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CDKL5 promotes proliferation, migration, and chemotherapeutic drug resistance of glioma cells via activation of the PI3K/AKT signaling pathway

Running title: CDKL5 promotes proliferation of glioma cells

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List of Abbreviations

CDKL5: Cyclin-dependent kinase-like 5

IHC: Immunohistochemistry

FBS: Fetal bovine serum

TBS: Tris-buffered saline

RT: reverse transcription

RNAi: Transfections and RNA interference

FC: flow cytometry

WB: Western blotting

SEM: Standard error of the mean

KD: knock-down

Abstract

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Gliomas, the most prevalent cancer in the central nervous system, are characterized by high morbidity and mortality, emphasizing the need to understand their etiology. Here, we report that cyclin-dependent kinase-like 5 (CDKL5) is highly expressed in gliomas, and CDKL5 over-expression promotes invasion, proliferation, migration, and drug (β-lapachone) resistance of glioma cells. *In vitro*, CDKL5 over-expression enhanced invasion, growth, and migration of glioma cells and stimulated the PI3K/AKT axis. Furthermore, CDKL5 over-expression *in vivo* promoted glioma proliferation, while CDKL5 knockdown had opposing effects. The effect of CDKL5 on drug resistance was eliminated if the PI3K/AKT axis was suppressed, and cisplatin combined with the PI3K/AKT suppressor XL147 remarkably prohibited proliferation in xenografts over-expressing CDKL5. Collectively, our findings suggest that CDKL5 acts through the PI3K/AKT axis in glioma cells, and suggest a possible role for CDKL5 in glioma therapy.

Key words: CDKL5; glioma; migration; PI3K/AKT; drug resistance

Introduction

As the most prevalent cancer in the central nervous system because of the noticeable incidence rate, fast relapse, and limited survival [1], gliomas are characterized by persistent growth, reinforced migration and invasion, and multiple molecular and cytogenetic aberrances [2]. Standard glioma therapy consists of operation [3] prior to radiotherapy[4] and chemotherapy [5]. Nevertheless, drug resistance is a difficult challenge to overcome [6]. Despite progress in malignancy therapy, the clinical outcome of glioma patients is far from satisfactory and less than five percent of patients survive for five years after diagnosis [7]. Furthermore, the understanding of the molecular etiology of gliomas is insufficient[8]. Consequently, it is urgent to elucidate the etiology and to recognize innovative targets for the treatment of gliomas [9, 10].

As a serine-threonine kinase, cyclin-dependent kinase-like 5 (CDKL5) was recognized via transcriptional mapping research focusing on the recognition of genes that brought about illness in Xp22 region 1[11]. The recognition of CDKL5 mutations in patients suffering from the Hanefeld variant of Rett syndrome of infantile epileptic encephalopathy in the early stage implicated the activity of CDKL5 in the human cerebra [12-14]. Accordingly, two present murine models with CDKL5 knock-outs featured reduced studying and recollection, characteristics resembling autism, and motor defects that complied with some aspects of clinical spectrum in patients displaying CDKL5 mutations [15, 16]. CDKL5/CDKL5 gene transcription is prevalent and proteins can be examined in most tissues and cells, not only in the nucleus but also in the cytoplasm [17]. Since the expression of CDKL5 reaches peak levels in the cerebra because of obvious cerebra-related activities, most research has aimed at the neuronal influence of CDKL5 [18-20]. Nevertheless, the understanding of its influence on gliomas is insufficient.

We investigated CDKL5 expression in gliomas and evaluated CDKL5 functions in the modulation of the biological activities of gliomas. Moreover, the promising etiology of gliomas was recognized.

Materials and methods

Cell lines and tissue samples

The human glioma cell lines U87 (glioblastoma of unknown origin, BNCC337885) and U251 were acquired from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and subsequently preserved in 5% CO_2 in DMEM (HyClone, USA) containing 10% fetal bovine serum (FBS, Gibco) at 37°C.

27 patients received clinical and histological diagnosis of gliomas at the First Affiliated Hospital of Dalian Medical University. Fully written informed consent was acquired, and our research was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University. The study methodologies conformed to the standards set by the Declaration of Helsinki. Cerebral tissue specimens were acquired from five patients who had encountered intracerebral hemorrhage. All samples were kept at −80°C.

Immunohistochemistry (IHC)

Paraffin slices (5 μm) of glioma and normal cerebral tissues were subjected to dehydration using a graded concentration series of ethanol before incubation in H_2O_2 with 1% bovine serum albumin in Tris-buffered saline (TBS). The specimens were incubated overnight with murine IgG isotype antibody or mouse anti-human CDKL5 monoclonal antibody at 4°C in a humid chamber. The slices were covered with goat anti-mouse IgG antibody conjugated with peroxidase (SP-9002; Golden Bridge International, Inc., Beijing, China) after three washes with TBS.

RNA isolation and quantitative polymerase chain reaction (qPCR)

TRIzol (Life Technologies) was used to isolate total RNA from tissues, which was purified using the RNeasy Mini kit (Qiagen, Hilden, Germany). Superscript III kit (Life Technologies) was applied for reverse transcription (RT). Complementary DNA was evaluated by qPCR. Transcription was quantified and evaluated by RT-PCR using the SYBR Green PCR Supermix kit (Bio-Rad Laboratories, Hercules, CA, USA). Every procedure was carried out in triplicate. Real-Time StatMiner (Integromics, Madrid, Spain) was used to assess gene expression.

