Phosphorylation of Pit-1 by Cyclin-dependent Kinase 5 at Serine 126 is Associated With Cell Proliferation and Poor Prognosis in Prolactinomas

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Abstract

Background

Pit-1 is a POU-homeodomain transcription factor, Cyclin-dependent kinase (CDK5) is a protein kinase that can phosphorylate many key transcription factors, but the mechanism of CDK5 phosphorylates Pit-1 is unclear.

Methods:

We generated an antibody that specifically recognizes phosphorylated serine at position 126 of Pit-1 (Ser126-Pit-1); we used western blotting to detect the level of Pit-1 phosphorylation and observed the proliferation and apoptosis of GH3 cells with different levels of Pit-1 phosphorylation by clone formation experiments, cell viability assays, and flow cytometry. ELISA was used to measure the level of PRL in the supernatant of GH3 cells. Tissue microarrays and immunohistochemistry were used to evaluate the expression of phosphorylation level of Ser126-Pit-1 (pSer126-Pit-1) in prolactinomas.

Results

Our data indicated that Ser126-Pit-1 is specifically phosphorylated by CDK5. pSer-126-Pit-1 can promote cell proliferation and PRL secretion. In addition, a higher level of pSer-126-Pit-1 correlates with a worse prognosis in patients with prolactinoma.

Conclusions

CDK5-mediated Ser126-Pit-1 phosphorylation regulates prolactinoma progression and PRL secretion.

Background

Pituitary adenomas (PAs) constitute approximately 15% of all intracranial neoplasms. Prolactinoma is the most common subtype of hormone-secreting pituitary tumors, accounting for approximately 45%-50% of cases. Due to the dysfunctional production of hormones, patients with prolactinomas often suffer from severe disorders affecting growth and development. However, the mechanism of the biological behavior of some prolactinomas has not yet been fully defined.

Pit-1 is a POU-homeodomain transcription factor, and it was described in the pituitary gland. Pit-1 is expressed exclusively in somatotrophs, lactotrophs, and thyrotrophs, and it is necessary for the establishment and maintenance of these differentiated cell types, as well as for proliferation of somatotrophs and lactotrophs(1). It is also expressed in breast, pancreatic, and prostate cancer, and its overexpression promotes tumor growth and metastasis(2-4). In the process of signal transduction regulation, the level of Pit-1 phosphorylation and the duration of activity regulated by its phosphorylation state are very important. Studies have shown that Pit-1 phosphorylation plays an important role in regulating the expression of the target gene. The most important phosphorylation sites found on Pit-1 are

serine 115 (S115) and threonine 229 (T220). Augustijn and others have shown that PKA, PKC, and cell cycle-dependent kinases can phosphorylate Pit-1 at the T220 site of the homologous domain(5). Phosphorylation or mutation of this site will change its relationship with Ets-1 binding segment RIII (AA190-257), which reduces the ability of Pit-1 to bind to PRL and the TSH promoter, and it activates cAMP, resulting in Pit-1 participation in the regulation of cell proliferation, apoptosis, and tumorigenesis(6-8).

Cyclin-dependent kinase 5 (CDK5) is a vital member of the serine/threonine kinase family; CDK5 activity is highest in the central nervous system and participates in a variety of neural system functional activities, including neuron migration, neuron apoptosis, survival, and synaptic plasticity(9). Its deregulation is directly involved in diverse pathological events, such as enhanced neurodegenerative and neuropsychiatric disorders and cancer(10). CDK5 is not activated by cyclins but by its activators p35 and p39(11, 12), whose constitutive expression is largely restricted to cells of neural crest origin. Our group found for the first time that CDK5 exists in normal human pituitary and pituitary adenomas and found that CDK5 can promote the growth and invasion of pituitary adenomas(13, 14), but the true regulatory mechanism of CDK5 in pituitary tumors is far from clear; specifically, it is unknown whether CDK5 is involved in the regulation of hormone synthesis, secretion, and apoptosis in prolactinoma, which requires in-depth research.

In the present study, the role of CDK5 was further studied regarding its regulation pSer126-Pit-1. We explored the function of pSer126-Pit-1 on the proliferation, hormone secretion and apoptosis of prolactinomas in vitro. Then, we investigated the clinical significance of the expression level of pSer126-Pit-1 using surgical specimens.

