The long non-coding RNA PANDAR regulates cell proliferation and epithelial-to-mesenchymal transition in glioma

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The long non-coding RNA PANDAR regulates cell proliferation and epithelial-to-mesenchymal transition in glioma

Running title: The prognostic marker of PANDAR in glioma cells

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Abstract

Glioma is one of the most aggressive intracranial tumors in the central nervous system. The long non-coding RNA P21 associated ncRNA DNA damage activated (PANDAR) has been reported to be an oncogene or tumor suppressor in several cancers. However, the prognostic value and biological function of PANDAR in glioma have not been described. Here, we report that expression of PANDAR is significantly up-regulated in glioma tissues and cell lines. PANDAR expression was correlated with tumor size ($p = 0.044$) and World Health Organization (WHO) grades ($p = 0.005$), as shown by chi-squared test. Moreover, significant upregulation of PANDAR was found to correlate with poor prognosis in glioma, as shown using Kaplan–Meier method and Cox multivariate survival analysis. Furthermore, PANDAR knockdown suppressed cell proliferation, G1/S transition, migration and invasion, and promoted apoptosis in glioma cell lines (U251 and U87). PANDAR knockdown decreased expression of CDK4, Bcl-2, N-cadherin and Vimentin, but increased E-cadherin expression in glioma cells. In conclusion, our data suggest PANDAR as a potential prognostic biomarker and therapeutic candidate for glioma.

Key words: glioma, PANDAR, proliferation, EMT
**Introduction**

Glioma is one of the most aggressive intracranial tumors in the central nervous system with 4~5/100000 annual incidence (Wen *et al.*, 2010). Glioma derived from the neuroglial stem or progenitor cells is histologically classified into high-grade (III/IV) and low-grade (I/II) glioma according to the WHO Classification of Tumors (Weller *et al.*, 2015; Louis *et al.*, 2016). Glioblastoma is a high-grade glioma (grade IV) that accounts for ~3% of all tumors (Le Rhun *et al.*, 2019). During recent decades, great advances have been achieved in standard treatment strategies, including surgical resection, immunotherapy, radiotherapy or chemotherapy (Ghotme *et al.*, 2017; Ganipineni *et al.*, 2018; McGranahan *et al.*, 2019). Unfortunately, the prognosis of glioma patients still remains unsatisfactory due to recurrence and metastasis (Wen and Reardon, 2016). Thus, better understanding of the molecular mechanism driving glioma progression is necessary for investigating more relevant molecular targets.

Long non-coding RNAs (lncRNAs) are a class of transcripts without protein-coding potential at a length of more than 200 nucleotides, which have an important regulatory role in multiple biological processes, including proliferation, differentiation and metastasis (Ponting *et al.*, 2009). Accumulating evidence indicates that deregulated lncRNAs are frequently identified as tumor suppressors or oncogenes in glioma. For example, downregulated MATN1-AS1 was correlated with poorer prognosis of glioblastoma patients and its overexpression results in significant inhibition of tumor growth in glioblastoma cells (Han *et al.*, 2019). NEAT1 acts as a tumor suppressor in glioma cells by suppressing proliferation and promotes apoptosis of glioma cells (Liu...
More lncRNAs, such as RGMB-AS1 (Pan et al., 2019), AWPPH (Dai et al., 2019) and SNHG5 (Meng et al., 2019) act as oncogenes in glioma progression by promoting tumor cell proliferation, migration and invasion.

P21-associated ncRNA DNA damage activated (PANDAR) was initially reported to possess 1,506 nucleotides in length mapping to 6p21.2 (Hung et al., 2011), which has been recently found to known as biomarkers in cancers by participating 112 in cancer cells (Li et al., 2017a). As demonstrated by Han et al (Han et al., 2015), decreased PANDAR expression was negatively correlated with poor prognosis and PANDAR overexpression repressed the proliferation in vitro and in vivo in non-small cell lung cancer. On the contrary, significant upregulation of PANDAR was found to indicate a poor prognosis in bladder cancer (Zhan et al., 2016), gastric cancer (Ma et al., 2016), hepatocellular carcinoma (Peng and Fan, 2015), colorectal cancer (Li et al., 2017b; Lu et al., 2017), renal cell carcinoma (Xu et al., 2017b), cervical cancer (Huang et al., 2017) and oral squamous cell carcinoma (Huang et al., 2018). Using loss-of-function assays, the suppressive effects of PANDAR knockdown on cell proliferation, migration and invasion have been demonstrated in thyroid cancer (Li et al., 2017c), breast cancer (Li et al., 2019) and osteosarcoma (Kotake et al., 2017). To our best knowledge, the prognostic value and biological function of PANDAR on cell proliferation and metastasis in glioma have not been reported yet to date.

