Dishevelled-associated antagonist of β-catenin homolog 3 (DACT3) suppresses glioma progression though Notch1 signaling pathway in β-catenin-dependent manner

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A B S T R A C T

The disheveled-associated antagonist of β-catenin homolog 3 (DACT3) has been recognized as a tumor suppressor in various cancers. However, the function of DACT3 on glioma malignant progression along with potential molecular mechanisms is poorly clarified. This research aimed to investigate how DACT3 contributes to suppressing the progression of glioma. In our investigation, a pronounced decrease in DACT3 expression was observed in glioma tissues. Through the over-expression of DACT3, we noted a significant suppression in the proliferation, invasion, and migration of glioma cells, while concurrently observing an increase in cell adhesion. Our exploration into the molecular mechanisms revealed that DACT3 executes its tumor-suppressive role by impeding the expression of notch 1 intracellular domain (NICD) and translocating into the nucleus by downregulating the expression of β-catenin. Consequently, this process leads to the suppression of Notch1 signaling. To summarize, our findings reveal the function of DACT3 to inhibit glioma progression via the Notch1 signaling pathway in β-catenin dependent manner. This study stands as the pioneer in examining the role of DACT3 in glioma progression and comprehensively elucidating its molecular mechanisms in glioma development. Therefore, our results suggest that DACT3 holds promise as both a prognostic factor and a potential biomarker for guiding treatment strategies in glioma patients (Graphical Abstract).

1. Introduction

Malignant glioma, is the most common malignant tumor of the central nervous system (CNS), accounting for 25.1 % of all CNS tumors and 80.8 % of all CNS malignancies [1], pose a significant challenge due to their highly lethal nature [2]. Gliomas present

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difficulties in treatment due to their infiltrative growth and rapid proliferation, resulting in therapeutic resistance and swift recurrence [2,3]. Despite significant advancements in treatments have been made over the past decade, the prognosis for glioma patients remains discouraging [4,5], with a median survival of about 14–17 months for malignant glioma [6]. While considerable progress has been made in identifying genes and pathways crucial to glioma progression, a comprehensive understanding of the molecular mechanisms is still lacking, hindering the development of effective therapies [7]. Therefore, urgent exploration of new therapeutic strategies and targets is essential to enhance anti-glioma efficacy.

The DACT (Dishevelled-associated antagonist of β-catenin) family, encompassing DACT1, DACT2, and DACT3 [8], serves as a negative regulator of Wnt/β-catenin signaling pathway, playing critical roles in cell homeostasis maintenance and development [9]. Over the past decade, research has identified DACT's role as a tumor suppressor gene in various cancers [10–13]. Notably, our prior study revealed a decreased in DACT2 expression in glioma, correlating with tumor grade and prognosis [14]. This study demonstrated that DACT2 overexpression inhibited glioma cell proliferation, while enhancing cell apoptosis and sensitivity to temozolomide [14]. These data built the platform for the research of DACT functions in glioma cells that require detailed mechanistic studies and functional assignment of DACT proteins in glioma. Despite evidence suggesting DACT3's involvement in tumor progression through the regulation of biological processes such as autophagy, proliferation, and invasion [15–17], its specific role in glioma progression remains unclear and necessitates further exploration.

The Notch signaling pathway, an evolutionarily conserved pathway, governs essential cellular processes, such as proliferation, apoptosis, and maintaining homeostasis [18,19]. Due to its pleiotropic functions, genetic alterations in the components of the Notch pathway have been implicated in many aspects of tumor development [20–22]. In glioma, Notch signaling has been shown to significantly impact aggressiveness [23,24]. Importantly, there exists an intricate relationship between the Wnt and Notch signaling pathways, often co-expressing their genes [25,26]. This intricate crosstalk occurs at multiple cellular levels, including receptor, nucleus, and cytoplasm, impacting cell fate decisions [26–29]. Although it is well-documented that Notch signaling is influenced by Wnt components [30,31], the extent to which DACT3 modulates Notch signaling by suppressing β-catenin remains a relatively unexplored area.

In this study, we further establish DACT3 as a tumor-suppressive gene of glioma and elucidate its role in inhibiting glioma progression through the Notch1 signaling pathway in a manner dependent on β-catenin. These findings suggest that DACT3 might serve as a prognostic factor and potential biomarker for glioma treatment.

2. Materials and method

All antibodies, reagents, vendors, catalog numbers, and concentrations or dilution ratios are shown in detail in Supplementary Table 1.

2.1. Patients and tissue Preparation

A total of 40 glioblastomas (GBM) and 8 brain tissues were obtained between 2019 and 2021 in the Guizhou Provincial People's Hospital for immunohistochemistry. Additionally, eight GBM and the corresponding para-tumor tissues were also obtained for Western blot analysis. Inclusion criteria: 1) GBM patients with confirmed pathology, 2) GBM patients with complete data records. Exclusion criteria: 1) GBM patients with unconfirmed pathology, 2) GBM patients with incomplete data records. They were approved by the Ethics Committee of Guizhou Provincial People's Hospital (Number: (2019)122, date: 2019/5/6). All the patients didn't receive chemotherapy or radiotherapy before surgery. Chinese Glioma Genome Atlas (CGGA) database analysis, gene expression data of glioma tissues, and baseline clinical data were downloaded from the publicly available CGGA website (http://www.cgga.org.cn/).

2.2. Plasmids and antibodies

Expression plasmids for DACT3, NICD, and β-catenin were generated by polymerase chain reaction (PCR) and cloning into LentiORF pLEX-MCS Vector (Invitrogen, USA). The shRNA system (pLKO-neo from Addgene) was used to knockdown endogenous DACT3 and β-catenin. The sequences of sense and antisense of DACT3 shRNA were 5′-AATTGTTCGGGTTCTCTCA

AGGTCACTCGAGTGACCTTGAGAGAACCCGAACTTTTTTTAT-3′ and 5′-AAAAAAAGTT-
CGGGTTCTCTCAAGGTCACTCGAGTGACCTTGAGAGAACCCGAAC-3'; The sequences of sense and antisense of β-catenin shRNA were: 5′-AATTGCTGCTATGTTCCCTGAGACACTCG-
AGTGTCTCAGGGAACATAGCAGCTTTTTTTAT-3′ and 5′-AAAAAAAGCTGCTAT-GTTCC-
CTGAGACACTCGAGTGTCTCAGGGAACATAGCAGC-3'. Constructs were confirmed by DNA sequencing and by immunoblotting for protein expression. p-β-catenin, Notch 1, NICD, β-catenin, Hes-1, MMP2, and GAPDH antibodies were purchased from Cell Signaling Technology (USA); VCAM-1, ICAM-1, and DACT3 antibodies were purchased from Santa Cruz (USA); MMP9 and Ki67 antibodies were purchased from Abcam (UK); Alexa Fluor 488-conjugated secondary antibodies and HPR-conjugated secondary antibodies (anti-mouse and rabbit) were purchased from Invitrogen (USA) and Cell Signaling Technology (USA), respectively.

2.3. Cell culture and transfections

GBM cell lines U118 (TCHu216), U251 (TCHu58), and U87 (TCHu138) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). GBM cell line T98 (CL-0583) was obtained from Procell Life Science &Technology (Wuhan, China). Above

four GBM cell lines were maintained in DMEM (Gibco, USA) supplemented with 10 % FBS (Gibco, USA) and 1 % penicillin/streptomycin (Beyotime, China) at 37 °C in 5 % CO2. Recombinant lentiviral particles were produced following a standard protocol [14,32]. For transduction, U118 and U251 GBM cell lines were maintained at 60 % confluence in six-well plates and viral solutions were added into a cell culture medium that contains 8 μg/mL polybrene (Solarbio, China). Over-expressing cells were selected under puromycin (Beyotime, China), and knockdown cells were selected using neomycin (Beyotime, China).

2.4. CCK8 assay

Cell proliferation ability was measured by CCK-8 assay (Beyotime, China). According to a previous study [14]. Glioma cells were resuspended and seeded into a 96-well plate at 5000 cells/well. Glioma cells were incubated at 37 °C for 1 h after the addition of 10ul CCK-8 working solution in each well. The absorbance at 450 nm was confirmed at 0, 24, 48, 72, and 96 h with an enzyme-linked instrument.

2.5. Colony formation assay

Following a previous study [11], glioma cells were cultured in 6-well plates at a density of 500 cells/well. After being cultured for 10–14 days, they were stained with crystal violet (Beyotime, China) after 4 % paraformaldehyde fixation. ImageJ software was used to count visible colonies (× 50 cells).

