Research Article

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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: Pit-1 (POU1F1) is a POU-homeodomain transcription factor, and it is one of the most important tissue-specific transcription factors in pituitary development. Cyclin-dependent kinase 5 (CDK5) is a protein kinase that can phosphorylate many key transcription factors, but the mechanism under which CDK5 phosphorylates Pit-1 is unclear. To investigate whether CDK5 can regulate cell proliferation and promote hormone secretion through phosphorylation of Ser126-Pit-1 in prolactinomas, 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 the phosphorylation level of Ser126-Pit-1 (pSer126-Pit-1) in

prolactinomas. Our data indicated that Ser126-Pit-1 is specifically phosphorylated by CDK5 and high-level pSer126-Pit-1 can promote cell proliferation and PRL secretion. In addition, a higher level of pSer126-Pit-1 correlates with a worse prognosis in patients with prolactinoma. Our results show that CDK5 mediated Ser126-Pit-1 phosphorylation and regulated prolactinoma progression and PRL secretion.

Keywords: Pit-1, CDK5, prolactinoma, phosphorylation, proliferation

1 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 the 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

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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, whose constitutive expression is largely restricted to cells of neural crest origin [11,12]. Our group found for the first time that CDK5 exists in normal human pituitary and PAs and found that CDK5 can promote the growth and invasion of PAs, but the true regulatory mechanism of CDK5 in pituitary tumors is far from clear [13,14]. 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.

2 Materials and methods

2.1 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 (<1 cm diameter), macroadenomas (>1 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

Table 1: Clinical and pathological characteristics of the patients

Characteristics	Patients
Numbers	48
Age (mean $\pm$ SD, years)	39.3 $\pm$ 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 $\pm$ SD, range)	4.8 $\pm$ 1.17, 2.5–7

F, female; M, male.

from the date of surgery to the date of tumor recurrence. Patients were collected 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 all patients signed the informed consent forms. The project ethics approval Number is KY2019-136-01. The patient characteristics are summarized in Table 1.

2.2 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 μ m sections using a serial microtome, and the 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.

2.3 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) were commercially developed using standard methods by the 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) was recorded, and an H-score was calculated as follows:

$$\text{H-score} = (\% \text{ cells } 1+) + 2(\% \text{ cells } 2+) + 3(\% \text{ cells } 3+).$$

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).

2.4 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.

2.5 Plasmid construction and CDK5 inhibitor

A CDK5 siRNA (SR507441) and CDK5 expression plasmid (NM_004935) constructs were purchased from OriGene Technologies (Rockville, MD, USA). Mutant Pit-1 (GFP-Ser126A-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).

2.6 Cell counting kit-8 (CCK-8) assay

Cells were seeded in 96-well plates at the density of 1×10^4 cells per well in 100 µL of cell culture medium for 24 h 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.

2.7 Colony formation test

The treated cell lines were seeded into six-well plates at 1,000 cells per well and incubated for 2 weeks. After incubation, the cells were fixed in 4% paraformaldehyde for 15 min and stained in 1 mL of a 0.1% crystal violet solution for 30 min. The culture plate was photographed. Visible colonies in each well were quantified by ImageJ software.

2.8 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 h 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.

2.9 Cell apoptosis assay

Cell apoptosis was determined using Annexin V-FITC/PI kits (BD Biosciences, Franklin Lakes, NJ). Cells were seeded for 48 h after transiently transfected with the indicated plasmids. Then, the 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).

2.10 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 mixture was transferred onto Biotodyne® B nylon membranes (Pall Corporation). Signals were visualized with reagents included

in the kit and with a ChemiDoc XRS system (Bio-Rad Laboratories, USA).

2.11 Luciferase reporter assay

GH3 cells were cultured at a density of 2×10^4 cells per well in 96-well culture plates. The cells were transfected with 0.2 µg of dual-luciferase reporter construct 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 a medium containing 6 µM 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.

2.12 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 rpm 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 h, followed by incubation with anti-β-actin (1:5,000; 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 h. 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.

2.13 Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.00 statistical software. Experimental data are reported as the mean ± SD (standard deviation) 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 <0.05 was considered to be statistically significant.

3 Results

3.1 CDK5 phosphorylates Ser126-Pit-1 in GH3 cells

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 phospho-specific antibody detected an ~32 kDa protein from (Flag)-tagged Pit-1 expressed in GH3 cells but did not recognize Flag-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 Ser126A-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).

3.2 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 Figure 1c, after inhibiting the activity of CDK5, the

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-1 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 showed that Ser126-Pit-1 phosphorylation was reduced by CDK5 siRNA treatment at the indicated concentrations. β -Actin served as a loading control. **, $P < 0.01$; ***, $P < 0.001$. The bar represents the mean $\pm$ SD.

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 the western blot. As shown in Figure 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.