Materials And Methods

Patients

In the study, we retrospectively reviewed 48 patients who had undergone pituitary surgery at Beijing Tiantan Hospital between 2008 and 2012. All patients had plasma prolactin (PRL) levels >200 ng/ml, and positive immunostaining for PRL. Medical therapy was interrupted at least 2 months before surgery. Tumor size was determined by MRI, and tumors were classified as microadenomas (<1cm diameter), macroadenomas (>1 cm and <4 cm), and giant adenomas (>4 cm). The mean postoperative follow-up was 4.8 years (range: 2.5–7 years). Recurrence-free survival was measured from the date of surgery to the date of tumor recurrence. Patients were censored at the date of the last neuroimaging follow-up. Normal human anterior pituitaries of people who died of non-neurological or non-endocrine diseases were obtained from a donation program. The Ethics Committee of Beijing Tiantan Hospital study approved the protocol, and informed consent was obtained from all patients. The patient characteristics are summarized in Table 1.

Tumor samples and tissue microarray construction

Formalin-fixed paraffin-embedded tissue blocks were sectioned and stained with hematoxylin and eosin (H&E). Three 2.0 mm diameter core biopsies were selected from the paraffin-embedded tissue blocks and transferred to tissue microarrays (TMAs) using a Mini core Tissue Arrayer (Mitogen, UK). Tissue microarrays were cut into 4 µm sections using a serial microtome and samples were randomly ordered and anonymized on the TMA slides. To minimize loss of antigenicity, the microarray slides were processed within 1 week of cutting.

IHC techniques and antibodies

In advance of IHC, TMA slides were stained with H&E and evaluated for quality and tumor content. TMAs were processed in a Leica BOND-III (Leica Biosystems, Germany) automated, random, and continuous-access slide staining system that simultaneously performed several IHC assays. A Bond Polymer Refine Detection System (Leica Biosystems, Germany) was used for the detection of primary antibodies. Appropriate positive and negative controls were used for each antibody, and TMAs were stained for each antibody in the same run to avoid interassay variability. The immunostained slides were examined for expression using an Aperio AT2 digital scanner (Leica Biosystems, Germany). Primary antibodies anti-pSer126-Pit-1 (4 µg/ml, Abmart), was commercially developed using standard methods by injection of specific Pit-1 -phosphothreonine peptide Ac-VVL(pS) PSHGIE-amide into a rabbit at the Abmart antibody production facility, Shanghai, China. The optimal titer of primary antibodies had been determined in previous experiments. The percentage of immunostaining and the staining intensity (0, negative; 1+, weak; 2+, moderate; and 3+, strong) were recorded and an H-score was calculated as follows:

H-score = (% cells 1+) + 2(% cells 2+) + 3(% cells 3+). Based on the H-score, pSer126-Pit-1 staining in the tissue sections was categorized as low (H-score of ≤ 168), or high (H-score > 168).

Cell culture

Rat pituitary cells (GH3) were obtained from the China Infrastructure of Cell Line Resources (Beijing, China) and cultured in 35 mm dishes, we use ATCC-formulated F-12K medium (Invitrogen) containing 2.5% fetal bovine serum (Gibco) and 15% horse serum (Gibco) in a 37°C incubator with a humidified atmosphere of 95% air and 5% CO₂. The culture medium was replaced every other day.

Plasmid construction and CDK5 inhibitor

A CDK5 siRNA (SR507441) construct was purchased from OriGene Technologies (Rockville, MD, USA). Mutant Pit-1 (GFP-S126A-Pit-1) and Pit-1 were generated by GenScript Biotech (Nanjing, China). All constructs were confirmed by DNA sequencing (Shanghai Shenggong Bio, China). Roscovitine was obtained from Sigma-Aldrich (R7772; St. Louis, MO, USA).

Cell counting kit-8 (CCK-8) assay

Cells were seeded in 96-well plates at the density of 1×10^4 cells/well in 100 µl of cell culture medium for 24 hours and were then transiently transfected with the indicated plasmids and short interfering RNA Cell

viability was measured using the CCK-8 assay kit (Dojindo, Japan). Following incubation, 10 µl of CCK-8 solution was added to each well of the 96-well plate and cultured for 3 h in an incubator. The optical density was measured at 450 nm and a proliferation curve based on time and absorbance was generated.

