

PSMC2/E2F1 Axis Promotes the Development and Progression of Glioma

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Research

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Abstract

Background

Glioma is the most common type of malignant brain tumor with limited treatment strategy and poor prognosis. Proteasome 26S subunit ATPase 2 (PSMC2) is a key member of the 26S proteasome 19S regulatory subunit, whose role in glioma is still not clear. In this context, we aim to explore the role of PSMC2 in glioma *in vitro* and *in vivo*.

Methods

Expression of PSMC2 in glioma tissues and normal tissues are detected by immunohistochemical (IHC) analysis. The proliferation assays (MTT assay and Celigo cell counting assay), flow cytometry and migration assays (wound-healing assay and Transwell) are used to detect the effects of PSMC2 knockdown on glioma cells. The influence of PSMC2 knockdown on tumor growth *in vivo* was evaluated by mice xenograft models. In addition, the downstream target of PSMC2 is determined by human GeneChip and Ingenuity Pathway Analysis (IPA).

Results

PSMC2 is overexpressed in glioma tissues than normal tissues. Moreover, knockdown of PSMC2 can inhibit the proliferation, migration and arrest cell cycle in G2 phase of glioma cells. Additionally, PSMC2 knockdown promotes glioma cell apoptosis by increasing expression of caspase3, caspase8, IGFBP-1, while reducing expression of IGF-I, Survivin, TRAILR-4. *In vivo* findings reveal that PSMC2 knockdown inhibit the tumorigenicity of glioma cells. Furthermore, downstream of PSMC2 is explored, identifying E2F transcription factor 1 (E2F1) as a potential target. Notably, E2F1 knockdown exhibits similar effects on the development of glioma with PSMC2, which could strengthen the inhibition effects of PSMC2 knockdown on glioma synergistically.

Conclusions

PSMC2 is closely associated with glioma development by targeting E2F1, and might be considered as a novel therapeutic target in patients with glioma.

Background

Glioma is the most common primary craniocerebral malignancy resulting from the canceration of glial cells in the brain and spinal cord, which has the characteristics of high incidence, high recurrence, high mortality and low cure rate [1]. According to the World Health Organization (WHO) classification of central nervous system tumors, gliomas can be divided into four grades (I-IV), of which grade IV is also known as

glioblastoma (GBM) [2]. In addition, molecular classification of gliomas based on gene expression has been proposed, including anterior nerve, neurological, classical and mesenchymal subtypes [3]. Although these different subtypes have been identified, effective targeted therapies for gliomas have not been developed in recent decades to improve prognosis, and most low-grade gliomas (I and II) inevitably relapse and develop into high-grade gliomas (III and IV) [4]. Currently, only 5.5% of patients can survive 5 years after diagnosis. Even with multimodal therapy including surgery, radiation, and chemotherapy, the overall median survival is still only 14.5–16.6 months, and the efficacy of treatment is still frustrating [5, 6]. Lacking of effective therapeutic targets pose major challenges to prolong the survival and improve the quality of life of patients with glioma. Therefore, there is an urgent need to thoroughly understand molecular mechanism of glioma.

A large amount of evidence indicated that the 26S proteasome is involved in a lot of biological processes, such as cell cycle progression, apoptosis, metabolic regulation, and signal transduction [7–9]. Given its importance, the 26S proteasome is a multiple target for anti-cancer therapy [10]. Proteasome 26S subunit ATPase 2 (PSMC2) located in the genome 7q22.1-q22.3, is a key member of the 26S proteasome 19S regulatory subunit [11]. Partial genome deletion of PSMC2 was found in more than 3000 tumors, and PSMC2 is closely related to cancer cells, indicating that PSMC2 may be a potential target for cancer treatment [11]. Recently, the expression correlation and molecular mechanism of PSMC2 in various cancers have been explored. For example, Qin *et al.*, proposed that in pancreatic cancer, PSMC2 expression is up-regulated, and promotes cell proliferation, inhibits apoptosis [12]. He *et al.* indicated that in colorectal cancer, PSMC2 is related to survival rate, and silencing of PSMC2 can regulate the biological activities of cancer cells [13]. Nevertheless, functional validation and mechanistic studies for PSMC2 in glioma is still lacking.

To the best of our knowledge, this study is the first attempt to illustrate the potential carcinogenic activity of PSMC2 in glioma. First, clinical specimens were applied to IHC staining to reveal the differential expression of PSMC2 in glioma tissues and normal tissues. Subsequently, the correlation of PSMC2 expression with tumor characteristics in glioma patients was statistically analyzed to clarify that PSMC2 may be associated with the progression and development of glioma. More importantly, potential roles and mechanisms of PSMC2 in glioma cells *in vitro* and *in vivo* were explored. Notably, downstream of PSMC2 was investigated by RNA sequencing and IPA.

Results

Relationship between the expression of PSMC2 and glioma characteristics

IHC analysis was performed to show that the expression of PSMC2 in the tumor sample was significantly higher than that in the normal sample (Fig. 1A). In addition, the mRNA level of PSMC2 was highly abundant in glioma cell lines including U87, U251, U373 and SHG-44 (Fig. 1B). The high expression of PSMC2 in glioma was further confirmed with reference to TCGA-GBM database (Fig. 1C). More

importantly, based on the results of tissue microarray determination, the expression of PSMC2 may be associated with age ($P < 0.01$), recurrence ($P < 0.05$) and pathological stage ($P < 0.01$) of glioma (Table 1). In detail, the increased expression of PSMC2 was accompanied by the deepening of tumor malignancy in patients with glioma. In general, it can be known that PSMC2 may be related to the progression and development of glioma.

