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Overexpression of Protease Serine 8 Inhibits Glioma Cell Proliferation, Migration, and Invasion via Suppressing the Akt/mTOR Signaling Pathway

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Protease serine 8 (PRSS8), a serine peptidase, has a widespread expression in normal epidermal cells. Recently, many researchers demonstrated downregulation of PRSS8 in cancer tissues as well as its tumor suppressor role in cancer development. However, the biological functions of PRSS8 in glioma remain unclear. In the current study, we demonstrated a decreased expression of PRSS8 in glioma tissues and cell lines. PRSS8 upregulation inhibited glioma cell proliferation, migration, and invasion. In addition, xenograft experiments showed that PRSS8 overexpression suppressed glioma cell growth in vivo. We also found that upregulated PRSS8 reduced the protein expression levels of p-Akt and p-mTOR in glioma cells. Taken together, our study demonstrated that overexpression of PRSS8 inhibited glioma cell proliferation, migration, and invasion via suppressing the Akt/mTOR signaling pathway. Therefore, PRSS8 may act as a novel therapeutic target for glioma.

Key words: Protease serine 8 (PRSS8); Proliferation; Migration; Invasion; Glioma

INTRODUCTION

Glioma, a common and lethal brain tumor, accounts for almost 70% of malignant tumors in the central nervous system[1–3]. In accordance with the WHO classification system, glioma is divided into different types, among which glioblastoma multiforme (GBM) is classified as grade IV and is the most aggressive form[4,5]. Patients with GBM have a low survival rate, and their median survival is only about 15 months[6–9]. Despite the advancement in therapeutic approaches, including surgery, chemotherapy, and radiotherapy, the situation of patients with high-grade glioma is not improving due to drug resistance, metastasis, and recurrence[10–12]. Thus, it is essential to identify new biomarkers specific to glioma stages and to improve the understanding of the mechanisms underlying glioma development, which may help to make therapies more effective.

Protease serine 8 (PRSS8), a trypsin-like serine peptidase, is known as prostasin[13–15]. It was first identified in 1994 by Yu et al., who separated it from seminal fluid and found its localization in prostatic ducts[13]. There have been studies demonstrating that PRSS8 abundantly exists in normal epidermal cells and plays an important role in terminal epithelial differentiation[16,17]. In addition, PRSS8 was found to be expressed in tissues such as kidney, bronchi, lung, colon, and liver, showing its role in other biological processes[13]. Recently, some researchers found a downregulation of PRSS8 in cancer tissues and its tumor suppressor role in various cancers[18–22]. However, other researchers suggested a contrary role for PRSS8 in cancer progression[23,24]. Despite the extensive study of PRSS8 in cancers, the biological functions of PRSS8 in glioma remain unclear.

The present study investigated the expression pattern and biological significance of PRSS8 in glioma. The study results showed that PRSS8 was downregulated in glioma tissues and cell lines. The in vitro experiments indicated that PRSS8 upregulation inhibited glioma cell proliferation, migration, and invasion. The xenograft experiments showed that PRSS8 overexpression suppressed glioma cell growth in vivo. We also demonstrated that PRSS8 exerted its inhibitory effect on glioma cells through suppressing the Akt/mTOR signaling pathway.

MATERIALS AND METHODS

Tissue Samples

The study was performed with the approval of the ethics committee of Huai'an First People's Hospital (P.R. China). Twenty pairs of glioma samples and normal brain tissues were acquired from patients who were hospitalized in the Huai'an First People's Hospital and participated in the study with informed consent. No patients received radiotherapy or chemotherapy before surgery. Tissues samples

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were collected, frozen in liquid nitrogen, and stored at −80°C before use.

