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RESEARCH ARTICLE

Expression of PDK1 in malignant pheochromocytoma as a new promising potential therapeutic target

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Abstract

Purpose Phosphoinositide-dependent kinase 1 (PDK1) is highly expressed in many solid tumors. And several studies have demonstrated that PDK1 has been an emerging and promising target for anti-cancer therapies. However, the role of PDK1 has not been studied so far in malignant pheochromocytoma (PCC).

Methods In this study, immunohistochemical staining was performed to investigate the protein level of PDK1 in 63 PCC tissue samples, of which 49 were benign and 14 were malignant. In addition, we evaluated the effect of inhibition of PDK1 with siRNA on cell growth, apoptosis and invasive capacity in PC12 cells and identified the underlying mechanisms.

Results We found that PDK1 was overexpressed in malignant PCC tissues, and knockdown of PDK1 with siRNA significantly inhibited cell proliferation, increased apoptosis induction, and attenuated cell migration and invasive capacity in PC12 cells. We also showed that knockdown of PDK1 significantly reduced the phosphorylation of Akt at threonine 308 (p-Akt T308) but did not alter the serine phosphorylation of Akt on the S473 site (p-Akt S473). Furthermore, we found that the p-Akt expression was noticeably decreased after knockdown of PDK1, but the t-Akt expression did not show a significant decrease.

Conclusion We have demonstrated for the first time that PDK1 is overexpressed in human malignant PCC and plays an important role in the malignant biological behaviors of PC12 cell. Specifically, we have revealed that knockdown of PDK1 could attenuate activation of the Akt signaling. These data suggest that PDK1 could be a new promising potential therapeutic target in human cancer treatment for malignant PCC.

Keywords Phosphoinositide-dependent kinase 1 · PDK1 · Adrenal gland · Malignant pheochromocytoma · Target

Introduction

Pheochromocytoma (PCC) is a rare neuroendocrine tumor, and most of those tumors arise from the medulla of the adrenal gland. Although up to 10–15% of patients with PCC shows malignancy, the malignancy for extra-adrenal tumors of PCC with certain risk factors are up to 50–60% [1, 2]. Early surgical excision is considered to as the only curable means. The prognosis for malignant PCC is poor with the 5-year survival rate lower than 50%, and recurrence rate up to 60% within 5 years [1, 2]. There are a lot of therapeutic strategies in the treatment of malignant PCC, such as radionuclide therapy with ^{131}I-MIBG and chemotherapy [3, 4]. However, there is no effective therapeutic strategy, because the outcomes show less favourable and frequently short-lived through those treatments [3–5]. There is no doubt that discovering new and effective therapeutic strategies to improve prognosis is an urgent need for malignant PCC.

Phosphoinositide-dependent kinase 1 (PDK1) is now widely investigated in malignant tumors due to PDK1 can act as an important junction point for multiple cell signaling pathways [6–8]. PDK1 is commonly overexpressed in many types of cancer, such as breast cancer, lung cancer, acute myeloid leukemia and ovarian cancer [8–11]. And several studies have demonstrated that PDK1 has been a promising therapeutic target for anticancer therapies [8–11]. However, the role of PDK1 has not been studied so far in malignant PCC. In the current study, we present data to show that PDK1 is overexpressed in malignant PCC specimens, and knockdown of PDK1 can suppress cell proliferation, promote cell apoptosis, and attenuate cell migration and

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invasive capacity of PC12 cells in vitro. These results indicate that PDK1 could be a new promising potential therapeutic target in human cancer treatment for malignant PCC.