Transfections and RNA interference (RNAi)

Plasmids CDKL5-pcDNA3.1 (CDKL5) and pcDNA3.1 (vector) were acquired from Shanghai Genepharma Co. Ltd. (Shanghai, China). U251 cells were seeded in 6-well plates one day prior to

transfection. Transfection admixture was generated by adding 4 μg of plasmid DNA and 3 μg of Turbofect reagent (Fermentas, Glen Burnie, MD, USA) to DMEM/F12 without serum. The admixture was supplemented to the culture media and the cells were further incubated for 6 h. In terms of siRNA transfection, U251 cells were transfected with 20 nmol of control GL2 siRNA targeting the luciferase gene (shCDKL5#1: CUA UGG AGU UGU ACU UAA AUU; shCDKL5#2: GCA GAG UCG GCA CAG CUA UUU; siCtr. 5'CGU ACG CGG AAU ACU UCG AUU3') or siRNA oligonucleotides targeting CDKL5 using Lipofectamine TM RNAiMAX (Life Technologies).

Cell survival

Cell survival was evaluated using the MTT assay. Cells were seeded in 96-well plates (5×10^4 cells/mL). MTT assay was carried out 48 h after transfection. Cell survival was assessed by adding 10 μL of MTT reagent to every well and cells were further incubated for 4 h at 37°C. The cells were examined using a microplate reader at 570 nm (Thermo Scientific). Every experiment was carried out three times independently.

Cell migration

The Transwell assay was performed to evaluation cell migration. Cells were suspended in DMEM containing 1 mg/mL mitomycin C. They were then seeded on the top chambers of 24-well polycarbonate Transwell filters (Millipore, Bedford, MA, USA). Cells in DMEM containing 10% FBS were seeded to the bottom chambers. Cells at the top surface were scraped off after 48 h of incubation while those at the bottom were fixed, stained, and quantified.

Cell invasion

Transwell chambers that were covered with Matrigel were utilized to assess cell invasion. Transfected cells were seeded (1×10^5 cells in each chamber) in the top chambers and incubated for 24 h. FBS (20%) served as a chemoattractant and was added to the chambers below. A cotton swab was used to eliminate non-invading cells on the top surface after incubation, while invading cells at the bottom were fixed with 100% methanol prior to staining with 1% crystal violet. Invading cells were quantified using a microscope, and six randomly selected visual fields were assessed for every assay.

Cell cycle

Cells were starved for 12 h in preparation for synchronization prior to re-activation for 24 h with 10% FBS. Cells were fixed in 75% ethanol and treated with the Cell Cycle Detection Kit. A FACS Calibur flow cytometer (Beckman, CA, USA) was used to categorize the cells. Flowjo software (Treestar Inc., USA) was used to assess the distribution of cell phase.

Cell apoptosis

Apoptosis was assessed by flow cytometry (FC). The supernatant was eliminated after centrifugation for 5 min at 100 rpm. The sediment was re-suspended with binding buffer. Propidium iodide and FITC-Annexin V were supplemented while the mixture was incubated for 10 min at 37°C. A FACScan flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used to evaluate fluorescence signals.

In vivo malignant xenografts

Procedures related to animals were approved by the Ethical Committee of the First Affiliated Hospital of Dalian Medical University. The CDKL5 plasmid was constructed into the lentivirus vector LV-3 (GenePharma). Stable cell lines with CDKL5 overexpression were selected with 4 μg/ml puromycin incubation for 7 days. Nude male BALB/c mice aged four weeks were subcutaneously injected with approximately the stable clones of U251 cells (4-6 × 106 cells), transfected with CDKL5, in their flanks regardless of the presence of infection. Mice were treated with 5 mg/kg cisplatin and 40 mg/kg XL147 (Selleckchem, Houston, TX) two times/week for 30 days by i.p. Every group consisted of five mice, which were executed after 30 days, and the malignancies were cut off and weighed.

Western blotting (WB)

Lysis buffer (Beyotime, China) was applied to homogenize cell lysates and tissues while protein quantification was carried out using the Bradford assay (Bio-Rad, Hercules, CA, USA). Protein evaluation was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Tris-HCl polyacrylamide gels (8–15%, Bio-Rad) were used to isolate proteins, which were moved to polyvinylidene fluoride membranes (Millipore, Bedford, MA). The blots were incubated overnight with primary antibodies (anti-PI3K, anti-AKT, anti-p-PI3K, anti-p-AKT, anti-CDKL5, anti-β-actin, Cell Signaling Technology, Beverly, MA, USA) at 4°C in TBS/Tween. Thereafter, the blots were

incubated with secondary antibodies conjugated with horseradish peroxidase. Enhanced chemiluminescence plus detection reagent (Pierce, Rockford, IL, USA) was used to examine immunoreactive bands, which were assessed by the Omega 16ic Chemiluminescence Imaging System (Ultra-Lum, CA, USA).