Cell Lines and Cell Culture

Human glioma cell lines T98G and HS683 as well as normal human astrocytes NHA were purchased from the Cell Bank of the Shanghai Branch of Chinese Academy of Sciences (Shanghai, P.R. China). Cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum (FBS; Sigma-Aldrich).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA isolation from tissue samples or cultured cells, PrimeScript™ RT Reagent Kit (TakaRa, Dalian, P.R. China) for cDNA synthesis, and SYBR Green II PCR Kit (TakaRa) for RT-PCR. Reaction conditions were 95°C for 10 min, 45 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 10 s. The primers used were as follows: PRSS8, 5′-AGAGGACATGGTGTGTGCTG-3′ (sense) and 5′-GAGGCTGGAGTTCTGTCACC-3′ (antisense); GAPDH, 5′-GTCAAGGCTGAGAACGGGAA-3′ (sense) and 5′-AAATGAGCCCCAGCCTTCTC-3′ (antisense). The relative changes in the mRNA expression of PRSS8 were normalized to the expression level of GAPDH and calculated through the $2^{-\Delta\Delta}$CT method[25].

Western Blot

Total protein was extracted from tissues or cells using RIPA buffer. Protein samples were separated with 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated at 4°C overnight with primary antibodies against PRSS8, p-Akt, Akt, p-mTOR, mTOR, or GAPDH (Invitrogen). After washing three times with TBST, the membranes were incubated for 1 h with HRP-conjugated secondary antibodies (Invitrogen). The protein was visualized using enhanced chemiluminescence (GE Healthcare, Milwaukee, WI, USA). Protein expression levels were normalized with GAPDH and quantified via ImageJ software.

Plasmid Constructs and Transfection

The PRSS8 expression plasmid (pcDNA3.1-PRSS8) was obtained from Life Technologies (Grand Island, NY, USA). Cells were transfected with pcDNA3.1-PRSS8 or pcDNA3.1 (empty vector) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours later, Western blot analysis was performed to confirm the transfection efficiency.

MTT Assay

The MTT assay was carried out to measure cell proliferation. In brief, cells were cultured in 96-well plates for 24 h at a density of 1×10^3 cells/well. MTT (20 μl; 5 mg/ml) was added to each well, and cells were further cultured for 4 h. The medium was then discarded, and 100 μl of DMSO (Sigma-Aldrich) was added. The absorbance was measured with a microplate reader at a wavelength of 570 nm.

Colony Formation Assay

Cells were seeded into six-well plates at a density of 500 cells per well. After culturing for 2 weeks, cells were fixed with 4% paraformaldehyde for 10 min and then stained with 0.1% crystal violet for 20 min. Subsequent to washing with PBS (Sigma-Aldrich), plates were air dried. The number of stained colonies containing 50 cells or more was counted under a microscope.

Transwell Assay

Transwell chambers (Corning Inc., Tewksbury, MA, USA) were used to measure cell migration and invasion. For the migration assay, cells were suspended in serum-free DMEM and added to the upper chamber at a density of 2×10^4 cells/well. DMEM containing 10% FBS was added to the bottom chamber. After cells were cultured for 24 h at 37°C, nonmigrating cells were removed with cotton wool, and cells that migrated were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of migrating cells was counted under a light microscope. The invasion assay was performed according to the above procedure, except that Transwell membranes were precoated with Matrigel.

In Vivo Tumorigenicity Assay

Female BALB/c mice (4 to 5 weeks old) were obtained from the Experiment Animal Center of Nanjing Medical University (P.R. China). Mice were kept under specific pathogen-free (SPF) conditions and used for studies with the approval of the Institutional Animal Care and Use Committee of Huai'an First People's Hospital. All mice were divided into two groups, and each group contained eight mice. For the in vivo tumorigenicity assay, 2×10^5 T98G cells (transfected with indicated plasmids) were subcutaneously injected into the right flank of nude mice. The tumor volume was measured every week with a vernier caliper. Five weeks later, mice were euthanized, and tumor tissues were stripped and weighed.

Statistical Analysis

Data are presented as means ± standard deviation (SD). Statistical analysis was performed using SPSS 13.0 and GraphPad Prism 4.0 software. Comparisons between

different groups were made using Student's *t*-test. A value of $p<0.05$ indicated significant difference.

RESULTS

PRSS8 Was Expressed in Low Amounts in Glioma Tissues and Cell Lines

In order to evaluate the role of PRSS8 in glioma, we performed RT-PCR and Western blot assays to measure the expression of PRSS8 in glioma tissues and cell lines. The results indicated that PRSS8 was expressed in low amounts in glioma tissues compared with corresponding normal tissues (Fig. 1A and B). PRSS8 showed a lower expression in T98G and HS683 cells than in the NHA cells at both mRNA and protein levels (Fig. 1C and D).

