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RPA3 promotes the proliferation, migration, and invasion of gliomas by activating the PI3K-AKT-mTOR pathway

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ABSTRACT

Gliomas are the most common primary malignant brain tumors, with a poor prognosis and high mortality, and there is no effective treatment regimen. A number of studies have shown that replication protein A3 (RPA3) can regulate DNA replication and that the abnormal expression of RPA3 can lead to genomic instability and induce the development of a variety of tumors. However, the relationship between RPA3 and gliomas and the mechanism of action remains unclear. In this study, we investigated the role of RPA3 in the development of gliomas and the possible mechanism. The Chinese Glioma Genome Atlas (CGGA), The Cancer Genome Atlas (TCGA), and the Gene Expression Omnibus (GEO) databases were used to analyze the expression level of RPA3 and its correlation with clinical prognosis. A univariate Cox regression model was established to predict the prognosis of glioma patients and analyze the correlation between RPA3 and immune cell infiltration and activation. Immunohistochemistry, RT–PCR, and Western blot (WB) were used to detect the expression of RPA3 in glioma specimens. After knocking down and overexpressing RPA3 with plasmids, effects on glioma cell proliferation, migration and invasive capacity were investigated in vitro. The possible molecular mechanisms were analyzed using WB. Results showed that the expression of RPA3 in glioma tissue and cells was significantly higher than that in normal glial cells and was positively correlated with the poor prognosis of patients with gliomas. The overexpression of RPA3 expression activated the phosphatidylinositol 3-kinase (PI3K)-AKT-mammalian target of the rapamycin (mTOR) pathway by promoting the phosphorylation of PI3K, AKT, and mTOR, thereby promoting the proliferation, migration and invasion of glioma cells. In conclusion, RPA3 is highly expressed in gliomas and promotes the proliferation, migration and invasion of gliomas by activating the PI3K-AKT-mTOR pathway. Therefore, RPA3 may be a prognostic biomarker and therapeutic target for gliomas.

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Introduction

Gliomas are the most common primary malignant brain tumors and can occur in any part of the central nervous system. The incidence of gliomas is 5.26/10, 000 per year (1), accounting for 50%-60% of primary tumors in the central nervous system (2). Gliomas usually originate from glial cells or precursor cells and develop into astrocytomas, oligodendrogliomas, oligoastrocytomas or ependymomas (3). Despite improvements in treatment methods that have prolonged the survival of glioma patients in recent years, the majority of glioma patients still have poor prognoses. According to The Chinese Glioma Genome Atlas (CGGA), the median survival time of glioma patients is only 14.4 months (4). However, the exact molecular mechanism underlying glioma formation is still not clear. Therefore, it is very important to explore the molecular mechanisms of glioma occurrence and development.

Replication protein A (RPA) is a highly conserved multisubunit single-stranded DNA-binding protein complex that is composed of 3 subunits: p70 (RPA1), p34 (RPA2) and p14 (RPA3). The 3 subunits interact with other DNA replication proteins and are mainly responsible for regulating DNA replication, cell cycle checkpoints and DNA recombination (5-7). Defects in these cellular processes may lead to genomic instability, gene mutations and chromosomal mismatches, resulting in the development of cancer (8, 9). Recently, the expression level of RPA protein in a variety of malignant tumors has become an important prognostic indicator. For example, blood anti-RPA2 antibodies can be used as a useful molecular marker for the early diagnosis of breast cancer. The expression of RPA1 and RPA2 proteins is a valid prognostic indicator for patients with colon cancer and bladder urothelial carcinoma (10, 11). Through bioinformatics strategies and public online resource prediction, we found that RPA3 is highly expressed in gliomas and is closely related to the prognosis of patients with gliomas. In this study, we investigated the relationship between RPA3 expression and the occurrence of gliomas and the effect of RPA3 expression on the occurrence and development of gliomas by verifying the effect of RPA3 regulation on the phosphatidylinositol 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway.

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Materials and Methods

Clinical tissue specimens

A total of 38 patient specimens collected at Baoding First Central Hospital, China from January 2021 to November 2021 were analyzed, including 30 glioma specimens (7 grade II, 13 grade III, and 10 grade IV) and 8 nontumor brain tissue specimens (collected from patients with a craniocerebral injury who underwent internal decompression) that were used as controls. Two neuropathologists confirmed the histological diagnosis of each specimen based on World Health Organization (WHO) guidelines. Glioma tissue and nontumor brain tissue specimens were collected immediately after resection and stored in liquid nitrogen for future use. This study was approved by the Medical Ethics Committee of Baoding First Central Hospital. All patients' families were informed of the study and signed an informed consent form prior to surgery.