Materials and methods

The expression of PDK1 by immunohistochemistry stain (IHC)

The present study included paraffin-embedded tissue samples from 63 patients with PCC who were diagnosed and treated by surgery from January 2001 to December 2015. Of those tissue samples, 49 were benign, and 14 were malignant. According to the World Health Organization, malignancy was defined as lymph node and distant metastasis at the initial intervention or recurrent PCC during follow-up. For IHC, the slides were incubated at 4 °C overnight with PDK1 primary antibodies (1:500, Abcam, England) and incubated with an HRP-conjugated secondary antibody at room temperature for 1 h. Diaminobenzidine (DAB) was applied for color development. Under high power microscopy at **100 × magnification, 5 visual fields were randomly selected per section and 200 cells were counted in each high power field. The IHC results were measured using a semi-quantitative score for both intensity and percentage of positive staining. Briefly, the intensity of staining was scored as: 1 (no stain), 2 (weak stain), 3 (medium stain), 4 (strong stain) and the percentage was scored as: 1 (< 25%), 2 (25–50%), 3 (50–75%), 4 (> 75%). The slides were scored independently by two experienced pathologists.

Cell lines, cell culture and small interfering (siRNA) transfection

The pheochromocytoma PC12 cell line was obtained from the Shanghai Institute of Cell Biology (Shanghai, China), and cultured in DMEM medium (GIBCO) supplemented with 10% inactivated horse serum (GIBCO) under an atmosphere of 5% CO_2 at 37 °C. Experimentally verified rat PDK1 siRNA and negative control siRNA were chemically synthesized by GenePharma Co., Ltd (Shanghai, China). The following sequences were used for RNA interference: PDK1, 5′-AATCTTCTCAGGACACCATCCGTTCAATT-3′; negative control, 5′-UUCGUGCUCCGAAUCACGUTT AACGUUAA-3′. Cells were transfected with the siRNA at 60–70% confluence. Transfection of siRNAs was carried out at a concentration of 50 nmol/l using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the transfection protocol provided with the product. The cells were harvested for analysis 48–72 h following transfection.

Cell viability assay by CCK-8 assays

Cell viability was measured by CCK-8 assay as previously described [12]. Cells were seeded on a 96-well plate at a density of 2×10^3 per well with 100 µl complete medium and incubated for 0–72 h. Then 10 µl of Cell Counting Kit-8 (CCK-8, Dojindo, Japan) solution was added into each well and incubated for 2 h. Cell viability was examined using a microplate reader. Absorbance in each well was then measured at a wavelength of 450 nm. These experiments were independently performed three times.

The detection of apoptotic cells by flow cytometry

Annexin V-FITC/PI Apoptosis Detection Kit was performed according to the manufacturer's protocol [13]. Cells were harvested and resuspended in 0.5 mL Annexin V-binding buffer. And then, those cells were stained with FITC-conjugated Annexin V and PI following the manufacturer's instruction (Invitrogen; Thermo Fisher Scientific, Inc.). After incubation for half an hour in the dark, evaluation of the level of apoptosis was analyzed by flow cytometer (BD FASCanto) and CellQuest software. The number of apoptotic cells divided by the number of total cells was the apoptosis rate. In this experiment, both early apoptotic cells and late apoptotic cells were counted as apoptotic cells. These experiments were independently performed in three times.

Cell migration assay by wound healing assay

Wound healing assay were utilized to evaluate cell migration ability according to the manufacturer's protocol [12]. Briefly, cells were seeded in 6-well plates. After being grown to over 90% confluence of monolayer cells overnight, cells were scratched using a sterile 200 µl pipette tip. To eliminate nonadherent cells, those cells were rinsed three times with PBS. Images of the wounds were acquired, and gap distances were measured under a light microscope by microscope (Nikon, Japan, × 200) at 0 and 48 h. These experiments were independently performed three times.

Cell invasion assay by a transwell approach

Invasion chambers (Costar, USA) were utilized to evaluate cell invasion ability according to the manufacturer's protocol [12]. Briefly, cell invasion assays were carried out in Matrigel-coated Transwell inserts containing polycarbonate filters with 8-µm pores. 100 µl of a matrigel solution was placed on a Transwell insert, allowed to gel at 37 °C for 2 h, and dried on a clean bench for 6 h. Cells were isolated and resuspended in serum-free medium at a concentration

of 1×10^5 cells/ml. Approximate 2×10^4 cells were plated onto the upper chamber. 600 µl complete medium with 10% FBS in the lower chamber was used as a chemoattractant. After 24 h of incubation, non-invaded cells were scraped off using a cotton swab. Invaded cells on the underside of membrane were fixed with 4% methanol, stained with 0.5% crystal violet, and then counted by an inverted microscope in five different fields at 200 × magnification and in duplicate wells, in at least three independent experiments.