PRSS8 Overexpression Inhibited In Vitro Proliferation of Glioma Cells

To investigate the role of PRSS8 in glioma cell growth, PRSS8 expression plasmids were transfected into glioma cells. The Western blot analysis was performed to confirm upregulated PRSS8 in T98G and HS683 cells (Fig. 2A and B). The effect of PRSS8 on the proliferation of T98G and HS683 cells was assessed by MTT and colony formation assays. The MTT assay showed that PRSS8 overexpression significantly inhibited the proliferation of

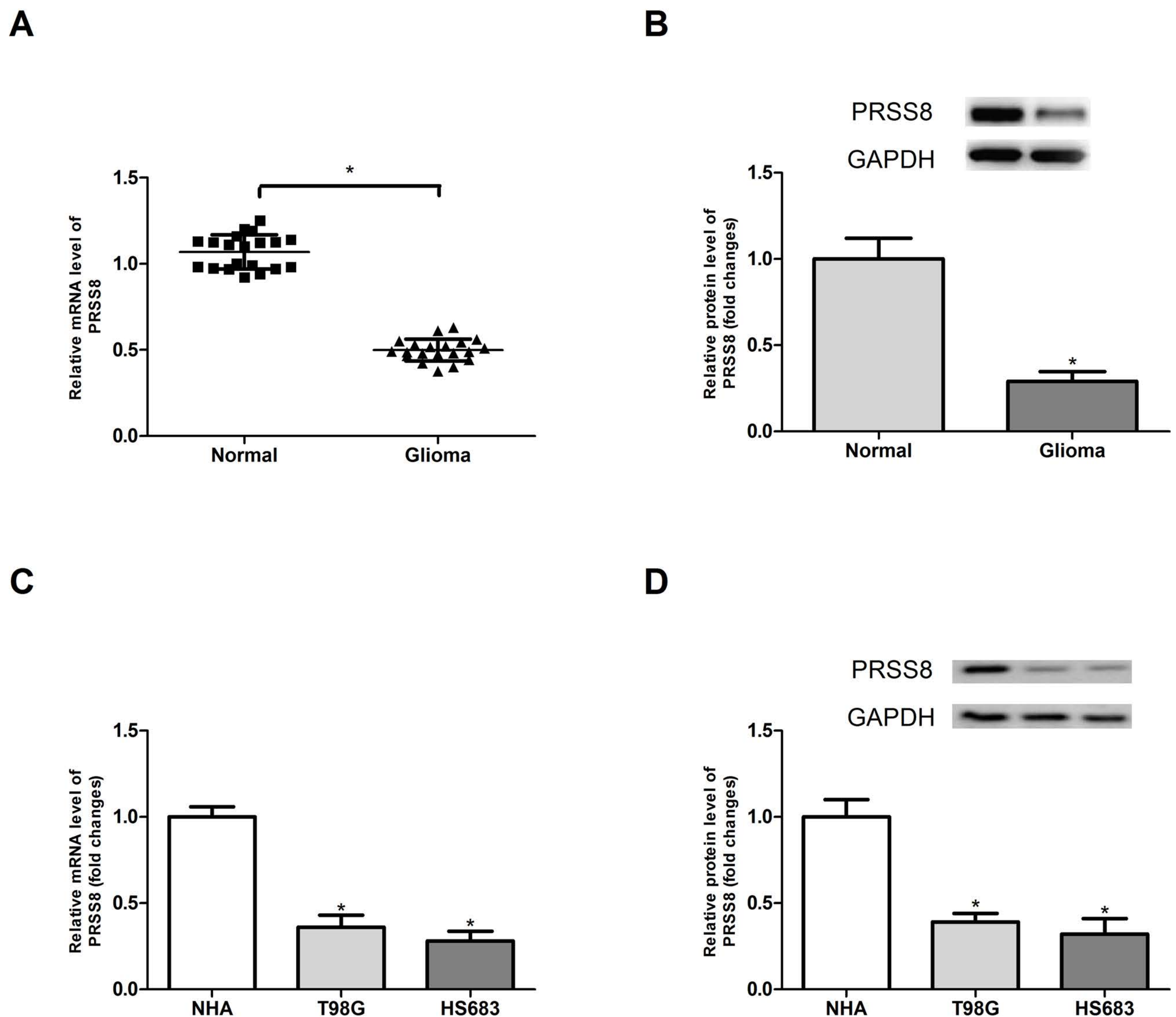


Figure 1. PRSS8 was expressed in low amounts in glioma tissues and cell lines. (A, B) RT-PCR and Western blot analysis of PRSS8 expression in glioma tissues compared with normal tissues ($n=20$). (C, D) The mRNA and protein expression levels of PRSS8 were decreased in T98G and HS683 cells in comparison with the NHA cells. *$p<0.05$.