Bioinformatics analysis

The pan-cancer dataset in The Cancer Genome Atlas (TCGA), which consists of 33 types of cancer and adjacent tissue samples, and RPA3 expression matrices were analyzed with UCSCXenaShiny (https://hiplot.com.cn/advance/ucsc-xena-shiny). In this study, we analyzed both glioblastomas(GBMs) and low-grade gliomas (LGGs). All the glioma datasets were obtained from Gliovis (http://gliovis.bioinfo.cnio.es/), including 6 datasets from 9 databases: TCGA, CGGA, Gravendeel, Rembrandt, LeeY, Freije, Kamoun, Murat, and Phillips. Survival analysis was then performed to identify the relationship between RPA3 expression and glioma prognosis. To determine the association between RPA3 and immune infiltration, we conducted gene set enrichment analysis (ssGSEA) to assess the presence of 24 types of immune cells in the glioma microenvironment; gene set signatures were obtained from a previous study (12). We then estimated the relationship between RPA3 expression and 18 cancer-related pathways using GSVA analysis. The gene datasets of 18 cancer-related pathways were downloaded from the Molecular Signatures Database (MSigDB, http://www.gsea-msigdb.org/).

Cell culture

Human brain glial cell lines (U251 and normal human astrocytes) were purchased from Procell Life Science & Technology Co., Ltd. The cells were cultured in RPMY-1640 (Gibco) medium containing 10% fetal bovine serum (FBS, Gibco) and 100 U/ml penicillin and streptomycin (Pen-Strep Solution, BI) at 37 °C in 5% CO2.

RNA extraction and quantitative RT–PCR

TRIzol reagent (Invitrogen) was used to extract RNA from U251 cells, normal human astrocytes and clinical specimens. RNA was reverse-transcribed into cDNA. Quantitative PCR was performed using ABI 7500 RT–PCR in a thermal cycler (Applied Biosystems). The PCR conditions were as follows: 1 cycle of 95 °C for 30 s, and then a 2-step cycle repeated 40 times (60 °C for 30 s and 72 °C for 30 s). The 2-ΔΔCt method was used to calculate relative expression. SYBR Premix Ex Taq TM Kit Mix (Takara) and primers 5'GTTCCGCCCCTATCCAAGTC and 3'GGAAGTGGAGATTGGCTGCT were used in the reaction.

Cell transfection

siRNA for the RPA3 sequence was synthesized by Zhongshi Tontau Co.; the siRNA sequence was CGGTTTCCGGAAAGAAGGTTCTCCA. The empty plasmid and the recombinant RPA3 expression plasmid were purchased from HanBio Biotechnology Co., Ltd. U251 cells in the logarithmic growth phase were inoculated into T25 cell culture flasks. After the cell density reached a confluence of 50%-60%, the cells were transfected following the protocol provided with the Lipofectamine 3000 reagent. The cells were harvested 24 h or 48 h after transfection.

Immunohistochemistry (IHC) staining

Tissue specimens were fixed and embedded in paraffin. After antigen retrieval at 95 °C for 20 min, the slides were blocked with goat serum for 1 h, incubated with primary antibodies at 4 °C overnight, and then incubated with rabbit secondary antibodies at room temperature for 2 h. Specimens for the negative control group (nontumor brain tissue specimens) were stained and visualized using the same method.

Protein extraction and Western blot (WB) assay

Total protein was extracted from glioma cells with whole cell lysis buffer, and equivalent amounts of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% skim milk for 2 h, the membrane was incubated with primary antibodies at 4 °C overnight, washed with 0.1% TBST 3 times (5 min/wash), and then incubated with pure goat anti-rabbit antibodies (1:10000; Abcam) for 1.5 h. The bands were detected using an Odyssey infrared scanner. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control, and the band intensity was quantified using ImageJ software.

Transwell invasion and migration assays

The invasion assay was performed using a Transwell chamber with a filter membrane pore size of 8 μm (Corning, 3422). In the invasion assay, 100 μl of Matrigel (Corning) was diluted 1:8 with serum-free RPMI-1640 and stored in a Transwell chamber for 4 h in a fridge for later use. For the migration assay, a Transwell chamber without Matrigel (Corning) was directly used. Two hundred microliters of serum-free culture medium containing 105 cells were added to the top chamber of the Transwell insert, and 750 μl of RPMI-1640 containing 10% FBS was added to the bottom chamber. The cells were incubated at 37 °C in 5% CO2 for 24 h; glioma cells in the top chamber were removed using a cotton swab. After fixation with 4% paraformaldehyde, the transmembrane cells were stained with crystal violet, and the cells in each well were counted under 5 different high-power microscope fields.