Western blotting analyses

Expression of PDK1, AKT, p-Akt, p-Akt Ser473, and p-Akt Thr308 were detected by Western blot as previously described [11]. After knockdown of PDK1, the whole cell extracts were collected, washed and lysed completely on ice. Protein concentrations were measured using a BCA assay kit (KeyGEN Biotech, Nanjing, China). Appropriate amounts of protein (20 µg/sample) were separated using 10% SDS–polyacrylamide gels, transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 5% nonfat milk. PVDF membranes were incubated overnight at 4 °C with the following primary antibodies: PDK1 (1:4000), AKT(1:4000), p-Akt (1:1000), p-Akt Ser473 (1:1000), p-Akt Thr308 (1:1000), and GAPDH (1:15,000) (all from Cell Signaling Technology, Beverly, USA) at dilutions specified by the manufacturer. Subsequently, the membranes were washed 3 times in Tris-buffered saline-Tween 20 (0.1% by volume, TBST) and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h, followed by enhanced chemiluminescence detection (NENTM Life Science Products Inc, USA).

Statistical analyses

The quantitative data were expressed as the mean ± SD. Student's *t* test, followed by Dunnett's multiple comparison test, was used to compare quantitative data. The χ^2 test was adopted to compare categorical data. The statistical analyses were performed using SPSS 20.0 statistical software (SPSS, Chicago, IL, USA). Value of $p < 0.05$ was considered to as significant.

Results

PDK1 is highly expressed in malignant tissue of PCC patients

To investigate the expression of PDK1, immunohistochemical staining was performed to examine the protein level of PDK1 in 63 PCC tissue samples, of which 49 were benign and 14 were malignant. We found that PDK1 was localized to the cytoplasm, with positive signals observed as yellow or brown patches (Fig. 1). The results showed that 12 of 14 (85.7%) malignant tissues were strongly positive expression of PDK1 (Fig. 1c). However, only 3 (6.1%) were strongly positive expression of PDK1 in the 49 benign tissues, and most of them (81.6%) were moderately positive expression (Fig. 1b). The results revealed that PDK1 levels was significantly higher expressed in malignant PCC tissues than that in benign PCC tissues.

Knockdown of PDK1 attenuates activation of the Akt signaling

Knockdown of PDK1 was carried out by transfected with PDK1 siRNA in pheochromocytoma PC12 cell. The effects of knockdown of PDK1 on activation of the Akt signaling in pheochromocytoma PC12 cell line was examined by western blot. The western blot analysis showed that PDK1 siRNA markedly decreased protein levels of PDK1 (Fig. 2). We observed that knockdown of PDK1 significantly reduced the phosphorylation of AKT at threonine 308 (p-AKT T308) but did not alter the serine phosphorylation of AKT on the S473 site (p-AKT S473) (Fig. 2). Also, we found that the p-Akt expression was noticeably decreased after knockdown of PDK1, but the t-Akt expression did not show a significant decrease. These results suggest that knockdown of PDK1 could attenuate activation of the Akt signaling.

Knockdown of PDK1 suppresses PC12 cell proliferation

To analyze the role of PDK1 in PC12 cell viability, the effect of knockdown of PDK1 on cell proliferation was investigated by transfected with siRNA for 0–72 h. As seen from the cell proliferation curve (Fig. 3), at 12, 24, 48 and 72 h following transfection, cells transfected with PDK1 siRNA gradually lost viability, and the cell numbers were notably reduced at 72 h, while cells in the control group maintained healthy growth. These results suggest that knockdown of PDK1 was able to suppress cell viability.