Colony formation test

The treated cell lines were seeded into six-well plates at 1000 cells per well and incubated for 2 weeks. After incubation, the cells were fixed in 4% paraformaldehyde for 15 minutes and stained in 1 mL of a 0.1% crystal violet solution for 30 minutes. Photograph the culture plate. Visible colonies in each well were quantified by Image J software.

ELISA

PRL protein levels were determined using a rat PRL ELISA kit from BioVision (K4688-100) according to the manufacturer's instructions. GH3 cells were harvested 72 hr after treatment with plasmid. The total protein content of the cells was determined for standardization of PRL production with a BCA protein assay kit (Pierce Biotechnology). The culture supernatants were collected and normalized to the cell numbers.

Cell apoptosis assay

cell apoptosis was determined using Annexin V-FITC/PI kits (BD Biosciences, Franklin Lakes, NJ). Cells were seeded for 48h after transiently transfected with the indicated plasmids. Then cells were harvested and stained with annexin V-FITC and PI according to the instructions of the manufacturer. Cells were analyzed using BD Accuri™ C6 (BD Biosciences). Data analysis was performed using CFlow® software (BD Biosciences).

Electrophoretic mobility shift assay (EMSA)

We used a 5'-biotinylated oligonucleotide as a probe. The probes were incubated with the recombinant protein at room temperature for 30 min. The entire reaction mixture was run on a nondenaturing 0.5×TBE 6% polyacrylamide gel at 60 V for 1 h at 4°C, and then the mix was transferred onto Biodyne® B nylon membranes (Pall Corporation). Signals were visualized with reagents included in the kit and with a ChemiDoc XRS system (Bio-Rad Laboratories, UAS).

Luciferase reporter assay

GH3 cells were cultured at a density of 2×10^4 cells/well in 96-well culture plates. The cells were transfected with 0.2 µg of dual-luciferase reporterconstruct p1, or they were cotransfected with 0.2 µg of the luciferase reporter construct p2 and the internal control vector pRL-TK, pRL-SV40, or pRL-CMV (Promega, Madison, WI) at a ratio of 20:1 (reporter construct: control vector); transfections were performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's

instructions. Five hours posttransfection, the transfection medium was removed and replaced with medium containing 6 μ M curcumin (Sigma-Aldrich, St. Louis, MO) solubilized in 100% dimethyl sulfoxide (Sigma). Forty-eight hours posttransfection, luciferase activity was measured using a Dual-Luciferase® Reporter Assay System (Promega). Renilla luciferase activity was normalized to firefly luciferase activity in cells transfected with the dual-luciferase reporter construct p1, and firefly luciferase activity was normalized to Renilla luciferase activity in cells cotransfected with the reporter construct p2 and the control vector.

Protein extraction and Western blot analysis and antibodies

Collected cells were washed with 1× PBS buffer, prepared with RIPA buffer supplemented with protease/phosphatase inhibitor cocktail, and centrifuged at 12,000 r/min for 5 min at 4°C to yield the total protein extract in the supernatants. The protein concentration was measured with a BCA assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Equal amounts of protein were separated by 8% SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween®20 (TBST) for approximately 1 hr, followed by incubation with anti- β -actin (1:5000; A1978, Sigma-Aldrich), and rabbit polyclonal anti-CDK5 (ab40773, 1/200) overnight at 4°C. After washing with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) at room temperature for 1 hr. ImageJ (NIH) was used to quantify the protein band densities. Primary antibodies anti-CDK5 (ab40773, 1/200) were obtained from Abcam (Cambridge, MA, USA). Anti-Pit-1 (sc-393943, 1/100) was sourced from Santa Cruz Biotechnology (Dallas, TX, USA). The optimal titer of primary antibodies had been determined in previous experiments.

Statistical analysis

All statistical analyses used GraphPad Prism 7.00 statistical software. Experimental data are reported as the mean $\pm$ SD (standard error of measurement) of at least three independent experiments, as indicated in the respective figure legends and methods. Statistical analysis was performed by one-way ANOVA or Student's t-test. A P-value $\leq$ 0.05 was considered statistically significant.

Results

CDK5 phosphorylates Ser126-Pit-1 in GH3 cells.