Statistical analysis

Results are displayed as mean ± standard error of the mean (SEM). Differences between groups were measured by two-tailed, unequal-variance Student's t-test and analysis of variance prior to Tukey's posthoc analysis. Statistical significance is indicated by $P < 0.05$.

Results

CDKL5 expression was promoted in glioma tissues

We examined CDKL5 in 27 normal cerebral specimens and gliomas to investigate the promising influence of CDKL5 on glioma generation and progression. CDKL5 transcription was noticeably upregulated in glioma tissues compared with that in normal tissues (**Fig. 1A**). IHC and WB were performed to evaluate CDKL5. IHC revealed that CDKL5 translation was reinforced in glioma tissues in comparison with that in control tissues (**Fig. 1B**), which was confirmed by WB of fresh specimens (**Fig. 1C and 1D**). These findings suggested that CDKL5 expression was promoted in gliomas and that CDKL5 could be correlated to gliomas.

CDKL5 enhanced migration invasion of glioma cells

We then examined the impact of CDKL5 on the migration and invasion of glioma cells. Non-coated Transwell chambers were used to evaluate the migration capability of U251 cells. It was shown that excessive CDKL5 expression remarkably promoted cell migration (**Fig. 2A and 2B**). The invasion capability of U251 cells was examined using polycarbonate Transwell filters coated with Matrigel. The number of U251 cells that invaded the Matrigel-coated filters and arrived at the bottom surface of the membrane was elevated after transfection of CDKL5 in comparison with that of the blank control (**Fig. 2C and 2D**). In addition, CDKL5 over-expression significantly increased the expression of

MMP9 and MMP2 in U251 cells (**Fig. 2E**). These findings suggested that CDKL5 enhanced the migration and invasion capability of U251 cells.

CDKL5 promoted proliferation and drug resistance of glioma cells in vitro

We explored the influence of CDKL5 on cell proliferation. The findings of the MTT assay proved that glioma cells with CDKL5 knock-down (KD) displayed remarkably reduced proliferation in comparison with that of the control group (**Fig. 3A**), while excessive CDKL5 expression promoted cell proliferation. The results of FC showed that the proportion of U251 cells in the S phase was noticeably elevated when CDKL5 was excessively expressed compared with control cells (**Fig. 3B and 3C**) and was obviously suppressed in CDKL5-KD cells.

We additionally explored whether CDKL5 regulated the malignancy counteraction activity of β-lapachone (β-lap), which served as an innovative malignancy-counteracting agent that has been proven to stimulate various reactions of apoptosis in malignant cells. We evaluated cytotoxicity using the CCK8 and observed that cell survival was suppressed in the presence of β-lap (**Fig. 3D**). Excessive CDKL5 expression clearly reduced the percentage of cell death. We also evaluated cell death using FC to better confirm the influence of CDKL5 on resistance to β-lap. Supplementation with β-lap evidently promoted cell death and excessive CDKL5 expression prohibited this promotion (**Fig. 3E and 3F**). These findings indicated that CDKL5 participated in modulating the chemosensitivity to β-lap in glioma cells.

CDKL5 promoted stimulation of PI3K/AKT axis in glioma cells

The PI3K/AKT axis participates in the modulation of cell proliferation and invasion[21]. We subsequently explored the impact of CDKL5 on the phosphorylation of the PI3K/AKT axis using WB. As shown in **Fig. 4**, CDKL5 overexpression obviously increased the phosphorylation level of PI3K, AKT and GSK3, whereas CDKL5 knockdown significantly inhibited the phosphorylation level of PI3K, AKT and GSK3. These findings proved that CDKL5 reinforced the stimulation of the PI3K/AKT axis in glioma cells.

CDKL5 quickened glioma generation through PI3K/AKT axis in vivo

Nude male BALB/c mice were injected with U251 cells with CDKL5 plasmid in the flanks and malignancies were weighed 30 days later to explore whether CDKL5 reinforced glioma proliferation in vivo. PI3K inhibitor XL147 significantly reduced CDKL5-induced the phosphorylation level of AKT in vivo (**Fig. 5A**). Mice that injected with U251 cells with CDKL5 plasmid exhibited noticeably larger malignancies in comparison with those in the vector, which were attenuated via prohibition of the PI3K/AKT axis (**Fig. 5B–5D**). Moreover, IHC evaluation of Ki67, a biomarker of growth, was carried out in malignant xenograft tissues. Excessive expression of CDKL5 remarkably elevated Ki67 concentration, which was counteracted via prohibition of the PI3K/AKT axis (**Fig. 5E**). These results indicated that CDKL5 promoted glioma carcinogenesis via stimulation of the PI3K/AKT axis.

Discussion

Our research showed that CDKL5 expression was promoted in glioma specimens in comparison with that in normal specimens. The activity of CDKL5 in glioma cells was studied by excessive expression and KD assays. We discovered that excessive CDKL5 expression reinforced drug resistance, migration, proliferation, and invasion in glioma cells whereas CDKL5 KD resulted in the opposite effects CDKL5 enhanced the stimulation of the PI3K/AKT axis, which subsequently participated in glioma generation. Collectively, our findings reveal that CDKL5 can modulate glioma proliferation and that the PI3K/AKT axis participates in this reaction. The results of this research throw light upon both the influence of CDKL5 on gliomas and strategies of glioma therapy.

