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RESEARCH PAPER

LncRNA PITPNA-AS1 stimulates cell proliferation and suppresses cell apoptosis in glioblastoma via targeting miR-223-3p/EGFR axis and activating PI3K/AKT signaling pathway

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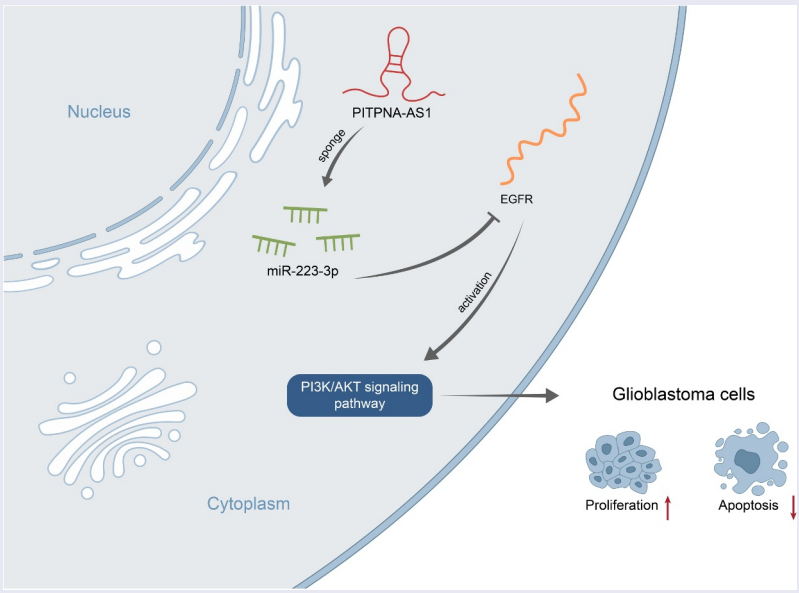
ABSTRACT

Glioblastoma (GBM) is a kind of malignant primary brain tumor, which is difficult to cure. Continuous researches have underlined that long non-coding RNAs (lncRNAs) get widely involved in the occurrence and progression of tumors, and glioblastoma is included. In this paper, we identified lncRNA PITPNA antisense RNA 1 (PITPNA-AS1) and explored its in-depth regulatory mechanism in glioblastoma cells. Firstly, RT-qPCR examined that PITPNA-AS1 was highly expressed in glioblastoma. Then, PITPNA-AS1 role in glioblastoma was assessed via functional assays. The results demonstrated that depletion of PITPNA-AS1 inhibited the proliferation and promoted the apoptosis of glioblastoma cells. After confirming that PITPNA-AS1 mainly existed in cell cytoplasm, we conducted mechanism assays which disclosed that PITPNA-AS1 sequestered microRNA-223-3p (miR-223-3p) and modulated epidermal growth factor receptor (EGFR) expression, thereby participating in the activation of PI3K/AKT signaling pathway. Eventually, rescue assays validated PITPNA-AS1 sponged miR-223-3p to promote EGFR expression, thus activating PI3K/AKT signaling pathway to accelerate proliferation and inhibit apoptosis of GBM cells. Overall, PITPNA-AS1 played an oncogenic role in glioblastoma which might be developed as a potential biomarker for glioblastoma diagnosis and treatment in the future.



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Supplemental data for this article can be accessed here

Introduction

It is commonly considered that glioma is one of the most prevalent malignant tumors of the central nervous system, among which, glioblastoma (GBM) is the most malignant [1]. Though many efforts have been made in improving the therapeutic effect, the incidence or mortality of GBM remains high [2]. Hence, it is especially pivotal to explore valuable biomarkers to develop effective methods for GBM diagnosis and treatment [3].

Currently, most efforts of GBM treatment are focused on the development of molecular targeted therapies [4]. Aberrant expression of long non-coding RNAs (lncRNAs), with longer than 200 nucleotides, has been widely correlated with tumor initiation and progression [5]. Moreover, some lncRNAs have been suggested as significant biomarkers for various malignancies [6]. More specifically, the oncogenic function of lncRNA TUC338 in bladder cancer has been raveled out [7]. LncRNA SNHG1 has been widely identified to be a biomarker for the diagnosis, prognosis, and treatment of cancers [8]. It is also learnt from existing literature that HOXA11-AS, up-regulated in cervical cancer tissues, is ascertained as a facilitator in modulating cervical cancer cells [9]. Furthermore, recent studies have demonstrated that lncRNAs exert vital functions in GBM [10]. For example, it is proposed in the research by Liu et al. that lncRNA LINC00152 is a positive modulator in glioblastoma by sponging miR-107 [11]. Li et al. have validated the LINC01446/miR-489-3p/TPT1 axis in glioblastoma [12].

The oncogenic role of lncRNA PITPNA-AS1 has been explored in hepatocellular carcinoma, lung squamous cell carcinoma, and cervical cancer [13–15]. However, whether PITPNA-AS1 participates in GBM remains to be clarified. Moreover, the involvement of signaling pathways in the regulatory mechanism of lncRNAs has been somewhat raveled out. Specifically, PI3K/Akt/mTOR signaling pathway is suggested as a therapeutic target for GBM [16]. Additionally, FoxD2-AS1 is ascertained to target miR-185-5p/HMGA1 axis and regulate PI3K/AKT signaling pathway, thus exerting its promoting impacts on GBM progression [17]. Whether certain pathway is involved in PITPNA-AS1 regulatory mechanism in GBM still remains to be elucidated.