CDK5 is specifically activated in human prolactinomas(14), and the pituitary has high levels of Pit-1. However, whether CDK5 affects the physiological function of Pit-1 by phosphorylation is unknown. The phosphorylation of Pit-1 is the key activation step for trafficking. CDK5 belongs to the serine/threonine kinase family and has specific sequence requirements for its phosphorylation substrate. It can only phosphorylate serine or threonine sites containing S/TPXX (K/R/H) conserved sequences. According to a

bioinformatics search, serine at position 126 of Pit-1 was the only potential typical CDK5 phosphorylation site (Figure 1a). To study Ser-126 activation, we designed a custom Pit-1 antibody that specifically recognizes phosphorylation at position 126 of Pit-1 to detect pSer126-Pit-1 in tumor tissue and cell lines. We utilized an alanine acid mutation to mimic phosphorylation of Ser126-Pit-1. The Ser126-Pit-1 phosphospecific antibody detected an ~34 kDa protein from wild-type green fluorescent protein (GFP)-tagged Pit-1 expressed in GH3 cells, but it did not recognize GFP-Pit-1 with a Ser (S) 126 to Ala (A) mutation. To test the specificity of the antibody, we transfected GH3 cells with WT-Pit-1 and S126A-Pit-1 (a nonphosphorylatable mutation), and the results indicated that the Ser126- Pit-1 phospho-antibody specifically recognized Pit-1 phosphorylated at Ser126 (Figure 1b).

CDK5 inhibition reduces pSer126-Pit-1

To determine the influence of CDK5 on the phosphorylation of Ser126-Pit-1, we cultured GH3 cells with different concentrations of roscovitine (a CDK5 inhibitor). As shown in Fig. 1c, after inhibiting the activity of CDK5, the protein level of Pit-1 was not obviously changed; however, as the concentration of roscovitine increased in a certain dose range, the phosphorylation level of Ser126-Pit-1 gradually decreased. We transfected GH3 cells with short interfering RNA (siRNA) targeting CDK5 mRNA, and the knockdown efficiency was verified by western blot. As shown in Fig. 1d, the phosphorylation level of Ser126-Pit-1 was decreased by CDK5 knockdown in GH3 cells. In particular, we found that both CDK5 inhibition and depletion significantly decreased pSer126-Pit-1.

pSer126-Pit-1 promotes GH3 cell proliferation and PRL secretion

To determine the influence of the phosphorylation level of Pit-1 on cell proliferation and apoptosis, we transfected GH3 cells with WT-Pit-1 and S126A-Pit-1 (MT- Pit-1). We detected cell proliferation with a colony formation assay, and the results are presented as the percentage of clones formed as a function of time. After two weeks of incubation, we clearly observed that the proportion of clones formed in GH3 cells in the WT- Pit-1 groups was higher than that in the MT- Pit-1 groups (Figure 2a). Similarly, we found that cell viability was significantly higher in the WT- Pit-1 groups than it was in the MT- Pit-1 groups (Figure 2c), suggesting that S126A-Pit-1 suppresses GH3 cell proliferation. To determine whether apoptosis was a contributing factor in cell survival inhibition, we performed flow cytometric analysis of cells pretreated with WT-Pit-1 and MT- Pit-1. Apoptosis assays showed that the apoptosis rates of GH3 cells increased in the MT-Pit-1 groups compared with the WT-Pit-1 groups after incubation for 72 h (Fig. 2b).

Pit-1 binds to the proximal PRL promoter and induces PRL expression(15, 16). To explore whether a mutation of the Ser126 phosphorylation site in Pit-1 would affect the ability of Pit-1 to bind to the PRL promoter, we performed an electrophoretic mobility shift assay (EMSA) using nuclear extracts from GH3 cells. As shown in Fig. 3a, Pit-1 could bind to the PRL promoter as expected, but when Ser 126 was mutated, the binding was almost completely abolished. HEK293 cells that transiently coexpressed the PRL promoter, WT-Pit-1, or S126A-Pit-1 were analyzed by luciferase reporter assay. WT-Pit-1 demonstrated stronger PRL transcriptional activation than S126A-Pit-1 (Fig. 3b). These results indicated that Ser126 in

Pit-1 is the key site for the combination of Pit-1 with the PRL promoter. To determine whether pSer126-Pit-1 affects the ability of GH3 cells to secrete PRL, an ELISA was used to measure the level of PRL in the supernatant of GH3 cells. The ELISA results show that the level of PRL was significantly higher in the Pit-1 groups than it was in the S126A-Pit-1 groups, which indicates that high pSer126-Pit-1 promoted PRL secretion in GH3 cells (Fig. 3c).