Gliomas are one of the most fatal malignancies in the central nervous system, with glioblastoma being the most common form, which features poor median survival of fifteen months and extreme aggressiveness [22, 23]. Despite rapid progress in glioma diagnosis and therapy, the five-year survival has not improved significantly, emphasizing the need to recognize and investigate the molecular etiology of glioma generation [24-26]. In the past ten years, genetic damage has been discovered in patients suffering from neurologic diseases, which appear as an early attack of usually refractory epilepsy, mental retardation, and suppressed motor regulation[27, 28]. Since mutations in CDKL5 have a noticeable influence on cerebral activities, most studies aim to study the influence of this kinase on neurons, but little has been revealed in terms of its effect on growing cells [29, 30]. Almost

no information has indicated that CDKL5 participates in cell growth [31]. Excessive expression of CDKL5 triggers cell cycle arrest of neuroblastoma cells whereas CDKL5 suppression via RNAi or aimed gene disturbance was shown to promote the incorporation of bromodeoxyuridine [32, 33]. Nevertheless, understanding of the effect of CDKL5 on gliomas is insufficient. Our research has revealed some novel aspects. We proved that CDKL5 expression was reinforced in glioma tissue samples and that CDKL5 enhanced glioma migration and invasion. Next, elevation in CDKL5 expression was shown to stimulate drug resistance and cell growth not only in vivo but also in vitro. Furthermore, CDKL5 KD stimulated counteracting effects on malignancy growth. These findings can assist us in understanding the etiology of the oncogenic effect of CDKL5 and alter our perspective of its impact as a candidate treatment agent. In vivo findings indicated that CDKL5 enhanced glioma generation by stimulating the PI3K/AKT axis.

The PI3K/AKT axis is related to metastasis and glioma development [34-38]. Previous research has shown that SRPK1 enhanced metastasis and vessel generation in gliomas via the PI3K/AKT axis [39]. As a conventional Chinese herbal medicine, Shikonin was demonstrated to suppress invasion and migration of glioblastoma cells by targeting the PI3K/AKT axis [40, 41]. We discovered that excessive CDKL5 expression promoted the phosphorylation of PI3K and AKT, which was prohibited by CDKL5 KD, indicating that CDKL5 stimulated the PI3K/AKT axis. As a serine-threonine kinase, CDKL5 may activate PI3K via interact with PI3K. Moreover, we found that prohibition of this axis attenuated the effect of excessive CDKL5 expression on glioma generation in vivo. The results of our research suggested that CDKL5 reinforces the generation of gliomas through the PI3K/AKT axis.

Conclusions

In summary, our research demonstrated that CDKL5 expression is reinforced in gliomas and that it affects the proliferation, migration, drug resistance, and invasion of glioma cells. Furthermore, CDKL5 enhances the generation of gliomas in vivo by stimulating the PI3K/AKT axis. This research emphasizes the promising effect of CDKL5 on the assessment of clinical outcome and treatment application of gliomas.

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None.

Conflict of Interest statement

There are no competing financial interests.

Authors' contributions

In this work, Zhenfu Jiang and Hong Wei conceived the study and designed the experiments. Zhenfu Jiang, Tongtong Gong and Hong Wei contributed to the data collection; performed the data analysis and interpreted the results. Zhenfu Jiang wrote the manuscript; Zhenfu Jiang and Hong Wei contributed to the critical revision of article. All authors read and approved the final manuscript.

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Figure legends

Fig. 1 CDKL5 expression was enhanced in glioma specimens. A, qRT-PCR was utilized to examine CDKL5 transcription in glioma and normal cerebral specimens (n=10). **B,** Representative photomicrographs displaying CDKL5 expression in glioma and normal specimens (n=8). Scale bar: 100 μm. **C–D**, Representative immunoblots (**C**) and quantification of CDKL5 (**D**) in normal and glioma specimens (n=9). Results are presented as means ± SEM. Student's t-test, $^{**}P < 0.01$ vs. normal group.

Fig. 2 CDKL5 enhanced migration and invasion of glioma cells. U251 cells transfected with empty vector or CDKL5 for 48 h. **A,** Images displaying migration of U251 cells to the bottom surface. Scale bar: 100 μm. **B,** Migrating U251 cells of various groups from five randomly selected visual fields visualized using a microscope. **C,** Images displaying the invasion of U251 cells on the bottom surface. Scale bar: 100 μm. **D,** Invading U251 cells of various groups from five randomly selected visual fields visualized using a microscope. **E,** Representative immunoblots of MMP9 and MMP2 in U251 cells. Results are presented as means ± SEM, Student's t-test, n = 3. $^{**}P < 0.01$ vs. vector group.