Table 1
Relationship between PSMC2 expression and tumor characteristics in patients with glioma

| Features | No. of patients | PSMC2 expression | | P value |
|--------------|-----------------|------------------|------|----------|
| | | low | high | |
| All patients | 173 | 92 | 81 | |
| Age (years) | | | | 0.0022** |
| < 41 | 79 | 52 | 27 | |
| ≥ 41 | 94 | 40 | 54 | |
| Gender | | | | 0.064 |
| Male | 107 | 51 | 56 | |
| Female | 66 | 41 | 25 | |
| Recurrence | | | | 0.0155* |
| Yes | 92 | 41 | 51 | |
| No | 81 | 51 | 30 | |
| Stage | | | | 0.0046** |
| I | 23 | 20 | 3 | |
| II | 78 | 40 | 38 | |
| III | 50 | 23 | 27 | |
| IV | 22 | 9 | 13 | |

Knockdown of PSMC2 inhibited the development of glioma cells in vitro

To further investigate the effects of PSMC2 on the development of glioma, cell lines U87 and U251 were selected to construct cell models. Lentivirus expressing PSMC2-targeting shRNA was used as shPSMC2 group and shCtrl as negative control group. The green fluorescence signal inside U87 and U251 cells was observed in > 80% cell popular, indicating successful transfection (Figure S1). The successful knockdown of PSMC2 was verified by Western Blot (WB) analysis (Fig. 1D). Subsequently, MTT assay (Fig. 2A), colony formation assay (Fig. 2B), and flow cytometry (Fig. 2C) were used to evaluate the proliferation,

colony formation, apoptosis of U87 and U251 cells. Apparently, results indicated that the knockdown of PSMC2 dramatically inhibited cell proliferation (> 2 folds, $P < 0.001$) and promoted cell apoptosis (> 2 folds, $P < 0.001$). Moreover, human apoptosis antibody array (Fig. 2D) was used to detect the differential expression of apoptosis-related proteins induced by PSMC2 knockdown in U251 cells. The results showed that the protein expression of caspase3, caspase8, IGFBP-1 was significantly up-regulated, while expression of IGF-I, Survivin, TRAILR-4 was significantly down-regulated ($P < 0.05$). Taken together, we concluded that PSMC2 knockdown inhibited cell proliferation and promoted apoptosis of glioma cells.

Knockdown of PSMC2 suppressed tumor growth in vivo

Furthermore, mice xenograft models were constructed by subcutaneous injection of U87 cells to further verify the role of PSMC2 in glioma. Results indicated that the tumor growth of glioma was significantly inhibited. Specifically, smaller size and lighter weight of tumors were observed in shPSMC2 group compared with shCtrl group ($P < 0.001$) (Fig. 3A-3C). In addition, bioluminescence imaging also proved that the bioluminescence intensity of shPSMC2 group was significantly lower than that of the shCtrl group ($P < 0.05$) (Fig. 3D, 3E), suggesting that proliferation ability of tumor tissue was weaker than that in negative group. Moreover, Ki67 expression in xenografts was also detected by IHC analysis, showing obvious lower expression in shPSMC2 group, which was consistent with the PSMC2-induced inhibition of tumor growth (Fig. 3F). In summary, knockdown of PSMC2 can inhibit the development of glioma cells, which was consistent with data from *in vitro* experiments.

Exploration of downstream molecular mechanism of PSMC2 in glioma cells

To further investigate the molecular mechanism of the effects of PSMC2 on glioma development, shPSMC2 ($n = 3$) and shCtrl ($n = 3$) transfected U251 cells were used for RNA sequencing analysis. In general, total of 2553 differentially expressed genes (DEGs) were identified based on $|\text{Fold Change}| \geq 2.0$ and $\text{FDR} < 0.05$, including 1132 up-regulated DEGs and 1421 down-regulated ones (Fig. 4A, S2A and S2B). Subsequently, typical signaling pathway, as well as disease and function were performed based on Ingenuity Pathway Analysis (IPA) to determine gene enrichment induced by knockdown of PSMC2 (Figure S2C and S2D). As indicated, the DEGs had significant correlation with cell viability, cell cycle progression, cell invasion and malignant tumor development by analyzing the enrichment of DEGs in IPA disease and function (Figure S2D). More importantly, considering our purpose to seek for tumor promotor factors, several down-regulated DEGs were selected for verification by qPCR and WB (Fig. 4B, 4C). Further combining the PSMC2 related interaction network predicted by IPA analysis, it was shown that E2F1 may be a potential downstream target of PSMC2 (Fig. 4D). Additionally, results of IHC indicated that E2F1 expression levels were obviously higher in glioma tissues than that in adjacent normal tissues (Fig. 4E). Indeed, the expression of E2F1 showed a similar pattern with PSMC2 in glioma tissues and cell lines (Fig. 4F). Taken together, E2F1 was identified as a potential target of PSMC2 in the regulation of glioma.

Knockdown of E2F1 aggravated the inhibition of glioma cells by PSMC2 depletion

To verify the hypothesis that PSMC2 regulates glioma development through E2F1, we investigated their functional roles in U251 cells. Herein, cells were divided into the following groups, including shCtrl (transfected with shCtrl, as negative control), shE2F1 (transfected with shE2F1 lentivirus for down-regulating E2F1 expression) and shPSMC2 + shE2F1 (transfected with shE2F1 and shPSMC2 lentivirus for simultaneously down-regulating E2F1 and PSMC2 expression). First, shE2F1 (RNAi-3) with the highest knockdown efficiency was screened by qPCR and used in the following experiments (Figure S3A). Fluorescence inside U251 cells transfected with shCtrl, shE2F1 and shPSMC2 + shE2F1 were observed by microscope and confirmed the successful transfection (Figure S3B). Moreover, results of qPCR (Figure S3C) and WB (Figure S3D) showed that E2F1 was down-regulated in the shPSMC2 group and PSMC2 also was down-regulated in the shE2F1 group, which indicated that there is a relationship between E2F1 and PSMC2 in U251 cells.