2.6. Cell adhesion assay

According to previous studies [33,34]. The 24-well plates were coated with 400ul 20ug/ml fibronectin (Solarbio, China) and were incubated for 2h. After washing with PBS, added 500ul 1 % sterile BSA (Beyotime, China) to the 24-well plate and incubated for another 2h. Glioma cells were detached by trypsin and suspended in a DMEM medium. Then, cells were added into 24-well plates and left for 60 min. Subsequently, the remaining adherent glioma cells were fixed with 4 % paraformaldehyde after washing off non-adherent cells. Adherent glioma cells were counted microscopically.

2.7. Transwell migration assay

The suspension containing 1×10^5 glioma cells were added to the upper chamber of 24-well (Corning, USA). Medium containing 1 % penicillin/streptomycin (Beyotime, China) and 10 % FBS (Gibco, USA) was added to the lower chamber. After incubation for 24h at 37 °C, glioma cells were fixed with 4 % paraformaldehyde, then stained with 0.1 % crystal violet (Beyotime, China). Migrated glioma cells were counted under the microscope.

2.8. Transwell invasion assay

Following previous studies [14,32]. The upper chambers were coated with Matrigel (BD, USA) and then maintain at 37 °C for half an hour. 2×10^5 glioma cells were resuspended with serum-free growth medium and added to the upper chambers. The left steps are the same as the transwell migration assay. The number of invasive glioma cells was counted microscopically.

2.9. Western blot

Total proteins were extracted from homogenized cells in a lysis buffer (Solarbio, China) containing a protease inhibitor cocktail (Solarbio, China). Nuclear and cytoplasmic proteins were extracted from homogenized cells using nuclear and cytoplasmic protein extraction kits (Solarbio, China), respectively. Equal amounts of protein per sample were separated by SDS-PAGE (8 % or 10 %) and electrotransferred to a polyvinylidene difluoride membrane (Millipore, USA). Membranes were blocked with 5 % BSA (Beyotime, China) for 1h at room temperature, and rinsed three times (10 min/time) with 0.5 % PBST (0.5 mL Tween-20 in 1L 0.01 M PBS). Next, incubated with the specified primary antibodies at 4 °C overnight, rinsed three times (10 min/time) with 0.5 % PBST, and then 1 h with HRP-conjugated goat anti-rabbit or anti-mouse IgG, rinsed three times (10 min/time) with 0.5 % PBST, followed by detection using an enhanced chemiluminescence kit (Millipore, USA).

2.10. Immunohistochemistry

The glioma tissues were embedded with paraffin, then glioma tissue sections were incubated with specific antibodies (DACT3, Ki67, MMP2, MMP9, VCAM-1, ICAM-1). Pictures were obtained with a digital camera (Olympus, Japan).

2.11. Immunofluorescence

Glioma cells were seeded on slides in 12-well plates for 24h. Cells were fixed with 4 % paraformaldehyde, incubated in 0.3 % Triton X-100 (Solarbio, China), then blocked with 5 % goat serum (Beyotime, China). Slides were stained with primary antibodies, followed by Alexa Fluor 488-conjugated secondary antibodies (Invitrogen, USA) incubation and covered with mounting medium containing

DAPI (Invitrogen, USA) in a dark environment. Images were captured under a fluorescent microscope (Olympus, Japan).

2.12. Mouse xenografts

Following previous studies [14,27]. Glioma Cells were subcutaneously injected into 5-week-old male nude mice. Tumor volumes were measured using a caliper every 7 days (Formula: $V = 0.52 \times L \times W^2$), and 5 nude mice were measured in each group at each time point. The mice were perfused after 28 days and tumor tissues were excised and weighed, which was used for immunohistochemistry.

2.13. Statistical analysis

All data were analyzed by SPSS 23.0 software and GraphPad Prism 9. All data were presented as mean $\pm$ SD and tested for normal distribution by Shapiro–Wilk test. For normally distributed data, differences between the two groups were compared by independent-sample t-tests. For gaussian distributed data, differences between the two groups were compared by non-parametric equivalent. Meanwhile, differences among multiple means were assessed by one-way, two-way, or repeated-measure of ANOVA, followed by a Bonferroni test whenever appropriate. The survival rate was analyzed by the Kaplan–Meier method and compared by the log-rank test. $p < 0.05$ was considered statistically significant.
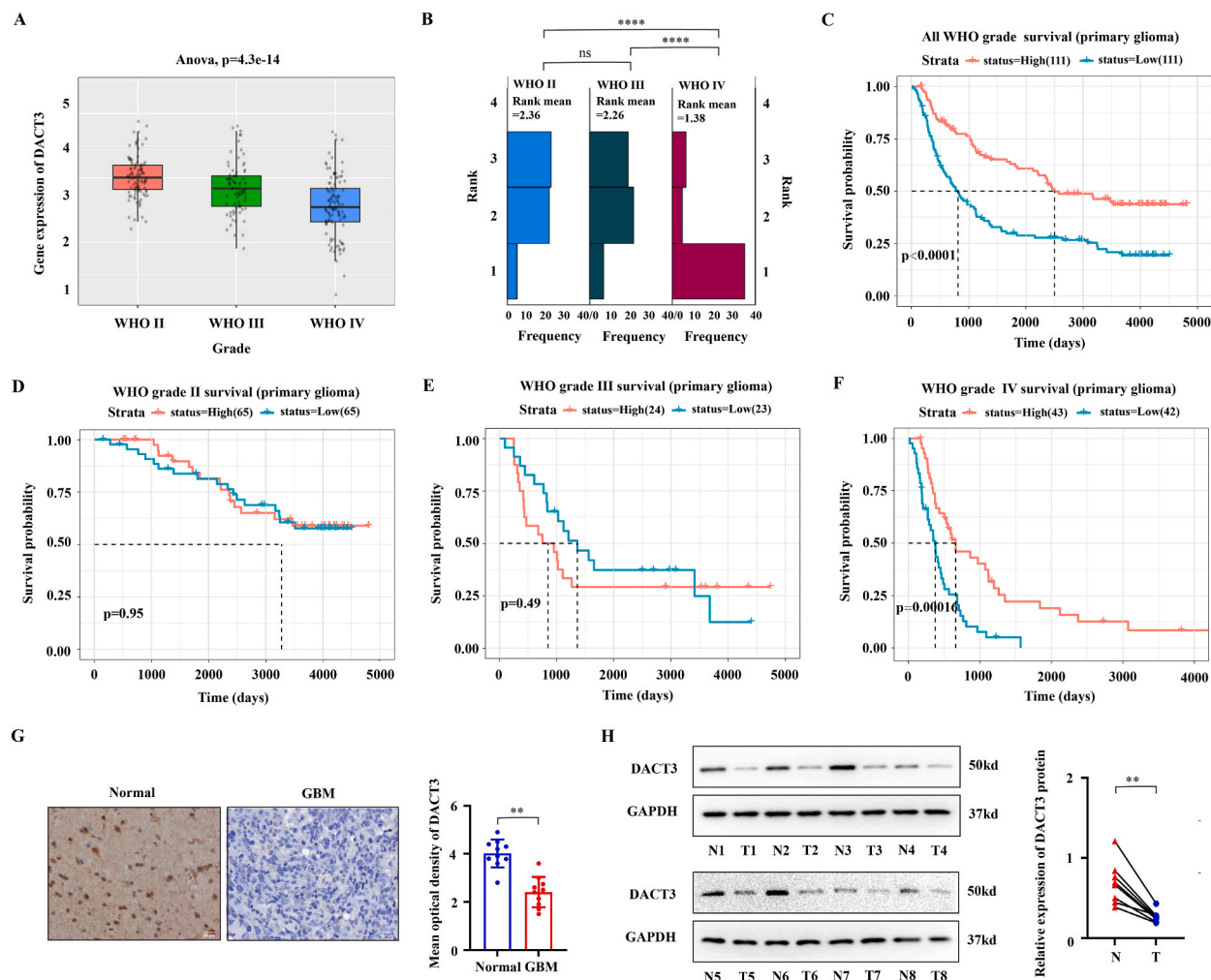