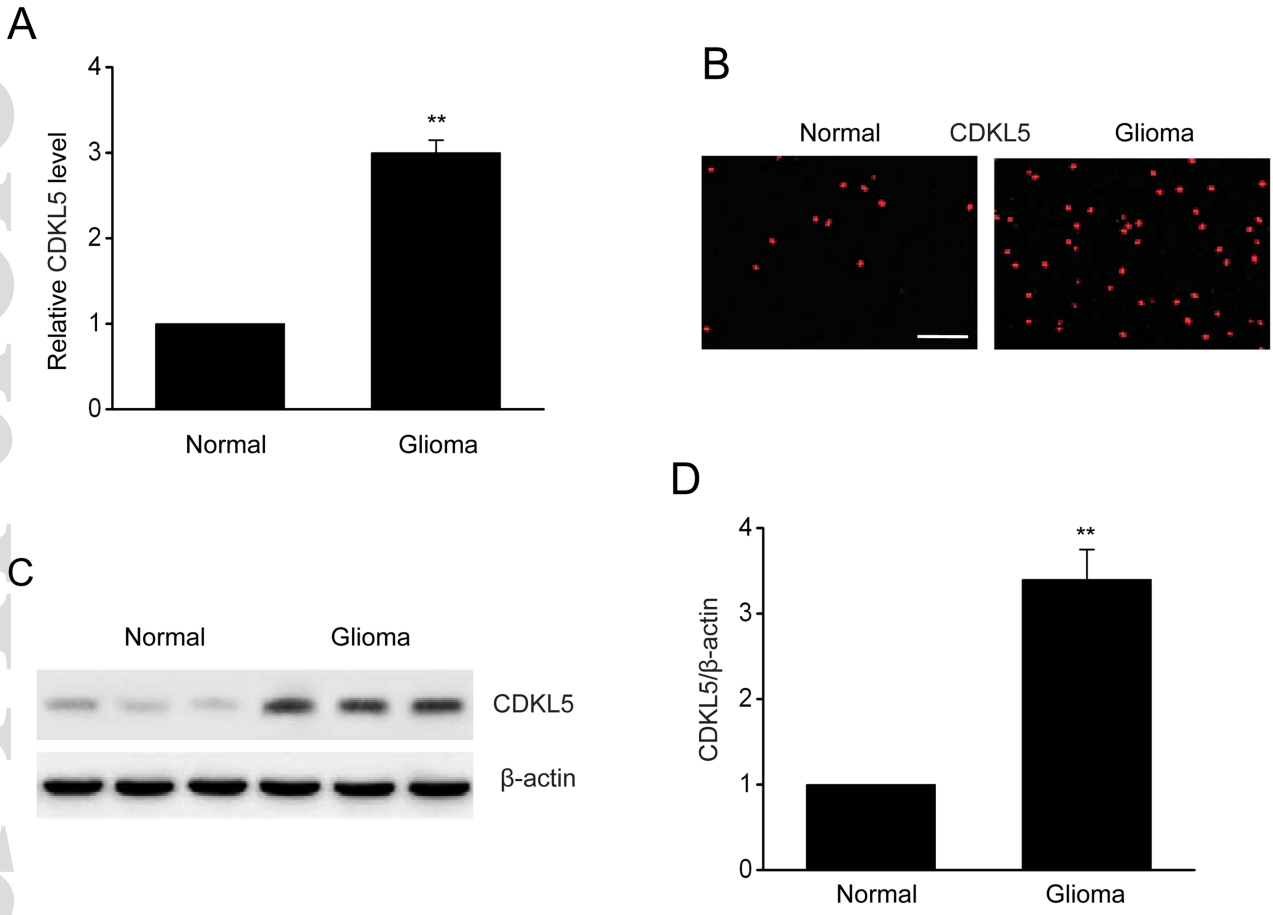
Fig. 3 CDKL5 reinforced drug resistance to β-lap and glioma proliferation in vitro. U251 cells were transfected with CDKL5 plasmid (CDKL5) or CDKL5 siRNA (si-CDKL5) for 48 h. **A,** MTT assay was performed to examine cell survival. **B–C,** Distribution of cell cycle phase was evaluated using FC. $^{**}P < 0.01$ vs. control group. **D–F,** CDKL5 promoted drug resistance to β-lap. U251 cells were transfected with CDKL5 plasmid (CDKL5) and supplemented with β-lap. MTT assay **(D)** was performed to examine cell survival. FC **(E–F)** was performed to examine cell death. Results are presented as means ± SEM, one way ANOVA, n = 3. $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$ vs. β-lap group.

Fig. 4 CDKL5 reinforced stimulation of PI3K/AKT axis in glioma cells

A–D, Representative immunoblots (**A**) and quantification of phosphorylation of PI3K (**B**), AKT (**C**) and GSK3β (**D**) in U251 cells after transient transfection with CDKL5 plasmid (CDKL5) or empty vector (Vector) for 48 h. Results are presented as means ± SEM, n =3. $^{**}P < 0.01$ vs. vector group. **E–H,** Representative immunoblots (**E**) and quantification of phosphorylation of PI3K (**F**), AKT (**G**) and GSK3β (**H**) in U251 cells after transient transfection with CDKL5 siRNA (si-CDKL5) or negative control (NC) for 48 h. Results are presented as means ± SEM, Student's t-test, n =3. $^{**}P < 0.01$ vs, NC group.