In our research, we laid emphasis on exploring the influence of PITPNA-AS1 on glioma cell malignant behaviors and also probed into the underlying regulatory mechanism.

Materials and methods

Cell culture

Human glioblastoma cell lines (H4, A172, SHG44, and LN229), normal cell line (HEB) and human embryonic kidney 293 T (HEK-293 T) cells were selected for this study. GBM cells H4, A172, and LN229, together with HEK-293 T cells, were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Besides, HEB and SHG44 cells were obtained from Huatuo Biological Technology Co., Ltd. (Shenzhen, China). All the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) except that LN229 was maintained in 5% FBS. All mediums were cultured at 37°C with 5% CO_2.

Cell transfection

The shRNAs targeting PITPNA-AS1 were procured from GenePharma (Shanghai, China) for knockdown of PITPNA-AS1. Besides, pcDNA3.1 vectors were inserted with the sequences of EGFR for overexpression. MiR-223-3p mimics/inhibitor and their corresponding negative controls were all obtained from RiboBio (Shanghai, China). Cell transfection was completed with Lipofectamine 2000 (Invitrogen).

Quantitative reverse transcription PCR (RT-qPCR) analysis

According to the manufacturer's instructions, TRIzol Reagent (Introgen, Carlsbad, CA, USA) was utilized to obtain total RNA. Then, reverse transcription of RNAs into cDNA was accomplished with RevertAid First-Strand cDNA

Synthesis Kit (Thermo Fisher, IL, USA). RNA levels were measured by utilizing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). GAPDH or U6 were regarded as the internal control according to $2^{-\Delta\Delta Ct}$ method.

Colony formation assay

Briefly, cells were inoculated in a 6-well plate and subjected to 14-day incubation. Then, cells were fixed in ethanol. Crystal violet was acquired to stain cells and the number of colonies was counted manually.

5-ethynyl-20-deoxyuridine (EdU) staining assay

Cells (5×10^4) at logarithmic growth phase were put in 96-well plates. Cell nucleus was double-stained with EdU and DAPI dye (Beyotime, Shanghai, China). Fluorescence microscope (Olympus, Tokyo, Japan) was obtained for observing cell proliferation.

Caspase-3/6/9 activity assay

With caspase-3/6/9 activity kit, caspase-3/6/9 activity assay was achieved as required by supplier (Beyotime). Protein extracts were cultured with caspase-3/6/9 substrate in provided reaction buffer. Caspase-3/6/9 activity was detected at 405 nm.

Subcellular fractionation

Via PARIS™ Kit (Ambion, Austin, TX), cytoplasmic and nuclear elements were separated. GAPDH or U6 was respectively regarded as cytoplasmic and nuclear controls.

Fluorescent in situ hybridization (FISH)

Before being permeabilized with 0.5% Triton X-100, GBM cells were firstly subjected to 15-min fixation with 4% PFA at room temperature. Then, cells were hybridized with the RNA FISH probe mix for PITPNA-AS1 in buffer which was synthesized by RiboBio. Hoechst solution was used to dye nuclei.

RNA pull down assay

GBM cells were treated with biotinylated PITPNA-AS1 probe (Bio-PITPNA-AS1). Magnetic beads were then added into cells. The pull-downs collected by beads were purified for PCR analysis.

RNA immunoprecipitation (RIP)

In accordance with Z-Magna RIPTM RNA-binding Protein Immunoprecipitation kit (Millipore Corporation, USA), RIP assay was conducted. Antibodies against Ago2 and IgG from Abcam were obtained for incubation with cell lysates to acquire immunoprecipitate. RT-qPCR analysis was finally done for the detection of purified RNA complexes.

Luciferase reporter assay

The sequence of PITPNA-AS1 or EGFR mRNA 3′-untranslated region (3′-UTR) containing wild-type (Wt) and mutant type (Mut) of miR-223-3p was inserted into pmirGLO dual-luciferase vector to form pmirGLO-PITPNA-AS1 Wt/Mut and pmirGLO-EGFR 3′-UTR-Wt/Mut respectively. Later, miR-223-3p mimics and control mimics were co-transfected with the reporter gene into GBM cells. After 48 h, the Dual-Luciferase Reporter Gene Assay Kit (Yeasen, Shanghai, China) was applied to measure luciferase activity. For detecting the influence of PITPNA-AS1 on luciferase activity of various signaling pathways, HEK-293 T was transfected with sh-PITPNA-AS1 and Cignal Reporter Assay Kit was applied for luciferase activity measurement.

Western blot analysis

Separated protein samples were transferred onto PVDF membranes (Thermo Fisher, IL, USA). After being blocked with skimmed milk, the membranes went through incubation with the following primary antibodies against BCL2, Bax, cleaved caspase-3, total caspase-3, EGFR, p-PI3K, PI3K, p-AKT, AKT, and GAPDH. Afterward, the blots were incubated with secondary antibody. At last, Chemiluminescence system (GE Healthcare,

Chicago, USA) was procured for proteins quantification.

Statistical analysis

Experimental data were analyzed by SPSS version 22.0 statistical software package. All data were expressed as mean ± standard deviation (SD). The differences between two groups or more groups were processed by employing Student's t-test or ANOVA. All the experiments were at least triplicated independently. Differences were considered statistically significant when $P < 0.05$.