In this study, we compared the expression of PANDAR between glioma tissues and adjacent para-carcinoma tissues. Then, we assessed whether PANDAR serves as an independent prognostic factor for glioma patients. Next, we constructed PANDAR
knockdown glioma cell lines to investigate the effects of PANDAR on cell proliferation, cell cycle progression, apoptosis, migration, invasion and epithelial to mesenchymal transition (EMT) in glioma.

Materials and methods

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and the approval from the Ethics Committee of The First Affiliated Hospital of Xiamen University and written informed consent was signed by each patient.

Clinical tissue collection

Paired tumor tissues and adjacent para-carcinoma tissues were collected through surgical removal from 48 glioma patients after histological diagnostic analysis at The First Affiliated Hospital of Xiamen University (Xiamen, China) and immediately placed in liquid nitrogen. All tumor tissues were classified based on the 2016 World Health Organization (WHO) Classification of Tumors of the Central Nervous System. The relevant clinical information, including age and gender is summarized in Table 1. No patient received antitumor treatments, such as chemotherapy or radiotherapy before surgery. All patients underwent five-year follow-up period for the survival status after surgical treatment. This study was conducted in accordance with the Declaration of Helsinki (1975) and approved by The First Affiliated Hospital of Xiamen University (Approval no. XUA-88DA, Xiamen, China).
Cell culture and transfection

Two human glioma cell line (U251 and U87) and normal human astrocyte cell line (NHA) were purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China). These cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, HyClone, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and maintained under a humidified atmosphere containing 5% CO$_2$ at 37 °C.

Different small interfering RNAs (siRNAs) against PANDAR (si-PANDAR#1: 5′-GTTGTCGTACTAACAGCAA-3′, si-PANDAR#2: 5′-CTAAGCACAGGCACCATTG-3′ and si-PANDAR#3: 5′-GAAUUGCUGGUAAUUCACU-3′) and siRNA negative control (si-NC: 5′-UUCUCCGAACGUGUCACGUTT-3′) were synthesized by GenePharma Co., Ltd (Shanghai, China). For PANDAR knockdown, U251 and U87 cells were transfected with the above oligonucleotides at a concentration of 50 nM for 48 h following the manufacturer’s protocol of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Quantitative real time PCR

Total RNA of tissue samples or cell lines was extracted using TRIzol Reagent (Invitrogen) and cDNA was synthesized with SuperScript First Strand cDNA System (Invitrogen) following the manufacturer's instructions. Quantitative real time PCR was conducted using SYBR Prime Script RT-PCR kits (Takara) with the synthesized primer sequences by Invitrogen as follows: PANDAR primers, forward: 5′-CGGTGGTGTTAAGGTCATTG-3′, reverse: 5′-CTGGAGGCTCATTAGGCTGAT-3′;
GAPDH primers, forward: 5′-ATCAGCCACATCGCGACA-3′, reverse: 5′-TGCGACGGACTCCATACT-3′. The relative expression of PANDAR was calculated using the average value in each triplicate with 2^-ΔΔCt method.

**Cell counting kit-8 (CCK-8) assay**

Transfected U251 or U87 cells at exponential phase were harvested and seeded into 96-well plates at a density of 3.5 × 10^3 cells per well. The cells in each well were added with 10 μL of CCK-8 solution (Beyotime Inst Biotech, China) at 0, 24, 48 and 72 h, respectively. After additional 2 h incubation, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). Each sample was prepared in triplicate and analyzed for three times independently.

**Colony formation assay**

After different transfections, U251 or U87 cells at a density of 500 cells per well were plated onto six-well plates and incubated for two weeks at 37 °C. Then, the visible colonies were fixed with 4% paraformaldehyde for 30 min, stained with 0.1% crystal violet and counted under a light microscope. Each sample was prepared in triplicate and analyzed for three times independently.