Fig. 5 CDKL5 quickened glioma generation through PI3K/AKT axis in vivo. Mice were

subcutaneously injected with stable U251 cells with CDKL5 plasmid (CDKL5) and administered with cisplatin and PI3K/AKT suppressor XL147 (CDKL5+CP+XL147). **A,** Representative immunoblots of p-AKT in tumor tissues. **B–C,** Representative images of malignancies (B) and quantification of malignancy volume (C) and weight (D) four weeks after subcutaneous xenografting. **E,** Representative IHC images of Ki67 in slices. Scale bar: 40 μm. Results are presented as means ± SEM, one way ANOVA, n = 5. $^{*}P < 0.05$ vs. vector group, $^{\#}P < 0.05$ vs. CDKL5 group.



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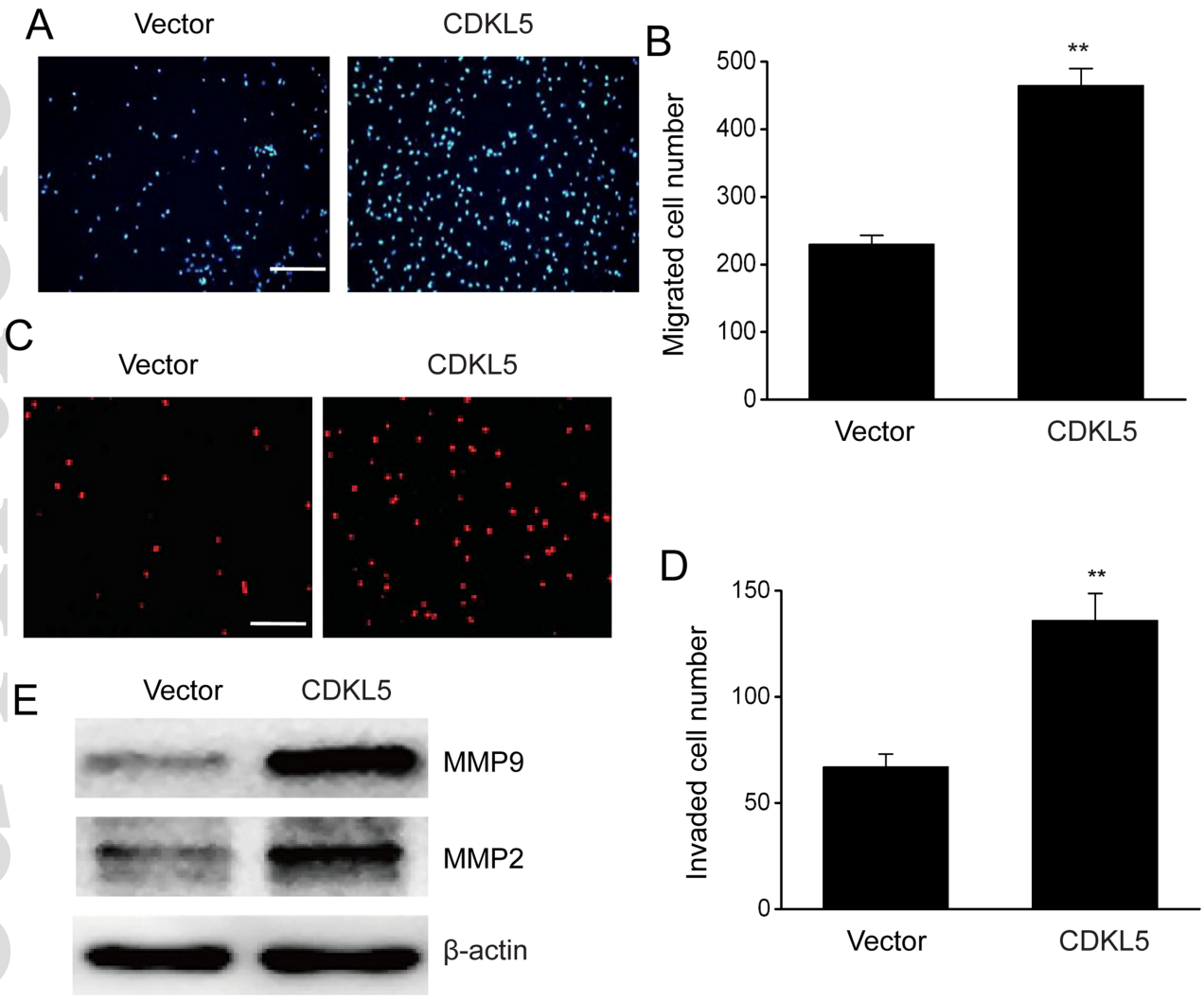