Results

LncRNA PITPNA-AS1 is up-regulated in GBM

Based on GEPIA data (http://gepia.cancer-pku.cn), compared with normal tissues, lncRNA PITPNA-AS1 expression was relatively high in GBM tissues (Figure 1a). Through RT-qPCR analysis, PITPNA-AS1 was also confirmed to be overexpressed in GBM cell lines (H4, A172, SHG44, and LN229) in comparison with normal cell line (HEB) (Figure 1b). The distribution of PITPNA-AS1 in SHG44 and LN229 cells was estimated by subcellular fractionation and FISH assays. According to the results, major cytoplasm distribution of PITPNA-AS1in GBM cells was ascertained (Figure 1c-d). Overall, PITPNA-AS1 is overexpressed in GBM.

PITPNA-AS1 knockdown hinders cell proliferation and boosts cell apoptosis in GBM

For the investigation into PITPNA-AS1 function on the biological behaviors of GBM cells, sh-PITPNA-AS1#1/2/3 plasmids were transfected into SHG44 and LN229 cells to knock down the expression of PITPNA-AS1 (Fig. S1A). Subsequently, colony formation and EdU assays revealed that PITPNA-AS1 silencing obviously inhibited cell proliferation in GBM as the colony number and EdU positive cell number both declined with PITPNA-AS1 depletion (Figure 2a-b). Western blot analysis uncovered that when PITPNA-AS1 expression was reduced, the BCL2 was decreased while Bax and cleaved caspase-3 were elevated, which indicated that PITPNA-AS1 down-regulation stimulated cell apoptosis (Figure 2c). At the same time, knockdown of PITPNA-AS1 also augmented the activities of Caspase 3/6/9 (Figure 2d). Taken together, PITPNA-AS1 boosts cell proliferation and restrains cell apoptosis in GBM.

MiR-223-3p bound with PITPNA-AS1 impedes GBM cell proliferation but facilitates cell apoptosis

After confirming the prominent existence of PITPNA-AS1 in the cytoplasm of GBM cells, we made the assumption that PITPNA-AS1 might play a competing endogenous RNA (ceRNA) role by sequestering certain miRNA. As a result, through starBase (http://starbase.sysu.edu.cn/index.php), we screened out three potential miRNAs (miR-223-3p, miR-524-5p, and miR-520d-5p) under-indicated conditions (CLIP Data ≥ 5, Degradome Data ≥ 1). Then RNA pull down assay verified the affinity between PITPNA-AS1 and miR-223-3p (Figure 3a). Thereafter, miR-223-3p was chosen for further study. RT-qPCR result implied that down-regulating PITPNA-AS1 in GBM cells could hardly change miR-223-3p expression (Fig. S1B). RT-qPCR analysis also suggested that miR-223-3p expression was markedly low in GBM cells (Figure 3b). The alignment between PITPNA-AS1 and miR-223-3p obtained from starBase was exhibited in Figure 3c. MiR-223-3p expression was augmented in cells by miR-223-3p mimics (Figure 3d). Luciferase reporter assay confirmed that overexpression of miR-223-3p lessened the luciferase activity in the PITPNA-AS1-Wt group (Figure 3e). Subsequently, functional experiments were conducted for the investigation into miR-223-3p influence on GBM cells. Via colony formation and EdU, miR-223-3p overexpression was testified to impede GBM cell proliferation (Fig. S1C-D). Moreover, an overt decline in BCL2 protein level was discovered as a result of the transfection of miR-223-3p mimics into cells while miR-223-3p overexpression contributed to a notable increment in protein levels of Bax and cleaved caspase-3, reflecting that miR-223-3p overexpression facilitated GBM cell apoptosis (Fig. S1E). To be

