Knockdown of NUSAP1 Inhibits Cell Proliferation and Invasion Through Down-Regulation of TOP2A in Human Glioblastoma

Yaotian Hu

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China

Zhiyi Xue

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China

Chen Qiu

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China. Department of Radiation Oncolog

Zichao Feng

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China

Qichao Qi

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China

Jiwei Wang

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China

Wenxing Jin

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China

Zhaoyang Zhong

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China

Xiaofei Liu

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China

Wenjie Li

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China

Qing Zhang

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China

Bin Huang

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China

Anjing Chen

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China

Jian Wang

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China. Department of Biomedicine, Univ

Ning Yang

Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, Jinan, China. Shandong Key Laboratory of Brain Function Remodeling, Jinan, China

Wenjing Zhou (✉ zhwjsdu@163.com)

Shandong Provincial Hospital affiliated to Shandong First Medical University   https://orcid.org/0000-0002-4932-9312

Research

Abstract

Background: Nucleolar and spindle associated protein 1 (NUSAP1) is an indispensable mitotic regulator, which has been reported to be involved in the development, progression, and metastasis of several types of cancer. Here, we investigated the expression and biological function of NUSAP1 in human glioblastoma multiforme (GBM).

Methods: The expression of NUSAP1 on GBM tissues and cells were determined by database analysis, immunohistochemistry and Western blot. EdU assay, transwell assay and flow cytometric analysis were performed to evaluate the effect of NUSAP1 knockdown on GBM cell proliferation, cell invasion and cell apoptosis. RNA sequencing was used to screen for downstream molecules altered in GBM cells after NUSAP1 depletion. An intracranial mice model and bioluminescent imaging were used to assess the effect of NUSAP1 on tumor growth and survival time *in vivo*.

Results: Analysis of the molecular data in CGGA, TCGA and Rembrandt datasets demonstrated that *NUSAP1* was significantly up-regulated in GBM relative to low grade gliomas and non-neoplastic brain tissue samples. Kaplan-Meier analysis indicated that patients with tumors showing high *NUSAP1* expression exhibited significantly poorer survival in both CGGA (P = 0.002) and Rembrandt cohorts (P = 0.017). Analysis of RNA sequencing data from P3-cells with stable knockdown of NUSAP1 revealed topoisomerase 2A (TOP2A) as a possible molecule down-regulated by the loss of NUSAP1. SiRNA knockdown of either NUSAP1 or TOP2A in U251, T98 and GBM derived patient P3 cells inhibited GBM cell proliferation and invasion, and induced cell apoptosis. Finally, stable knockdown of NUSAP1 with shRNA led to decreased tumor growth in an orthotopic xenograft model of GBM in mice.

Conclusions: Taken together, NUSAP1 gene silencing induced apoptosis possibly through the down-regulation of the candidate downstream molecule TOP2A. Interference with the expression of NUSAP1 might therefore inhibit malignant progression in GBM, and NUSAP1 might thus serve as a promising molecular target for GBM treatment.

Background

Glioblastoma multiforme (GBM) is the most malignant type of primary human brain tumor characterized by a high proliferation rate and an unfavorable prognosis [1–3], where the 5-year survival rate remains dismally at less than 5%[4–6]. Over the past 40 years, numerous therapeutic modalities have been implemented to enhance survival. However, due to the highly invasive nature of GBM, tumor tissue cannot be completely removed with surgery, which leads to tumor recurrence. Rigorous molecular analysis of GBM is ongoing to understand the development of the disease and its property of invasion for the identification of novel biomarkers and efficacious therapeutic avenues.

Nucleolar and spindle associated protein 1 (NUSAP1), a 55-kD vertebrate protein is localized to spindle microtubules during mitosis and an indispensable mitotic regulator[7,8]. In the normal cell cycle, the expression of NUSAP1 protein reaches its peak in the G2 phase and decreases after the cell enters the

division phase. However, high expression of NUSAP1 has been reported in several types of cancer. NUSAP1, for example, has been found to be associated with metastasis of cervical carcinoma[9] and involved in tumor recurrence in prostate cancer[10]. In breast cancer, NUSAP1 has been shown to affect the DNA damage response by controlling BRCA1 protein levels. NUSAP1 has also been identified as potential marker for breast ductal carcinoma in situ and a cause of resistance to therapy[7]. Knockout of the NUSAP1 gene inhibited cell proliferation, migration and invasion in colorectal cancer[11], indicating that up-regulation of NUSAP1 promotes cancer progression. However, whether increased NUSAP1 also has a role in driving the development of GBM remains unclear.

In this study, we investigated the expression and function of NUSAP1 in human GBM. We first analyzed molecular and clinical data in publicly available databases and found that NUSAP1 expression is significantly up-regulated in GBM tissue samples and GBM cell lines. We subsequently used knockdown strategies in models in vitro and in vivo to explore the function of NUSAP1 in GBM and to identify co-regulated molecules, such as topoisomerase 2A (TOP2A), that might mediate the function of NUSAP1. Our results thus provide a potential strategy for a new targeted therapy for the treatment of GBM.

Materials And Methods

Databases used

The Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/), the Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/), Rembrandt (http://www.betastasis.com/glioma/rembrandt/), and TargetScanHuman 5.2 (http://www.targetscan.org/vert_50/) databases were mined for relevant molecular data.

Cell lines and cultures

Human GBM cell lines (U251, T98, U87, and A172) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). P3, the in vivo propagated primary GBM tumor cell line and the GFP-luciferase stable U251 (GFP$^+$ U251) glioma cell line was kindly provided by Prof. Rolf Bjerkvig, University of Bergen. U251, T98, U87, and A172 Cells were grown in Dulbecco's modified Eagle's medium (DMEM; SH30022.01B, Gibco; Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (10082147 Hyclone; GE Healthcare Life Sciences; Pittsburgh, PA, USA), whereas P3 cells were grown in Neurobasal Medium (#21103-049, NBM; ThermoFisher Scientific; Waltham, MA,) supplemented with 2% B27 (#A3653401, ThermoFisher Scientific), 1% L-glutamine (#BE17-605E, BioNordika; Oslo, Norway), 1% penicillin/streptomycin (#17-603E, BioNordika) and 20 ng/mL EGF (#236-eg-200, R&D Systems; Minneapolis, MN) in 5% CO_2 in a humidified incubator at 37°C.