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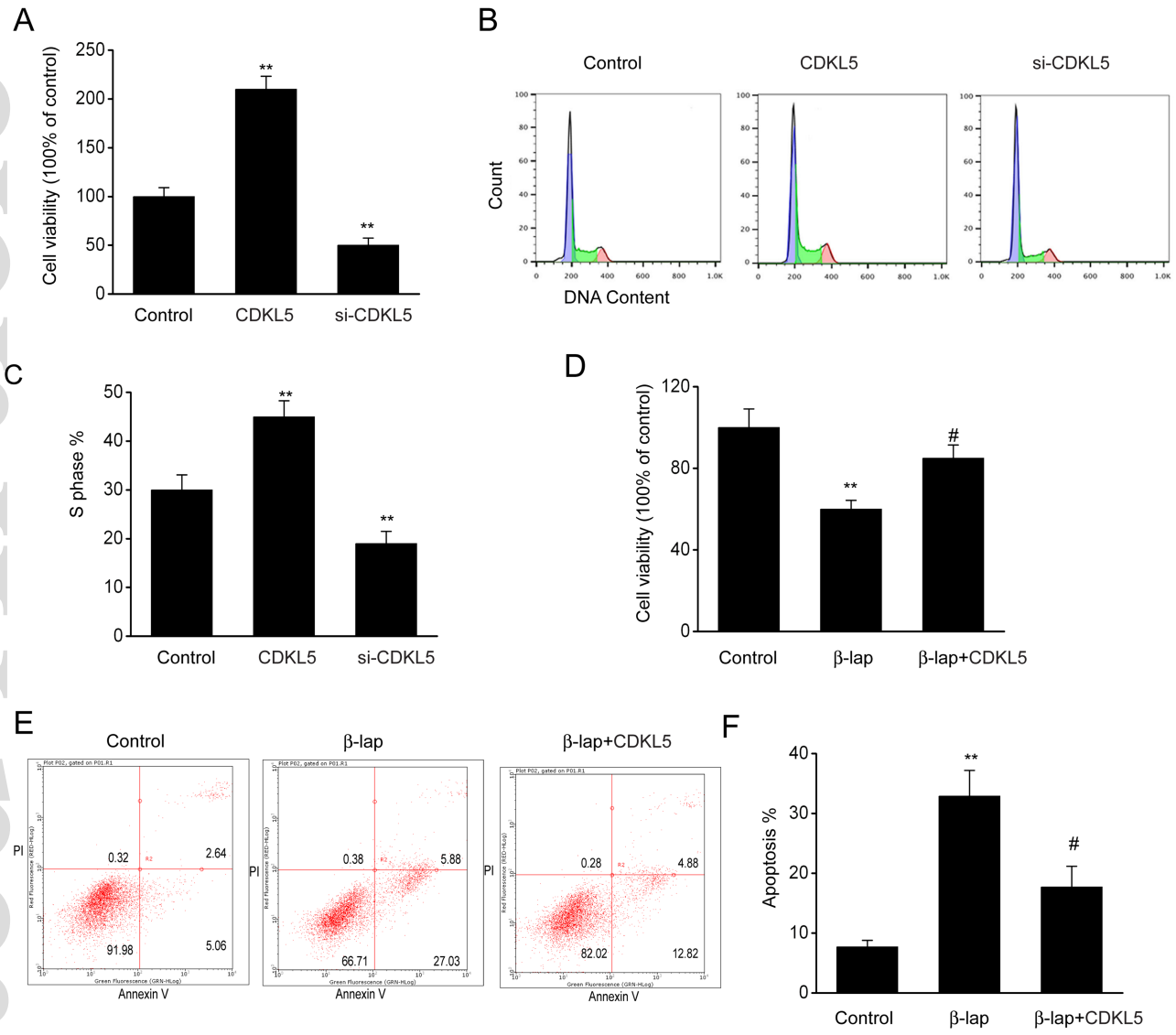

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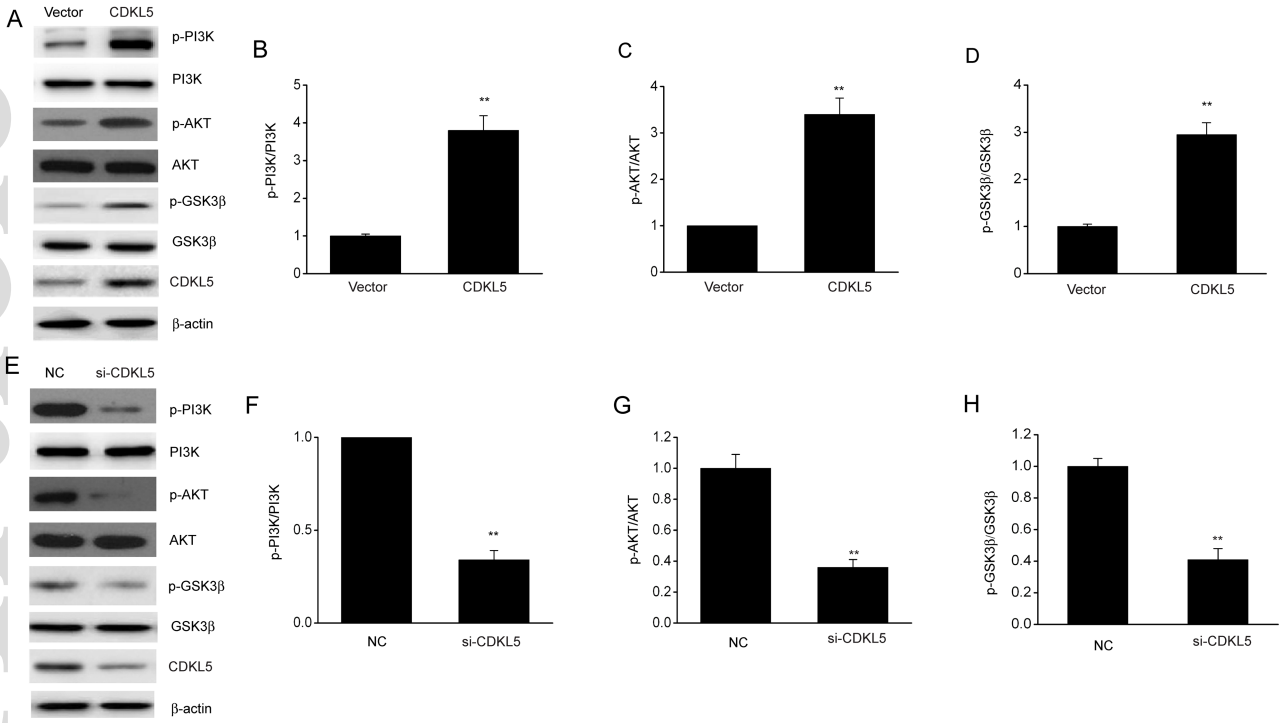