**Flow cytometry**

For cell cycle analysis, approximately 1 × 10^6 transfected U251 or U87 cells were trypsinized, pelleted and treated with pre-cooled 70% ethanol overnight at 4 °C. Next
day, cells were cultivated with 10 µl of RNase and stained with 5 µl propidium iodide (PI; 100 µg/ml, BD Biosciences) for 30 min in the dark. The cell cycle distribution was analyzed using a FACSscan flow cytometer (BD Biosciences). For apoptosis analysis, cells were stained with Annexin V-FITC and PI for 20 min in the dark according to the instructions provided with the Apoptosis Detection Kit (Nanjing KeyGen Biotech Co., Ltd.). Finally, apoptosis rate was determined using a FACSscan flow cytometer (BD Biosciences) and presented as a combination of early and late-stage apoptosis. Each sample was prepared in triplicate and analyzed three times independently.

**Transwell assay**

The migration and invasion ability of transfected glioma cells was assessed with 24-well insert transwell chambers (Corning, NY, USA). Different from migration, the transwell chambers were pre-coated with 30 µl of Matrigel (BD Biosciences) at 37 °C for 4 h for invasion assay. The other similar measurements for both migration and invasion were briefly described as follows: Transfected U251 or U87 cells (5 × 10⁴ cells) were resuspended in 100 µl serum-free DMEM and transferred to the upper chambers. A total of 500 µl of DMEM containing 10% FBS was added to the lower chamber. After incubation at 37 °C for 24 h, the cells on the bottom surface were stained with 0.1% crystal violet for 15 min and counted under a light microscope (×200 magnification) in five randomly selected fields.
Western blot analysis

Total protein was extracted from transfected U251 or U87 cells by lysed in RIPA lysis buffer (Thermo Fisher Scientific, Inc.) and centrifugation at 12,000 rpm for 15 min at 4 °C. Following quantification by BCA protein assay kit (Beyotime Institute of Biotechnology), a total of 30 µg total protein per lane was separated by 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with TBST containing 5% nonfat dry milk for 2 h at room temperature (RT) and incubated with primary antibodies against CDK4 (1:1000, 11026-2-AP, Proteintech, Chicago, IL, USA), Bcl-2 (1:1000, #2724, Cell Signaling Technology, Danvers, MA, USA), E-cadherin (1:1000, 20874-1-AP, Proteintech), N-cadherin (1:1000, #4061, Cell Signaling Technology), Vimentin (1:1000, #5741, Cell Signaling Technology) and GAPDH (1:500000, 10494-1-AP, Proteintech) overnight at 4 °C. After washing three times with TBST, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (1:5000, SC-2054, Santa Cruz, Dallas, TX, USA) for 2 h at RT. Subsequently, protein bands were detected by Enhanced Chemiluminescence (Nanjing KeyGen Biotech Co., Ltd.) with GAPDH as the internal control.

Statistical analysis

All data in this study were analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as mean ± standard deviation (SD) from three independent experiments. The chi-squared test was carried out to analyze
the association between PANDAR expression and clinicopathological characteristics of glioma patients. Kaplan–Meier method followed by log-rank test was used to perform survival curves. The Cox regression analysis was employed to perform univariate and multivariate analyses of survival data. The significance of the differences between groups was estimated using Student’s t-test or one-way analysis of variance (ANOVA), followed by Dunnett’s test. A p-value of less than 0.05 was indicated to be statistically significant.

Results

PANDAR was upregulated in glioma tissues and predicted poor prognosis in glioma patients

To assess the clinical significance of PANDAR in glioma, the expression levels of PANDAR were first determined in paired glioma and adjacent para-carcinoma tissues from 48 patients using quantitative real time PCR analysis. As shown in Figure 1A, PANDAR was significantly increased in tumor tissues compared with the adjacent para-carcinoma tissues. According the expression level of PANDAR in each tumor sample calculated by 2−ΔΔCt method, we obtained the median value by the formula “median”. Using the median expression level of PANDAR as a cut-off, all glioma patients were divided into high PANDAR expression group (n = 30) and low PANDAR expression group (n = 18). The chi-squared test showed that high PANDAR expression level was significantly associated with tumor size (p = 0.044) and WHO grades (p = 0.005), but not correlated with age (p = 0.377) and gender (p = 0.644) (Table 1). Next,
we analyzed the association between PANDAR expression and prognosis of glioma patients by Kaplan–Meier method followed by log-rank test. As displayed in Figure 1B, patients with high PANDAR expression level exhibited shorter five-year survival rate compared with low PANDAR expression of patients \( (p = 0.0069) \). Moreover, Cox multivariate survival analysis indicated WHO grade \( (p = 0.008) \) and PANDAR expression \( (p = 0.026) \) were independent risk prognostic factors of glioma (Table 2).