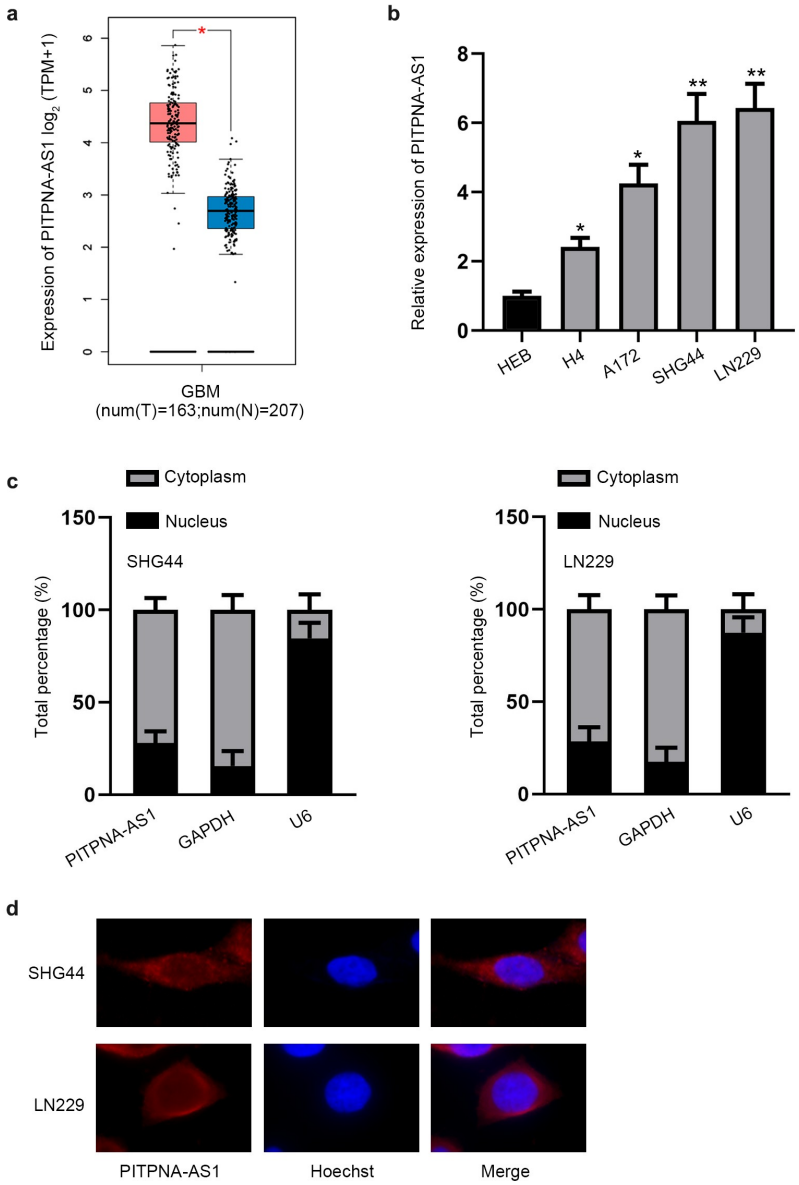

Figure 1. LncRNA PITPNA-AS1 is up-regulated in GBM. (a) Expression pattern of PITPNA-AS1 in GBM tissues and normal tissues was obtained from GEPIA. (b) RT-qPCR analysis was utilized for the detection of PITPNA-AS1 expression in GBM cell lines (H4, A172, SHG44 and LN229) and normal cell line (HEB). (c-d) Subcellular fractionation and FISH assays were applied to discover the cellular location of PITPNA-AS1 in GBM cells. * $P < 0.05$, ** $P < 0.01$.

summarized, PITPNA-AS1 sequesters miR-223-3p and miR-223-3p functions as a negative modulator of GBM cell phenotype.

PITPNA-AS1 sponges miR-223-3p to modulate EGFR expression and activates PI3K/AKT signaling pathway

From the experimental results of luciferase reporter assay, PITPNA-AS1 was found to be related to PI3K/AKT signaling pathway for that PITPNA-AS1 inhibition led to an overt decrease in the luciferase activity of PI3K/AKT signaling pathway instead of other common signaling pathways (Notch, Wnt, Hedgehog, MAPK, and NF-κB signaling pathways) (Figure 4a). More intriguingly, we discovered that EGFR, a key signaling molecule of PI3K/AKT signaling pathway, was a downstream of miR-223-3p based on starBase. In addition, EGFR has been certified to be a clinical marker in GBM and promote cell proliferation, migration, and invasion [18,19].