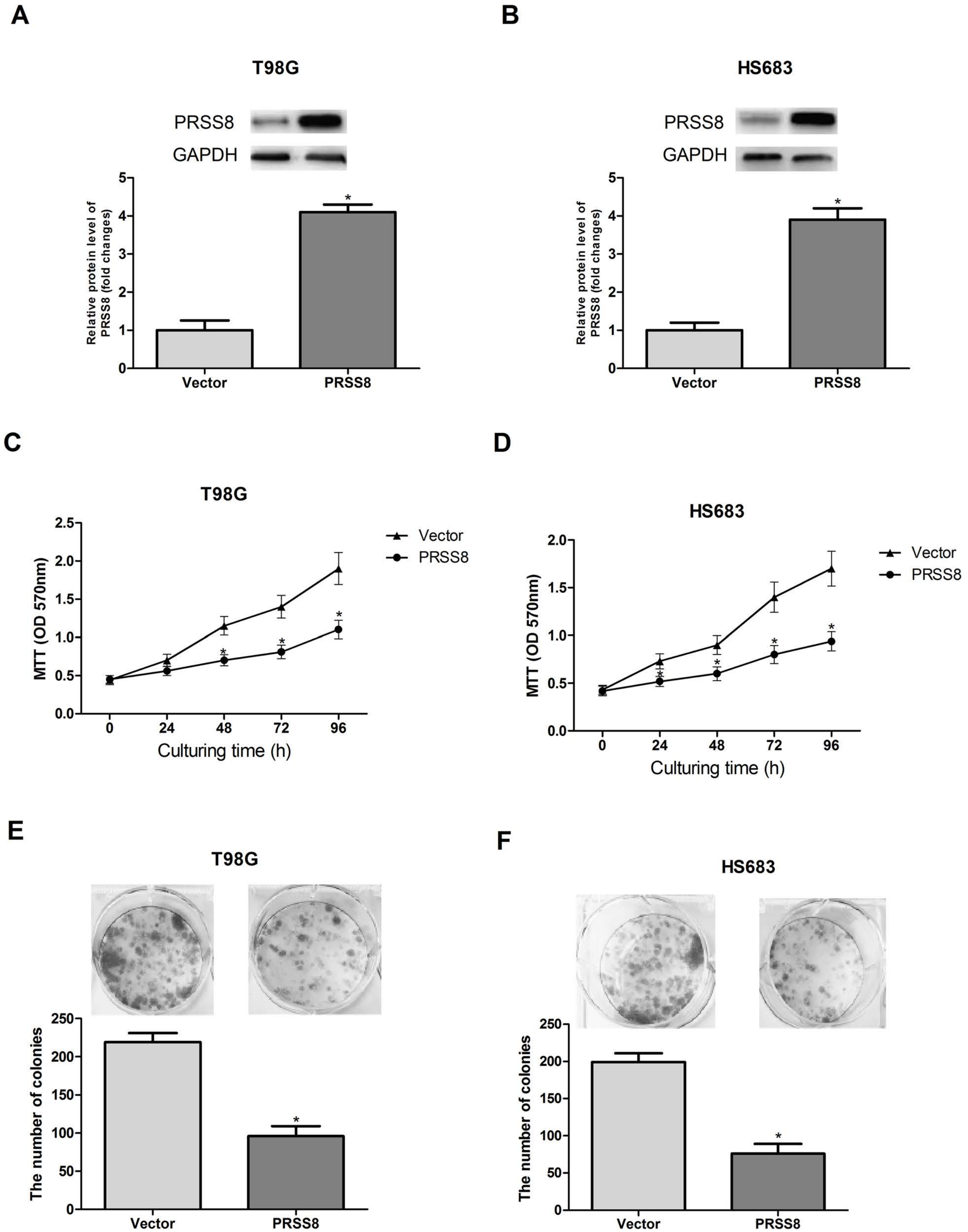


Figure 2. PRSS8 overexpression inhibited in vitro proliferation of glioma cells. (A, B) The Western blot analysis was used to detect the expression of PRSS8 in T98G and HS683 cells after transfection with pcDNA3.1-PRSS8 or empty vector. (C, D) PRSS8 overexpression markedly inhibited the proliferation of T98G and HS683 cells. (E, F) PRSS8 overexpression obviously reduced the number of colonies formed by T98G and HS683 cells. *$p<0.05$.

T98G and HS683 cells (Fig. 2C and D). Consistently, the colony formation in T98G and HS683 cells was also inhibited by PRSS8 overexpression (Fig. 2E and F).

PRSS8 Overexpression Inhibited In Vitro Migration and Invasion of Glioma Cells

The Transwell assay was performed to investigate the role of PRSS8 in the migration and invasion of glioma cells. PRSS8 overexpression significantly inhibited the migration and invasion of T98G cells after 24 h of culturing (Fig. 3A and B). The migratory and invasive abilities of HS683 cells were similarly suppressed by PRSS8 overexpression (Fig. 3C and D).

PRSS8 Overexpression Inhibited Glioma Development via Suppressing Akt/mTOR Signaling

There is evidence demonstrating the connection between PRSS8 and the Akt signaling in cancer progression[26]. Moreover, numerous studies have found the important role of Akt signaling in the development of various cancers, including glioma[27,28]. Based on the above findings, we supposed that PRSS8 might affect glioma development by regulating the Akt signaling. Therefore, we investigated the status of Akt and its downstream molecule mTOR. PRSS8 overexpression reduced the protein expression levels of p-Akt and p-mTOR in glioma cells, indicating decreased Akt signaling activity (Fig. 4A). In addition, we treated PRSS8-transfected T98G cells with 100 nM rapamycin (mTOR inhibitor) and found that treatment with rapamycin augmented PRSS8-suppressed glioma cell proliferation (Fig. 4B) and invasion (Fig. 4C).

PRSS8 Overexpression Inhibited In Vivo Growth of Glioma Cells

We confirmed the effect of PRSS8 on glioma growth in vivo. T98G cells transfected with PRSS8 expression