SiRNA transfections and lentiviral transduction

Gene-specific siRNAs against NUSAP1 and TOP2A (GenePharma; Shanghai, China) were transfected into U251, T98 and P3 cells for 48 h using Lipofectamine 2000 (Invitrogen, 11668-027) according to the

manufacturer's instructions. Nonspecific random sequences were used as the non-specific negative control (Si-NC). Lentiviral vectors expressing human shRNA targeting NUSAP1 (shNUSAP1, LV2017-18615, GenePharma) or the scrambled-control (shNC) were used to generate stable cell clones expressing shNUSAP1 or a nonspecific shRNA as the control. Transfected clones were selected using 1 mg/mL of puromycin (Selleckchem). Western blot analysis was used to evaluate siRNA and shRNA knockdown efficiency. Sequences of the siRNAs used are the following: NUSAP1#1, 5'-GCA GGA UCA UUC AGA GAU ATT UAU CUC UGA AUG AUU CUG CTT-3'; NUSAP1#2, 5'-GCA CCA AGA AGC UGA GAA UUU AUU CUC AGC UUC UUG GUG CTT-3'; and NUSAP1#3, 5'-GGA AAU GGA GUC CAU UGA UTT AUC AAU GGA CUC CAU UUC CTT-3'; TOP2A#1, 5'-GAC UGU CUG UUG AAA GAA UTT AUU CUU UVA ACA GAC AGU CTT -3'; TOP2A #2, 5'- CUC CUA ACU UCU AGU AAC UTT AGU UAC UAG AAG UUA GGA GTT-3'; and TOP2A #3, 5'-GAU CCA CCA AAG AUG UCA AUU UUG ACA UCU UUG GUG GAU CTT-3'; and the non-targeting control, 5'- UUC UCC GAA CGU GUC ACG A-3'.

Cell proliferation and invasion assays

The EdU incorporation assay (C103103, Ribobio; Guangzhou, China) was used to determine cell proliferation according to the manufacturer's protocol. Briefly, cells were seeded into 24-well plates at a density of 5.0×10^4 cells per well and incubated overnight. After treatment, EdU was incorporated into proliferating cells and detected through a catalyzed reaction with a fluorescently labeled azide. Five random views from images acquired with a fluorescence microscope were used to count EdU positive cells.

For invasion assays, inserts in transwell migration plates (8 μm pore size, Corning; Sigma-Aldrich; St. Louis, MO, USA) were coated with matrigel (Becton-Dickinson; Bedford, MA, USA) and incubated for 4 h at 37°C. Cells (2×10^4) in 100 μL DMEM without FBS were seeded into the upper chamber, and 600 μL of medium containing 10% FBS was added to the lower chamber. After 24 h at 37°C, cells remaining on top of the Matrigel in the upper chamber insert were removed with a cotton swab while cells that had migrated through the Matrigel to the lower side of the insert were fixed with 4% formalin for 15 min, rinsed twice with PBS, and stained with 0.1% crystal violet for 10 min. Five random views from images acquired under a light microscope were used to count migrated cells.

Immunohistochemistry and western blotting analysis

Immunohistochemical (IHC) staining of the target proteins and evaluations of the intensities of staining were performed as previously described[24]. Briefly, paraffin-embedded samples were sliced and mounted on microscopic slides. Heat-induced epitope retrieval was performed with a microwave in 10 mmol/L citric acid buffer at pH 7.2. The sections were blocked with goat serum, incubated with primary antibodies at 4°C overnight, rinsed with PBS and incubated with a horseradish peroxidase-linked goat anti-rabbit antibody. Results were visualized through the reaction with diaminobenzidine and sections were counter stained with Mayer's hematoxylin. Western blot analysis of the target proteins in glioma cells and human glioma samples was performed as previously described[25]. β-actin and GAPDH were used as the loading

controls. Primary antibodies used were the following: anti-NUSAP1 (#12024-1-AP, Proteintech; Chicago, IL, USA); anti-TOP2A (#MA5-32096; Invitrogen; Carlsbad, CA, USA); anti-MKI67 (#ab15580, Abcam; Cambridge, UK); mouse anti-β-actin (#TA-09, monoclonal, ZSGB-BIO; Beijing, China); and anti-GAPDH (#BA2913, BOSTER; Wuhan, China).

Flow cytometric analysis of apoptosis

GBM cells were seeded in 6-well plates and incubated for 72h. Floating cells were harvested, re-suspended in 1´ binding buffer, and incubated with Annexin V-FITC (BD Biosciences; San Jose, CA, USA) and propidium iodide according to the manufacturer's instructions. Apoptotic cells were detected with flow cytometry (ACEA Biosciences; San Diego, CA, USA), and the software Flowjo (Tree Star; Ashland, OR, USA) was used to analyze the data.

Intracranial xenograft model

The xenograft tumor model was generated and evaluated as previously described. Briefly, athymic mice (male; 4 weeks old; 20–30 g; Shanghai SLAC Laboratory Animal Co., Ltd; Shanghai, China) were randomly divided into four groups: U251-shNC (n = 6), U251-shNUSAP1 (n = 6), P3-shNC (n = 6) and P3-shNUSAP1 (n = 6). The mice were anesthetized with chloral hydrate and securely placed on a stereotactic frame. A longitudinal incision was made in the scalp and a 1 mm-diameter hole was drilled 2.5 mm lateral to the bregma. P3-shNC, P3-shNUSAP1, GFP-luciferase stable U251/ GFP$^+$ U251-shNC or GFP-luciferase stable U251/GFP$^+$ U251-shNUSAP1 glioma cells (2×10^5) in 10 μL of PBS were implanted 2.5 mm into the right striatum using a Hamilton syringe. GFP-luciferase stable/GFP$^+$ U251 injected mice were monitored with bioluminescence imaging every week. Mice were injected with 100 mg luciferin (Caliper/PerkinElmer; Waltham, MA, USA), anesthetized with isoflurane, and subsequently imaged with a cooled charge-coupled device camera (IVIS-200, Xenogen; Alameda, CA, USA). Bioluminescence values of tumors were quantitated using the Living Image 2.5 software package (Xenogen). Mice were euthanized when their weight dropped by more than 20% and perfused with 4% paraformaldehyde in PBS. Excised tumour tissues were formalin-fixed, paraffin-embedded, and sectioned. Sections were either stained with hematoxylin and eosin (H&E) or used for IHC.

Statistical analysis

Three independent experiments were performed, and all statistical analyses and experimental graphs were performed using GraphPad Prism 8 software (San Diego, CA, USA). X^2-tests were used to compare categorical variables, and continuous variables were analyzed using ANOVA and the Student's t-test. P-values determined from different comparisons are indicated as follows: *P < 0.05; ** P < 0.01; and *** P < 0.001. P-values 0.05 were considered statistically significant.