A series of subsequent cell function assays clarified that E2F1 knockdown affected glioma cell progression, such as inhibiting proliferation ($P < 0.001$, fold change = 3.1) (Fig. 5A), stimulating apoptosis ($P < 0.001$, fold change = 16.3) (Fig. 5B), arresting cell cycle in the G2 phase ($P < 0.001$) (Fig. 5C) and repressing migration ($P < 0.001$) (Fig. 5D, 5E). Thus, it can be speculated that E2F1 and PSMC2 have similar roles in glioma cells. More importantly, it was obviously demonstrated that further knockdown of PSMC2 in E2F1 knockdown cells could aggravate all the effects of it on cellular functions including cell proliferation ($P < 0.001$, fold change = 3.3), cell apoptosis ($P < 0.001$, fold change = 20.9), cell cycle ($P < 0.001$) and migration ($P < 0.001$, fold change > 2) (Fig. 5A-E). In conclusion, knockdown of E2F1 exhibited similar effects on the development of glioma with PSMC2, which could also strengthen the inhibition effects of PSMC2 knockdown on glioma synergistically.

Discussion

In this study, the upregulation of PSMC2 in glioma was demonstrated by IHC and qPCR analysis. Additionally, the data of *in vitro* experiments clarified that knockdown of PSMC2 may inhibit the proliferation, migration and arrest cell cycle. *In vivo* findings also revealed that PSMC2 knockdown inhibited the tumorigenicity of glioma cells. More importantly, PSMC2 knockdown promoted apoptosis of glioma cells by increasing expression of caspase3, caspase8, IGFBP-1, while reducing expression of IGF-I, Survivin, TRAILR-4.

Notably, apoptosis is a well-organized cellular mechanism that mediates programmed cell death through apoptosis-related protein cascades [14]. Therefore, the protein levels of apoptosis-associated factors were determined in PSMC2-transfected glioma cells. The protein expression of caspase3, caspase8, IGFBP-1 was significantly up-regulated in U251 cells with PSMC2 silencing. Conversely, the expression levels of IGF-I, Survivin, TRAILR-4 was down-regulated by PSMC2 knockdown. Shinoura and Fianco *et al.*, demonstrated caspase3 and caspase8 have abnormal activity in glioma cells and can regulate apoptosis [15, 16]. Studies have shown that IGFBP-1 inhibited tumor activity by inducing apoptosis in human cancer, such as prostate cancer and breast cancer [17, 18]. Furthermore, it was previously suggested that IGFBP-1 might be responsible for the reduced proliferative capacity of malignant glioma cells [19, 20].

Zumkeller *et al.*, proposed IGF-I was thought to play a pivotal role in the proliferation promotion of glioma [21]. Fenstermaker *et al.*, supported that Survivin is an inhibitor of apoptosis protein that is highly expressed in human glioma cells [22]. Meusch *et al.*, manifested that abnormalities of TRAIL and its receptor TRAIL-1, TRAILR-2, TRAILR-3, and TRAILR-4 appear to collectively promote the resistance of monocytes to TRAIL-induced apoptosis [23]. These findings were supported by the fact that PSMC2 silencing induced abnormal protein abundance associated with cleavage apoptosis.

Moreover, downstream of PSMC2 was explored by RNA sequencing followed by IPA, and E2F1 was identified as a potential target. Genome-wide mapping studies have revealed that E2F transcription factor 1 (E2F1) with genes from hundreds of promoter regions is involved in numerous cellular pathways [24–27]. Including cell cycle control [28], apoptosis [29], senescence [30], and DNA damage response [31]. As the primary regulator of cell fate, the complex role of E2F1 has been extensively investigated [32, 33]. Interestingly, recent data suggested that a certain correlation between the expression of this factor and cancer. Farra *et al.*, proposed that the role of E2F1 in HCC is not a trivial aspect as it may have a significant impact on the development of new treatment options targeting E2F1 [34]. Additionally, Rouaud *et al.*, reported the key role of E2F1 in controlling melanoma cell death and drug sensitivity [35]. Recently, Zhi *et al.*, elucidated the new mechanism of ECT2 affecting the proliferation of glioma cells by regulating the expression of the deubiquitinating enzyme PSMD14 to affect the stability of E2F1 [36]. Strikingly, this study elucidated that knockdown of E2F1 has a similar effect on the development of glioma by detecting cell proliferation, apoptosis, cell cycle and migration and can also synergistically enhance the inhibitory effects of PSMC2 on glioma cells.

Conclusion

In conclusion, PSMC2 was closely associated with proliferation, apoptosis, cell cycle and migration of glioma cells by targeting E2F1, and might be considered as a novel prognostic indicator in patients with glioma.

Methods

Immunohistochemical (IHC) staining

Brain glioma tissue and paired normal tissue microarray chips were purchased from Shanghai Outdo Biotech Co. Ltd. (Cat. # HBraG180Su01). Meanwhile pathological characteristics of these clinical samples were collected. All patients signed the informed consent form.

Briefly, after tissue sections were deparaffinized, repaired and blocked with citric acid antigen, they were incubated with anti-PSMC2 (1:100, SANTA CRUZ, Cat # sc-166972) and anti-E2F1 (1:100, Abcam, Cat # ab179445) respectively, overnight. After elution with PBS, secondary antibody IgG (1: 400, Abcam, USA, Cat # ab6721) was added and incubated. Tissue sections were then stained with DAB and hematoxylin for visualization. All tissues in the chip were pictured with microscopic and all slides were viewed with

ImageScope and CaseViewer. IHC scores were determined by staining percentage scores (classified as: 1 (1%-24%), 2 (25%-49%), 3 (50%-74%), 4 (75%-100%)) and staining intensity scores (scored as 0: Signalless color, 1: brown, 2: light yellow, 3: dark brown). To distinguish between high and low expression, the median was selected as cut off-value to reduce the impact of outliers.

Cells culture

Human glioma cell lines U87 and U251 were purchased from BeNa Technology. U87, U251 cells were cultured in CM1-1 medium containing 90% DMEM-H medium supplemented with 10% FBS. All culture medium was changed every 3 days and cells were humid maintained in a 37°C 5% CO₂ incubator.