CCK-8 analysis

Cells (5×103 cells/well) were seeded in 96-well plates. At 0, 1, 2, 3, and 4 days after cell inoculation, CCK-8 solution was added to each well, and the cells were incubated for another 2 h. The optical density was measured at 450 nm using a microplate reader.

Scratch assay

Cells were cultured in 6-well plates for 24 h. Then, a wound was made on the cell monolayer in each well; the monolayer was then washed twice with phosphate-buffered saline (PBS) and incubated in a serum-free RPMI-1640 medium. The cells were photographed under a microscope at 0 h and 24 h and analyzed using ImageJ software.

Data analysis

Data were analyzed using SPSS (version 20.0) (IBM Corp., Armonk, NY, USA) and Prism 8 (GraphPad Inc, USA). All results are expressed as the mean ± SD of 3 independent assays. Differences between groups were analyzed using the Student's t-test and one-way analysis of variance (ANOVA). $P<0.05$ was used as the threshold for statistical significance.

Results

RPA3 was highly expressed in gliomas and was associated with poor patient prognosis

To determine whether RPA3 was overexpressed in glioma cells, we first analyzed the mRNA expression of RPA3 in tumor tissue. Tumor databases were used to analyze the differential expression of genes. We used the TCGA database to assess RPA3 mRNA expression in cancer tissues and normal clinical specimens. The results showed that compared with that in normal tissue, RPA3 mRNA expression was significantly upregulated in some tumor tissues. Among tumor tissue, RPA3 mRNA expression was high in both LGGs and high-grade gliomas (Figure 1), suggesting that the transcript level of RPA3 correlates with tumor type. Subsequently, we used a TCGA dataset to analyze the expression level of RPA3 mRNA. We observed that the expression level of RPA3 was high in gliomas with a high degree of malignancy and was associated with patient age but not sex (Figure 2). Additionally, we divided tumor tissue into a high-expression group and a low-expression group based on the level of RPA3 expression. Survival analysis was used to clarify the prognostic differences. The results suggested that overall survival (OS), disease-specific survival (DSS) and progression-free survival (PFS) were all lower in the RPA3 mRNA high-expression group than in the low-expression group in both LGGs and GBMs (Figure 3). To further clarify the correlation between RPA3 mRNA expression and the prognosis of glioma patients, we performed Kaplan–Meier curve and log-rank test analyses using 9 tumor databases. The results indicated that the survival time of patients with high RPA3 mRNA expression was significantly shorter than that of patients with low RPA3 mRNA expression (Figure 4). To further validate these results, we performed IHC, WB, enzyme-linked immunosorbent assay (ELISA), and qRT–PCR for RPA3 to assess the difference in RPA3 expression in gliomas and normal tissue. As expected, WB and qRT–PCR results confirmed that the expression level of RPA3 in glioma cells (U87, U251, and U137) was significantly higher than that in normal glial cells. The IHC results revealed that the expression of RPA3 exhibited a progressive increase with the increase in glioma grade, based on the WHO classification, suggesting high RPA3 expression in gliomas and thus a poor prognosis (Figure 5).

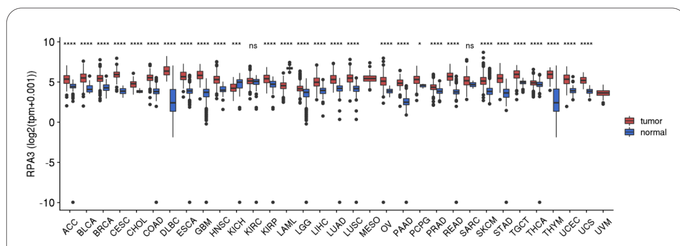

Figure 1. Compared with that in normal tissue, RPA3 mRNA expression was higher in both LGGs and high-grade gliomas OS (overall survival), DFS (disease-free survival), DSS (disease-specific survival), PFS (progression-free survival).

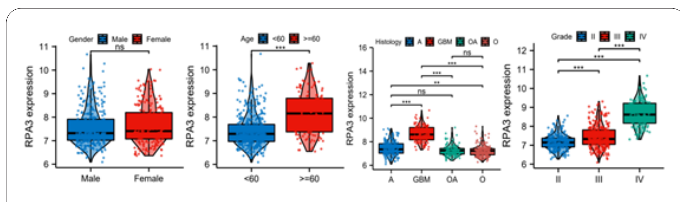

Figure 2. The expression level of RPA3 was high in gliomas with a high degree of malignancy and was associated.