Ser126-Pit-1 phosphorylation in human prolactinoma tissue

To investigate the clinical significance of Ser126-Pit-1 phosphorylation, we carried out IHC staining of human prolactinoma tissue from 48 patients with the pSer126-Pit-1 phosphoantibody. Based on the IHC staining (Fig. 4a), we divided human prolactinoma tissue into the pSer126-Pit-1 high expression group (mean H-score: 195) and the pSer126-Pit-1 low expression group (mean H-score: 138). The prognostic value of pSer126-Pit-1 for recurrence-free survival in prolactinoma

patients was evaluated by comparing the patients with low and high pSer126-Pit-1 expression. According to Kaplan-Meier survival analysis, patients with high pSer126-Pit-1 expression had a distinctly shorter recurrence-free survival time than those with low pSer126-Pit-1 expression (Fig. 4b). These data suggest that pSer126-Pit-1 might serve as a prognostic biomarker for predicting the outcome of prolactinoma.

Table 1. Clinical and pathological characteristics of the patients.

Characteristic	Patients
Numbers	48
Age (mean ± SD, years)	39.3 ±10.7, 14-62
Sex (M/F)	22/26
Macroadenoma (%)	23 (47.9%)
Microadenoma (%)	4 (8.3%)
Giant adenoma (%)	21 (43.8%)
Mean follow-up, years (mean ± SD, range)	4.8 ± 1.17, 2.5–7

F, female; M, male;.

Discussion

The data presented here have shown that both inhibition and depletion of CDK5 reduce pSer-126-Pit-1 in GH3 cells, and the high level of phosphorylated Pit-1 can promote cell proliferation and inhibit apoptosis. Meanwhile, Ser126 in Pit-1 is the key site enabling the association of Pit-1 with the PRL promoter. We found that a higher level of pSer-126-Pit-1 correlates with worse prognosis in patients. These results

suggested that CDK5 phosphorylates Ser126-Pit-1 to regulate prolactinoma progression and PRL secretion.

CDK5 is a unique member of the cell cycle-dependent kinase family. It plays a key regulatory role in the nervous system's transcriptional activity. In response to changes in the external environment or in hormone levels, CDK5 will undergo translocation from the cytoplasm to the nucleus and will phosphorylate different transcription factor substrates. Previous studies have shown that CDK5 can participate in the regulation of transcriptional activity through phosphorylation of key transcription factors, such as STAT3 (signal transducer and activator of transcription 3), MEF2 (myocyte enhancer factor 2), and mSds3(17, 18). CDK5 can phosphorylate serine 727 of STAT3 and regulate its transcriptional activity, which affects the downstream expression of c-fos, junB, and Foxp3 and regulates T cell development(19, 20). Although CDK5 has been investigated in some types of cancers, the functional role of CDK5 in proliferation and apoptosis of pituitary adenoma cells remains to be elucidated. In our previous study(13, 14, 21), we found that CDK5 activity was upregulated in pituitary adenomas and was associated with p35. Here, we have shown that both proliferation and apoptosis of pituitary cells were regulated by pSer126-Pit-1. After we treated cells with the CDK5 inhibitor and CDK5 siRNA, CDK5 activity and expression levels were reduced, which decreased the level of Ser126-Pit-1 phosphorylation. Our results reveal that CDK5 can exert its biological function by specifically regulating Ser126-Pit-1 phosphorylation.

Pit-1 contains 291 amino acids, including an N-terminal transcription activation domain, a POU-specific domain (POU-specific), and a POU homology domain (POU homeodomain). POU-specific domains and POU-homeodomains are high affinity DNA binding domains, and they also interact with other transcriptional regulators. Ser126 is located at the beginning of the POU-specific domain. Ser126-Pit-1 phosphorylation can change its affinity for DNA and affect its DNA binding site. After CDK5 specifically and appropriately phosphorylates Pit-1 in the nucleus, Pit-1 may be overactivated to produce excessive GH or PRL. Studies have shown that the R271W mutation of Pit-1 converts arginine to tryptophan at position 271 in the C-terminal POU-H domain, leading to the loss of a positive charge in the basic amino acid region. R271W-mutated Pit-1 can bind to DNA, thus competitively inhibiting wild-type Pit-1(21). According to our experimental results, a mutation in the Ser126 site in Pit-1 impacts its effective binding to the PRL promoter and reduces CDK5 phosphorylation of Pit-1.