Target gene knockdown cell models

Short hairpin RNAs (shRNA) of human PSMC2 and E2F1 and related control sequence were designed by Shanghai Bioscienceres, Co., Ltd. for the knockdown experiment. The target sequences were inserted into BR-V-108 vector using the T4 DNA ligase enzyme (NEB). Plasmids were extracted by EndoFree Maxi Plasmid Kit (Tiangen) and qualified plasmid was packaged with 293T cells. U251 and U87 cells at a density of 2×10^5 cells/ml were seeded in a six-well plate. 24 h later, cells were infected with 100 μ L lentiviral vectors (1×10^7 TU/well) additive with ENI.S and polybrene (10 μ g/ml, Sigma-Aldrich). After cultured at 37°C with a 5% CO₂ for 72 h, the fluorescence was observed by microscope.

qPCR analysis

U87 and U251 cells with shPSMC2, shE2F1, shPSMC2+shE2F1, and the corresponding negative controls grown in the 6-well plate were harvested with TRIZOL reagent (Invitrogen, Carlsbad, USA) and then provided by the manufacturer with a standard procedure to isolate the total RNA. Next, RNA samples were washed with ethanol and then dissolved in DEPC H₂O. Reverse transcription was applied to M-MLV reverse transcriptase and RNase inhibitor (Promega Corporation, Madison, Wisconsin, USA). qPCR was performed using SYBR Master Mix (Takara, Japan), where GAPDH was used as the internal control. Primer information was shown in the Table S1. Finally, the $2^{-\Delta\Delta Cq}$ method was used for quantification and dissolution curve was drawn.

WBanalysis

U87 and U251 cells with shPSMC2, shE2F1, shPSMC2+shE2F1, and the corresponding negative controls grown in the 6-well plate were harvested with Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime, Shanghai, China). Proteins were degenerated in Sodium dodecyl sulfate (SDS) sample buffer, followed by separating on SDS-polyacrylamide gelelectrophoresis (PAGE) gel using electrophoresis. After that, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane, and incubated with primary antibodies (Table S2) overnight at 4°C. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies IgG (Goat Anti-Rabbit) at room temperature for 1 h. Protein expression was then determined using ECL kit (Thermo Fisher Scientific, NY, USA).

MTT assay

Cell viability was measured using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-h-tetrazole bromide (MTT) for U87 and U251 cells with shPSMC2 and the corresponding negative controls for 48 h. Briefly, cells were plated at 2×10^3 per milliliter. MTT solution 5 mg/ml was added to each well of the 96-well plates for 4 h. After incubation, 100 μ l dimethyl sulfoxide (DMSO) were added to each well. The formazan was then quantified by measuring the 490 nm absorbance.

Colony formation assay

U87 and U251 cells with shPSMC2, shE2F1, shPSMC2+shE2F1, and the corresponding negative controls were trypsinized, resuspended, counted, and seeded in 6-well plate (2 ml/well) and incubated for 8 days to form colonies. Subsequently, cells were fixed by paraformaldehyde. After Giemsa staining, residue was washed multiple times and finally photographed with a digital camera. Numbers of colonies (> 50 cells/colonies) were counted under a microscope (MicroPublisher 3.3 RTV; Olympus, Tokyo, Japan). All determinations were repeated three times.

Cell apoptosis and cycle assay

U87 and U251 cells with shPSMC2, shE2F1, shPSMC2+shE2F1, and the corresponding negative controls cells were inoculated in a 6-well plate until cell density reached 85%. Cells were harvested, centrifuged ($1200 \times g$), and resuspended. For cell apoptosis, 10 μ l Annexin V-APC (eBioscience) was added and incubated at room temperature without light for 10 min. For cell cycle, cells were stained by PI staining solution (BD Biosciences). Apoptosis analyses and cell cycle distribution was detected Guava easyCyte HT FACS Calibur. (Millipore).

Celigo cell counting assay

After U251 cells with shPSMC2, shE2F1, shPSMC2+shE2F1, and the corresponding negative controls were cultured in 96-well plate 5 days. After plating, numbers of cells were evaluated using Celigo® image cytometer (Nexcelom, Lawrence, Mass, USA) by scanning green fluorescence every day for 5 days at room temperature.

Migration assays

After transfected with shPSMC2, shE2F1, shPSMC2+shE2F1, and the corresponding negative controls 72 h of U251 cells (2×10^3 cells/well) were scraped a monolayer at the scheduled time (0 h, 24 h, 48 h) for wound-healing assay and under 5% CO₂ incubated at 37°C.

Suspension of U251 cells transfected with shPSMC2, shE2F1, shPSMC2+shE2F1, and the corresponding negative controls were adjusted to 5×10^5 cells/ml, and then the cells were seeded in Transwell (Corning, NY, USA). The cells were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet

solution. Finally, the cells passing through the filter were photographed by an inverted fluorescence microscope.

Human apoptosis antibody array

Differentially expressed apoptosis-related proteins in U251 cells with shPSMC2, and the corresponding negative controls were detected by human apoptosis antibody array (RayBio, Norcross, GA, USA). First, the membrane was placed in the 8-well trays provided in the kit. Incubations with sample and Biotin-conjugated Anti-Cytokines should be performed overnight at 4°C. Overnight blocking and wash steps are useful for reducing background signal intensities even with completed membranes. Wash with Wash Buffer II, followed by repeating incubation with Streptavidin-HRP and chemiluminescent detection. Finally, Gelpro Analyzer software (Media Cybernetics, Rockville, MD, USA) was used to analyze protein expression levels.