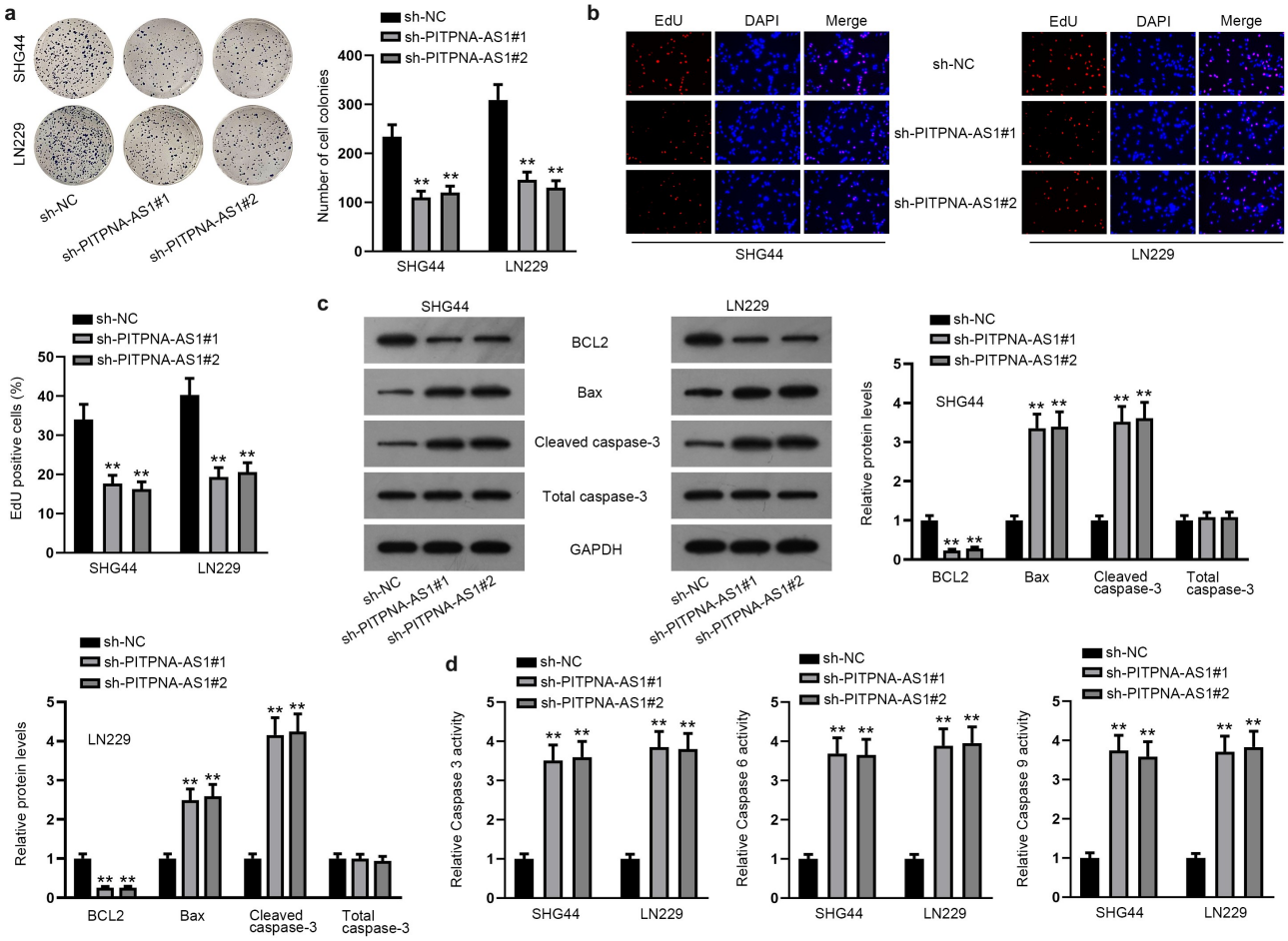

Figure 2. PITPNA-AS1 knockdown hinders cell proliferation and boosts cell apoptosis in GBM. (a-b) Colony formation and EdU assays were done for the assessment of GBM cell proliferation when PITPNA-AS1 was silenced. (c) With the aim to fathom out PITPNA-AS1 influence on GBM cell apoptosis, western blot was done to analyze the protein levels of apoptosis-related factors after the transfection of sh-PITPNA-AS1#1/2 in GBM cells. (d) Caspase 3/6/9 activity was tested when PITPNA-AS1 was down-regulated in SHG44 and LN229 cells. ** $P < 0.01$.

According to the data obtained from GEPIA, EGFR also displayed relatively high expression in GBM tissues (Fig. S1F). Thence, we made a conjecture that PITPNA-AS1 motivated PI3K/AKT signaling pathway by sponging miR-223-3p and modulating EGFR expression. According to western blot analysis, we found that after PITPNA-AS1 was depleted, protein levels of EGFR, p-PI3K, and p-AKT all declined (Figure 4b). At the same time, EGFR expression was also decreased on account of miR-223-3p up-regulation (Figure 4c). Moreover, an overt decline in levels of p-PI3K and p-AKT was discovered from western blot analysis, which resulted from up-regulation of miR-223-3p (Fig. S1G). The binding region between miR-223-3p and EGFR was demonstrated in Figure 4d. The abundant enrichment of PITPNA-AS1, miR-223-3p, and EGFR in Ago2 antibody was demonstrated in RIP assay, indicating the interaction among the three indicated genes (Figure 4e). Additionally, the interplay between miR-223-3p and EGFR was attested by luciferase reporter assay (figure 4f). MiR-223-3p inhibitor transfection into GBM cells was to hamper miR-223-3p expression (Figure 4g). Additionally, EGFR expression at mRNA level and protein level was dramatically knocked down on account of PITPNA-AS1 depletion, which was then restored in response to the down-regulation of miR-223-3p (Figure 4h-i). In a word, PITPNA-AS1 activates EGFR/PI3K/AKT signaling pathway via sequestering miR-223-3p.