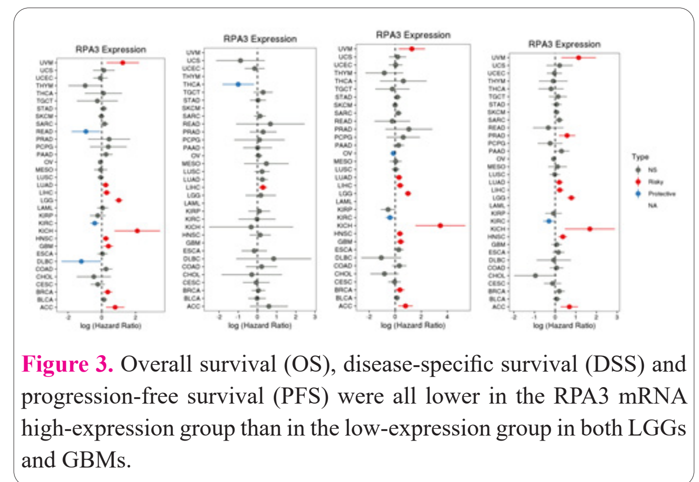

Figure 3. Overall survival (OS), disease-specific survival (DSS) and progression-free survival (PFS) were all lower in the RPA3 mRNA high-expression group than in the low-expression group in both LGGs and GBMs.

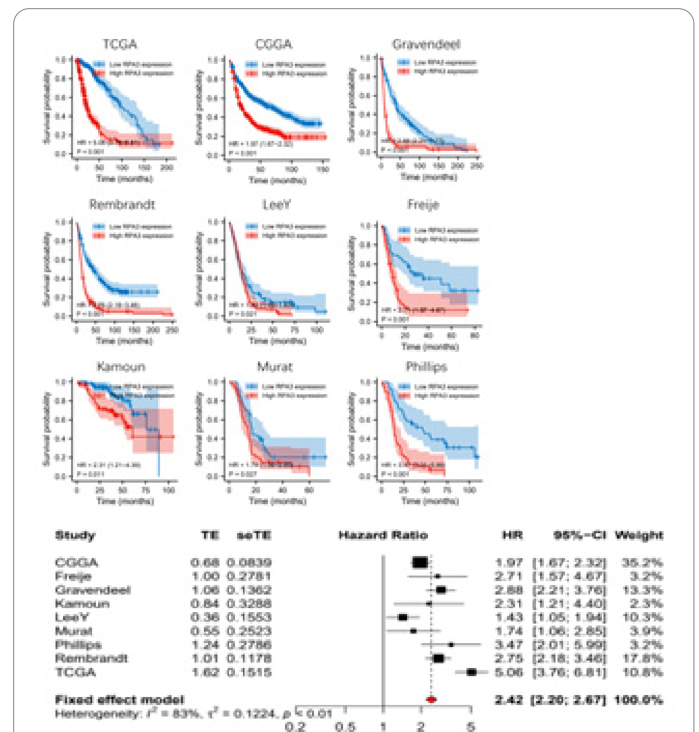

Figure 4. The survival time of patients with high RPA3 mRNA expression was significantly shorter than that of patients with low RPA3 mRNA expression.