3.3 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 Ser126A-Pit-1 (MT-Pit-1). Western blot results showed that pSer126-Pit-1 in GH3 cells with WT-Pit-1 is significantly higher than GH3 cells

with MT-Pit-1 (Figure 2a). 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 2 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 2b). 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 2d), suggesting that Ser126A-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 (Figure 2c). In order to confirm that whether CDK5 affects cell proliferation via p-Ser126-Pit-1 dominantly, GH3 cells were co-transfected with CDK5 and Pit-1 siRNA to assess whether the effect of CDK5 could be reversed by Pit-1 siRNA. The results indicated that the cell viability in CDK5 groups

Figure 2: pSer126-Pit-1 promotes GH3 cell proliferation and suppresses apoptosis. (a) Western blot showed pSer126-Pit-1 in GH3 cell with WT-Pit-1 or Pit-1-126A. (b) 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. (c) The apoptosis rates of the Pit-1-126A groups were significantly higher than those of the Pit-1 groups. (d) Higher cell viability was observed in the Pit-1 groups. (e) The effect of CDK5 could be reversed by Pit-1 siRNA. **, $P < 0.01$; ***, $P < 0.001$. The bar represents the mean $\pm$ SD.

was significantly higher than CDK5 + Pit-1 siRNA groups, and the cell viability of Pit-1 siRNA decreased (Figure 2e). These data suggest that CDK5-mediated pSer126-Pit regulates cell proliferation.

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 Figure 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 Ser126A-Pit-1 were analyzed by luciferase reporter assay. WT-Pit-1 demonstrated stronger PRL transcriptional activation than Ser126A-Pit-1 (Figure 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 Ser126A-Pit-1 groups, which indicates that high pSer126-Pit-1 promoted PRL synthesis and secretion in GH3 cells (Figure 3c).

3.4 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 (Figure 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

Figure 3: WT-Pit-1 promotes PRL secretion and induces PRL expression. (a) Pit-1-PRL complexes were detected. (b) Ser126A-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 groups was higher than that of the Pit-1-126A groups. **, $P < 0.01$; ***, $P < 0.001$. The bar represents the mean $\pm$ SD.

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 (Figure 4b). These data suggest that pSer126-Pit-1 might serve as a prognostic biomarker for predicting the outcome of prolactinoma.

4 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 a 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. It is specifically activated in human prolactinomas, and the pituitary has high levels of Pit-1. However, whether CDK5 affects the physiological function of Pit-1 by phosphorylation is unknown. 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. In response to changes in the external environment or 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 the proliferation and apoptosis of PA cells remains to be elucidated.

Figure 4: pSer126-Pit-1 is associated with poor prognosis. (a) Representative images of pSer126-Pit-1 staining of a tissue microarray, including high pSer126-Pit-1 levels (left panel) and low pSer126-Pit-1 levels in prolactinomas (right panel). Insets show 200 $\times$ 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, *, $P < 0.05$.

In our previous study, we found that CDK5 activity was upregulated in PAs and was associated with p35 [13,14,21]. 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.

5 Conclusion

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 the poor prognosis of prolactinomas. Additionally, our findings showed that CDK5 inhibitors could directly or indirectly block cell proliferation in prolactinomas.

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

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

References

- [1] Tatsumi K, Amino N. PIT1 abnormality. *Growth Horm IGF Res.* 1999;9(Suppl B):18–22. discussion 3.
- [2] Martinez-Ordonez A, Seoane S, Cabezas P, Eiro N, Sendon-Lago J, Macia M, et al. Breast cancer metastasis to liver and lung is facilitated by Pit-1-CXCL12-CXCR4 axis. *Oncogene.* 2018;37(11):1430–44.
- [3] Feldmann G, Mishra A, Hong SM, Bisht S, Strock CJ, Ball DW, et al. Inhibiting the cyclin-dependent kinase CDK5 blocks pancreatic cancer formation and progression through the suppression of Ras-Ral signaling. *Cancer Res.* 2010;70(11):4460–9.
- [4] Wissing MD, Dadon T, Kim E, Piontek KB, Shim JS, Kaelber NS, et al. Small-molecule screening of PC3 prostate cancer cells identifies tilorone dihydrochloride to selectively inhibit cell growth based on cyclin-dependent kinase 5 expression. *Oncol Rep.* 2014;32(1):419–24.
- [5] Augustijn KD, Duval DL, Wechselberger R, Kaptein R, Gutierrez-Hartmann A, van der Vliet PC. Structural characterization of the