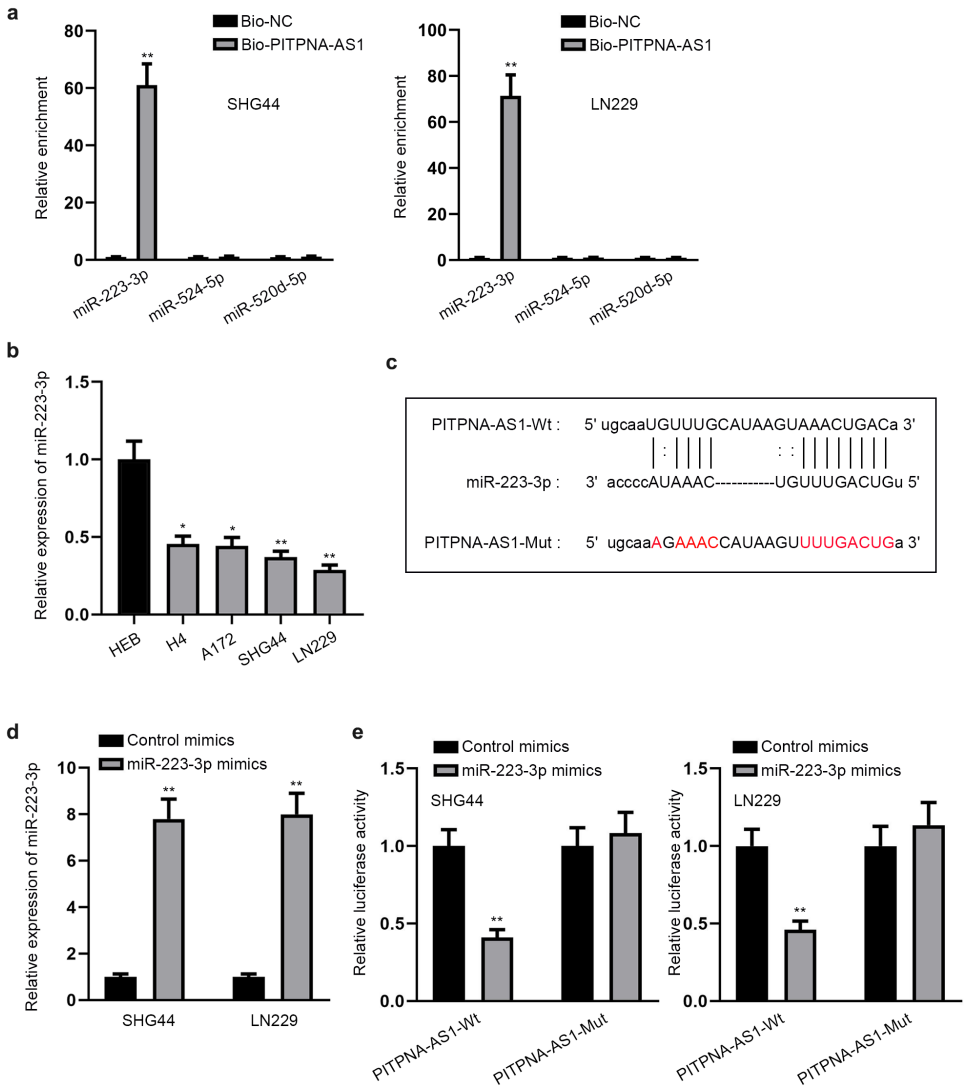

Figure 3. PITPNA-AS1 binds to miR-223-3p. (a) RT-qPCR analyzed the enrichment level of miR-223-3p, miR-524-5p and miR-520d-5p in RNA pull down assay. (b) RT-qPCR analysis was applied for the quantification of miR-223-3p expression in GBM cell lines and normal cell line. (c) StarBase predicted the binding sequence between PITPNA-AS1 and miR-223-3p. (d) RT-qPCR analysis was applied for the quantification of miR-223-3p expression in GBM cells with or without miR-223-3p mimics transfection. (e) Luciferase reporter activity confirmed the affinity between PITPNA-AS1 and miR-223-3p based on the detection of the relative luciferase activity in SHG44 and LN229 cells co-transfected with PITPNA-AS1-Wt/Mut and miR-223-3p mimics. * $P < 0.05$, ** $P < 0.01$.

PITPNA-AS1 modulates GBM cell proliferation and apoptosis by targeting miR-223-3p/EGFR axis

To substantiate the PITPNA-AS1/miR-223-3p/EGFR axis in GBM cells, rescue assays were carried out. Firstly, EGFR was overexpressed in LN229 cells after the transfection of pcDNA3.1/EGFR (Figure 5a). Later, cell proliferation was investigated via colony formation and EdU assays. The results revealed that miR-223-3p down-regulation or EGFR up-regulation restored the weakened proliferative capacity resulting from PITPNA-AS1 silence (Figure 5b-c). Western blot analysis unmasked that strengthened cell apoptosis induced by PITPNA-AS1 depletion was counter-vailed by miR-223-3p inhibition or EGFR over-expression (Figure 5d). Moreover, the enhanced Caspase 3/6/9 activities on account of PITPNA-