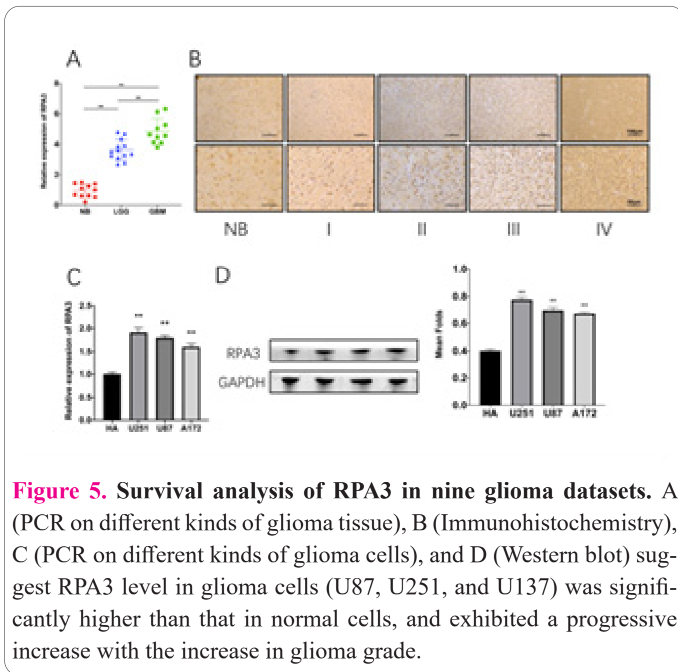

Figure 5. Survival analysis of RPA3 in nine glioma datasets. A (PCR on different kinds of glioma tissue), B (Immunohistochemistry), C (PCR on different kinds of glioma cells), and D (Western blot) suggest RPA3 level in glioma cells (U87, U251, and U137) was significantly higher than that in normal cells, and exhibited a progressive increase with the increase in glioma grade.

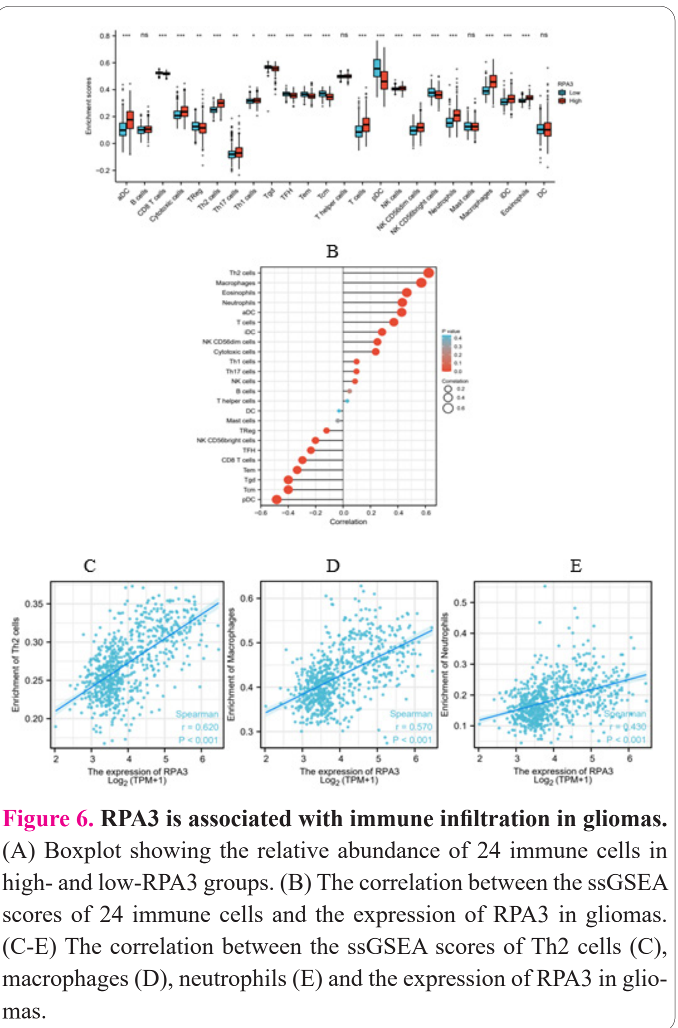

Figure 6. RPA3 is associated with immune infiltration in gliomas. (A) Boxplot showing the relative abundance of 24 immune cells in high- and low-RPA3 groups. (B) The correlation between the ssGSEA scores of 24 immune cells and the expression of RPA3 in gliomas. (C-E) The correlation between the ssGSEA scores of Th2 cells (C), macrophages (D), neutrophils (E) and the expression of RPA3 in gliomas.

Correlation between RPA3 and immune infiltration of gliomas

Accumulating evidence suggests that immunosuppressive cells may protect tumors from natural antitumor immune responses by inhibiting antitumor immune effector cells and facilitating immune escape. Here, we investigated the role of RPA3 in the immune microenvironment. Our results indicated that RPA3 expression was positively related to the level of multiple immunosuppressive cells, including Th2 cells, macrophages and neutrophils. These findings suggest that RPA3 may play a role in generating an immunosuppressive microenvironment by regulating immune infiltration (Figure 6).

Estimation of the association between RPA3 and cancer-related pathways

Various pathways have played significant roles in cancer development. We further investigated the relationship between RPA3 and 18 cancer-related pathways. Our results showed that RPA3 was positively correlated with the enrichment scores for the cell cycle and P53 signaling and PI3K-AKT-mTOR signaling pathways. These results suggest that RPA3 might affect glioma development by regulating these pathways (Figure 7).