Results

NUSAP1 expression is up-regulated in high-grade gliomas and inversely associated with glioblastoma patient prognosis

To begin to understand the role of NUSAP1 in the development of human gliomas, we first examined expression of the gene in primary tumor samples by analyzing molecular data in publicly available datasets, including the Chinese Glioma Genome Atlas (CGGA), the Cancer Genome Atlas (TCGA) and Rembrandt. We found *NUSAP1* to be significantly up-regulated in GBMs (grade IV, high grade gliomas, HGG) compared with low grade gliomas (grade II and grade III, LGG) and normal brain tissue (NBT) (Fig. 1A). Kaplan-Meier analysis of the expression data in the CGGA and Rembrandt datasets demonstrated that high *NUSAP1* expression in tumors predicted shorter overall survival in patients (Fig. 1B).

To determine whether NUSAP1 protein levels correspondingly increased, we performed immunohistochemistry (IHC) with an antibody against NUSAP1 on paraffin-embedded glioma (grade II, n = 10); grade III, n = 10; and grade IV, n = 15) and normal brain tissue (NBT; n = 5) samples. IHC staining and quantitative analyses of mean NUSAP1 IHC staining scores showed that the NUSAP1 protein levels also increased with increasing disease grade (Fig. 1C and 1D). Western blot analysis confirmed that expression of NUSAP1 protein was significantly up-regulated in GBMs (grade IV) compared with low grade gliomas (Fig. 1E).

We also performed western blot analysis to determine NUSAP1 protein levels in GBM (U87, A172, U251, and T98) and patient-derived GBM (P3) cell lines. U251, T98, and P3 cell lines displayed higher NUSAP1 protein levels than in U87 and A172 (~ 3-5x; Fig. 1F). All together, these data demonstrated that NUSAP1 was significantly up-regulated in glioma samples and enhanced expression was correlated with disease progression in clinical glioma patients.

Down-regulation of NUSAP1 inhibits cell proliferation and invasion and promotes apoptosis in glioma cells

Further analysis of the molecular data in the CGGA revealed a strong correlation between the expression of *NUSAP1* and proliferation-associated molecules, such as *MKI67* and *PCNA*, in primary gliomas samples and recurrent gliomas samples (Fig. 2A). Therefore, we next examined the possible function of NUSAP1 in the development of human glioma in siRNA knockdown studies of NUSAP1 in U251, T98, and P3 GBM cell lines in vitro. Each of the three siRNAs targeting NUSAP1 (siN1-1, siN1-2, and siN1-3) exhibited significant knockdown of NUSAP1 as demonstrated on western blot (Fig. 2B). In EdU assays, the number of EdU positive cells decreased by ~ 30% relative to the control in all three cell lines transfected with siN1-2 and siN1-3 demonstrating that loss of NUSAP1 inhibited cell proliferation (Fig. 2C and 2D). Silencing of NUSAP1 with siN1-2 and siN1-3 also significantly inhibited invasion capabilities of U251, T98, and P3 GBM cell lines in transwell assays (~ 3·; Fig. 2E and 2F).

To identify possible cellular mechanisms involved in the inhibition of cell proliferation with loss of NUSAP1, we used flow cytometry to determine whether NUSAP1 knockdown induced apoptosis.

Knockdown with siN1-2 and siN1-3 induced early (annexin V+/PI−) and/or late (annexin V+/PI+) stage apoptosis in all three cell lines (siN1-2/siN1-3 vs siNC, U251 ~ 2.7/2.23x; T98 ~ 6.4/9.7x; P3 ~ 1.4/1.5x) (Fig. 2G). Taken together, these results indicated that NUSAP1 might promote malignant progression of GBM and thereby function as a potential oncogene.

Down-regulation of NUSAP1 inhibits mRNA and protein expression levels of TOP2A in GBM cells

To further elucidate the molecular pathways mediating the putative oncogenic activity of NUSAP1, we first performed mRNA sequencing to identify gene expression changes following the silencing of NUSAP1 in P3 cells. The results demonstrated that loss of NUSAP1 led to significant down-regulation of 8 genes, including *MT1F*, *S1PR1*, *IGFN1*, *UTP14C*, *TOP2A*, *KRT6A*, *ANO1*, and *UFSP1*, and up-regulation of 5 genes, including *SERF1B*, *ADCY10P1*, *DIRC3*, *INMT-MINDY4*, and *TMLHE-AS1*, in P3 cells (Fig. 3A and 3B). We then used the bioinformatics analysis tool TargetScanHuman 5.2 to identify potential signaling pathways associated with these genes. TargetScanHuman is a bioinformatics tool developed to aid in the prediction of the targets involved in the activities of protein or molecules. This analysis yielded a potential relationship between NUSAP1 and TOP2A (Fig. 3C). Western blot analysis confirmed a possible relationship, demonstrating that knockdown of NUSAP1 led to decreased levels of TOP2A in U251 and P3 cell lines (~ 60% and 40%, respectively; Fig. 3D). These results demonstrated that loss of NUSAP1 directly altered expression of genes involved in cell proliferation, such as *TOP2A*.

Loss of TOP2A inhibits proliferation and invasion, and induces apoptosis in GBM cells

Analysis of molecular data from the CGGA showed a strong correlation between the expression of *TOP2A* and *NUSAP1* in primary and recurrent gliomas samples (Fig. 4A). Kaplan-Meier analysis of clinical and molecular data from the CGGA revealed that high *TOP2A* expression in primary and recurrent gliomas samples predicted shorter overall survival in patients (P < 0.0001 and P = 0.015, primary and recurrent glioma samples, respectively; Fig. 4B). We therefore further investigated the role of TOP2A in the development of human glioma by knocking down TOP2A expression in U251 and P3 GBM cells with three small-interfering RNAs, siTOP2A-1, siTOP2A-2, and siTOP2A-3. All three siRNAs achieved significant knockdown of TOP2A as assessed on western blot and we therefore used siTOP2A-1 and siTOP2A-2 in functional experiments (Fig. 4C). The percentage of EdU positive cells decreased by ~ 30% in U251 and P3 cells transfected with siTOP2A-1 and siTOP2A-2 relative to the control siRNA (Fig. 4D and 4E). The number of invading cells in transwell assays also decreased significantly in U251 and P3 cells with knockdown of TOP2A (~ 50%; Fig. 4F and 4G) of glioma cells. Finally, to determine possible mechanisms contributing to the inhibition of cell growth, we used flow cytometry to determine whether TOP2A knockdown induced apoptosis. SiTOP2A-1 and siTOP2A-2 knockdown in both U251 and P3 cell lines led to up to ~ 4x and ~ 2x increase in apoptosis (Fig. 4G). Taken together, these results indicated that loss of TOP2A in GBM cells inhibited GBM cell growth by triggering apoptosis.