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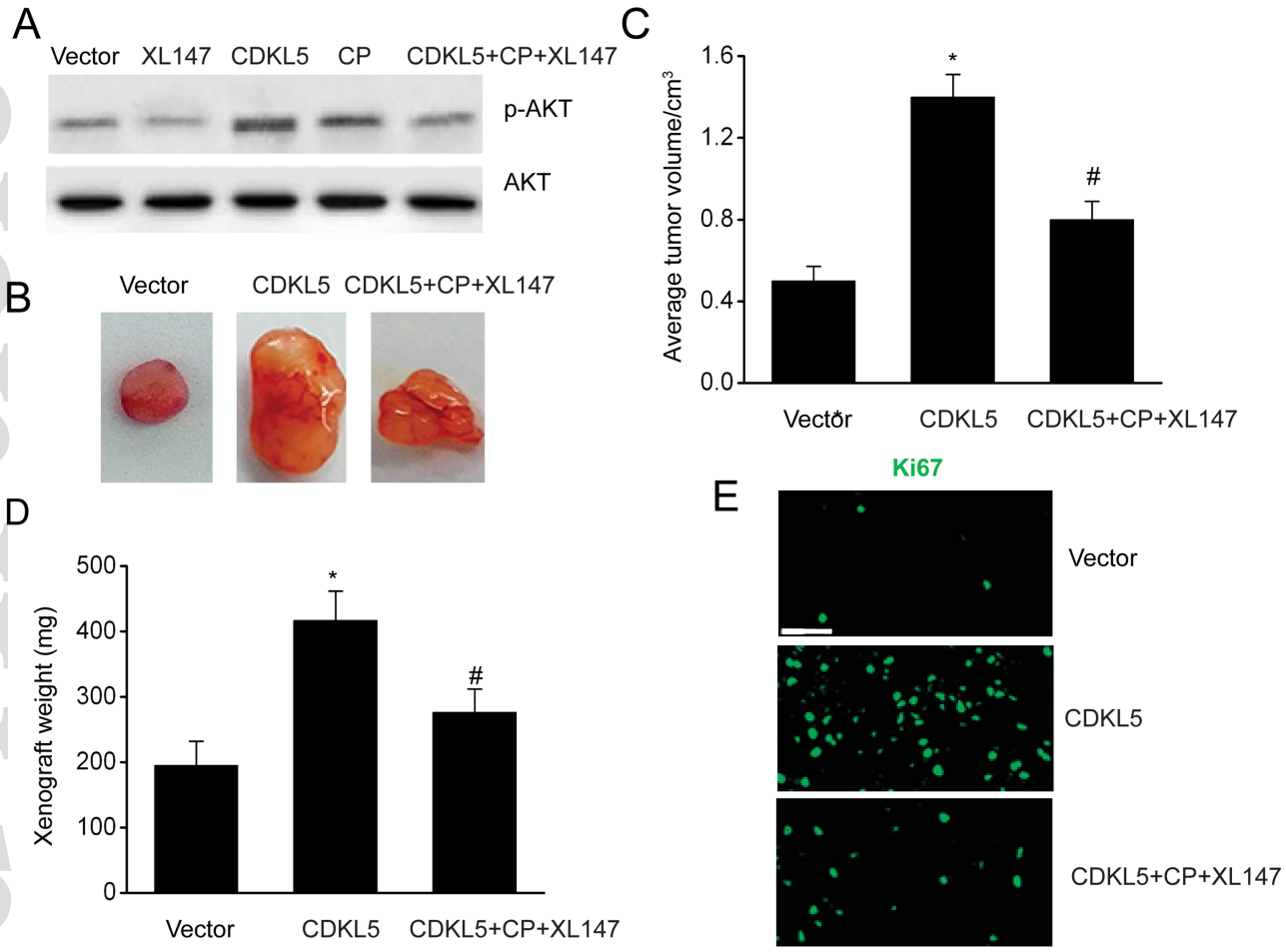

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