251 cells.
Data was exhibited as mean ± SD from three independent repeats. *P < 0.05.

clinical application. Secondly, the oncogenic effects of TC2N in gliomas need to be systematically elucidated by illustrating the underlying mechanisms, which will be essential for therapeutic development in the future. Nevertheless, our data unmask an ambivalent role of TC2N in glioma, providing evidence on its value as both prognostic predictive factor and therapeutic target.

In conclusion, high TC2N expression is significantly correlated with poor overall survival of glioma patients. In vitro and in vivo data demonstrated that TC2N can promote glioma progression at least partially by enhancing tumor growth.

Conflict of Interest

The authors declare no conflict of interest.

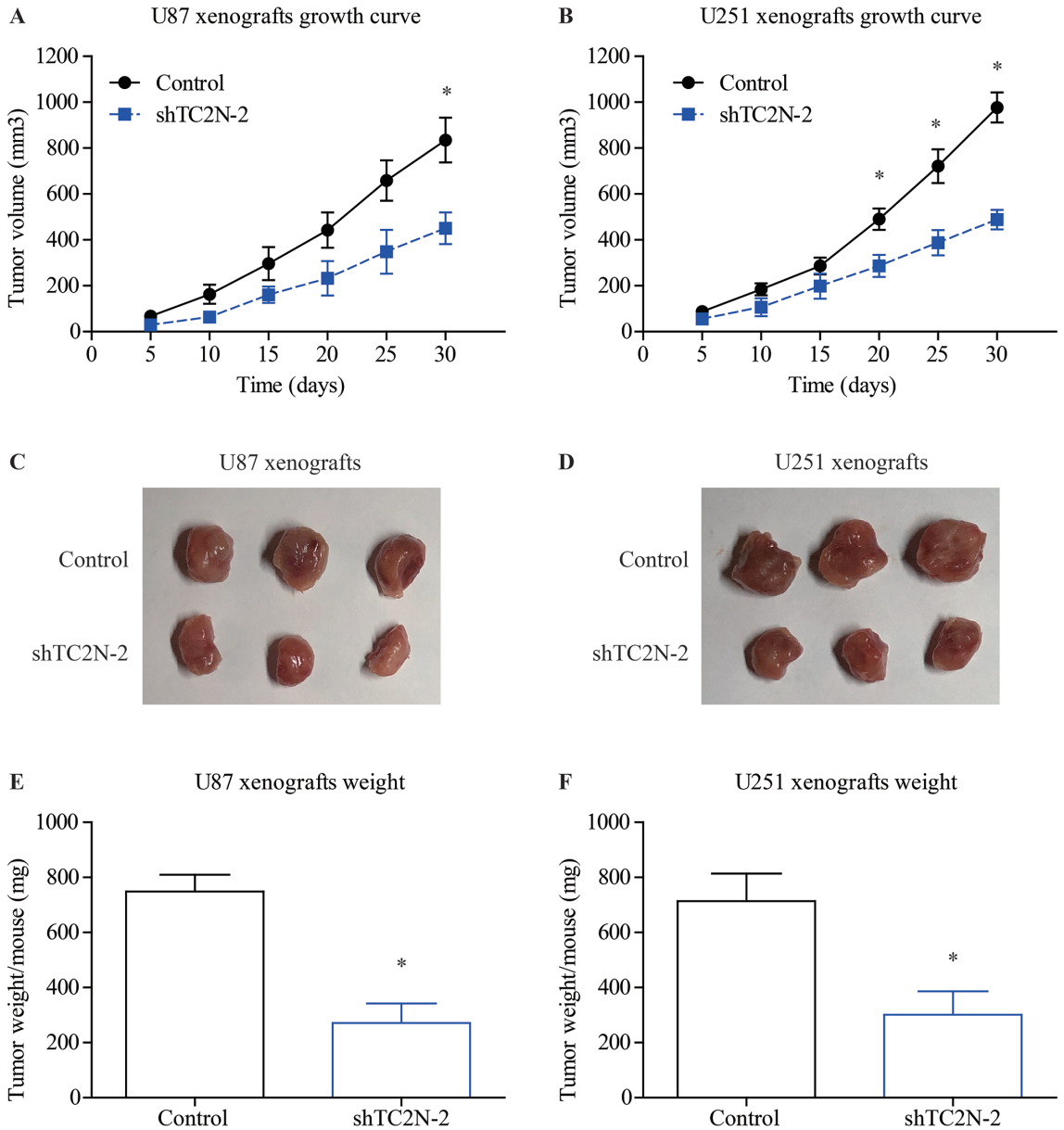

Fig. 4. Silencing TC2N suppresses glioma growth in vivo.
The growth of subcutaneous xenografts, which were generated by transfected U87 and U251 cells, were monitored and plotted. The growth curves indicated that TC2N interference (shTC2N-2) can significantly attenuate glioma growth (A, B). Thirty days after tumor cell injection, xenografts were isolated for picturing (C, D) and weighting (E, F), which showed remarkable smaller tumor size and tumor weight in the TC2N-knockdown groups.
*P < 0.05 by Student's t-test. Data was exhibited as mean ± SD from three independent repeats.

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