Down-regulation of NUSAP1 inhibits the expression of TOP2A and glioma growth and invasion *in vivo*

To investigate the effect of NUSAP1 on GBM tumor growth in vivo, we examined the effect of the loss of NUSAP1 in an orthotopic tumor model. We transduced U251 and P3 cells with a retrovirus containing an shRNA targeting NUSAP1, and implanted U251-shNC, U251-shNUSAP1, P3-shNC and P3-shNUSAP1 cells intracranially into nod-scid mice. Tumor size at 28 days was decreased in both U251- and P3-shNUSAP1 cells compared to controls based on animals' bioluminescence values (Fig. 5A). Overall survival was also enhanced in animals bearing U251- or P3-shNUSAP1 tumors compared to shNC control groups (P < 0.01, Fig. 5B). H&E staining of tumor sections showed that tumor sizes and invasion were markedly decreased in both U251- and P3-shNUSAP1 xenografts compared to controls (Fig. 5C).

Finally, immunohistochemical staining performed on xenograft sections demonstrated that MKI67 and TOP2A as well as NUSAP1 were decreased in U251- and P3-shNUSAP1 xenografts compared with U251- and P3-shNC xenografts (Fig. 5C and 5D).

Discussion

In this study, we demonstrated that NUSAP1, a gene involved in mitotic regulation, might also promote the malignant progression of human GBM. Analysis of the clinical and molecular data from CGGA, TCGA and Rembrandt databases revealed high expression of *NUSAP1* in high-grade glioma tissues relative non-neoplastic brain tissue samples. Immunohistochemistry performed on our own cohort of primary tumor samples confirmed that expression of NUSAP1 increased with increasing glioma grade. Moreover, NUSAP1 overexpression was significantly correlated with poor patient survival. In functional studies, knockdown of NUSAP1 inhibited GBM cell growth in vitro and in vivo. Finally, loss of NUSAP1 triggered apoptosis which might be due to the concomitant loss of TOP2A. These results indicate that NUSAP1 might have a role as an oncogene in the development of human GBM and may serve as a novel molecular target in therapeutic treatment of the disease.

NUSAP1 plays an important role in spindle formation, bundling microtubules, attachment to chromosomes and mitotic regulation[12,13]. It has been implicated in the development, progression, metastasis, and poor prognosis in a variety of cancer types, including non-small-cell lung cancer[14], bladder cancer[15], prostate cancer[10], astrocytoma[16], colorectal cancer[11], and cervical carcinoma[9], and is a biomarker in prostate cancer following surgery[10]. Knockout of NUSAP1 has been shown to inhibit cell proliferation, migration and invasion through loss of *DNMT1* expression in human colorectal cancer[11]. Our study also demonstrated that the knockdown of NUSAP1 with siRNA, as in the colorectal cancer model, inhibited proliferation and invasion in U251, T98, and P3 GBM cell lines. We also found that the cellular process underlying suppression of cell growth might be the induction of apoptosis.

To reveal possible pathways mediating this response, we used RNA sequencing to identify genes co-regulated with loss of NUSAP1 in the P3 GBM cell line. RNA sequencing showed that knockdown of NUSAP1 significantly down-regulated expression of 8 genes *MT1F, S1PR1, IGFN1, UTP14C, TOP2A, KRT6A, ANO1,* and *UFSP1,* and up-regulated 5 genes, including *SERF1B, ADCY10P1, DIRC3, INMT-MINDY4,* and *TMLHE-AS1.* Using TargetScanHuman 5.2, we identified potential signaling pathways associated

with these genes and showed that NUSAP1 expression was correlated with KIF11, TOP2A, NCAPG, DLGAP5, CDC20, CDK1 MKI67, NDC80, and CCNA2. Based on the results of our analysis of public databases and our RNA-seq data, we further investigated the relationship between NUSAP1 and TOP2A. Western blot analysis revealed that loss of NUSAP1 led to decreased protein levels of TOP2A in U251 and P3 GBM cells.

Topoisomerase 2A (TOP2A), a member of the DNA topoisomerases, is an enzyme that controls DNA superhelicity and unlinks double-stranded DNA segments during processes such as replication and transcription[17,18]. Many studies have shown that TOP2A plays a crucial role in the tumorigenesis and progression of a variety of malignancies, such as adrenocortical carcinoma[19], bladder cancer[20], prostate cancer[21], liver cancer[22], and breast cancer[18,23]. Analysis of the molecular data in the CGGA database confirmed that there was a strong correlation between *TOP2A* and *NUSAP1* expression in gliomas samples. High *TOP2A* expression in primary and recurrent gliomas predicted shorter overall survival in patients. In functional studies, knockdown of TOP2A in U251 and P3 GBM cells suppressed cell proliferation and invasion capabilities possibly through the induction of apoptosis. Taken together, our data indicated that TOP2A might act downstream of NUSAP1 in the development and progression of GBM.

Conclusions

In conclusion, we demonstrated that NUSAP1 inhibits proliferation and invasion of GBM cells in vitro and in vivo possibly through the triggering apoptosis. The induction of apoptosis might be due to the concomitant down-regulation of the expression of TOP2A. Thus, our study provides new insights into the possible oncogenic role of NUSAP1 in the development of human GBM and furthermore demonstrates the therapeutic relevance of silencing NUSAP1 in malignant human brain tumors.

List Of Abbreviations

Full name	Abbreviations
Nucleolar and spindle associated protein 1	NUSAP1
Glioblastoma multiforme	GBM
Topoisomerase 2A	TOP2A
siRNAs targeting NUSAP1	siN1

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Shandong University (Jinan, China). All experiments performed with human samples were approved by the Research Ethics Committee of Shandong University. Informed written consent was obtained from all participants.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare no conflicts of interest.

Funding

Authors' contributions

CQ and ZF analyzed and interpreted the expression of NUSAP1 on GBM tissues and cells. YH performed the effect of NUSAP1 knockdown on GBM cell proliferation, cell invasion and cell apoptosis. XZ evaluated the effect of TOP2A knockdown on GBM cell proliferation, cell invasion and cell apoptosis. YH, XZ and QC assessed the effect of NUSAP1 on tumor growth and survival time *in vivo.* WZ and YN analysed the RNA sequencing data in GBM cells after NUSAP1 knockout, and were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

# References

1. Alifieris, C. & Trafalis, D. T. Glioblastoma multiforme: Pathogenesis and treatment. *Pharmacol. Ther.* **152**, 63–82 (2015).