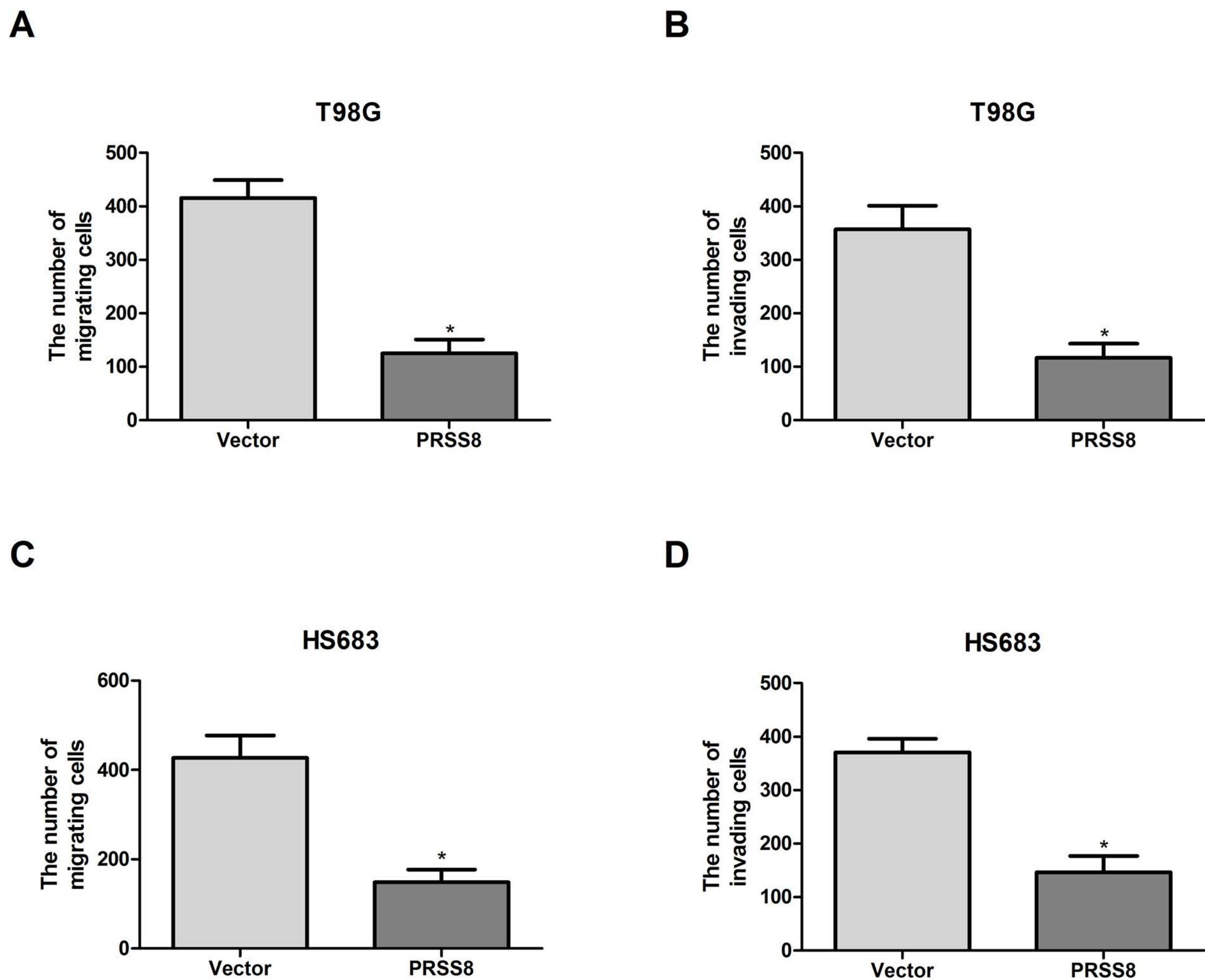