Mice xenograft model

The animal experiments performed have been approved by the Animal Protection and Use Committee of Sir Run Run Shaw Hospital. Nude mice (BALB/c males, 4 weeks old) were purchased from Shanghai Lingchang Experimental Animal Co., Ltd (Shanghai, China) and were pathogen-free. Twenty mice were randomly divided into two groups (shCtrl group and shPSMC2 group) before the experiments. Meanwhile, 2×10^6 U87 cells with or without knockdown of PSMC2 suspended in PBS were injected into mice under the right axillary skin to construct a mouse xenograft model. Cultured for another 20 days after injection and collected data from 10 days after injection. Mice were injected with cells 10 days after starting to collect data twice a week, including animal weighing, measuring tumor length short diameter. The mice were finally sacrificed by injection of pentobarbital sodium, and the tumor was removed for photographing and weighing.

Repaired and blocked with citrate antigen after tumor tissue was removed from sacrificial mice. Antibody Ki67 (1:200, Abcam, USA, cat # ab16667) was added to shPSMC2 or shCtrl, respectively. After PBS elution, secondary antibody IgG (1:400, Abcam, USA, cat # ab6721) was incubated at room temperature. Tissue sections were first stained with DAB and then with hematoxylin. Finally, images were collected and analyzed by optical microscope.

RNA sequencing analysis

First, RNA was extracted and tested for quality as described above. And then, RNA sequencing analysis was performed by Genechem (Shanghai, China). Library for RNA sequencing was constructed from TruSeq Stranded mRNA LT Sample Preparation Kit (Illumina, San Diego, California, USA) according to the instructions of manufacturer, and scan it by Affymetrix Scanner 3000 (Affymetrix, Santa Clara, California, USA). DEGs were determined between the two groups based on thresholds of $|\text{Fold Change}| \geq 2.0$ and $\text{FDR} < 0.05$. IPA (Qiagen, Hilden, Germany) based on all DEGs to analyze rich functional annotations. Z-

score ≥ 2 meant that the pathway was significantly activated; otherwise the pathway was significantly inhibited.

Statistical analysis

All experiments were performed in triplicate and data were shown as mean \pm SDs. Statistical analyses and graphs were performed by GraphPad Prism 6.01 (Graphpad Software) and P value < 0.05 as statistically significant. The significance differences between groups were determined using the two-tailed Student's t test or One-way ANOVA analysis. PSMC2 expression in glioma tissues and normal tissues revealed in IHC assay were analysis with Sign test. Mann-Whitney U analysis and Spearman rank correlation analysis were used while explaining the relationships between PSMC2 expression and tumor characteristics in patients with glioma.

Abbreviations

PSMC2: Proteasome 26S subunit ATPase 2; E2F1: E2F transcription factor 1; WHO: the World Health Organization; GBM: glioblastoma; DEGs: differentially expressed genes; IPA: Ingenuity Pathway Analysis; WB:Western Blot; IHC: Immunohistochemical; shRNA: Short hairpin RNAs; RIPA: Radio Immunoprecipitation Assay; SDS: Sodium dodecyl sulfate; PAGE: polyacrylamide gelelectrophoresis; PVDF: polyvinylidene difluoride; HRP: horseradish peroxidase; DMSO: dimethyl sulfoxide.

Declarations

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Author contributions

Shuxu Yang, Yirong Wang and Xueyong Zheng designed this program. Xuchen Qi, Qin Lu and Dajiang Xie operated the cell experiments, Junhui Lv and Yiwei Liu performed animal experiments. Xuchen Qi and Qin Lu conducted the data collection and analysis. Xuchen Qi and Qin Lu produced the manuscript which was checked and revised by, Shuxu Yang and Yirong Wang.

All the authors have confirmed the submission of this manuscript.

Conflict of interest

The authors declare no conflict of interest.

Ethics approval and consent to participate

The animal experiments performed have been approved by the Animal Protection and Use Committee of Sir Run Run Shaw Hospital.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Figures