Knockdown of PDK1 promotes PC12 cell apoptosis

To analyze the role of PDK1 in PC12 cell survival, the effect of knockdown of PDK1 on cell apoptosis was investigated by flow cytometry. After transfection with PDK1 siRNA for 48 h, we harvested and stained cells with annexin-V-FITC and PI, and apoptosis was analyzed using flow cytometry. In PC12 cell, the total apoptotic rate for PDK1 siRNA transfected cells was significantly increased compared to that of the control (Fig. 4). These results suggest that knockdown of PDK1 was able to promote cell apoptosis.

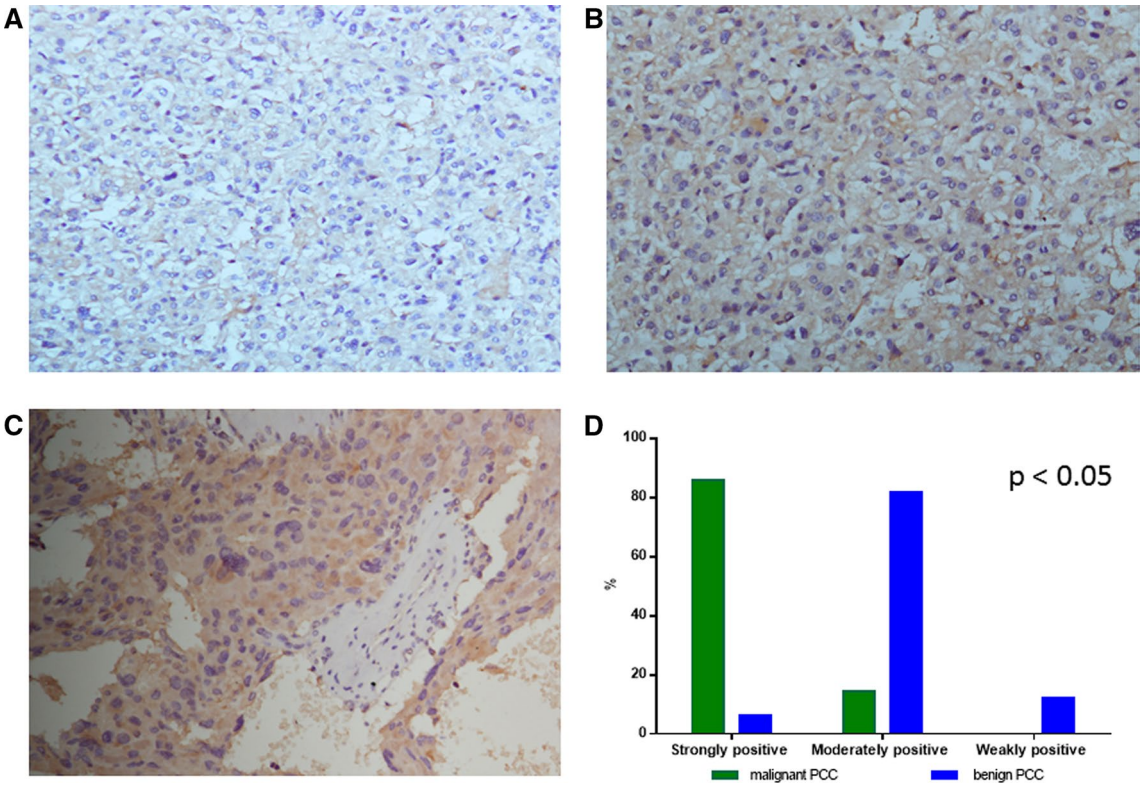

Fig. 1 The expression of PDK1 on human benign and malignant PCC tissues. PDK1 was localized to the cytoplasm, with positive signals observed as yellow or brown patches. **a** The expression of PDK1 was weakly positive in benign PCC. **b** The expression of PDK1 was moderately positive in benign PCC. **c** The expression of PDK1 was strongly positive in malignant PCC. **d** 12 of 14 (85.7%) malignant tissues showed strongly positive expression of PDK1. However, only 3 (6.1%) showed strongly positive expression of PDK1 in the 49 benign tissues, and most of them (81.6%) showed moderately positive expression