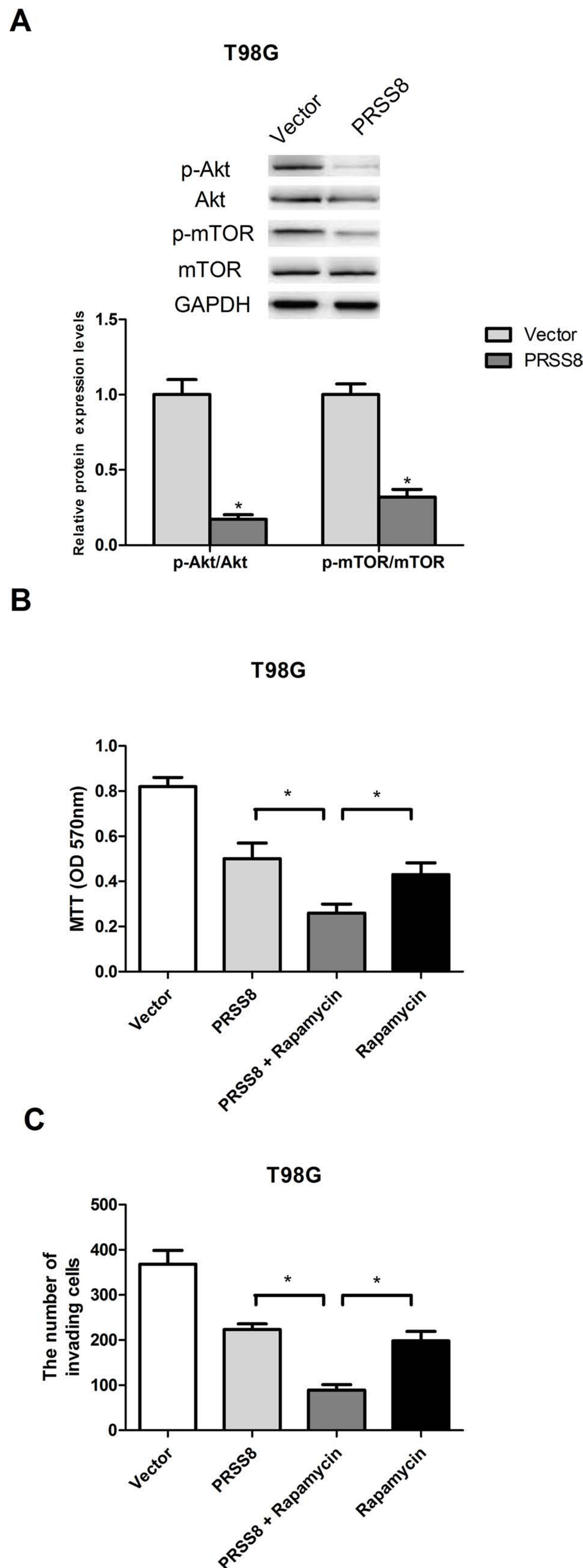