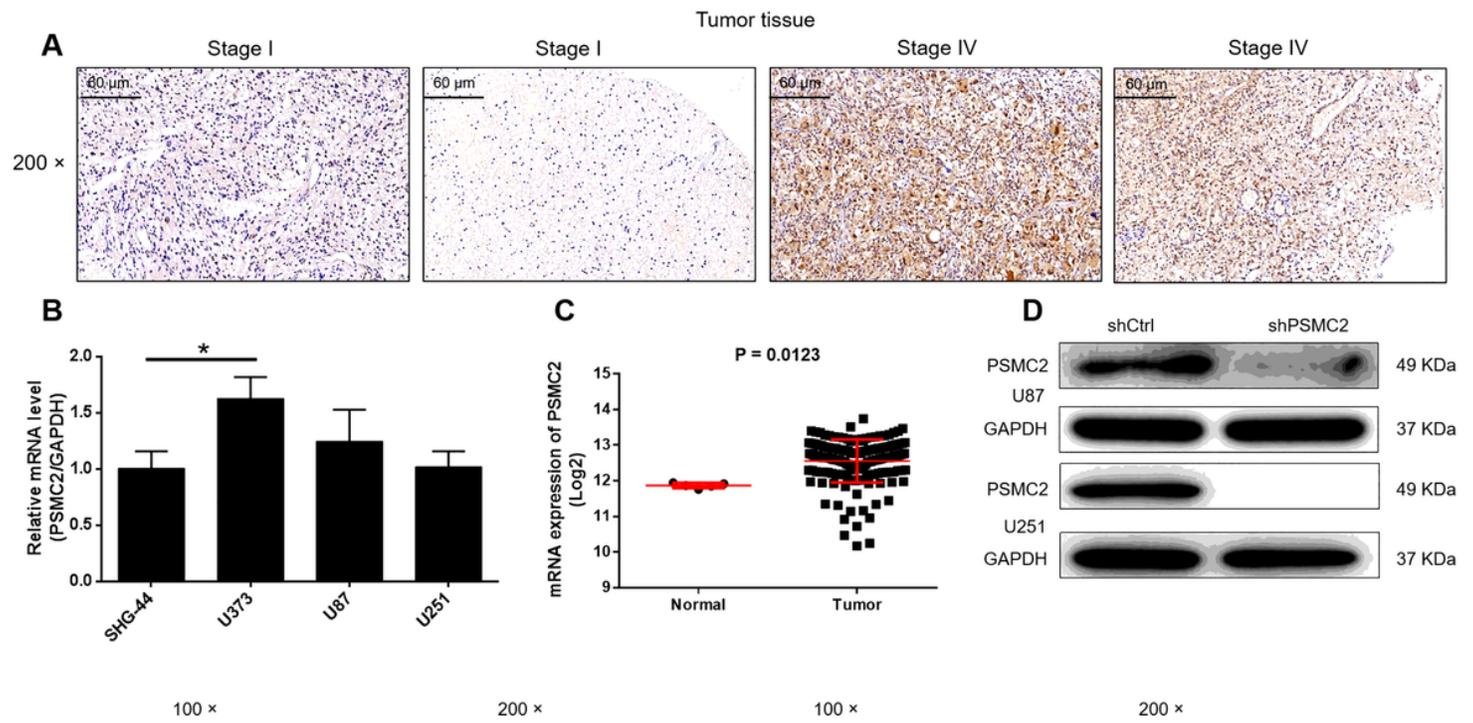


Figure 1

PSMC2 is highly expressed in glioma tissues and cells (A) The expression of PSMC2 in the normal and tumor detected by IHC (The magnification is 200×, scale bars is 60 μm). (B) The mRNA expression of PSMC2 in glioma cell lines. (C) The mRNA expression of PSMC2 in normal and tumor samples was evaluated from TCGA-GBM database. (D) The specificity and validity of the lentivirus-mediated shRNA knockdown of PSMC2 expression was verified by WB. The data was expressed as the mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.

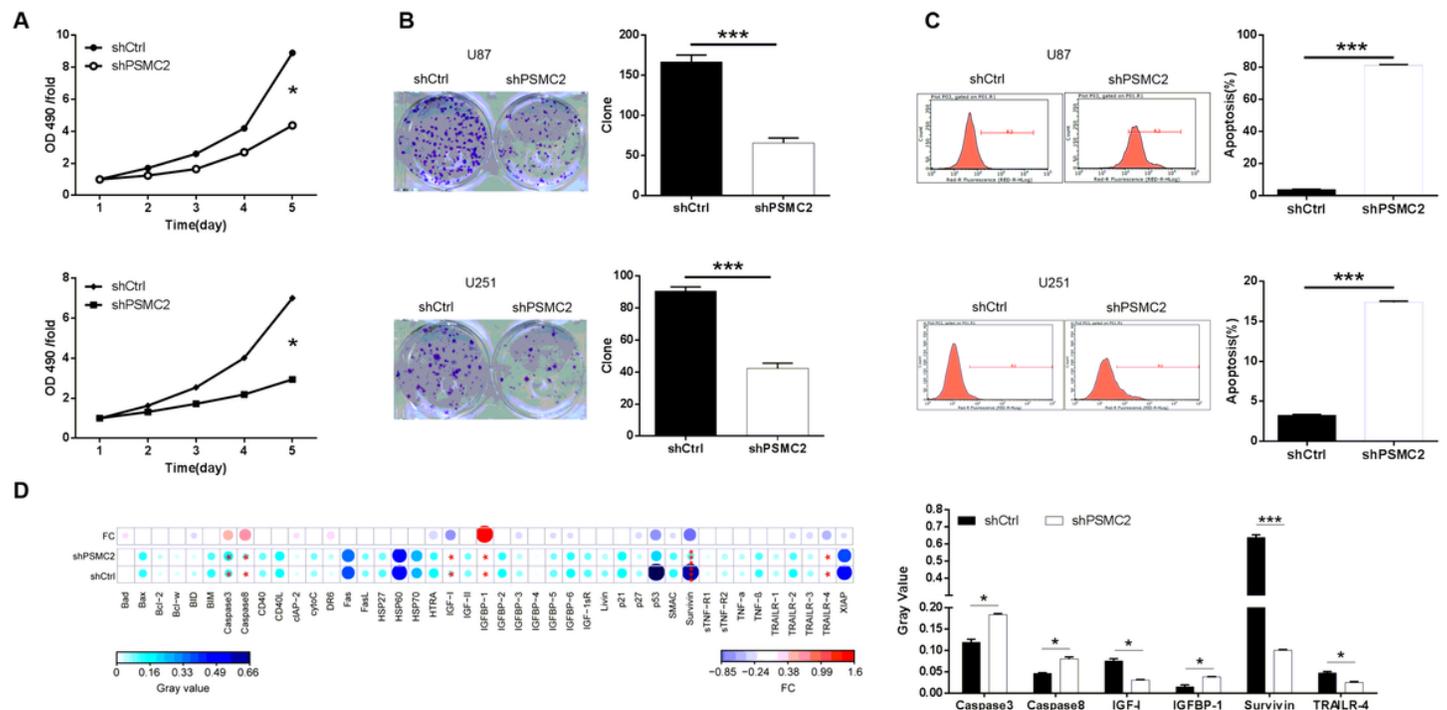


Figure 2

The effects of PSMC2 knockdown on proliferation, apoptosis of glioma cells (A) MTT assay was performed to detect the cell proliferation rate of U87 and U251 cells transfected with shCtrl or shPSMC2. (B) Colony formation assay was used to evaluate cell cloning capacity of U87 and U251 cells transfected with shCtrl or shPSMC2. (C) Flow cytometry analysis was performed to show the apoptotic cell percentage of U87 and U251 with or without PSMC2 knockdown (D) Human apoptosis antibody array was utilized to illustrate the regulation of the expression of apoptosis related proteins by PSMC2 knockdown. The data was expressed as mean \pm SD ($n \geq 3$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