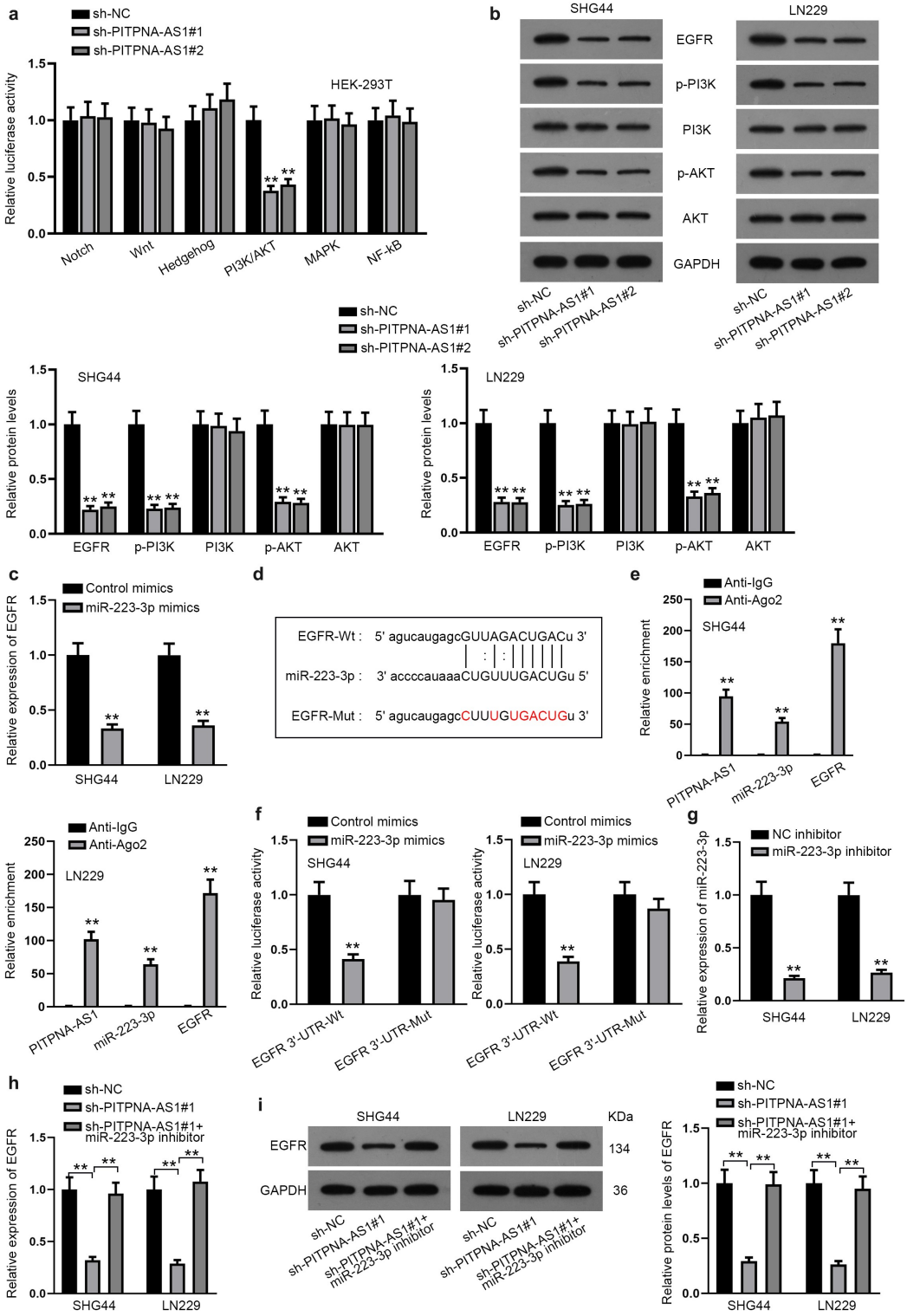

Figure 4. PITPNA-AS1 sequesters miR-223-3p to modulate EGFR expression and activates PI3K/AKT signaling pathway. (a) The luciferase activity of several common signaling pathways including Notch, Wnt, Hedgehog, PI3K/AKT, MAPK and NF-κB was detected in HEK-293 T cells with PITPNA-AS1 down-regulation. (b) The protein levels of key factors of PI3K/AKT signaling pathways (EGFR, p-PI3K, PI3K, p-AKT, AKT) were analyzed in sh-PITPNA-AS1 transfected cells by western blot. (c) RT-qPCR analysis was applied for the detection of EGFR expression in SHG44 and LN229 cells with miR-223-3p augment. (d) Binding regions between miR-223-3p and EGFR were obtained based on the prediction on starBase. (e) RIP assay disclosed the interactions among PITPNA-AS1, miR-223-3p and EGFR in GBM cells. (f) Luciferase reporter assay validated the binding between miR-223-3p and EGFR. (g) RT-qPCR analysis was applied for investigating miR-223-3p expression in GBM cells on account of miR-223-3p inhibitor transfection. (h–i) EGFR expression at mRNA and protein levels was examined by RT-qPCR analysis and western blot in SHG44 and LN229 cells transfected with indicated plasmids including sh-NC, sh-PITPNA-AS1#1 and sh-PITPNA-AS1#1+ miR-223-3p inhibitor. ** $P < 0.01$.