Figure 3. PRSS8 overexpression inhibited in vitro migration and invasion of glioma cells. (A, B) PRSS8 overexpression significantly inhibited the migration and invasion of T98G cells compared with the control cells. (C, D) PRSS8 overexpression remarkably inhibited the migration and invasion of HS683 cells compared with the control cells. *$p < 0.05$.



plasmid or empty vector were subcutaneously injected into nude mice. The tumor volume was measured every week. The tumor growth curves showed that PRSS8 overexpression significantly reduced tumor growth in comparison with the control group (Fig. 5A). Furthermore, the weight of tumors formed by PRSS8-transfected T98G cells was smaller than that of tumors formed by the control cells (Fig. 5B).

DISCUSSION

With high morbidity, glioma features high mortality and the prognosis of glioma patients remains dismal. To improve the poor outcome of glioma patients, novel and effective therapeutic approaches are desperately needed.

PRSS8, a serine peptidase, has a widespread expression in normal epidermal cells. Recently, many researchers demonstrated a decreased expression of PRSS8 in cancer tissues as well as its tumor suppressor role in cancer development. For instance, Bao et al. suggested that PRSS8 was downregulated in colorectal cancer and acted as a tumor suppressor during the progression of colorectal cancer[26]. In our study, we similarly found that PRSS8 was expressed in glioma tissues and cell lines at a low level. Furthermore, PRSS8 upregulation inhibited glioma cell proliferation, migration, and invasion in vitro. We also performed xenograft experiments in nude mice and found that PRSS8 overexpression suppressed glioma growth in vivo. All these findings were consistent with previous studies that supported the role of PRSS8 as a tumor suppressor. Unlike us, Tamir et al. reported upregulation of PRSS8 in ovarian cancer and suggested PRSS8 to be a potential biomarker for the early detection of ovarian cancer[29]. In addition, PRSS8 was found to have a high expression in bladder cancer. These results were contrary to ours and revealed the relation of PRSS8 to the mechanisms of tumor promotion. In combination with all these observations, we inferred that PRSS8 exerted different functions, depending on cancer type.