Knockdown of PDK1 attenuates cell migration and invasive capacity of PC12 cells

To evaluate the role of PDK1 in PC12 cell migration capacity, the effect of knockdown of PDK1 on cell migration was examined using a wound healing assay in a serum-free medium. As shown in Fig. 5a, cells transfected with PDK1 siRNA filled the gap more slowly than cells transfected with negative control siRNA did. The results suggest that knockdown of PDK1 was able to prevent cell migration. And then, Matrigel invasion assays were used to evaluate whether knockdown of PDK1 could attenuate invasive capacity in PC12 cell. As shown in Fig. 5b, after transfected with PDK1 siRNA, the numbers of invaded cells that had migrated through the pores and into the lower surface of the membrane were obviously reduced when compared with the control group, exhibiting a significant decrease in invasive ability as compared to the control. Taken together, these results suggest that knockdown of PDK1 was able to attenuate cell migration and invasive capacity of PC12 cells.

Discussion

Malignant pheochromocytoma (PCC) is a relatively rare neuroendocrine tumor that has a poor prognosis despite recent advances in chemotherapy and molecular-targeted therapeutics against malignant PCC [1–5]. Therefore, the development of a new therapeutic strategy for malignant PCC is urgently needed.

Phosphoinositide-dependent kinase 1 (PDK1) is now widely studied in malignant tumors because PDK1 can act as an important junction point for multiple cell signaling pathways. PDK1 is a type of serine/threonine kinase, which was first separated and identified by Alessi DR in 1997 [6–8]. It includes the kinase domain of n-terminal and platelet-white leucocyte C kinase substrate (PH domain). The PH domain of PDK1 can bind the product of PI3 K, three inositol phosphate (PI3P), allowing PDK1 to target the cell membrane and activate Akt [6–8]. The PDK1 pathway is always hyperactivated in human cancers. Therefore, PDK1 could be an ideal target in human cancer treatment. Although PDK1 has been reported to play an important role in regulating cell

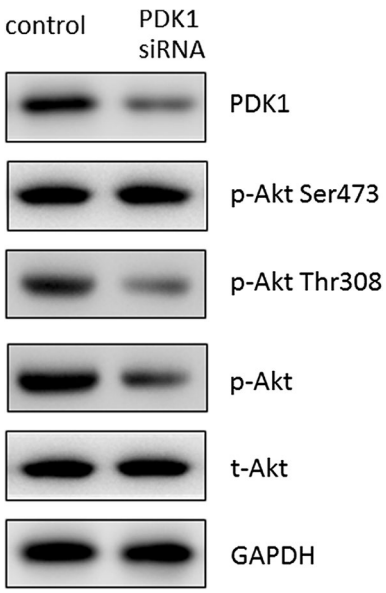

Fig. 2 Knockdown of PDK1 attenuates activation of the Akt signaling. PDK1 siRNA markedly decreased protein levels of PDK1. Knockdown of PDK1 significantly reduced the phosphorylation of Akt at threonine 308 (p-Akt T308) but did not alter the serine phosphorylation of Akt on the S473 site (p-Akt S473). The p-Akt expression was noticeably decreased after knockdown of PDK1, but the t-Akt expression did not show a significant decrease