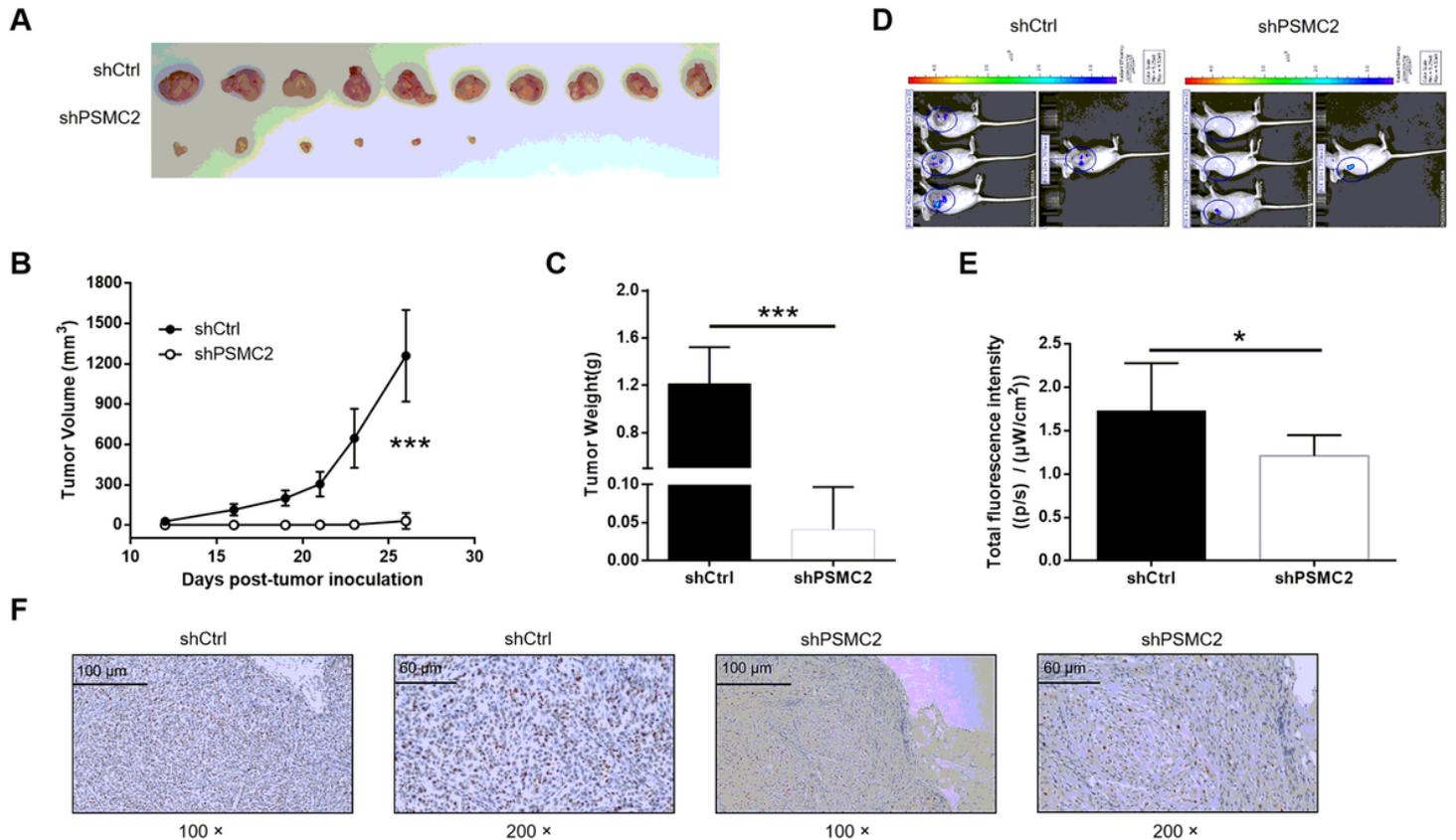


Figure 3

The knockdown of PSMC2 inhibits glioma growth in vivo. (A) The photos of xenograft tumors removed from the mice models in shCtrl and shPSMC2 groups. (B) The volume of tumors was measured and calculated during experiments. (C) The weight of tumors was measured after the sacrifice of mice models. (D) The total bioluminescent intensity of tumors in shCtrl group and shPSMC2 group. (E) The bioluminescence imaging of tumors in shCtrl group and shPSMC2 group. (F) The photos of Ki-67 staining of the removed tumors was evaluated by IHC analysis (The magnification is 100 \times and 200 \times , and the corresponding scale bars is 100 μm and 60 μm , respectively). The data was expressed as mean \pm SD ($n \geq 3$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