**PANDAR knockdown suppressed cell proliferation, G1/S transition and promoted apoptosis in glioma cells**

Consistent with the expression of PANDAR in glioma tissues, we found PANDAR expression was remarkably elevated in glioma cell lines (U251 and U87) compared with normal human astrocyte cell line NHA (Figure 2A). Next, we constructed PANDAR knockdown glioma cells by transfecting different siRNA targeting PANDAR into U251 and U87 cells. As manifested by quantitative real time PCR, si-PANDAR#1-3 caused a significant reduction of PANDAR expression in U251 (Figure 2B) and U87 (Figure 2C) cells, of which si-PANDAR#2 generated higher suppressive effects on PANDAR expression and was then selected for the subsequent loss-of-function assays. CCK-8 assay indicated that PANDAR knockdown significantly inhibited cell proliferation in U251 (Figure 2D) and U87 (Figure 2E) cells. Similarly, the number of colonies in si-PANDAR#2 group was obviously decreased when compared with si-NC group in both U251 and U87 cells (Figure 2F). Furthermore, we analyzed the effects of PANDAR knockdown on cell cycle progression and apoptosis using flow cytometry...
analysis. As shown in Figure 3A, PANDAR knockdown notably increased the percentage of cells at G0/G1 phase in U251 and U87 cells, accordingly decreased cells at S phase in U251 and U87 cells and cells at G2/M phase in U251 cells, which indicated that PANDAR knockdown blocked cell cycle progression at G0/G1 phase. In addition, we observed that the apoptotic cells were significantly increased in si-PANDAR#2 group compared with si-NC group in both U251 (si-PANDAR#2 vs. si-NC: 34.9% ± 1.0% vs. 14.3% ± 1.1%) and U87 (si-PANDAR#2 vs. si-NC: 23.6% ± 1.0% vs. 12.0% ± 1.7%) cells (Figure 3B). From these data, we concluded that the suppressive effects of PANDAR knockdown on cell proliferation might be through induction of G0/G1 arrest and apoptosis in glioma.

PANDAR knockdown inhibited the migration and invasion ability of glioma cells

In addition to proliferation, we further analyzed the effects of PANDAR on glioma metastasis in vitro. Using transwell migration assay, we found the number of migratory cells was significantly decreased from 102.0 ± 6.6 in si-NC group to 44.7 ± 6.5 in si-PANDAR#2 group in U251 cells and from 421.0 ± 10.1 in si-NC group to 215.0 ± 10.5 in si-PANDAR#2 group in U87 cells (Figure 4A). Similarly, PANDAR knockdown remarkably reduced the number of invasive cells from 97.3 ± 10.4 to 40.3 ± 4.5 in U251 cells and from 375.7 ± 6.8 to 157.7 ± 7.5 in U87 cells (Figure 4B). These findings suggested that PANDAR knockdown suppressed glioma cellular migration and invasion in vitro.
PANDAR knockdown regulated the protein expression associated with G1/S transition, apoptosis and EMT

To better understanding the role of PANDAR knockdown on regulating glioma cell proliferation, migration and invasion, we further investigated the related protein expression in glioma cells under PANDAR knockdown conditions using western blot analysis. The results showed that G1/S transition marker CDK4, anti-apoptotic Bcl-2 and mesenchymal markers (N-cadherin and Vimentin) were downregulated, while epithelial marker E-cadherin was upregulated in si-PANDAR#2 transfection group, in comparison with si-NC transfection in both U251 (Figure 5A) and U87 (Figure 5B) cells.