2. Minniti, G., Lombardi, G. & Paolini, S. Glioblastoma in Elderly Patients: Current Management and Future Perspectives. *Cancers* **11**, 336 (2019).

3. Avril, T. *et al.* CD90 Expression Controls Migration and Predicts Dasatinib Response in Glioblastoma. *Clin. Cancer Res.* **23**, 7360–7374 (2017).

4. Bush, N. A. O., Chang, S. M. & Berger, M. S. Current and future strategies for treatment of glioma. *Neurosurg. Rev.* **40**, 1–14 (2017).

5. Vollmann-Zwerenz, A., Leidgens, V., Feliciello, G., Klein, C. A. & Hau, P. Tumor Cell Invasion in Glioblastoma. *Int. J. Mol. Sci.* **21**, 1932 (2020).

6. Altinoz, M. A. & Elmaci, İ. Targeting nitric oxide and NMDA receptor-associated pathways in treatment of high grade glial tumors. Hypotheses for nitro-memantine and nitrones. *Nitric Oxide* **79**, 68–83 (2018).

7. Kotian, S. *et al.* NUSAP1 influences the DNA damage response by controlling BRCA1 protein levels. *Cancer Biol. Ther.* **15**, 533–543 (2014).

8. Raemaekers, T. *et al.* NuSAP, a novel microtubule-associated protein involved in mitotic spindle organization. *J. Cell Biol.* **162**, 1017–1029 (2003).

9. Li, H. *et al.* Nucleolar and spindle associated protein 1 promotes metastasis of cervical carcinoma cells by activating Wnt/β-catenin signaling. *J. Exp. Clin. Cancer Res.* **38**, (2019).

10. Gulzar, Z. G., McKenney, J. K. & Brooks, J. D. Increased expression of NuSAP in recurrent prostate cancer is mediated by E2F1. *Oncogene* **32**, 70–77 (2013).

11. Han, G. *et al.* NUSAP1 gene silencing inhibits cell proliferation, migration and invasion through inhibiting DNMT1 gene expression in human colorectal cancer. *Exp. Cell Res.* **367**, 216–221 (2018).

12. Wadia, P. P. *et al.* Antibodies specifically target AML antigen NuSAP1 after allogeneic bone marrow transplantation. *Blood* **115**, 2077–2087 (2010).

13. Nie, J., Wang, H., He, F. & Huang, H. Nusap1 is essential for neural crest cell migration in zebrafish. *Protein Cell* **1**, 259–266 (2010).

14. Xu, Z. *et al.* NUSAP1 knockdown inhibits cell growth and metastasis of non-small-cell lung cancer through regulating BTG2/PI3K/Akt signaling. *J. Cell. Physiol.* **235**, 3886–3893 (2020).

15. Gao, S. *et al.* Nucleolar and Spindle Associated Protein 1 (NUSAP1) Promotes Bladder Cancer Progression Through the TGF-β Signaling Pathway. *OncoTargets Ther.* **Volume 13**, 813–825 (2020).

16. Wu, X. *et al.* Nucleolar and spindle associated protein 1 promotes the aggressiveness of astrocytoma by activating the Hedgehog signaling pathway. *J. Exp. Clin. Cancer Res.* **36**, (2017).

17. Drlica, K. & Zhao, X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol. Mol. Biol. Rev. MMBR* **61**, 377–392 (1997).

18. Seoane, J. A., Kirkland, J. G., Caswell-Jin, J. L., Crabtree, G. R. & Curtis, C. Chromatin regulators mediate anthracycline sensitivity in breast cancer. *Nat. Med.* **25**, 1721–1727 (2019).

19. Jain, M. *et al.* TOP2A is overexpressed and is a therapeutic target for adrenocortical carcinoma. *Endocr. Relat. Cancer* **20**, 361–370 (2013).

20. Zeng, S. *et al.* Prognostic value of TOP2A in bladder urothelial carcinoma and potential molecular mechanisms. *BMC Cancer* **19**, (2019).

21. de Resende, M. F. *et al.* Prognostication of prostate cancer based on TOP2A protein and gene assessment: TOP2A in prostate cancer. *J. Transl. Med.* **11**, 36 (2013).

22. Liu, L.-M. *et al.* DNA topoisomerase 1 and 2A function as oncogenes in liver cancer and may be direct targets of nitidine chloride. *Int. J. Oncol.* (2018) doi:10.3892/ijo.2018.4531.

23. Yang, Z. *et al.* Suppression of PTEN/AKT signaling decreases the expression of TUBB3 and TOP2A with subsequent inhibition of cell growth and induction of apoptosis in human breast cancer MCF-7 cells via ATP and caspase-3 signaling pathways. *Oncol. Rep.* **37**, 1011–1019 (2017).

24. Zhou, W. *et al.* Matrine induces senescence of human glioblastoma cells through suppression of the IGF1/PI3K/AKT/p27 signaling pathway. *Cancer Med.* **7**, 4729–4743 (2018).