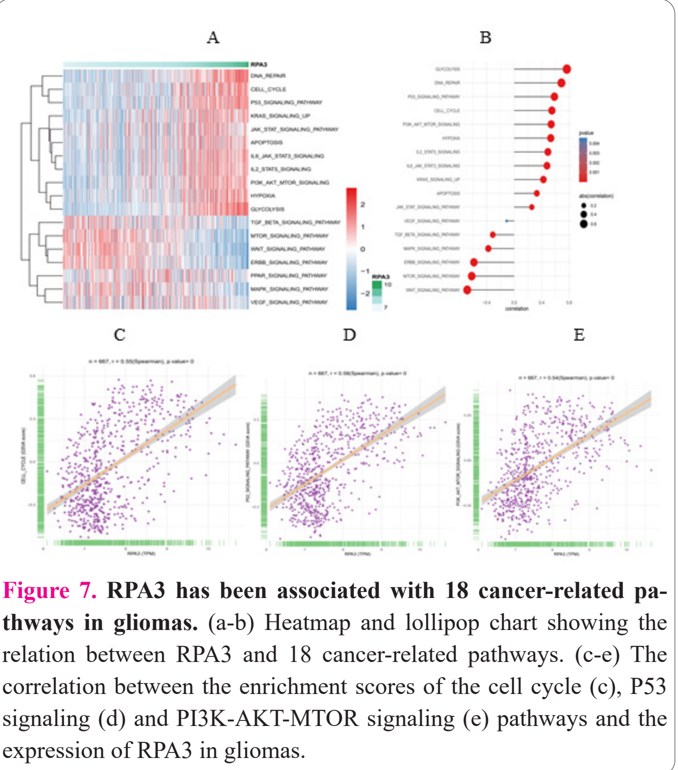

Figure 7. RPA3 has been associated with 18 cancer-related pathways in gliomas. (a-b) Heatmap and lollipop chart showing the relation between RPA3 and 18 cancer-related pathways. (c-e) The correlation between the enrichment scores of the cell cycle (c), P53 signaling (d) and PI3K-AKT-MTOR signaling (e) pathways and the expression of RPA3 in gliomas.

The effect of RPA3 knockdown or overexpression on the in vitro proliferation, migration and invasion ability of glioma cells

To evaluate the role of RPA3 in gliomas, we used 2 groups of RPA3 expression plasmids to stably transfect U87 cells to construct RPA3 knockdown cells and RPA3 overexpression cells. WB and RT–PCR results verified low and high RPA3 expression in glioma cells in the knockdown group and overexpression group, respectively, suggesting that the plasmid transfection was effective and that the cells could be used for subsequent in vitro experiments. The Transwell analysis results showed that after RPA3 knockdown, the invasive ability of U87 cells significantly decreased; in contrast, after RPA3 was overexpressed, the invasive ability of the glioma cells significantly increased. Subsequent CCK-8 and scratch assay results indicated that the proliferation and migration abilities of gliomas progressively increased with the increase in RPA3 expression and that RPA3 knockdown significantly inhibited the proliferation and migration of gliomas. The above results jointly suggest that RPA3 promotes the proliferation, migration and invasion of gliomas(Figure 8).