Discussion

Glioma, accounting for 50% to 60% of all brain malignancies, displays a poor prognosis and threatens human health (Morgan, 2015). In recent years, different lncRNAs have been demonstrated to be aberrantly expressed in glioma, implying the potential of lncRNAs as diagnostic biomarkers and therapeutic targets (Vecera et al., 2018; Ding et al., 2020). In the present study, we confirmed that PANDAR was significantly upregulated in glioma tissues and cell lines. Moreover, high PANDAR expression was positively associated with tumor size and WHO grades. According to the World Health Organization (WHO) Classification of Tumors (Louis et al., 2016), glioma can be divided into four grades of tumors, including grade I (pilocytic astrocytoma, pleomorphic xanthoastrocytoma, and subependymal giant cell
astrocytoma), grade II (oligodendroglioma and astrocytoma), grade III (anaplastic oligodendroglioma, anaplastic astrocytoma, anaplastic oligoastrocytoma, and anaplastic ependymoma) and grade IV (glioblastoma). The positive association between PANDAR and the WHO grades of glioma indicated that PANDAR might be closely correlated with malignancy of glioma. More importantly, we demonstrated that high PANDAR expression might be an independent prognostic factor affecting the survival rate in glioma patients. Consistently, PANDAR was overexpressed in acute myeloid leukemia (AML), and that higher PANDAR expression was associated with poor clinical outcome (Yang et al., 2018). Similar clinical prognostic values of PANDAR have also been reported in bladder cancer (Zhan et al., 2016), gastric cancer (Ma et al., 2016), hepatocellular carcinoma (Peng and Fan, 2015), colorectal cancer (Li et al., 2017b; Lu et al., 2017), renal cell carcinoma (Xu et al., 2017b), cervical cancer (Huang et al., 2017) and oral squamous cell carcinoma (Huang et al., 2018). In contrast, PANDAR was generally downregulated in NSCLC and decreased PANDAR expression negatively correlated with greater tumor size, advanced TNM stage and poor overall survival (Han et al., 2015). These differences on the role of PANDAR in tumor cells might be ascribed to different types of cancers.

Subsequent functional assays manifested that knockdown of PANDAR repressed proliferation, G1/S transition and induced apoptosis in U251 and U87 cells. At a molecular level, we found that PANDAR knockdown downregulated the expression of CDK4 and Bcl-2. Disruption of G1/S cell cycle control plays a crucial role in most human cancer development, which is regulated by cyclin D1/cyclin dependent kinase
Apoptosis is tightly regulated by pro-apoptotic proteins such as caspases and anti-apoptotic proteins like Bcl-2 family proteins. In line with our data, knockdown of PANDAR suppressed cell proliferation, G1/S transition and induced apoptosis in breast cancer (Sang et al., 2016), thyroid cancer (Li et al., 2017c) and renal cell carcinoma (Xu et al., 2017b). On the contrary, Han et al indicated that PANDAR overexpression significantly repressed the proliferation in vitro and in vivo in NSCLC (Han et al., 2015). Moreover, PANDAR was a direct transcriptional target of p53 in NSCLC cells and the p53/PANDAR/NF-YA/Bcl-2 interaction might serve as targets for NSCLC diagnosis and therapy (Han et al., 2015). We thus concluded that the different roles of PANDAR in Bcl-2 were closely associated with its regulation on cell proliferation in different tumor cells. Here, we made a hypothesis that p53 might be an upstream factor regulating PANDAR-mediating Bcl-2 in glioma cells, which will be further validated in our next work.

In addition, we observed that PANDAR knockdown suppressed migration and invasion in U251 and U87 cells. At molecular level, downregulation of PANDAR obviously upregulated E-cadherin, while it downregulated the expression of N-cadherin and Vimentin in both U251 and U87 cells. EMT plays an important role in cancer metastasis, which is characterized by reduced expression of epithelial marker E-cadherin and increased expression of mesenchymal markers (N-cadherin and Vimentin) (He et al., 2016). Consistent with our data, knockdown of PANDAR inhibited, while overexpression of PANDAR promoted cell migration, invasion and EMT in melanoma.
Knockdown of PANDAR can suppress EMT through inhibiting N-cadherin, vimentin, β-catenin, Snail and Twist expression and increasing the expression levels of E-cadherin in colorectal cancer (Lu et al., 2017). Additionally, the suppressive role of PANDAR on EMT pathway was also reported in cholangiocarcinoma (Xu et al., 2017a) and breast cancer (Li et al., 2019).