25. Zhou, W. *et al.* Oxidative stress induced autophagy in cancer associated fibroblast enhances proliferation and metabolism of colorectal cancer cells. *Cell Cycle* **16**, 73–81 (2017).
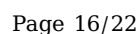
Figures
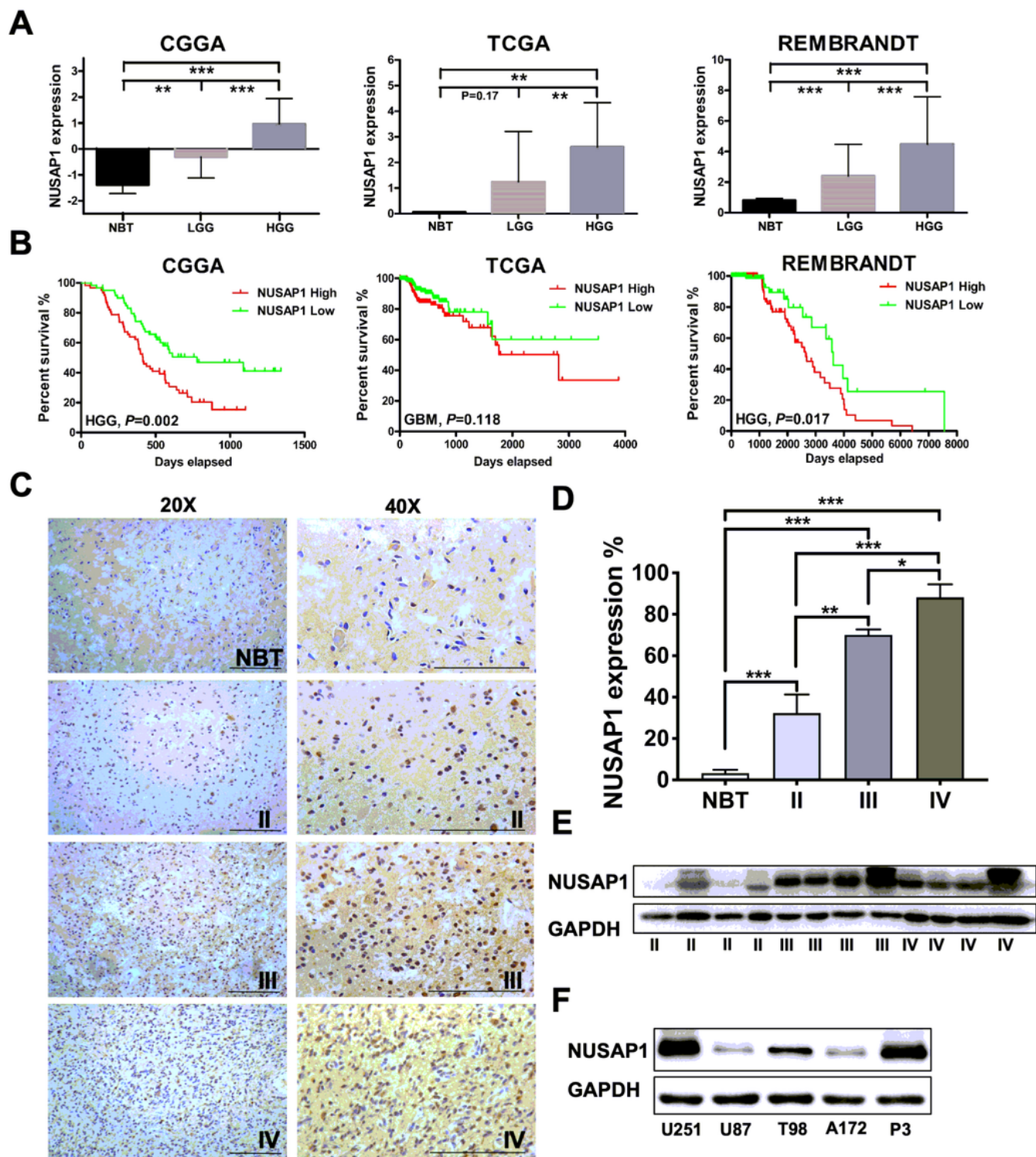
Figure 1

NUSAP1 expression is up-regulated in high grade gliomas and inversely associated with glioblastoma patient prognosis. A. Relative expression levels of NUSAP1 mRNA in normal brain (NBT), low grade glioma (LGG) and high grade glioma tissue samples from the CGGA, TCGA and Rembrandt databases. **P <0.01 and ***P <0 .001. B. Kaplan-Meier analyses showing differences in overall survival for patients with low and high NUSAP1 expressing gliomas from CGGA, TCGA and Rembrandt databases. C.

Representative images of immunohistochemical staining for NUSAP1 in 4-µm sections from normal brain tissue samples (NBT), and grade II, III and IV (GBM) gliomas. Scale bar = 100 µm. D. Immunohistochemical scores for NUSAP1 in normal brain tissue samples (NBT), and grade II, III, and IV (GBM) gliomas. Five random fields from each section were counted. Data are shown as the mean ± SD. *P < 0.05, **P <0 .01 and ***P < 0.001. E. Western blot analysis of NUSAP1 levels in grade II, III and IV gliomas. F. Western blot analysis of NUSAP1 levels in U87, A172, U251 ,T98 and GBM patient derived P3 cell lines.

Figure 2

Down-regulation NUSAP1 expression inhibits glioma cell proliferation and invasion, and induces apoptosis. A. Correlation between mRNA expression of NUSAP1 and proliferation-related molecules MKI67 and PCNA in primary and recurrent gliomas samples in the CGGA database. B. Western blot to confirm knockdown efficiency of NUSAP1 with siRNA in U251, T98, and P3 cells. C. Fluorescence microscopy of EdU incorporation in U251, T98, and P3-siN1-2,-3 siRNAs. Pollo 567 (red colour) detects EdU, while DAPI (blue colour) stains nuclei. Scale bar = 50 μm. D. Statistical analysis of the number of EdU-positive cells for U251, T98, and P3 cell lines transfected with siRNAs. All data are expressed as the mean ± SD of values from experiments performed in triplicate. **$P < 0.01$. E. Transwell invasion results of si-NC/N1-2,-3 in U251, T98 and P3 GBM cells for 24 h. Scale bar = 100 μm. F. Statistical results of the invasive ratio of si-NC/N1-2, -3 in U251, T98 and P3 GBM cells for 24 h. **$P < 0.01$. G. Flow cytometric analysis to detect apoptosis in U251, T98, and P3-siN1-2, -3 siRNA cells as determined by annexin V- and/or FITC and propidium iodide staining for DNA content. The percentages of annexin V-and/or FITC-positive cells are indicated.
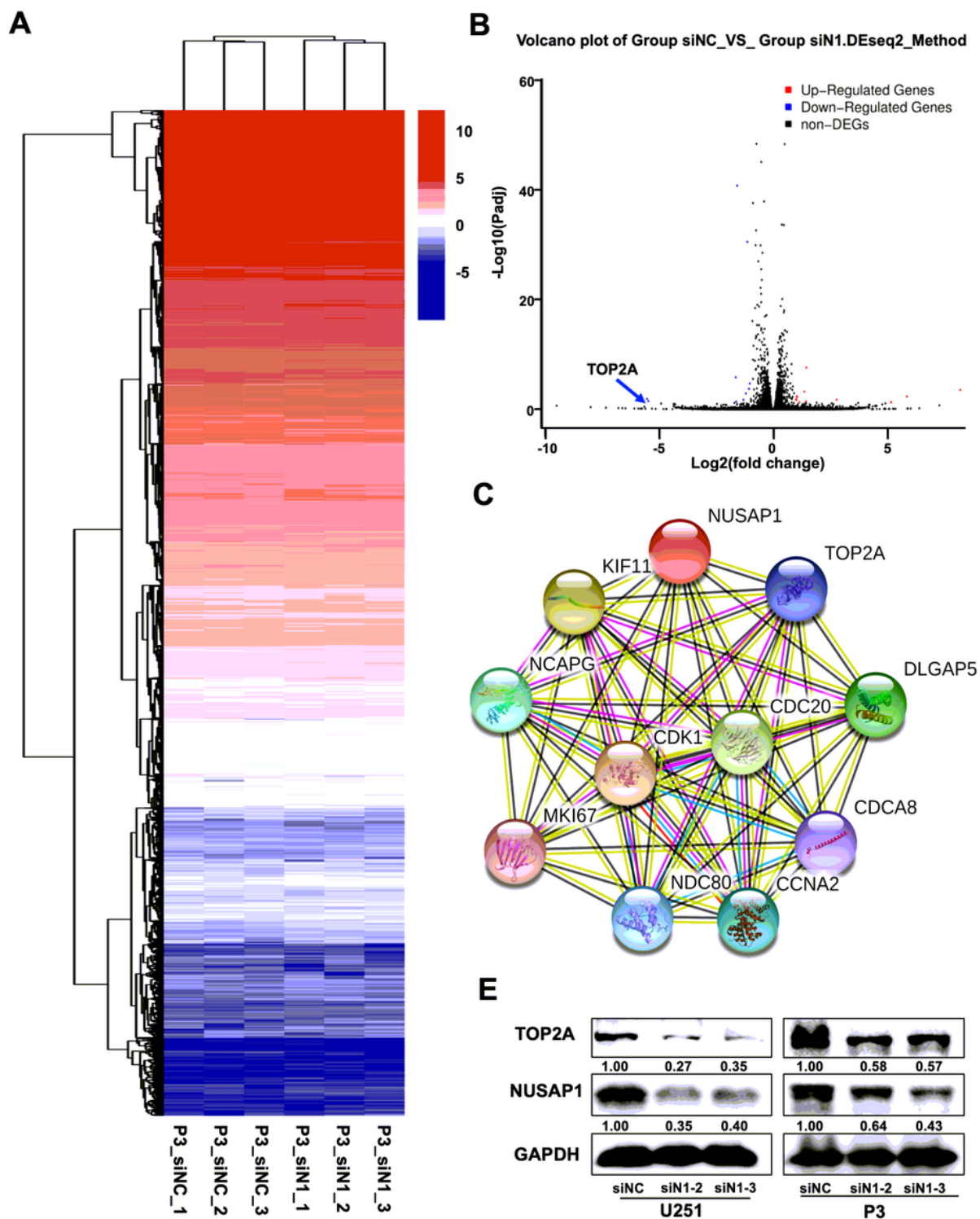
Figure 3