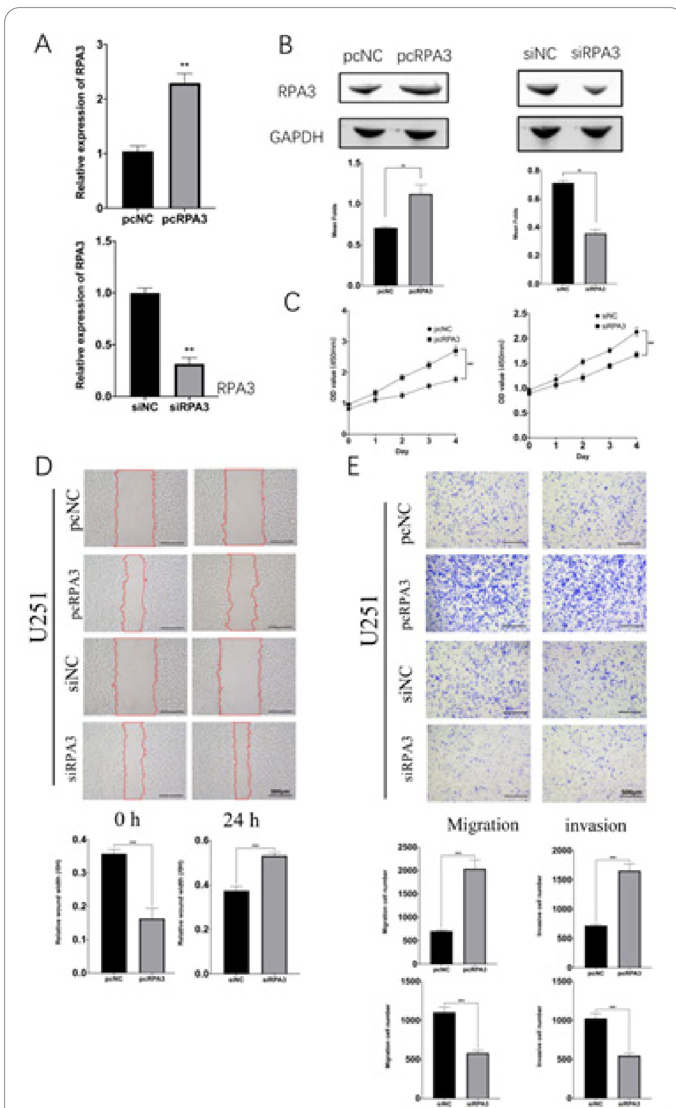

Figure 8. The regulation of RPA3 expression affects the proliferation and migration of glioma cells in vitro. (A) Relative RPA3 mRNA expression in U251 cells after upregulating or konckdowning of RPA3 determined by real-time PCR.(B)Western blot analysis of RPA3 expression in U251 cells after upregulating or konckdowning of RPA3.GAPDH is adopted as a loading control.(C)CCK8 assays were used to investigate the proliferation rates in RPA3-silenced and RPA3-activated U251 cells.(D)A wound-healing assay was adopted to investigate the migration capacity of the RPA3-silenced and RPA3-activated U251 cells. Representative images of the wound healing assay are shown in the above drawing and the percentage of wound width (%) in the drawing below.(E)Colony formation assay and Transwell assay were used to investigate the migration and invasion of the RPA3-silenced and RPA3-activated U251. Representative pictures are shown in the above drawing, and the number of migrated or invaded cells was counted in the drawing below

RPA3 regulated the proliferation of glioma cells through the PI3K-AKT-mTOR signaling pathway

RPA3 siRNA and overexpression plasmids were transfected into U87 cells. The possible tumor proliferation signaling pathways activated by RPA3 were analyzed by detecting the phosphorylated forms of PI3K, AKT, and mTOR via WB, to reveal the molecular mechanism by which RPA3 mediates cell proliferation. The WB results indicated that RPA3 knockdown significantly reduced the phosphorylation of PI3K, AKT, and mTOR, whereas the phosphorylation levels of PI3K, AKT, and mTOR in-

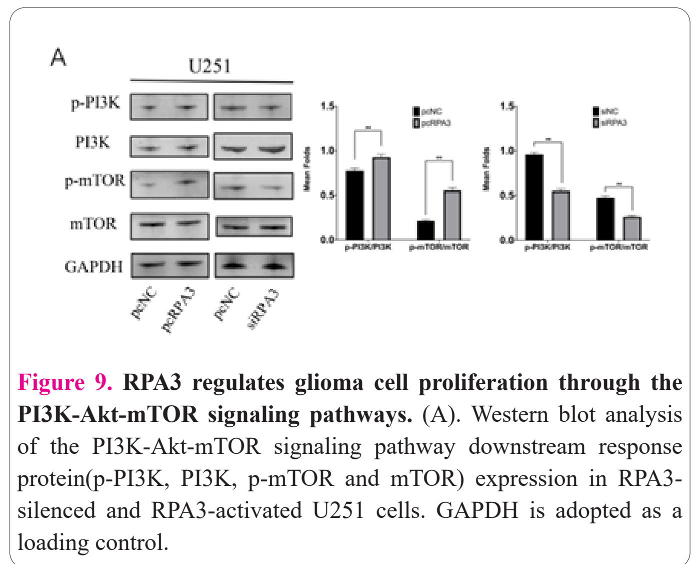

Figure 9. RPA3 regulates glioma cell proliferation through the PI3K-Akt-mTOR signaling pathways. (A). Western blot analysis of the PI3K-Akt-mTOR signaling pathway downstream response protein(p-PI3K, PI3K, p-mTOR and mTOR) expression in RPA3-silenced and RPA3-activated U251 cells. GAPDH is adopted as a loading control.

creased as RPA3 expression increased without a change in the total expression of PI3K, AKT, and mTOR. Therefore, RPA3 can promote the proliferation of glioma cells by activating the PI3K-AKT-mTOR signaling pathway (Figure 9).