- PIT-1/ETS-1 interaction: PIT-1 phosphorylation regulates PIT-1/ETS-1 binding. *Proc Natl Acad Sci U S A*. 2002;99(20):12657–62.
- [6] Jean A, Gutierrez-Hartmann A, Duval DL. A Pit-1 threonine 220 phosphomimic reduces binding to monomeric DNA sites to inhibit Ras and estrogen stimulation of the prolactin gene promoter. *Mol Endocrinol*. 2010;24(1):91–103.
- [7] Ben-Batalla I, Seoane S, Macia M, Garcia-Caballero T, Gonzalez LO, Vizoso F, et al. The Pit-1/Pou1f1 transcription factor regulates and correlates with prolactin expression in human breast cell lines and tumors. *Endocr Relat Cancer*. 2010;17(1):73–85.
- [8] Gil-Puig C, Seoane S, Blanco M, Macia M, Garcia-Caballero T, Segura C, et al. Pit-1 is expressed in normal and tumorous human breast and regulates GH secretion and cell proliferation. *Eur J Endocrinol*. 2005;153(2):335–44.
- [9] Ghose A, Shashidhara LS. Cyclin beyond the cell cycle: new partners at the synapse. *Dev Cell*. 2011;21(4):601–2.
- [10] Cortes N, Guzman-Martinez L, Andrade V, Gonzalez A, Maccioni RB, CDK5 A, et al. and Its Multiple Roles in the Nervous System. *J Alzheimers Dis*. 2019;68(3):843–55.
- [11] Mishiba T, Tanaka M, Mita N, He X, Sasamoto K, Itohara S, et al. Cdk5/p35 functions as a crucial regulator of spatial learning and memory. *Mol Brain*. 2014;7:82.
- [12] Asada A, Saito T, Hisanaga S. Phosphorylation of p35 and p39 by Cdk5 determines the subcellular location of the holokinase in a phosphorylation-site-specific manner. *J Cell Sci*. 2012;125(Pt 14):3421–9.
- [13] Xie W, Wang H, He Y, Li D, Gong L, Zhang Y. CDK5 and its activator P35 in normal pituitary and in pituitary adenomas: relationship to VEGF expression. *Int J Biol Sci*. 2014;10(2):192–9.
- [14] Xie W, Liu C, Wu D, Li Z, Li C, Zhang Y. Phosphorylation of kinase insert domain receptor by cyclin-dependent kinase 5 at serine 229 is associated with invasive behavior and poor prognosis in prolactin pituitary adenomas. *Oncotarget*. 2016;7(32):50883–94.
- [15] Pellegrini I, Barlier A, Gunz G, Figarella-Branger D, Enjalbert A, Grisoli F, et al. Pit-1 gene expression in the human pituitary and pituitary adenomas. *J Clin Endocrinol Metab*. 1994;79(1):189–96.
- [16] Pellegrini-Bouiller I, Morange-Ramos I, Barlier A, Gunz G, Figarella-Branger D, Cortet-Rudelli C, et al. Pit-1 gene expression in human lactotroph and somatotroph pituitary adenomas is correlated to D2 receptor gene expression. *J Clin Endocrinol Metab*. 1996;81(9):3390–6.
- [17] Hsu FN, Chen MC, Lin KC, Peng YT, Li PC, Lin E, et al. Cyclin-dependent kinase 5 modulates STAT3 and androgen receptor activation through phosphorylation of Ser(7)(2)(7) on STAT3 in prostate cancer cells. *Am J Physiol Endocrinol Metab*. 2013;305(8):E975–86.
- [18] Lin H, Chen MC, Chiu CY, Song YM, Lin SY. Cdk5 regulates STAT3 activation and cell proliferation in medullary thyroid carcinoma cells. *J Biol Chem*. 2007;282(5):2776–84.
- [19] Lam E, Choi SH, Pareek TK, Kim BG, Letterio JJ. Cyclin-dependent kinase 5 represses Foxp3 gene expression and Treg development through specific phosphorylation of Stat3 at Serine 727. *Mol Immunol*. 2015;67(2 Pt B):317–24.
- [20] Fu AK, Fu WY, Ng AK, Chien WW, Ng YP, Wang JH, et al. Cyclin-dependent kinase 5 phosphorylates signal transducer and activator of transcription 3 and regulates its transcriptional activity. *Proc Natl Acad Sci U S A*. 2004;101(17):6728–33.
- [21] Kishimoto M, Okimura Y, Fumoto M, Iguchi G, Iida K, Kaji H, et al. The R271W mutant form of Pit-1 does not act as a dominant inhibitor of Pit-1 action to activate the promoters of GH and prolactin genes. *Eur J Endocrinol*. 2003;148(6):619–25.
- [22] Wasyluk B, Hagman J, Gutierrez-Hartmann A. Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends Biochem Sci*. 1998;23(6):213–6.
- [23] Xu L, Lavinsky RM, Dasen JS, Flynn SE, McInerney EM, Mullen TM, et al. Signal-specific co-activator domain requirements for Pit-1 activation. *Nature*. 1998;395(6699):301–6.
- [24] Ohta K, Nobukuni Y, Mitsubuchi H, Fujimoto S, Matsuo N, Inagaki H, et al. Mutations in the Pit-1 gene in children with combined pituitary hormone deficiency. *Biochem Biophys Res Commun*. 1992;189(2):851–5.
- [25] Pfaffle RW, DiMattia GE, Parks JS, Brown MR, Wit JM, Jansen M, et al. Mutation of the POU-specific domain of Pit-1 and hypopituitarism without pituitary hypoplasia. *Science*. 1992;257(5073):1118–21.
- [26] Radovick S, Nations M, Du Y, Berg LA, Weintraub BD, Wondisford FE. A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency. *Science*. 1992;257(5073):1115–8.
- [27] Tatsumi K, Miyai K, Notomi T, Kaibe K, Amino N, Mizuno Y, et al. Cretinism with combined hormone deficiency caused by a mutation in the PIT1 gene. *Nat Genet*. 1992;1(1):56–8.