Fig. 1. Low DACT3 expression in glioma tissues predicts a poor prognosis. A. The CGGA datasets shows DACT3 expression was decreased in glioma as the grade increased. **B.** After conducting ANOVA, Bonferroni post-hoc analyses were performed to pinpoint the specific group differences. **C.** Survival analysis of patients with all WHO grade glioma from the CGGA datasets. **D. E. F.** Survival curves comparing DACT3 levels in WHO grade II-IV of glioma from the CGGA datasets. **G.** Immunostaining shows DACT3 expression in normal brain tissues GBM tissues (400×, n = 10). **H.** Western blot shows DACT3 expression in 8 pairs of GBM tissues (T) compared to paired adjacent brain tissues (N) (n=3, The full, non-adjusted images are shown in Supplement file). CGGA: Chinese Glioma Genome Altas; DACT3: Dishevelled-associated antagonist of β-catenin homolog 3; WHO: World Health Organization; GBM: glioblastoma; (ns : no significance; ** $P < 0.01$; **** $P < 0.0001$).
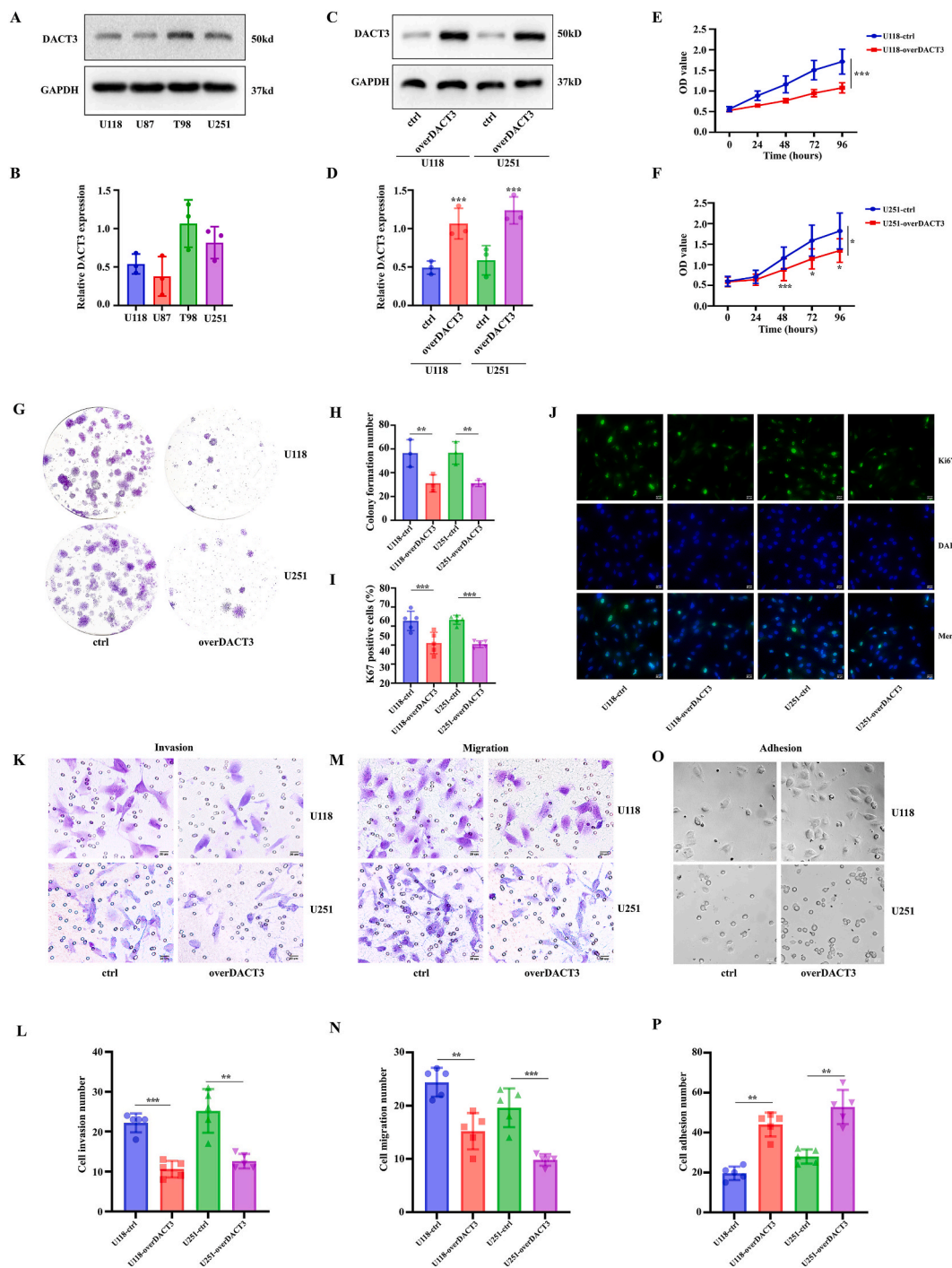
Fig. 2. Overexpression of DACT3 suppresses progression of glioma cells in vitro. A. Western blot analysis shows endogenous expression of DACT3 in four glioma cell lines (U87, U118, T98, U251, n=3, The full, non-adjusted images are shown in Supplement file). **B.** Expression levels of DACT3 proteins in four glioma cell lines were quantified and normalized to GAPDH levels (n=3). **C.** Western blot analysis demonstrates DACT3 over-expression in U118 and U251 glioma cells (n=3, The full, non-adjusted images are shown in Supplement file). **D.** Expression levels of DACT3 proteins in DACT3 over-expressing glioma cells were quantified and normalized to GAPDH levels (n=3). **E-J.** Cell proliferation was detected in U251 and U118 glioma cells by CCK-8 assay (**E. F**), colony formation assay (**G. H**) and Ki67 immunofluorescence assay (**I. J**) (400×, n=3). **K-N.** Invasive (**K. L**) and migrative (**M. N**) ability are tested in U251 and U118 glioma cells by transwell assay (400×, n=3). **O. P.** Adhesion ability is tested in U251 and U118 glioma cells by Cell adhesion assay (400×, n=3). DACT3: Dishevelled-associated antagonist of β-catenin homolog 3; ctrl: transfected with empty vectors; overDACT3: transfected with DACT3vectors. (* $P<0.05$, ** $P < 0.01$, *** $P < 0.001$; * ctrl vs overDACT3).

3. Result

The aim of this study is to test the hypothesis that DACT3 can regulate Notch signaling by suppressing β-catenin. We investigated the effects of DACT3 on glioma *in vitro* and *in vivo*, including cell proliferation, cell migration, and invasion. Furthermore, we demonstrated that DACT3 suppresses glioma progression by inhibiting the Notch1 signaling pathway in β-catenin dependent manner (The full, non-adjusted images of Western blot are shown in Supplement file).

3.1. DACT3 expression correlates with the prognosis of glioma patients

To examine DACT3 mRNA expression in clinical gliomas, the RNA-Seq data from the Chinese Glioma Genome Altas (CGGA) were analyzed (http://www.cgga.org.cn/analyse/RNA-data.jsp). The results revealed that the transcriptional level of DACT3 correlated
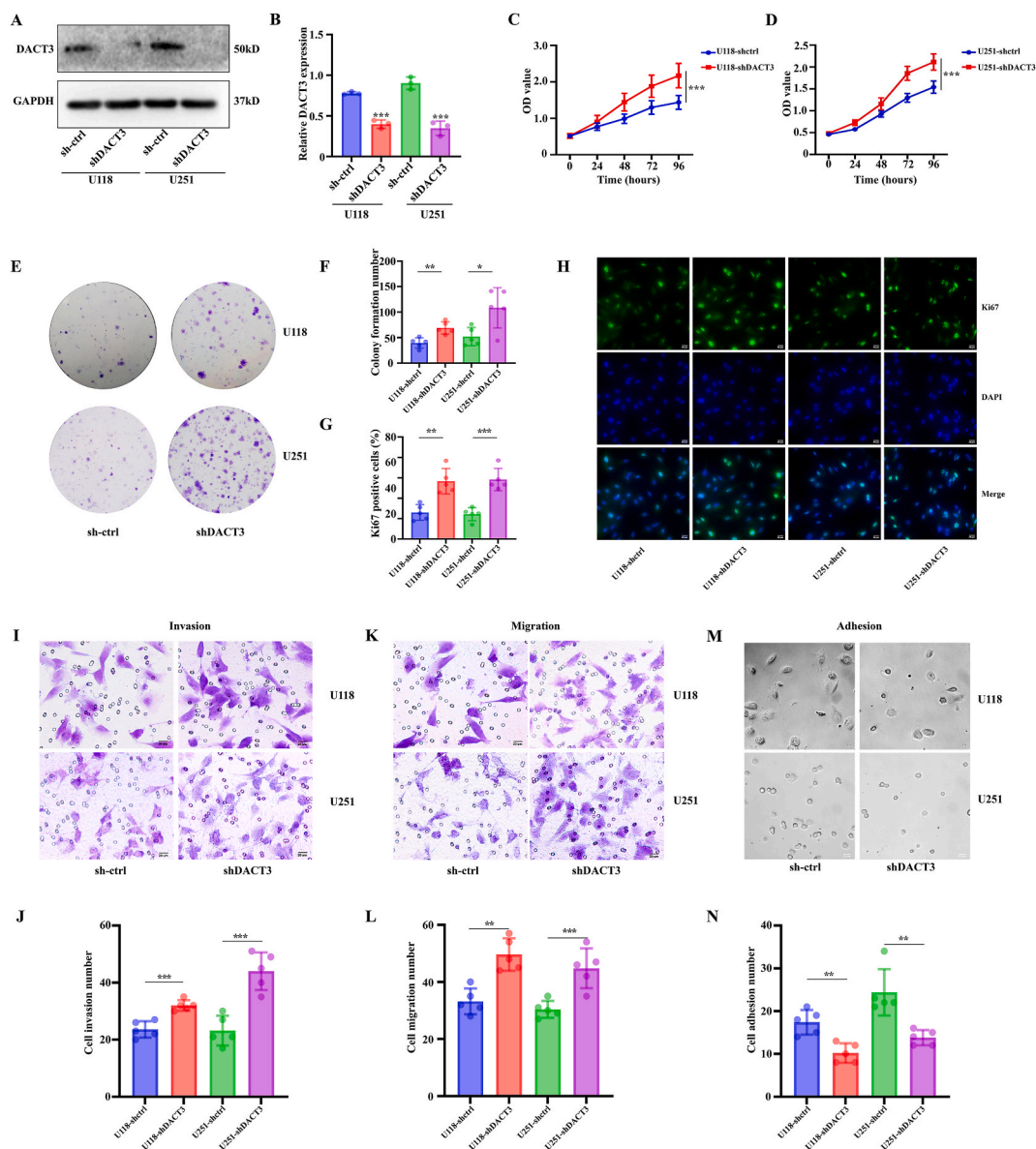