Discussion

Gliomas are nervous system tumors and the most common central nervous system malignant tumors(13). Because the pathophysiological mechanism of glioma occurrence is not fully understood, the progress in developing new treatment options for patients with gliomas has been limited. Elucidating the molecular mechanism of glioma development will facilitate the discovery of novel therapeutic targets and contribute to the development of therapeutic strategies for gliomas. The RPA complex is an important single-stranded DNA-binding protein. It functions through interactions with DNA and related proteins to counteract the harmful effects of DNA damage and plays an important role in the process of DNA metabolism. The abnormal expression of the RPA complex can affect the stability of the genome and induce tumorigenesis. As a subunit connecting RPA1 and RPA2, RPA3 can stabilize and support the RPA complex (14, 15) and directly bind to DNA (16); it is also involved in the recognition and repair of DNA damage (17, 18). Increasing evidence has shown that RPA3 protein expression is related to the patient survival rate and that it is highly expressed in a variety of malignant tumors. However, its role in glioma development has not been directly studied. The results from this study showed that RPA3 activated the PI3K-AKT-mTOR pathway, promoted the proliferation of glioma cells, and maybe a potential target for the treatment of gliomas.

In this study, we first investigated the expression of RPA3 in different grades of glioma and found that RPA3 was overexpressed in glioma tissue. Kaplan–Meier and Cox regression analyses indicated that high RPA3 expression was closely associated with the poor prognosis of glioma patients. Subsequently, fluorescence quantitative PCR, WB, and IHC were used to detect the mRNA and protein expression levels in glioma tissues and cells. The results suggested that the expression level of RPA3 was positively correlated with the degree of malignancy of gliomas. Next, we regulated the expression of RPA3

in glioma cells. The results from Transwell and scratch assays showed that the invasion and migration abilities of glioma cells were significantly reduced after RPA3 gene knockdown. To elucidate the mechanism of action of RPA3 on glioma cells, we confirmed that PI3K-AKT-mTOR is a putative target of RPA3 based on the results of bioinformatics analysis of a miRNA database.

The PI3K-AKT-mTOR signaling pathway is closely related to the mitogen-activated protein kinase (MAPK) pathway. It regulates cell metabolism and cytoskeletal reorganization. It is frequently activated in human cancers and is an important intracellular signaling pathway in the development of most tumors (19, 20). The mechanism of action includes the promotion of tumor cell proliferation, invasion, and migration and the inhibition of autophagy and senescence (21-24). Studies have shown that a variety of positive regulators of the PI3K-AKT-mTOR axis (for example, PI3CA (p110) and PIK3R1 (P85)) have carcinogenic potential (25, 26), leading to the activation of PI3K; then, AKT is phosphorylated and activated and localizes to the plasma membrane to phosphorylate and activate the key downstream target mTOR, initiating its protein synthesis function (27). Our study showed that the expression levels of P-PI3K, P-AKT and P-mTOR in U251 cells after RPA3 knockdown significantly decreased and that the expression levels of P-PI3K, P-AKT and P-mTOR in U251 cells after RPA3 overexpression significantly increased, without affecting the total amount of PI3K and AKT, suggesting that RPA3 activates the PI3K-AKT-mTOR pathway to promote the proliferation and invasion of tumor cells.

Although this study revealed this mechanism, there are still some details that need to be further elucidated. First, although we found that RPA3 activates the PI3K-AKT-mTOR axis and regulates the proliferation and migration of tumor cells, the detailed molecular mechanism by which RPA3 regulates the PI3K-AKT-mTOR pathway is not fully understood. Second, the effect of RPA3 on gliomas was only studied at the tissue and cell levels; therefore, further, in vivo studies are needed before clinical application.

In summary, our study showed that RPA3 plays a tumor-promoting role in gliomas and that high RPA3 expression is closely related to poor prognosis and can be used as an independent marker of poor prognosis. Additionally, the disruption of RPA3 expression can inhibit the activation of the PI3K-AKT-mTOR pathway, thereby inhibiting the proliferation, migration, and invasion of glioma cells. Therefore, RPA3 may become a potential target for the treatment of gliomas.

RPA3 is highly expressed in glioma tissue and cells, and high RPA3 expression is positively correlated with poor patient prognosis. It promotes the proliferation, migration, and invasion of gliomas by activating the PI3K-AKT-mTOR pathway. Therefore, a full understanding of the exact mechanism of action of RPA3 in human gliomas can facilitate the development of future glioma treatments based on RPA3 inhibition in human glioma cells, making it a potential prognostic biomarker and novel therapeutic target for gliomas.

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