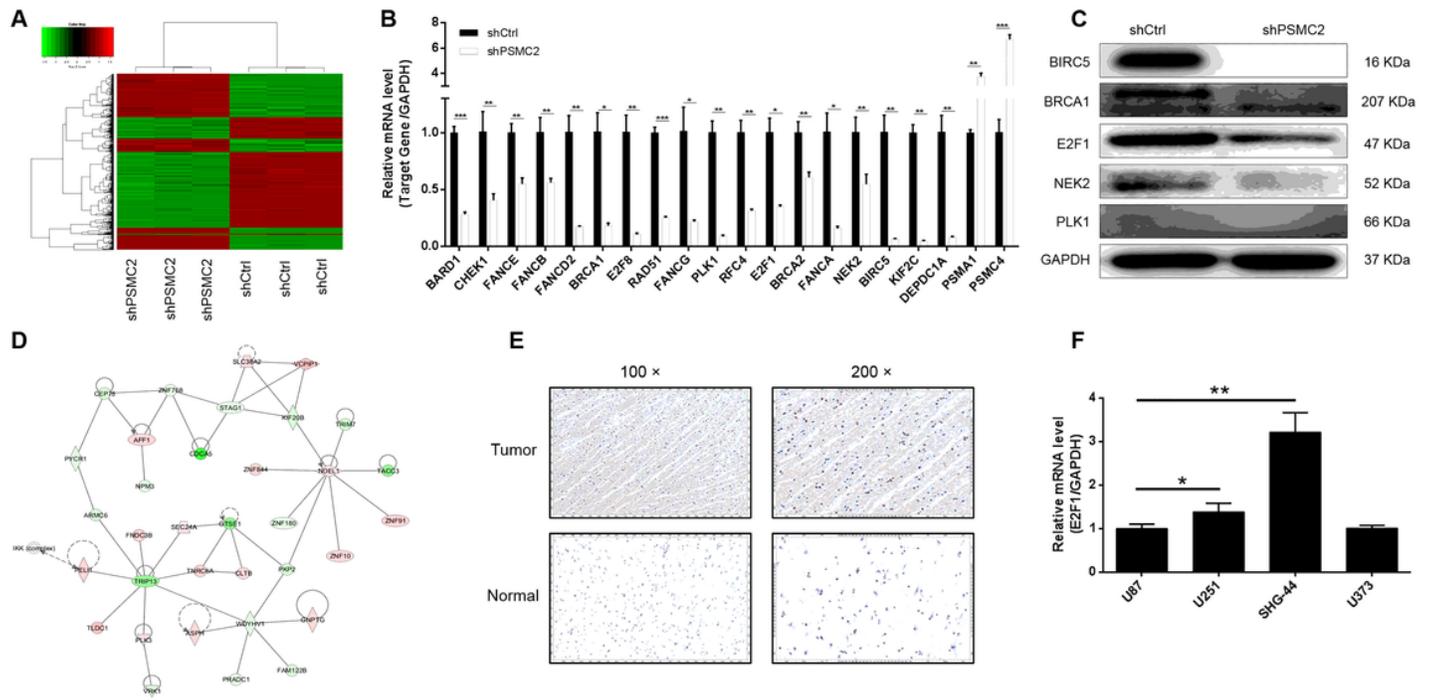


Figure 4

Underlying mechanism was explored by RNA sequencing and IPA analysis. (A) A Prime View Human Gene Expression Array was performed to identify the differentially expressed genes (DEGs) between shPSMC2 and shCtrl groups of U251 cells. qPCR (B) and WB (C) were used to detect the expression of several selected DEGs in U251 cells with or without PSMC2 knockdown. (D) A PSMC2-associated interaction network was established based on IPA analysis. (E) The expression of E2F1 in glioma tissues and normal tissues was evaluated by IHC analysis (The magnification is 100x and 200x, and the corresponding scale bars is 100 μm and 60 μm, respectively). (F) The mRNA expression of E2F1 in glioma cell lines was detected by qPCR. The representative images were selected from at least 3 independent experiments. The data was expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001

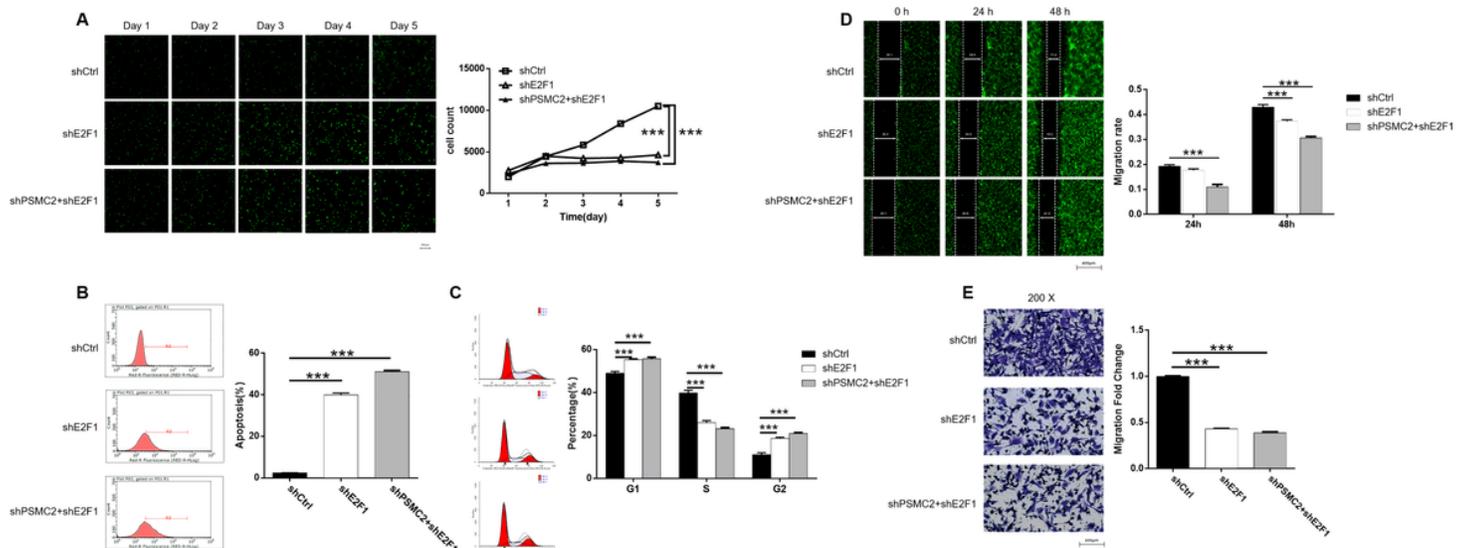


Figure 5

Effects of knockdown of E2F1 in coordination with PSMC2 in glioma cells The effects of U251 cells transfected with shCtrl, shE2F1, and shPSMC2+shE2F1 on proliferation, apoptosis, cell cycle and migration were examined by Celigo cell counting assay (scale bar = 200 μm) (A), flow cytometry (B, C), wound-healing assay (scale bar = 400 μm) (D) and Transwell assay (scale bar = 100 μm) (E), respectively. The representative images were selected from at least 3 independent experiments. The data was expressed as mean \pm SD ($n \geq 3$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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