Fig. 3. Down-expression of DACT3 promotes progression of glioma cells in vitro.A. B. Western blot analysis demonstrates DACT3 expression in U251 and U118 glioma cells (n=3, The full, non-adjusted images are shown in Supplement file). **C-H.** Cell proliferation was detected in U251 and U118 glioma cells by CCK-8 assay (**C.D**), colony formation assay (**E. F**) and Ki67 immunofluorescence (**G. H**) (400×, n=3). **I-L.** Invasive (**I. J**) and migrative (**K. L**) ability are tested in U251 and U118 glioma cells by transwell assay (400×, n=3). **M. N.** Adhesion ability is tested in U251 and U118 glioma cells by Cell adhesion assay. (400×, n=3). DACT3: Dishevelled-associated antagonist of β-catenin homolog 3; sh-ctrl: transfected with empty vectors; shDACT3: transfected with shDACT3 vectors. (* *P*<0.05, ** *P* < 0.01, *** *P* < 0.001; * shctrl vs shDACT3,).

with the glioma histopathologic grade, and DACT3 expression was significantly decreased in high-grade gliomas (Fig. 1A). After conducting ANOVA, post-hoc analyses were performed to pinpoint the specific group differences (Fig. 1B). Bonferroni post-hoc test revealed that WHO IV had significantly lower DACT3 expression compared to WHO III ($p < 0.0001$), and WHO II ($p < 0.0001$). However, there was no significant difference between WHO II and WHO III ($p > 0.05$). Survival analysis showed that patients with glioma and low DACT3 expression had shorter survival times (Fig. 1C). In higher-grade gliomas (WHO IV), patients with low DACT3 expression had significantly shorter survival times. However, in patients with WHO II or III grade, DACT3 expression did not show a clear link to patient survival (Fig. 1D–F).

We further compared DACT3 protein expression in GBM and brain normal tissues by immunohistochemistry (IHC) staining, which showed that DACT3 expression in GBM was notably lower than that in normal brain tissues (Fig. 1G). Similarly, Western blot results indicated that DACT3 protein in GBM was markedly decreased compared to the paired para tumor tissues (Fig. 1H). Collectively, these
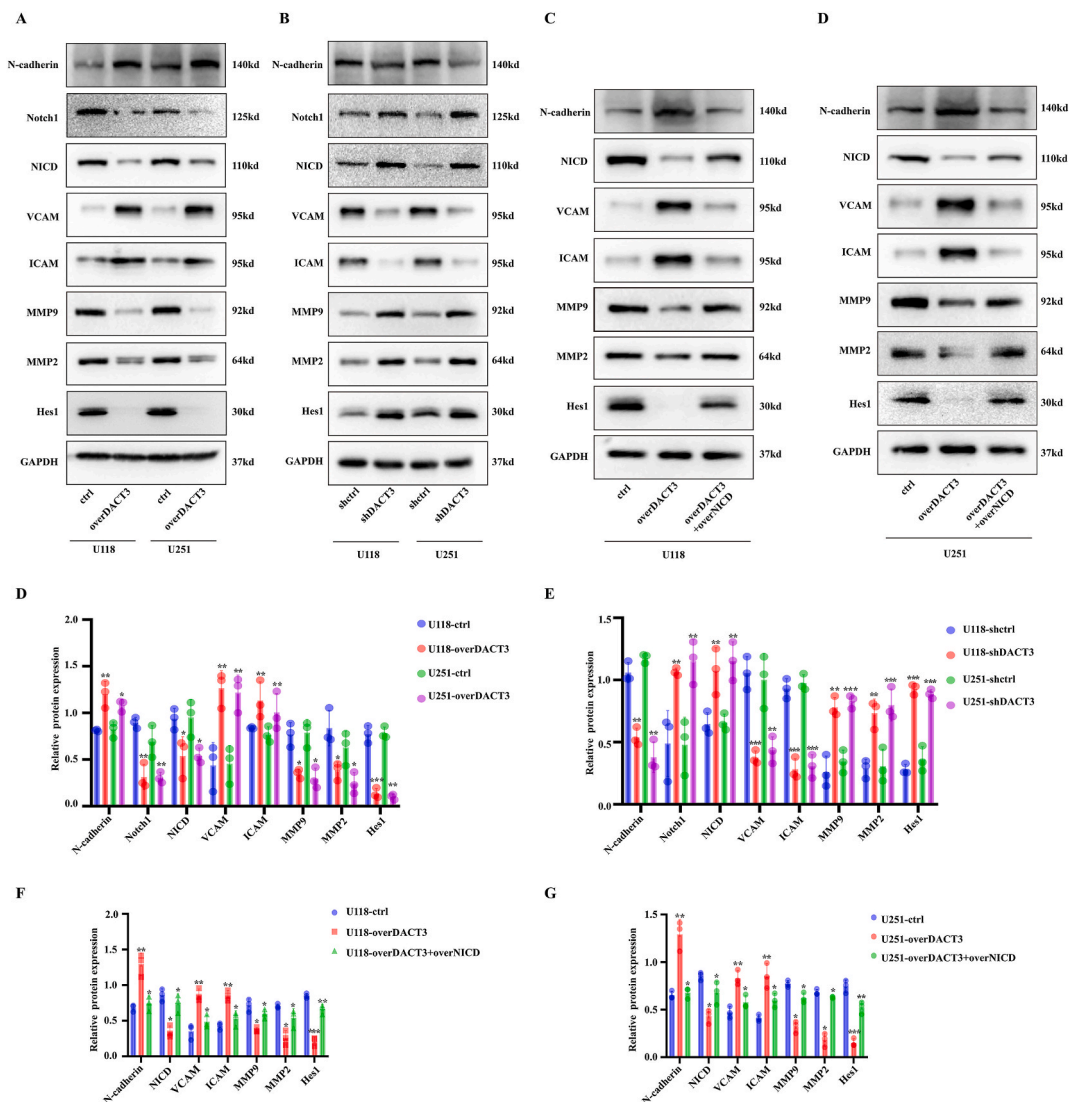

Fig. 4. Molecular mechanisms of DACT3 inhibits progression of glioma cells. A. D. Western blotting analysis of the expression of Notch1 signaling pathway related protein (Notch1, NICD, N-cadherin, VCAM-1, ICAM, MMP2, MMP9 and Hes1) in ctrl and overDACT3 glioma cells (n=3). **B. E.** Western blotting analysis of the expression of Notch1 signaling pathway related protein (Notch1, NICD, N-cadherin, VCAM-1, ICAM, MMP2, MMP9 and Hes1) in ctrl and shDACT3 glioma cells (n=3). **C-G.** Western blotting analysis of the expression of Notch1 signaling pathway related protein (NICD, VCAM-1, ICAM, MMP2, MMP9 and Hes1) in NICD-transfected U118 and U251 cells treated with DACT3 (n=3). DACT3: Dishevelled-associated antagonist of β-catenin homolog 3; NICD: notch 1 intracellular domain; VCAM: vascular cell adhesion molecule; ICAM: intercellular adhesion molecule; MMP: matrix metalloproteinase; ctrl: transfected with empty vectors; overDACT3: transfected with DACT3 vectors, overDACT3+overNICD: transfected with DACT3 vectors and NICD vectors; sh-ctrl: transfected with empty vectors; shDACT3: transfected with shDACT3 vectors. (* $P<0.05$, ** $P < 0.01$, *** $P < 0.001$; * ctrl vs overDACT3 or shctrl vs shDACT3, # overDACT3 vs overDACT3+overNICD, The full, non-adjusted images are shown in Supplement file)

data suggested that DACT3 may contribute to the malignant development of gliomas and decreased DACT3 expression was associated with poor prognosis of glioma patients.