Down-regulation of NUSAP1 inhibits mRNA and protein expression levels of TOP2A in GBM cells. A. Hierarchical clustering based on the differentially expressed genes obtained through RNA sequencing of P3 siNC and P3 siN1 cells. B. Significantly up- or down-regulated genes identified through analysis of RNA sequencing data obtained from stable knockdown of NUSAP1 in P3 cells. C. TargetScanHuman, a bioinformatics analysis tool was used to predict the targets involved in the activities of protein or

molecules. D. Western blot analysis of NUSAP1 and TOP2A levels in U251 and P3 cell lines transfected with NUSAP1 siRNAs.
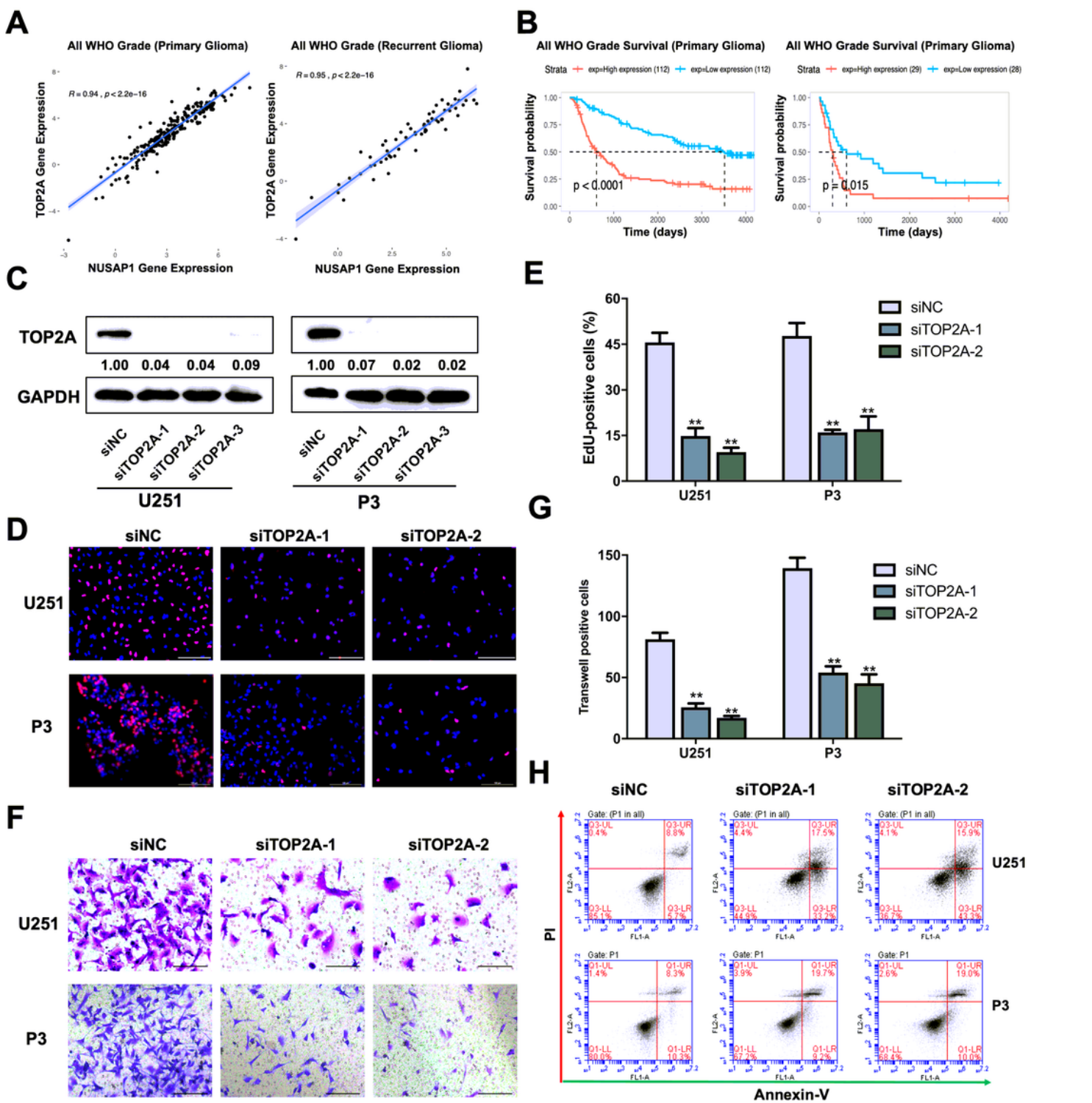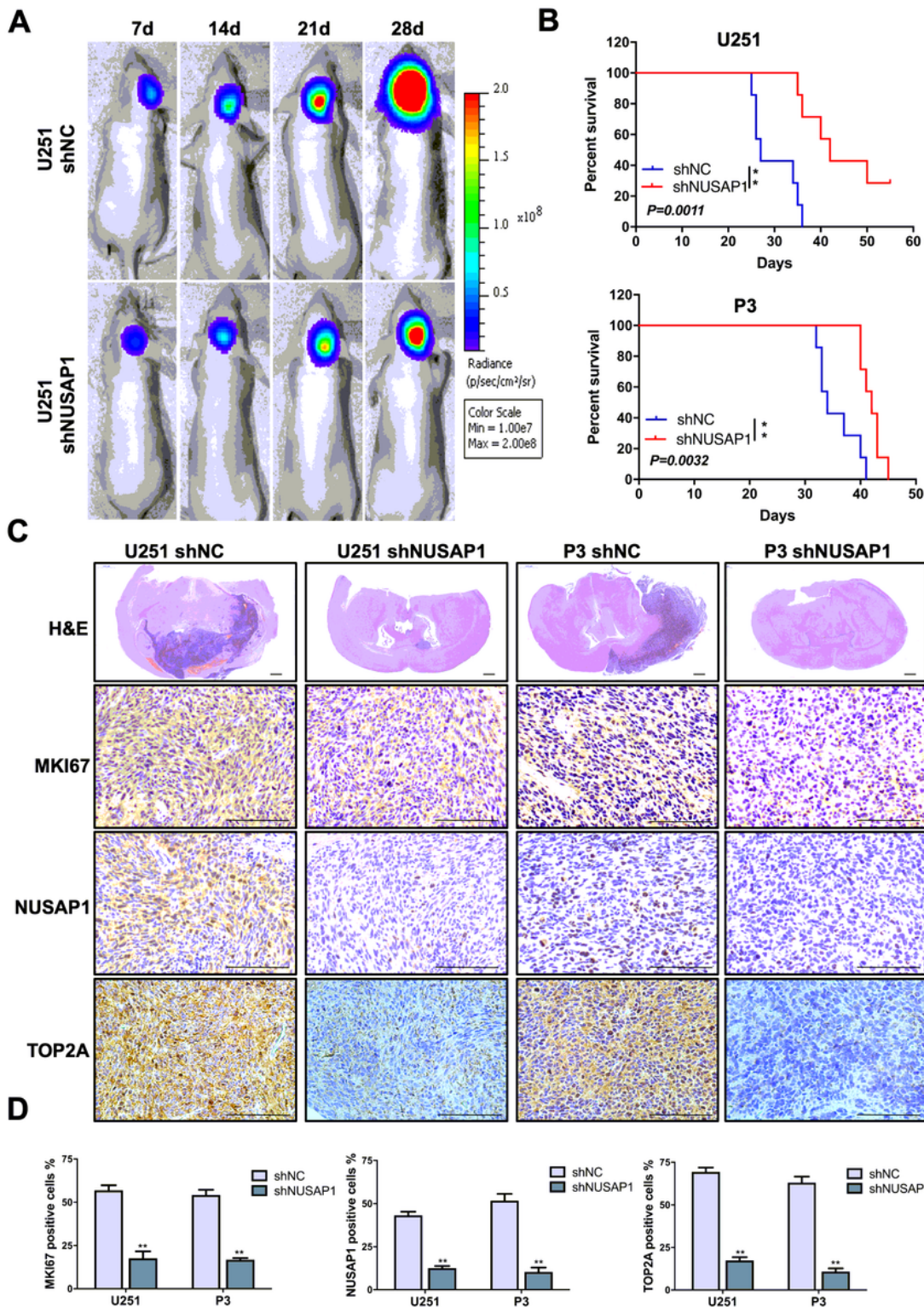

Figure 4

Down-regulation TOP2A expression inhibits glioma cell proliferation and invasion, and induces apoptosis in glioma cells. A. Correlation between NUSAP1 and TOP2A mRNA expression levels in primary and recurrent gliomas samples in the CGGA database. B. Kaplan-Meier analyses showing differences in

overall survival for patients with low and high TOP2A expressing gliomas. The data were obtained from CGGA databases. C. Western blot to confirm siRNA knockdown efficiency of TOP2A in U251 and P3 cells. D. Fluorescence microscopy of EdU incorporation in U251 and P3-siTOP2A-1, -2 siRNAs. Pollo 567 (red color) detects EdU, while DAPI (blue colour) stains nuclei. Scale bar = 50 µm. E. Statistical analysis of the number of EdU-positive cells for U251 and P3 cell lines transfected