In conclusion, we have demonstrated that PANDAR was upregulated in glioma tissues and an independent marker for predicting the clinical outcome of glioma patients. Functionally, our data showed that knockdown of PANDAR suppressed cell proliferation, migration, invasion and EMT in glioma cells. Taken together, these data suggest that PANDAR might be a promising therapeutic target for the treatment of glioma.

**Abbreviations**

PANDAR, P21-associated ncRNA DNA damage activated; WHO, World Health Organization; SD, standard deviation; CCK-8, Cell counting kit-8; EMT, epithelial to mesenchymal transition; siRNAs, small interfering RNAs; PI, propidium iodide; TBST, Tris buffered saline tween; DMEM, Dulbecco’s modified Eagle’s medium; NC, negative control; ANOVA, one-way analysis of variance

**Author’s contributions**

SCZ designed the experiments, provided the financial support and supervised laboratorial processes. GJF and XDY performed the experiments. XDY and LZJ
coordinated the research and analyzed the data. GJF and LZJ helped to draft the
manuscript. All authors read and approved the final manuscript.

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Data availability statement
The data during and/or analyzed during the current study are available from the
corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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References
Lncrna awpph promotes the migration and invasion of glioma cells by
activating the tgf-beta pathway. Oncol. Lett. 18, 5923-5929.
expression of long non-coding rnas (lncrnas) is involved in brain glioma
HISTOLOGY AND HISTOPATHOLOGY


Wen P.Y., Macdonald D.R., Reardon D.A., Cloughesy T.F., Sorensen A.G., Galanis E.,
Degroot J., Wick W., Gilbert M.R., Lassman A.B., Tsien C., Mikkelsen T., Wong
E.T., Chamberlain M.C., Stupp R., Lamborn K.R., Vogelbaum M.A., van den Bent
M.J. and Chang S.M. (2010). Updated response assessment criteria for high-
unfavorable prognosis and promotes tumorigenesis in cholangiocarcinoma.
Onco Targets Ther. 10, 2873-2883.
non-coding rna pandar is associated with poor prognosis in clear cell renal cell
carcinoma. BMC Cancer 17, 373.
Yang L., Zhou J.D., Zhang T.J., Ma J.C., Xiao G.F., Chen Q., Deng Z.Q., Lin J., Qian J. and
Zhan Y., Lin J., Liu Y., Chen M., Chen X., Zhuang C., Liu L., Xu W., Chen Z., He A., Zhang
pandar is associated with poor prognosis and promotes tumorigenesis in

Figure legends

Figure 1 PANDAR was overexpressed in glioma tissues and associated with poor
prognosis. (A) The PANDAR expression levels in 48 pairs of glioma and matched
adjacent para-carcinoma tissues were determined by quantitative real time PCR. (B)
Glioma patients were divided into a high-level group (n = 30) and a low-level group (n
= 18) based on the median value of PANDAR expression in glioma tissues. The Kaplan-
Meier method and log-rank test were used to evaluate the relationship between
PANDAR expression and overall survival time of glioma patients.

Figure 2 PANDAR knockdown suppressed cell proliferation in glioma cells. (A)
The expression of PANDAR in glioma cell lines (U251 and U87) and normal human
astrocyte cell line NHA was measured by quantitative real time PCR. ***p < 0.001,
compared with NHA; The transfection efficiency of siRNAs (si-NC, si-PANDAR#1, si-PANDAR#2 and si-PANDAR#3) was measured by quantitative real time PCR in U251 (B) and U87 (C) cells. The significance of the differences between groups was estimated using one-way analysis of variance (ANOVA), followed by Dunnett’s test.

Cell proliferation of transfected U251 (D) and U87 (E) cells was analyzed by CCK-8 assay. OD 450 values at indicated time points were displayed to indicate cell proliferation. (F) Colony formation assay was performed in transfected U251 and U87 cells. **p < 0.01, ***p < 0.001, compared with si-NC; Results are displayed as mean ± SD of three independent experiments. The significance of the differences between groups was estimated using Student’s t-test.