3.2. DACT3 weakens malignant behaviors of glioma cells

In order to better characterize the relationship between the expression of DACT3 and the biological roles of glioma cells, DACT3 expression was first analyzed by Western blot in four glioma cell lines, including U87, U118, T98 and U251 (Fig. 2A and B). U118 and U251 were selected to further precisely clarify the function of DACT3 on the biological roles of glioma cells via lentivirus infections. The overexpression efficiency was verified by western blotting (Fig. 2C and D). Then we investigated the changes in proliferation, colony formation, adhesion, migration, and invasion of glioma cells. As expected, the CCK8 assay revealed that overexpression of DACT3 in U118 and U251 glioma cells obviously suppressed cell viability (Fig. 2E and F). Meanwhile, we also found that DACT3 overexpression markedly suppressed the colony formation ability of U118 and U251 glioma cells (Fig. 2G and H). Moreover, the proportion of Ki67-positive cells was markedly decreased in overDACT3 U118 and U251 cells (Fig. 2I and J). These results indicated that DACT3 overexpression inhibits glioma cell proliferation. Considering that invasiveness and adhesion ability is a major cause that resulted in glioma rapid recurrence and poor survival of patients. We observed the role of DACT3 in the invasion, migration, and adhesion of glioma cells. Our data suggested that overDACT3 U118 and U251 cells displayed a decreased invasion and migration ability (Fig. 2K, L, 2 M, and 2 N), while cell adhesion ability in these cells strongly enhanced (Fig. 2O and P). Taken together, these findings revealed that DACT3 overexpression suppresses proliferation, invasion, and migration, and enhanced the adhesion ability of glioma cells.

To delineate DACT3-mediated tumor inhibitory role, we employed its specific shRNA to silence DACT3 expression in U118 and U251 glioma cells (Fig. 3A and B). Next, we assessed DACT3's impact on cell viability using CCK8, finding that reducing DACT3 increased the number of viable U118 and U251 glioma cells (Fig. 3C and D). Consistent with CCK8 findings, DACT3-knockdown U118 and U251 glioma cells demonstrated enhanced cell colony formation compared to control cells (Fig. 3E and F). Furthermore, reduced DACT3 expression led to a notable increase in Ki67-positive glioma cells (Fig. 3G and H). Through transwell assays, we observed that reduced DACT3 expression significantly increased the migratory and invasive capabilities of glioma cells *in vitro* (Fig. 3I, J, 3K, and 3L).
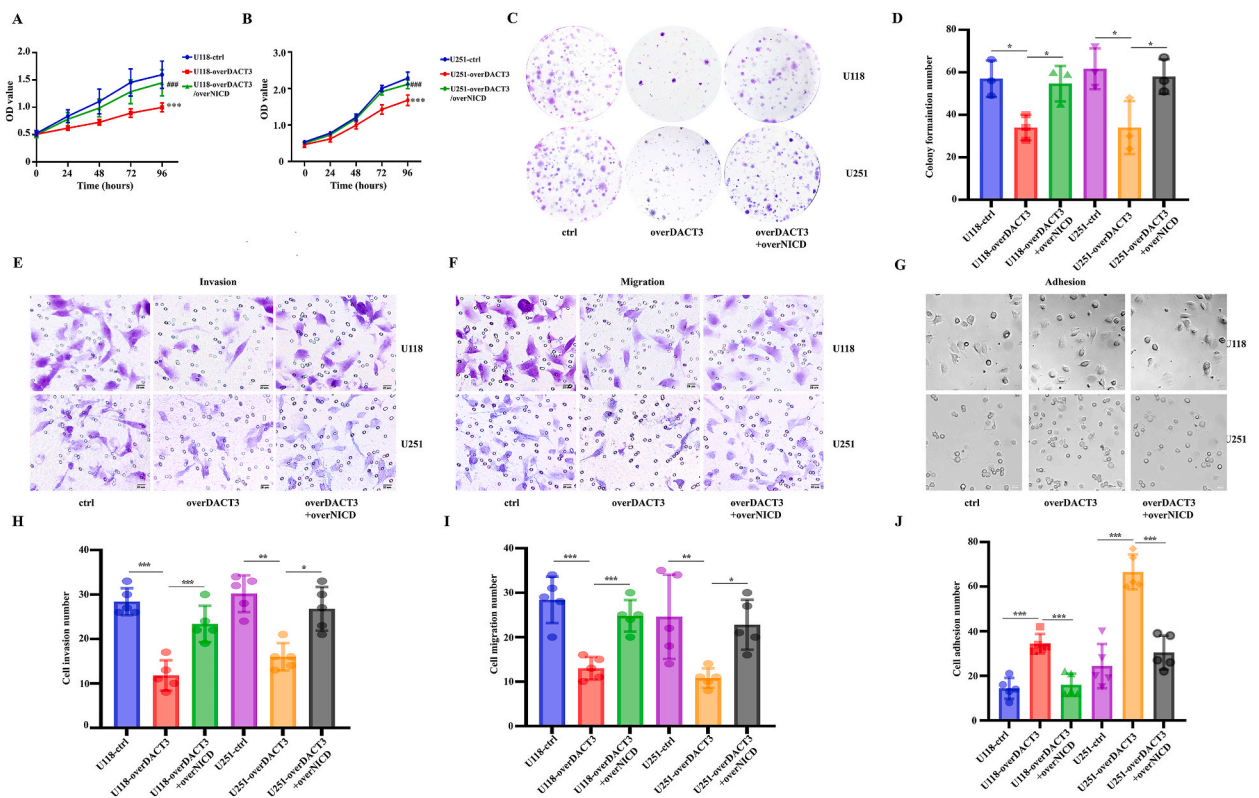

Fig. 5. NICD rescues DACT3 mediated effect of glioma cells. A-D. Cell proliferation was detected in U251 and U118 glioma cells by CCK-8 assay (**A. B**) and colony formation assay (**C. D**) (n=3). **E-I.** Invasive (**E. H**) and migrative (**F. I**) ability are tested in U118 and U251 glioma cells by transwell assay (400×, n=3). **G. J.** Adhesion ability is tested in U251 and U118 glioma cells by cell adhesion assay (400×, n=3). DACT3: Dishevelled-associated antagonist of β-catenin homolog 3; NICD: notch 1 intracellular domain; ctrl: transfected with empty vectors; overDACT3: transfected with DACT3 vectors, overDACT3+overNICD: transfected with DACT3 vectors and NICD vectors (* $P<0.05$, ** $P < 0.01$, *** $P < 0.001$, ### $P < 0.001$; * ctrl vs overDACT3, # overDACT3 vs overDACT3+overNICD).

Additionally, inhibiting DACT3 notably reduced glioma cell adhesion ability (Fig. 3M and N). These findings collectively indicate that DACT3 exhibits a tumor-suppressing role for glioma cells, and overexpression of DACT3 can inhibit the proliferation, migration, and invasion ability of glioma cells, while promoting cell adhesion.

3.3. DACT3 suppressed glioma progression through the notch signaling pathway

Considering the role of Notch signaling pathways on cell proliferation, adhesion, and invasion, we further analyzed the involvement of Notch signaling pathways in glioma progression. To confirm our hypothesis, we observed that overexpressing DACT3 reduced Notch1, NICD, Hes1, MMP9, and MMP2 levels in glioma cells, and upregulated adhesion-associated molecules, N-cadherin, VCAM-1, and ICAM-1 expression (Fig. 4A and D). However, knockdown of DACT3 expression activated Notch signaling and induced the expression of Notch1, NICD, Hes1, MMP2/9, and β-catenin, and downregulated adhesion-associated molecules, N-cadherin, VCAM-1, and ICAM-1 expression (Supplementary Figs. 1A–1B and Fig. 4B–E). In addition, overexpression of NICD sufficiently reversed the expression of Hes1, VCAM-1, ICAM-1, MMP9, and MMP2 (Fig. 4C, D, 4F, and 4G). Furthermore, overexpression of NICD significantly restored the proliferation ability of overDACT3 U118 and U251 cells (Fig. 5A, B, 5C, and 5D). Similarly, overexpression of NICD obviously restored the migration and invasion phenotype of glioma cells (Fig. 5E, F, 5H, and 5I). The adhesion ability of overDACT3 U118 and U251 cells was also reversed by overexpression of NICD (Fig. 5G and J). Collectedly, these findings revealed that DACT3 suppressed glioma cell proliferation, invasion, and migration, and enhanced cell adhesion mediated by the Notch1 signaling pathway.