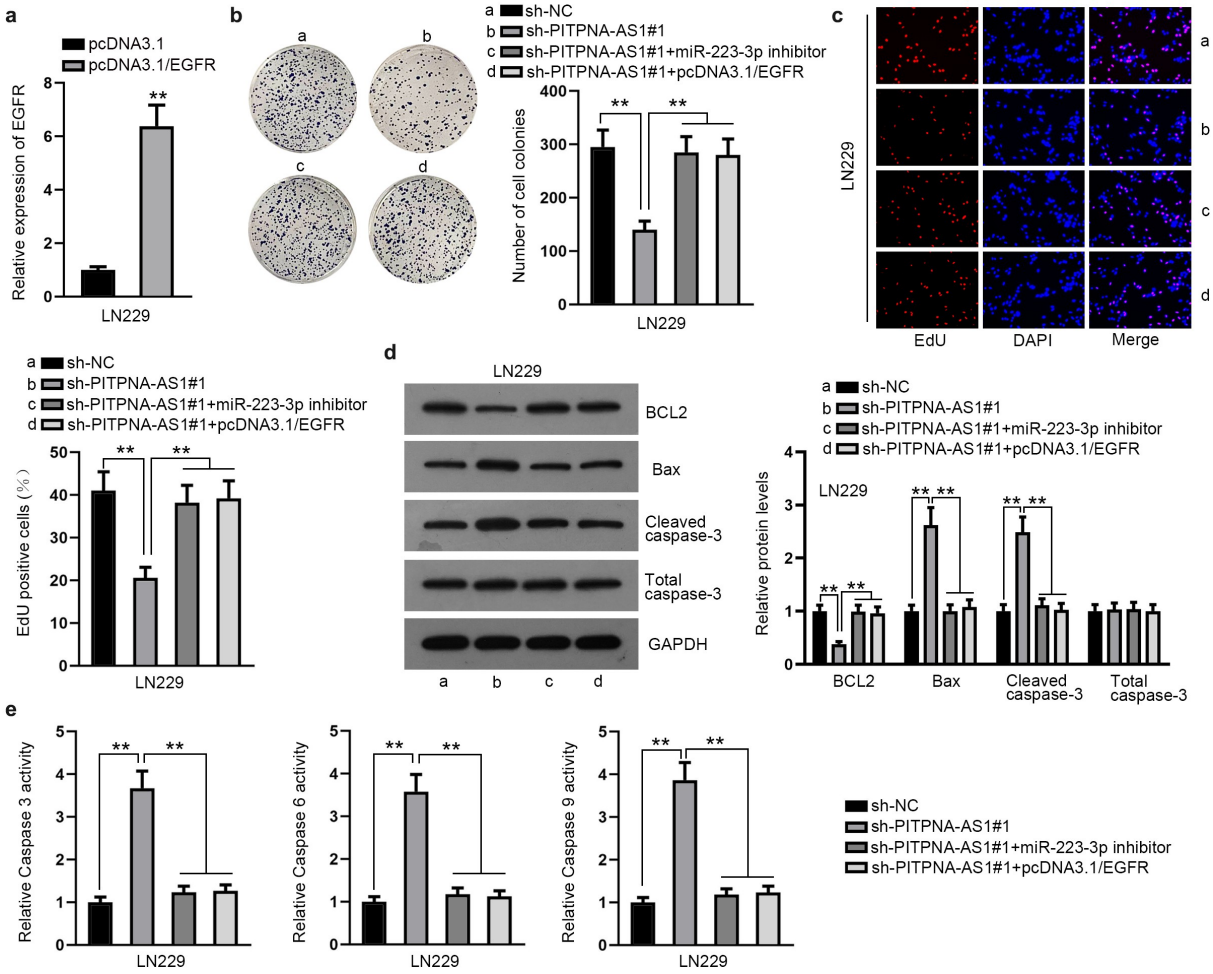

Figure 5. PITPNA-AS1 exacerbates the malignant behaviors of GBM cells by targeting miR-223-3p/EGFR axis. (a) EGFR expression in LN229 cells with pcDNA3.1/EGFR transfection was determined through RT-qPCR. (b-c) Colony formation and EdU assays were implemented to probe the proliferative ability of LN229 cells after the transfection of indicated plasmids including sh-NC, sh-PITPNA-AS1#1, sh-PITPNA-AS1#1+ miR-223-3p inhibitor and sh-PITPNA-AS1#1+ pcDNA3.1/EGFR plasmids. (d) Protein levels of BCL2, Bax, Cleaved caspase-3 and Total caspase-3 in the sh-NC group, sh-PITPNA-AS1#1 group, sh-PITPNA-AS1#1+ miR-223-3p inhibitor group and sh-PITPNA-AS1#1+ pcDNA3.1/EGFR group were measured via western blot. (e) Caspase 3/6/9 activity was detected in LN229 cells in different groups. ** $P < 0.01$.

AS1 inhibition were all reversed by down-regulation of miR-223-3p or up-regulation of EGFR (Figure 5e). Taken together, the PITPNA-AS1/miR-223-3p/EGFR axis exacerbates malignant behavior of GBM cells.

Discussion

Emerging evidence has proved that lncRNA-based accurate biomarkers are of great significance to improve the diagnosis, prognosis, and treatment of GBM [10]. LncRNA PITPNA-AS1 is a novel identified lncRNA in some malignancies. Specifically, it is learnt from a recent research of Sun et al. that the PITPNA-AS1/miR-876-5p/WNT5A axis is a crucial participant in modulating the development of hepatocellular carcinoma [13]. Additionally, based on a published literature of Ren et al., lncRNA PITPNA-AS1 is suggested to bind with TAF15 protein and stabilizes HMGB3 mRNA to facilitate cell proliferation and migration in lung squamous cell carcinoma [14]. Guo et al. have confirmed that lncRNA PITPNA-AS1 plays a promoting role in cervical cancer via sequestering miR-876-5p [15]. Consistently, it was discovered in the present study that PITPNA-AS1 was overexpressed in GBM tissues and cells. Functionally, inhibition of PITPNA-AS1 weakened the proliferative capacity while strengthened the apoptotic capacity of GBM cells.

LncRNA-associated ceRNA mechanism occupies an important position in the pathogenesis of GBM [20]. Subcellular fractionation and FISH assays disclosed the accumulation of PITPNA-AS1 in the cytoplasm of GBM cells, which indicated the possible existence of ceRNA mechanism. Through starBase and mechanism assays, miR-223-3p was testified to bind with PITPNA-AS1 and observably down-regulated in GBM cells. Moreover, it was determined that miR-223-3p expression could hardly be affected by PITPNA-AS1. As a crucial participant in the specific regulation mechanism of PITPNA-AS1 in GBM, miR-223-3p overexpression was validated to facilitate GBM cell apoptosis but suppress GBM cell proliferation.

It has been discovered that PI3K/AKT signaling pathway is a possible therapeutic target for GBM [16]. As an important participator in PI3K/AKT signaling pathway, the functions of EGFR cannot be ignored. EGFR has been implicated to be a therapeutic target in GBM [18,21]. Besides, EGFR has been attested to induce the development of GBM [19,22]. Through our investigation, EGFR was demonstrated to be targeted by miR-223-3p and influenced the activation of PI3K/AKT signaling pathway.

At last, rescue assays elaborated that down-regulation of miR-223-3p or overexpression of EGFR rescued the limited cell proliferation and accelerated cell apoptosis in GBM caused by PITPNA-AS1 depletion. Thus, the conclusion could be drawn that PITPNA-AS1 could promote cell proliferation and restrain cell apoptosis in GBM via targeting miR-223-3p and EGFR.

Overall, with all our experimental results, we could draw a conclusion that lncRNA PITPNA-AS1 enhanced EGFR expression by sponging miR-223-3p, thus contributing to GBM cell proliferation, and impeding cell apoptosis through the activation of PI3K/AKT signaling pathway. Though the mechanism in which PITPNA-AS1 regulated EGFR in GBM cells requires deeper investigation with other possible pathways taken into consideration and large-scale clinical samples need to be involved for further confirmation of PITPNA-AS1/miR-223-3p/EGFR axis, the roles and functions of PITPNA-AS1 in GBM cells were elucidated for the first time in this study. Hopefully, PITPNA-AS1 might serve as a biomarker and therapeutic target in GBM and be of great value for GBM treatment in the future.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Not applicable.

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