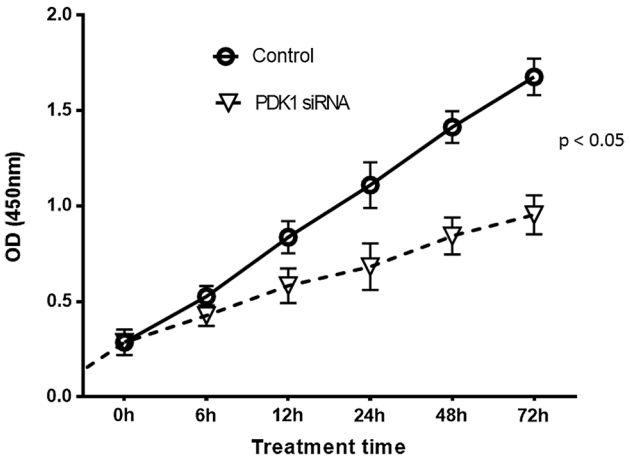

Fig. 3 Knockdown of PDK1 suppresses PC12 cell proliferation. At 12, 24, 48 and 72 h following transfection, cells transfected with PDK1 siRNA gradually lost viability, and the cell numbers were notably reduced at 72 h, while cells in the control group maintained healthy growth

proliferation and apoptosis in many other kinds of human normal cells and cancer cells [14–18], the role and molecular mechanism of PDK1 in controlling human malignant PCC remains unknown. First, to investigate the role of PDK1 in malignant PCC, PDK1 expression was detected by immunohistochemistry in 63 PCC tissue samples, of which 49 were benign and 14 were malignant. The results found that 12 of 14 (85.7%) malignant tissues showed strongly positive expression of PDK1. However, only 3 (6.1%) showed strongly positive expression of PDK1 in the 49 benign tissues, and most of them (81.6%) showed moderately positive expression. These results strongly suggest that PDK1 is overexpressed in malignant PCC tissues.

Since PDK1 levels is significantly higher expressed in malignant PCC tissues than that in benign PCC tissues, it is reasonable to think that PDK1 may play an important role in the pathogenesis and progression of malignant PCC. To test this hypothesis, we employed a downregulation strategy. In the current study, knockdown of PDK1 was carried out by transfected with PDK1 siRNA in pheochromocytoma PC12 cell. As shown in Fig. 2, the level of PDK1 was highly decreased in PDK1-transfected PC12 cells. We next evaluated the role of PDK1 on cell growth, apoptosis, migration and invasive capacity in PC12 cells. To analyze the role of PDK1 in PC12 cell viability, the effect of knockdown of PDK1 on cell proliferation was investigated by transfected with siRNA for 0–72 h. As shown in Fig. 3, cells transfected with PDK1 siRNA gradually lost viability, while cells in the control group maintained healthy growth. To analyze the role of PDK1 in PC12 cell survival, the effect of knockdown of PDK1 on cell apoptosis was investigated by flow cytometry. As shown in Fig. 4, the total apoptotic rate for PDK1 siRNA transfected cells was significantly increased compared to that of the control. To evaluate the role of PDK1 in PC12 cell migration capacity, the effect of knockdown of PDK1 on cell migration was examined using a wound healing assay. As shown in Fig. 5a, cells transfected with PDK1 siRNA filled the gap more slowly than cells transfected with negative control siRNA did. And then, Matrigel invasion assays were used to evaluate the role of PDK1 in PC12 cell invasive capacity. As shown in Fig. 5b, after transfected with PDK1 siRNA, the numbers of invaded cells that had migrated through the pores and into the lower surface of the membrane were obviously reduced when compared with the control group. Taken together, these data suggest that knockdown of PDK1 was able to suppress cell proliferation, promote cell apoptosis, and inhibit cell migration and invasion.

PDK1 activity leads to the phosphorylation of a number of targets including AKT, mTORC-1, P70-S6 K, and mTORC-2. Canonically, PDK1 directly phosphorylates Thr308 residues of Akt, but requires mTOR complex 2 (mTORC2) induced Akt phosphorylation on Ser473 to confer its full activation [6–8]. The fully activated Akt can phosphorylate a series of downstream substrates, such as Bad, Bax, FoxO3a, mTOR/p70S6K and Caspase, and further influence protein transcriptional translation, cell growth, cell migration and anti-apoptosis [6–8]. Because PDK1, as an upstream kinase of Akt, mediates and