3.4. β-catenin involved in DACT3-mediated inhibition of the notch signaling pathway

Considering the close relation between Notch and Wnt signaling pathways in cancer progression, we next performed experiments to analyze the effect of β-catenin on the Notch signaling pathway in glioma cells. The results revealed that DACT3 overexpression resulted in a remarkable decrease of NICD, Hes1, and β-catenin, and a notable increase of p-β-catenin in glioma cells (Fig. 6A and B), otherwise, DACT3 knockdown increased the expression of β-catenin (Supplementary Fig. 1). We next silenced the β-catenin expression in glioma cells, and the results revealed that the expression of NICD and Hes1 in glioma cells decreased (Fig. 6C and D). Moreover, β-catenin overexpression in overDACT3 U118 glioma cells also restored the NICD and Hes1 expression (Fig. 6E and F). Interestingly, DACT3 overexpression suppressed β-catenin levels in the nucleus by immunofluorescence assay, while overexpression of NICD obviously
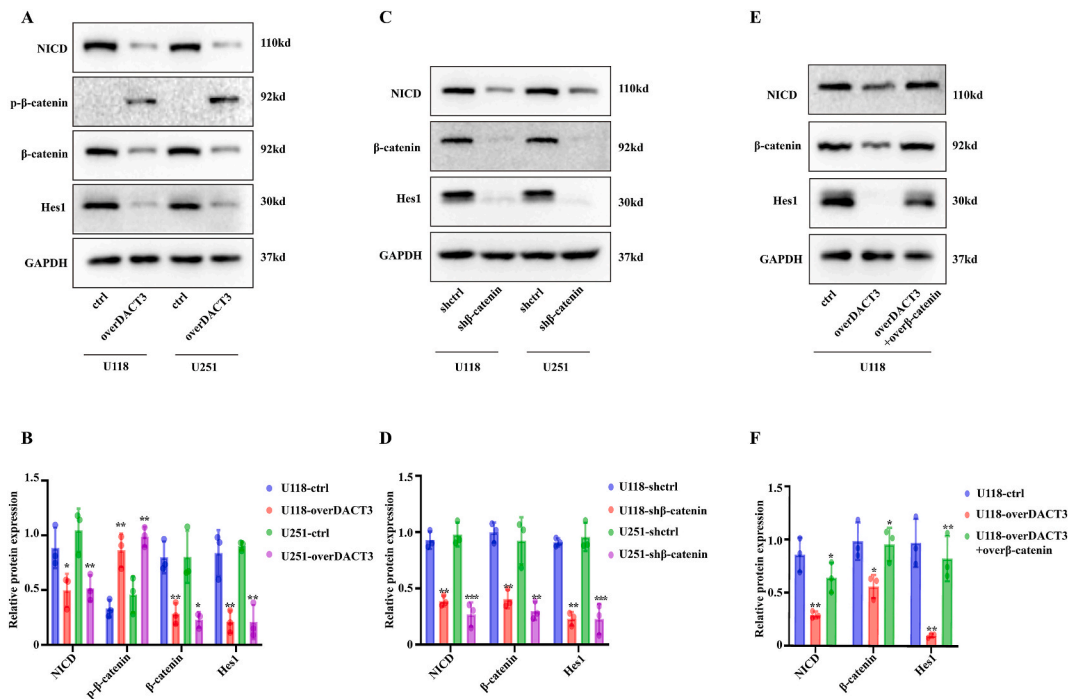

Fig. 6. β-catenin involved in DACT3-mediated inhibition of Notch1 signaling pathwayA. B. Western blotting analysis of the NICD, p-β-catenin, β-catenin and Hes1 expression in ctrl and overDACT3 glioma cells (n=3). **C. D.** Western blotting analysis of the NICD, β-catenin and Hes1 expression in shβ-catenin glioma cells (n=3). **E. F.** Western blotting analysis of the NICD and Hes1 in β-catenin-transfected U118 glioma cells treated with DACT3 (n=3). DACT3: Dishevelled-associated antagonist of β-catenin homolog 3; NICD: notch 1 intracellular domain; ctrl: transfected with empty vectors; overDACT3: transfected with DACT3 vectors, overDACT3+over-β-catenin: transfected with DACT3 vectors and β-catenin vectors, sh-ctrl: transfected with empty vectors, shβ-catenin: transfected with shβ-catenin (* $P<0.05$, ** $P < 0.01$, *** $P < 0.001$; * ctrl vs overDACT3 or shctrl vs shβ-catenin, # overDACT3 vs overDACT3+overβ-catenin, The full, non-adjusted images are shown in Supplement file).

increased β-catenin expression, and promoted the transport of β-catenin into the nucleus in overDACT3 U118 cells (Fig. 7A and Supplementary Figs. 2A–2F). Similarly, overexpression of β-catenin obviously restored NICD expression in the nucleus suppressed by DACT3 overexpression in U118 cells (Fig. 7B). These results suggested that DACT3 inhibited the Notch signaling pathway in a β-catenin dependent manner.

3.5. DACT3 suppresses glioma progression in vivo

To determine the effect of DACT3 in glioma progression *in vivo*, overDACT3 and control glioma cells were injected subcutaneously into nude mice. As shown in Fig. 8, overexpression of DACT3 remarkably suppressed the tumor growth of glioma cells, and tumor weight and volume results showed that the overDACT3 groups were markedly suppressed (Fig. 8A,B and 8C). The overexpression of DACT3 also prolonged the survival time of glioma-bearing mice (Fig. 8D and E). Additionally, IHC assay revealed that DACT3 overexpression reduced the expression of Ki67, MMP2, and MMP9, and upregulated ICAM-1 and VCAM-1 expression (Fig. 8F, G, and 8H). Conversely, the shDACT3 glioma cells grew faster than the sh-control cells (Fig. 9A). Tumor weight and volume in the shDACT3 group were significantly promoted (Fig. 9B and C) and subcutaneous injection of shDACT3 glioma cells reduced the survival time of glioma-bearing mice (Fig. 9D and E). DACT3 down-expression upregulated Ki67, MMP2, and MMP9 expression, and downregulated ICAM-1 and VCAM-1 expression (Fig. 9F, G, and 9H). Combined with the results from the *in vitro* analysis, these data verified the tumor-suppressing role of DACT3 in glioma progression *in vivo*.

4. Discussion

DACT3 is often considered a tumor-suppressive gene involved in various tumors [16,17], However, its specific role in glioma progression had not been previously explored. Our experimental data and analysis result from CGGA database revealed a significant association between DACT3 expression and GBM progression and survival. The functional experiments showed that DACT3 over-expression significantly suppress the proliferation, invasion, and migration of glioma cells, which strongly supported the anticancer properties of DACT3 in glioma cells. Additionally, the enforced expression of DACT3 extended the survival time of glioma-bearing mice. These findings suggest that DACT3 acts as a tumor-suppressing gene, actively inhibiting the malignant characteristics of glioma. This study presents compelling evidence of its antitumor effect in glioma.

The antitumor effect of DACT3 has been confirmed in various tumors. Building upon our previous findings, we have established that DACT2, a gene homologous to DACT3, impedes the progression of GBM [14]. To further understand DACT3's impact, we evaluated its expression levels in glioma using the CGGA database. The analysis results indicated that DACT3 expression inversely correlates with the glioma WHO grade. Further analysis results revealed an association between DACT3 expression and the survival of glioma patients, and a significantly correlation between WHO grade IV glioma and DACT3 and survival. Our experimental data revealed a significant decrease in DACT3 expression in GBM compared to normal brain tissues. Together, the results of this study underscored its potential as a prognostic biomarker.