We also explored the mechanism underlying the inhibitory effect of PRSS8 on glioma development. Considering that PRSS8 was found to regulate Akt signaling in order to effect functions in colorectal cancer and that Akt signaling was reported to be involved in the progression

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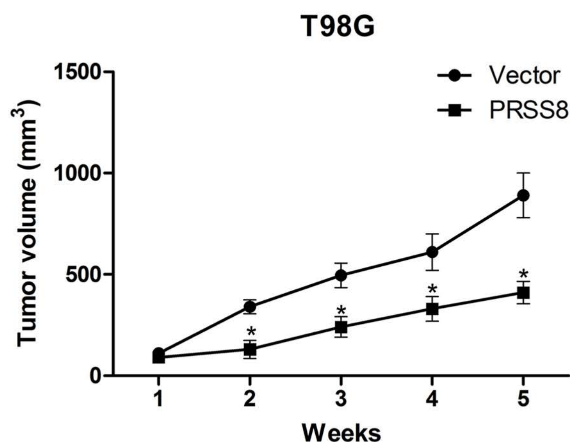
Figure 4. PRSS8 overexpression inhibited glioma development via suppressing the Akt/mTOR signaling. (A) Protein expression levels of p-Akt, Akt, p-mTOR, and mTOR, as determined by Western blot analysis, in T98G cells transfected with PRSS8 expression plasmid or empty vector. (B, C) Transfected T98G cells were treated with 100 nM mTOR inhibitor rapamycin for 24 h. The MTT and Transwell assays were performed to measure cell proliferation and invasion respectively. *$p<0.05$.



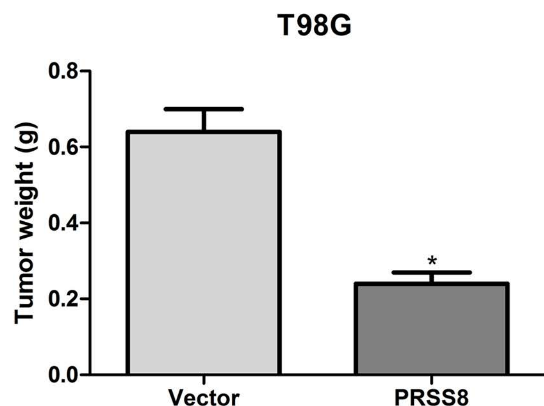


Figure 5. PRSS8 overexpression inhibited in vivo growth of glioma cells. (A) Tumor growth curves were established by measuring tumor volume every week after injection of T98G cells transfected with PRSS8 expression plasmid or empty vector. (B) Histograms showed the weight of tumors formed by PRSS8-transfected T98G cells and the control cells. $*p<0.05$.

of diverse cancers including glioma, we chose, among multiple signaling pathways, Akt signaling as a research objective. Akt plays a crucial role in a number of cellular processes such as cell proliferation and migration[30,31]. In addition, Akt is capable of phosphorylating and activating mTOR, whose dysregulation has been reported in many cancers[32,33]. Plenty of studies have found that the Akt/mTOR signaling pathway is frequently activated and subsequently influences 30% to 50% of cancers[27]. In a previous study, liver cancer cell proliferation and migration were attenuated after suppression of the Akt/mTOR signaling pathway[34]. This result was similar to that obtained by Zhang et al., who found that targeting the Akt/mTOR signaling pathway blocked the development of breast cancer[35]. These studies demonstrated the important role of the Akt/mTOR signaling pathway in cancer progression. In this study, we examined the status of Akt and mTOR, aiming to observe the effect of PRSS8 on the activity of Akt signaling. The Western blot analysis showed that the protein expression levels of p-Akt and p-mTOR in glioma cells were significantly decreased by PRSS8 overexpression, indicating inhibited Akt signaling activity. Moreover, treatment with rapamycin (mTOR inhibitor) augmented PRSS8-suppressed glioma cell proliferation and invasion. All these findings suggest that PRSS8 inhibits the glioma development via, at least in part, suppressing the Akt/mTOR signaling pathway. The additional effects exerted by rapamycin together with PRSS8 overexpression indicated the involvement of other signaling pathways on the effect of PRSS8. Therefore, we need to perform further studies.

In conclusion, the current study demonstrated a decreased expression of PRSS8 in glioma tissues and cell lines. PRSS8 upregulation inhibited glioma cell proliferation, migration, and invasion. In addition, the xenograft experiments showed that PRSS8 overexpression suppressed glioma cell growth in vivo. We also found that upregulated PRSS8 reduced the protein expression levels of p-Akt and p-mTOR. Taken together, we suggest PRSS8 as a novel therapeutic target for glioma.

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