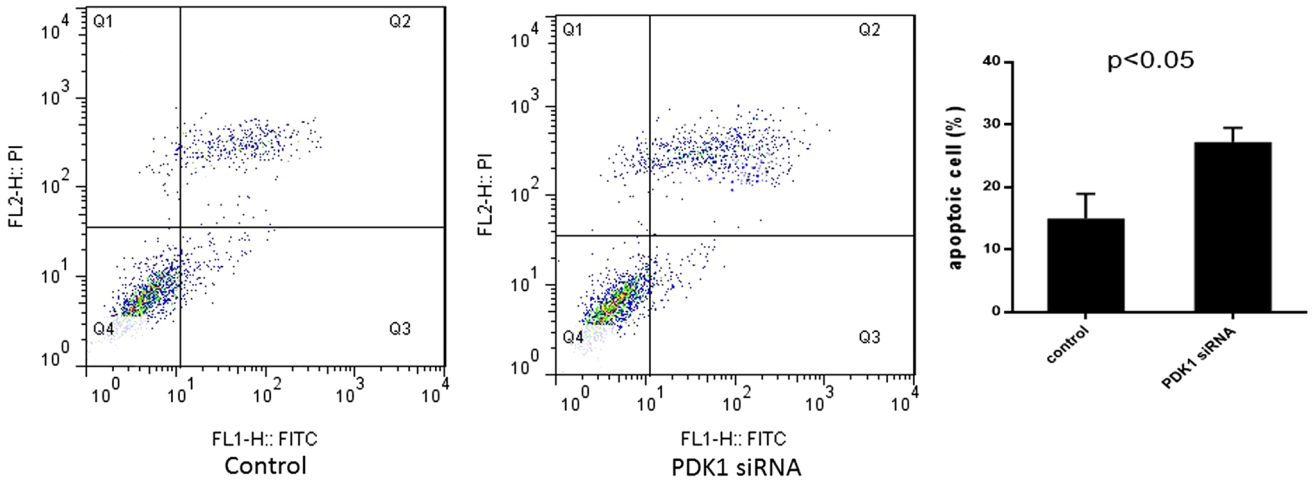

Fig. 4 Knockdown of PDK1 promotes PC12 cell apoptosis. PC12 cells transfected with negative control siRNA and PDK1 siRNA were subjected to flow cytometry for cell cycle analysis. The data used to create the bar chart was taken from triplicate experiments and was represented as the mean ± SD. $p < 0.05$ for comparison of PDK1 siRNA versus control