Previous studies underscore the collaboration between Wnt/β-catenin and Notch signaling pathway function in various tumors [35–39]. Notably, Notch1 signaling pathway is essential for regulating cell adhesion that causes progression of cancer cells. Hence, our investigation aimed to elucidate the impact of DACT3 on the activity of Notch1 signaling pathway, attempting to illustrate its role in
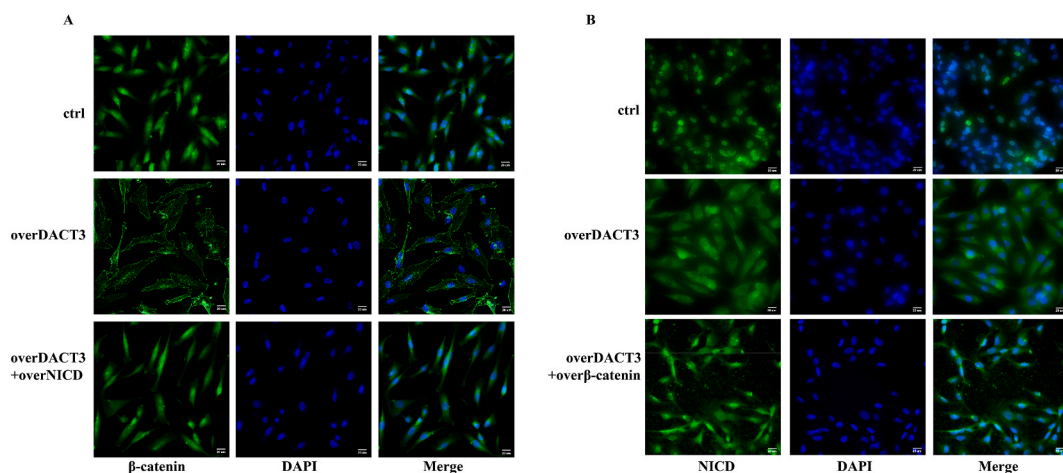

Fig. 7. β-catenin involved in DACT3-mediated NICD location.A. Immunofluorescence analysis of the location of β-catenin in ctrl, overDACT3 and overDACT3/overNICD U118 glioma cells (400×, n=3). **B.** Immunofluorescence analysis of the location of NICD in ctrl, overDACT3, over-DACT3/overβ-catenin (400×, n=3). DACT3: Dishevelled-associated antagonist of β-catenin homolog 3; NICD: notch 1 intracellular domain; ctrl: transfected with empty vectors; overDACT3: transfected with DACT3 vectors, overDACT3+over-β-catenin: transfected with DACT3 vectors and β-catenin vectors.
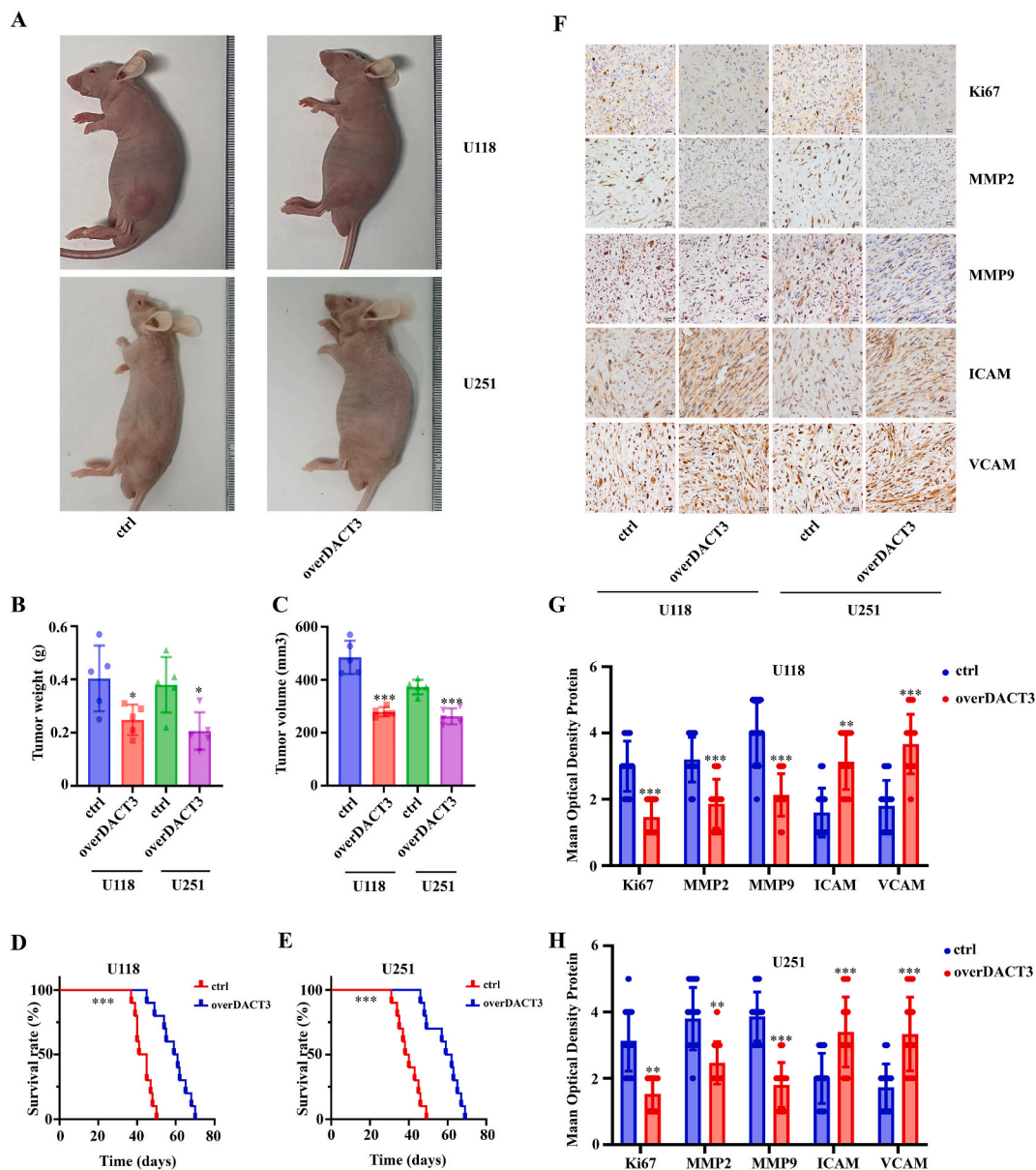
Fig. 8. The growth suppressive effect of DACT3 overexpressing on glioma in subcutaneous tumor model. A. DACT3 inhibited the growth of glioma in subcutaneous tumor model (n=5). **B. C.** Tumor weight and volume (n=5). **D. E.** The survival time of glioma-bearing mice (n=10). **F. G. H.** IHC analysis of the protein expression of Ki67, MMP2, MMP9, ICAM and VCAM-1 in transplanted tumors (400×, n=5). DACT3: Dishevelled-associated antagonist of β-catenin homolog 3; VCAM: vascular cell adhesion molecule; ICAM: intercellular adhesion molecule; MMP: matrix metalloproteinase; ctrl: transfected with empty vectors; overDACT3: transfected with DACT3 vectors (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; * ctrl vs overDACT3).

suppressing tumor progression. Our results found that Notch signaling pathway may be inhibited due to Notch1, NICD and Hes-1 were downregulated in DACT3 overexpressing cells. Intriguingly, DACT3 overexpression sharply stimulated N-cadherin, VCAM-1 and ICAM-1 expression, while inhibited MMP2/9 expression. Functional experiments further supported that DACT3 overexpression significantly promote adhesion of glioma cells. Particularly noteworthy, the expression of VCAM-1, ICAM-1, N-cadherin, MMP2/9, Notch1 and Hes1 were remarkably reversed by NICD overexpression. These findings suggested that DACT3 might regulate the expression of MMP2/9, N-cadherin, VCAM and ICAM by inhibiting Notch signaling, potentially promoting adhesion of glioma cells.