with siRNAs. All data are expressed as the mean ± SD of values from experiments performed in triplicate. **P < 0.01. F. Transwell invasion results of siNC/TOP2A-1, -2 U251 and P3 GBM cells for 24 h. Scale bar = 100 µm. G. Statistical results of the invasive ratio of siNC/TOP2A-1, -2 in U251 and P3 GBM cells for 24 h. **P < 0.01. H. Flow cytometric analysis of apoptosis in U251- and P3-siTOP2A-1,-2 siRNAs cells as determined with annexin V-and/or FITC and propidium iodide staining for DNA content. The percentages of annexin V-and/or FITC-positive cells are indicated.

Figure 5

Down-regulation of NUSAP1 inhibits the expression of TOP2A, and leads to reduced glioma growth and invasion in vivo. A. U251 cells expressing luciferase were orthotopically implanted into athymic nude mice. Tumor growth was monitored using the IVIS-200 imaging system for detection of bioluminescence. Bioluminescent signals were measured at days 7, 14, 21, and 28 after implantation. B. Kaplan-Meier survival analysis of overall survival, and log rank analysis to assess the statistical significance of the

differences. C. Representative images of H&E staining and immunohistochemical staining for NUSAP1, MKI67, and TOP2A in xenograft sections from mice injected with U251- and P3-shNC or U251- and P3-shNUSAP1 cells. Scale bar = 100 μm. D. Graphic representation of IHC scoring for NUSAP1, MKI67, and TOP2A expression in xenograft sections. All data are presented as the mean ± SD, **P < 0.01.