By interacting with different types of transcription factors, Pit-1 can also regulate different signaling pathways, such as PKA (protein kinase A) and PKC (protein kinase C), and Ras signaling pathways, thereby targeting the regulation of these pathways could occur via regulation of the Pit-1 promoter(22, 23). Previously, some research groups found that the Pit-1 gene was related to pituitary dysplasia and PRL, GH, and TSH secretion defects. The absence of the Pit-1 gene leads to complete PRL and GH secretion defects(24-27). Our results have confirmed that in comparison to the mutant Pit-1, WT-Pit-1 more stably binds to the PRL promoter and induces PRL expression, thus leading to excessive hormone secretion and enabling cells to grow aggressively and invade surrounding tissues.

Conclusion

We have demonstrated that CDK5 phosphorylates Pit-1 at Ser126 in GH3 cells, which is a step that is required for cell proliferation and apoptosis in vitro. Therefore, Pit-1 phosphorylation at Ser126 may play a critical role in prolactinoma progression, and it could be used in the prediction of poor prognosis of prolactinomas. Additionally, our findings showed that CDK5 inhibitors could directly or indirectly block cell proliferation in prolactinomas.

Abbreviations

CDK5: cyclin-dependent kinase 5;

Ser126-Pit-1: the serine at position 126 of Pit-1;

pSer126-Pit-1: phosphorylation level of Ser126-Pit-1

PRL: prolactin

IHC: immunohistochemistry

TMA: tissue microarrays

EMSA: Electrophoretic mobility shift assay

CCK-8: Cell counting kit-8

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Beijing Tiantan Hospital, Capital Medical University (Beijing, China).

Consent for publication

Not applicable.

Availability of data and materials

The authors can confirm that all relevant data and materials are available upon request from the authors.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

W.X. and Y.Z. conceived the project. Q.F. and W.X. designed the experiments, analyzed the data, and wrote the manuscript. C.L. and J.G. assisted with the management of clinical data and specimens. L.G. performed the experiments. All authors read and approved the manuscript.

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Figures

Figure 1

Both CDK5 inhibition and depletion affect the phosphorylation level of Ser126-Pit-1. a. Bioinformatics analysis reveals that Ser126 Pit-1 was the only typical CDK5 potential phosphorylation site. b. GH3 pituitary cells were transfected with WT-Pit or Pit-1-126A. Western blotting shows that the anti-pS126-Pit-1 antibody can specifically recognize the phosphorylation of Ser126-Pit-1. c. Western blot (left) and densitometry analysis (right) showed that S126-Pit-1 phosphorylation was reduced by roscovitine treatment at the indicated concentrations. The protein levels of Pit-1 was not clearly different. β-actin

served as a loading control. d. Western blot (left) and densitometry analysis (right) showed that S126-Pit-1 phosphorylation was reduced by CDK5 siRNA treatment at the indicated concentrations. β -actin served as a loading control. **, $P \leq 0.01$; ***, $P \leq 0.001$. The bar represents the mean $\pm$ SD.

Figure 2

pSer126-Pit-1 promotes GH3 cell proliferation and suppresses apoptosis a. The colony formation test shows that the percentage of colonies formed was reduced in the Pit-1-126A groups compared with that of the Pit-1 groups. b. The apoptosis rates of the Pit-1-126A groups were significantly higher than those of the Pit-1 groups. c. Higher cell viability was observed in the Pit-1 groups. **, $P \leq 0.01$; ****, $P \leq 0.0001$. The bar represents the mean $\pm$ SD.

Figure 3

WT-Pit-1 promotes PRL secretion and induces PRL expression. a. Pit-1-PRL complexes were detected. b. S126A-Pit-1 significantly inhibited the luciferase activity of the PRL 3'UTR relative to the levels seen with Pit-1 c. The PRL level of the Pit-1 group was higher than that of the Pit-1-126A group. **, $P \leq 0.01$. The bar represents the mean $\pm$ SD.

Figure 4

pSer126- Pit-1 is associated with poor prognosis. a. Representative images of pSer126-Pit-1 staining of a tissue microarray, including low pSer126-Pit-1 levels (left panel) and high pSer126-Pit-1 levels in prolactinomas (right panel). Insets show 200× magnifications of the low-power images, scale bar, 50 μ m. b. Significant differences in recurrence-free survival are shown based on pSer126-Pit-1 expression status in 48 patients after surgical removal of prolactinomas, * $P<0.05$.