**Figure 3** PANDAR knockdown suppressed G1/S transition and promoted apoptosis in glioma cells. (A) Cell cycle analysis revealed that down-regulation of PANDAR repressed G1/S transition in U251 and U87 cell lines. (B) Flow cytometry analysis of U251 and U87 cell lines indicated that down-regulation of PANDAR facilitated cell apoptosis. Results are displayed as mean ± SD of three independent experiments. **p < 0.01, ***p < 0.001, compared with si-NC. The significance of the differences between groups was estimated using Student’s t-test.

**Figure 4** PANDAR knockdown inhibited the migration and invasion ability of glioma cells. (A) Migration assay in U251 and U87 cell lines showed that knockdown of PANDAR decreased the cell migration compared with that in the si-NC group. (B)
Knockdown of PANDAR decreased the ability of cellular invasion compared with that in the si-NC group in U251 and U87 cell lines. Results are displayed as mean ± SD of three independent experiments. The significance of the differences between groups was estimated using Student’s t-test. **p < 0.01, ***p < 0.001, compared with si-NC; scale bar, 50 µm.

**Figure 5** PANDAR knockdown regulated the protein expression associated with G1/S transition, apoptosis and EMT. The protein expression of CDK4, Bcl-2, E-cadherin, N-cadherin and Vimentin was measured in U251 (A) and U87 (B) cells after transfection with si-PANDAR#2 or si-NC using western blot analysis. Results are displayed as mean ± SD of three independent experiments. The significance of the differences between groups was estimated using Student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001, compared with si-NC.
Table 1 Association between PANDAR expression and clinicopathological characteristics of glioma patients

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Table 2 Univariate and multivariate analysis for five-year overall survival in glioma patients

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Abbreviations: HR: hazard ratio; CI: confidence interval; NA, not analyzed
A) Relative PANDAR expression in adjacent and tumor tissues. The graph shows a significant difference with a p-value of < 0.001.

B) Kaplan-Meier survival curves for patients with low (n = 18) and high (n = 30) PANDAR expression. The log-rank test revealed a statistically significant difference in survival rates, with a p-value of 0.0069.
A) si-NC vs. si-PANDAR#2 on U251 and U87 cells. The histograms show the distribution of cells in different phases of the cell cycle (G0/G1, S, G2/M) and the percentage of apoptotic cells. The bar graphs illustrate the percentage of cells in each phase and the percentage of apoptotic cells.

B) Annexin V FITC-A analysis of U251 and U87 cells. The dot plots show the PI and Annexin V FITC-A staining for each group. The bar graphs indicate the percentage of apoptotic cells.
A

U251 si-NC si-PANDAR#2

U87 si-NC si-PANDAR#2

B

U251 si-NC si-PANDAR#2

U87 si-NC si-PANDAR#2

Migrated cells per field

Invasive cells per field

U251 U87

si-NC si-PANDAR#2
HISTOLOGY AND HISTOPATHOLOGY

**A**

<table>
<thead>
<tr>
<th>Protein</th>
<th>U251 si-NC</th>
<th>U251 si-PANDAR#2</th>
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<tbody>
<tr>
<td>CDK4</td>
<td></td>
<td>34 kDa</td>
</tr>
<tr>
<td>Bcl-2</td>
<td></td>
<td>26 kDa</td>
</tr>
<tr>
<td>E-cadherin</td>
<td></td>
<td>97 kDa</td>
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<tr>
<td>N-cadherin</td>
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<td>130 kDa</td>
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<tr>
<td>Vimentin</td>
<td></td>
<td>54 kDa</td>
</tr>
<tr>
<td>GAPDH</td>
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<td>35 kDa</td>
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**B**

<table>
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<tr>
<th>Protein</th>
<th>U87 si-NC</th>
<th>U87 si-PANDAR#2</th>
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<tr>
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<td>GAPDH</td>
<td></td>
<td>35 kDa</td>
</tr>
</tbody>
</table>

**Graphs**

- **CDK4**: si-NC > si-PANDAR#2
- **Bcl-2**: si-NC > si-PANDAR#2
- **E-cadherin**: si-NC > si-PANDAR#2
- **N-cadherin**: si-NC > si-PANDAR#2
- **Vimentin**: si-NC > si-PANDAR#2
- **GAPDH**: si-NC > si-PANDAR#2