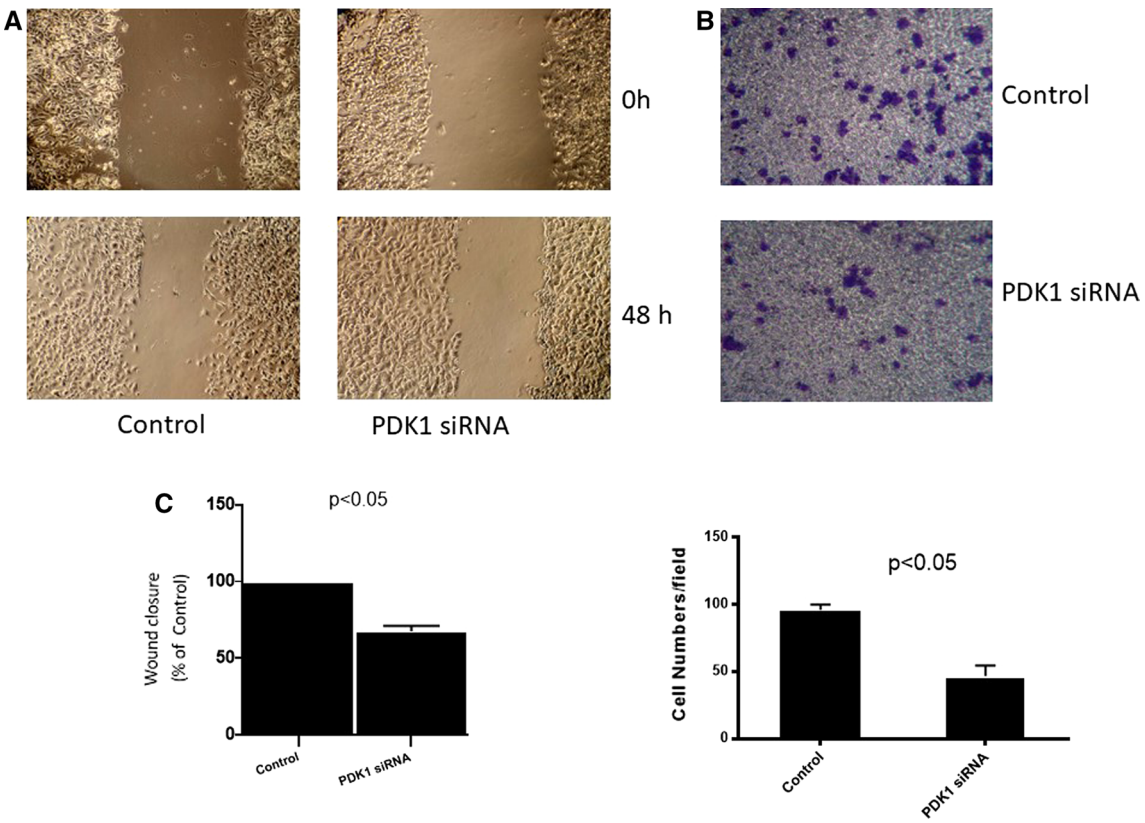

Fig. 5 Knockdown of PDK1 attenuates cell migration and invasive capacity of PC12 cell. **a** PC12 cells were transfected with negative control or PKD1 siRNA, and subsequently subjected to wound healing assay as described in "Materials and methods" section. **b** Transwell assay was performed on PC12 cells transfected with negative control or PDK1 siRNA. The numbers of cells that migrated to the bottom side of the membrane were calculated. Data are presented as mean ± SD of the migrated cells. $p < 0.05$ for comparison of PDK1 siRNA versus control. **c** Left panel: Measurement of wound closure based on initial and residual (48 h) gap lengths in wound healing assay (relative to the control group). Data were presented as mean ± SD; $p < 0.05$ for comparison of PDK1 siRNA group versus control. Right panel: The numbers of cells that migrated to the bottom side of the membrane were calculated. Data were presented as mean ± SD of the migrated cells. $p < 0.05$ for comparison of PDK1 siRNA group versus control

regulates the Akt signal pathway, it is reasonable to think that PDK1 may be a more effective treatment target than Akt. Thereby, targeting PDK1 may provide an opportunity for developing novel therapeutic target in human cancer treatment for malignant PCC. In this study, we further analyzed the effect of knockdown of PDK1 on intracellular molecules in the Akt signaling pathway. As shown in Fig. 2, western blot results showed that knockdown of PDK1 significantly reduced the phosphorylation of Akt at threonine 308 (p-Akt T308) but did not alter the serine phosphorylation of Akt on the S473 site (p-Akt S473). Also, we found that the p-Akt expression was noticeably decreased after knockdown of PDK1, but the t-Akt expression did not show a significant decrease. These results suggest that knockdown of PDK1 could attenuate activation of the Akt signaling, which indicated that PDK1 was potentially the molecular target in treatment for malignant PCC.

In summary, we have demonstrated for the first time that PDK1 is overexpressed in human malignant PCC and plays an important role in the malignant biological behaviors of PC12 cell. Specifically, we have revealed that knockdown of PDK1 could attenuate activation of the Akt signaling. These data suggest that PDK1 could be a new promising potential therapeutic target in human cancer treatment for malignant PCC.

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Authors' contributions ZY conceived and designed the experiments. XZ performed the experiments. ZY coordinated the research and analyzed the data. XZ wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval and consent to participate This study was in accordance with the ethical standards and was approved by The First Affiliated Hospital of Wenzhou Medical University.

Ethical statement All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Availability of data and materials The datasets generated and/or analysed during this study are available from the first author and corresponding author on reasonable request.

Consent for publication Not applicable.

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