To our knowledge, as a member of DACT family, DACT3 is commonly subject to epigenetic regulation and often implicated in tumor progression, such as colorectal cancer, lung cancer and breast cancer [15–17]. The Wnt/β-catenin signaling has been confirmed to be aberrantly activated in many tumors and involved in cancer progression regulation [40,41]. Moreover, it has been reported that the Wnt/β-catenin signaling pathway is closely linked with the tumorigenesis and progression of glioma [32,42]. DACT3's regulation
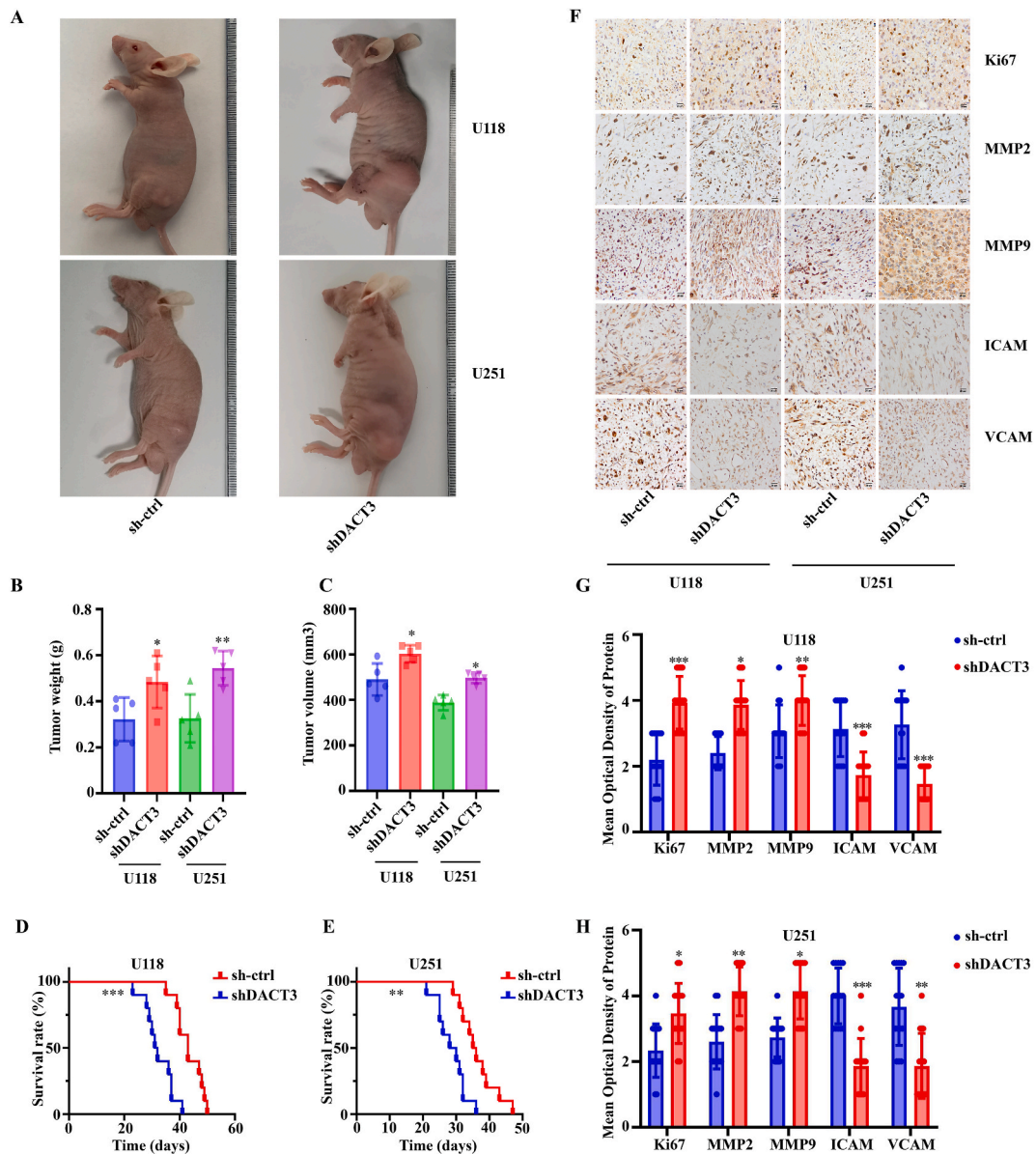
Fig. 9. The growth promotive effect of DACT3 down-expression on glioma in subcutaneous tumor model. A. DACT3 silencing promotes the growth of glioma in subcutaneous tumor model (n=5). **B. C.** The survival time of glioma-bearing mice (n=10). **D. E.** Tumor weight and volume (n=5). **F. G. H.** IHC analysis of the protein expression of Ki67, MMP2, MMP9, ICAM and VCAM-1 in transplanted tumors. (400×, n=5). DACT3: Dishevelled-associated antagonist of β-catenin homolog 3; VCAM: vascular cell adhesion molecule; ICAM: intercellular adhesion molecule; MMP: matrix metalloproteinase; sh-ctrl: transfected with empty vectors; shDACT3: transfected with shDACT3 vectors (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; * shctrl vs shDACT3).

of the Wnt signaling pathway and its involvement in glioma progression has been attributed to its role as a general Dishevelled antagonist [43]. Acting as a negative regulator of Wnt signaling, DACT inhibits Dishevelled from forming complexes with Axin, GSK-3, CKI, and β-catenin [43]. Consequently, any factor capable of reducing Dishevelled expression could potentially inhibit Wnt signaling. Colella et al. demonstrated that autophagy-induced degradation of Dishevelled 2 could negatively modulate of Wnt/β-catenin signaling, leading to accumulation of β-catenin in sub-plasma membrane areas and increased association between β-catenin and N-cadherin in glioma cells [44]. Furthermore, Fujii et al. reported that small-molecule antagonists targeting the PDZ domain inter-action of Dishevelled effectively down-regulate the β-catenin-dependent Wnt signaling pathway in lung cancer [45].

Previous studies have highlighted the role of Notch activation in enhancing the invasive and migratory properties of glioma cells through stimulating β-catenin signaling [19]. Furthermore, NICD overexpression led to the upregulated of several metastasis-associated molecules in glioma cells, which could be eliminated by the knockdown of β-catenin [46]. In fact, accumulating

evidence has elucidated the intricate intersection of Notch and Wnt/β-catenin signalings at various steps [26–28]. β-catenin can regulate the transcriptional activity of the Notch1 and NICD and interacts with NICD to translocate into the nucleus to maintain the proliferation during disease progression through activating the downstream target genes, including CyclinD1 and Hes1 [26]. In our study, we observed that DACT3 overexpression induced p-β-catenin expression, while resulting in decreased expression of β-catenin, Hes1 and NICD. Further experiments involving β-catenin knockdown decreased NICD and Hes1 expression in glioma cells. Moreover, the overexpression of β-catenin in DACT3-overexpressing U118 glioma cells restored the expression of NICD and Hes1. Additionally, overexpression of NICD obviously increased β-catenin expression and promoted the translocation of β-catenin into the nucleus in DACT3-overexpressing U118 cells. Similarly, the overexpression of β-catenin restored NICD expression in the nucleus affected by DACT3 overexpression in U118 cells. These results suggest that DACT3 may suppress NICD expression and its translocation into the nucleus by modulating expression of β-catenin.

The findings of present study indicated that DACT3 serves as a tumor-suppressive gene and regulates glioma progression through the Notch1 signaling pathway in a β-catenin-dependent manner, presenting potential therapeutic approach. Utilizing this knowledge, novel treatment strategies could be developed by targeting the DACT3-Notch1-Wnt/β-catenin axis to impede glioma progression and/ or reduce recurrence. Moreover, these insights might provide a foundation for exploring similar mechanisms in other cancers. Overall, this study offers a promising approach for potential therapeutic approaches in glioma and provides a broader framework for studying similar pathways in various cancers, potentially leading to more effective and targeted treatments in the future.

Although our study yielded some meaningful findings in therapeutic target of glioma and underlying molecular mechanisms, our study still has some limitations. First, our research might be limited to in cell and animal model investigations, potentially lacking the full complexity of the human disease. Second, the study might focus primarily on the DACT3-Notch1-β-catenin axis, overlooking potential interactions or pathways that could also contribute to glioma progression. Third, these findings translated into clinical applications, such as specific therapies or treatments, still requires diverse models or patient cohorts to confirm the specific role of DACT3 in glioma progression.

5. Conclusion

Taken together, our data revealed that DACT3 expression decreased in glioma tissues. Furthermore, DACT3 overexpression remarkedly hindered the proliferation, invasion, and migration, while enhanced the adhesion of glioma cells, achieved by suppressing the Notch1 signaling pathway. Notably, our research demonstrated that DACT3 downregulated NICD expression and translocated into the nucleus by reducing β-catenin expression. To our knowledge, this study stands as the first research to investigate the role of DACT3 in glioma progression and elucidate its molecular mechanisms in glioma development. Thus, our results indicated that DACT3 might serve as a prognostic factor and potential biomarker for glioma treatment.

Ethics statement

This article included human and animal material. The present study was performed in accordance with the Declaration of Helsinki and approved by Ethic Committee of Guizhou Provincial People's Hospital (Number: (2019)122) and written informed consent was obtained from all subjects. All experiments were conducted according to the relevant guidelines and regulations.

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Consent for publication

Not Applicable.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Jianhe Yue: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Jiqin Zhang:** Writing – review & editing, Funding acquisition, Conceptualization. **Rengzheng Huan:** Validation, Software, Methodology. **Yu Zeng:** Validation, Methodology, Data curation. **Ying Tan:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Yuan Cheng:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23511.

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