Inhibition of PKCα reduces the ability of migration of kidney cancer cells but has no impact on cell apoptosis

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Abstract. Kidney cancer is among the most important causes of cancer-associated mortality worldwide. The present study aimed to evaluate protein kinase C α (PKCα) expression in kidney cancer tissues and cell lines, and its significance in apoptosis and migration. Expression of PKCα was analyzed using quantitative polymerase chain reaction and western blotting. In addition, the inhibitor of PKCα (calphostin C and GO6976) was used to treat kidney cancer cells. The ACHN cell line was generated with PKCα-small-interfering RNA (siRNA) and a stable expression of PKCα, in order to facilitate the analysis of apoptosis and migration of PKCα during knockdown and inactivation. Flow cytometry was used to determine the rates of apoptosis. Immunohistochemical staining was used to identify the localization of PKCα in renal clear cell carcinoma and normal sections. PKCα expression in normal tissues was found to be greater than in cancerous tissues. Furthermore, apoptosis was not promoted with PKCα inhibitors or PKCα-siRNA treatment, and a decrease of the migration ability was observed following transfection with PKCα-dominant negative. The results indicated that inhibition of PKCα might not contribute to apoptosis progression in kidney carcinoma.

Introduction

Kidney cancer is the one of the leading causes of cancer-related mortality worldwide. Renal clear cell carcinoma (RCC) accounts for ~85% of kidney cancer diagnoses, particularly in the United States (1,2).

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Abbreviations: PKC, protein kinase C; RCC, renal clear cell carcinoma; ADM, adriamycin; PARP, poly-ADP-ribose polymerase; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PI, propidium iodide

Key words: protein kinase Cα, kidney carcinoma, apoptosis, migration, inhibitor

Protein kinase C (PKC) represents a family of serine/threonine kinases that are classified into three major groups: Classical (α, βI, βII and γ), novel (δ, ε, η and θ) and non-classical (μ, ζ and ι). These kinases may be involved in growth, differentiation, apoptosis, tumor promotion and migration (3-5). As a result, all of these PKC isozymes have distinct functions and sometimes they even have opposing roles in cancer (6,7). For instance, in glioma cells, PKCα enhances cell proliferation, indicating that it may have a role in tumor promotion. However, overexpression of PKCδ inhibits cell proliferation and may be associated with cell apoptosis (8). Previous results have suggested that PKCα may be closely associated to cancer progression (5). Notably, a high level of PKCα expression was strongly associated with a high migratory activity of colon cancer cells, and a translocation of the activated PKCα at the plasma membrane was observed (9). By contrast, cell apoptosis was induced in LNCaP cells of prostate carcinoma upon activation of PKCα, indicating tumor suppressive properties (10). Previous research from our laboratory revealed that high expression and activation of PKCα is associated with tumor progression in superficial bladder carcinoma, and abnormal activation of PKCα may result in endogenic resistance to chemotherapy drugs, such as adriamycin (11). Furthermore, it has been suggested that the unusual translocation of PKCα between the plasma membrane and cytosol could be involved in the progression of kidney carcinoma (12). However, the mechanism of PKCα in kidney cancer and its efficiency thus far remained unclear. Whether PKCα functioned as a tumor promoter or suppressor in kidney cancer was also unclear. Therefore, the present study investigated the expression of PKCα in both clinical specimens and kidney cancer cell lines, and used gene silencing technology in order to identify the function of PKCα in kidney cancer.

Materials and methods

Cell culture. All cell lines were purchased from Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). In total, four different types of kidney cell lines selected: ACHN (Obtained from carcinomatous pleural effusion of kidney cancer), 786-O (renal adenocarcinoma), caki-1 (skin metastasis of kidney cancer) and HKC (immortalized renal tubular epithelial cells). ACHN and 786-O were cultured in RPMI-1640 (Lonza, Verviers Sprl, Verviers, Belgium) basal medium. Caki-1 was grown

in McCOY's 5A medium (Gibco; Thermo Fischer Scientific, Inc., Grand Island, NY, USA), and F12 basal medium (Gibco; Thermo Fischer Scientific, Inc.) was selected for the HKC cells. Cells were also supplemented with 10% fetal bovine serum (FBS; EuroClone SpA, West York, UK) with antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in a humidified 5% CO_2 incubator at 37˚C.

PKCα inhibitors and antibodies. The PKCα inhibitor GO6976 and calphostin C were obtained from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany) and dissolved in dimethylsulfoxide (Sigma-Aldrich; Merck Millipore). Antibodies against PKCα (sc-8393) and poly-ADP-ribose polymerase 1 (PARP-1; sc-25780) were purchased from Santa Cruz Biotechnology, Inc.). Anti-caspase 3 was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; WH0002597M1) was obtained from Sigma-Aldrich (Merck Millipore) and anti-caspase-9 (WL01551) was supplied by Wanleibio Co., Ltd. (Shenyang, China).

Small-interfering RNA (siRNA), plasmid and cell transfection. The sequences of PKCα siRNA were as follows: Forward, 5'-GUGCCAUGAAUUUGUUACUTT-3' and reverse, 5'-AGUAACAAAUUCAUGGCACTT-3'. Inactivation of PKCα in ACHN cells was achieved when ACHN cells stably expressed the PKCα-dominant negative (DN; ACHN-DN) PKCα. PKCα-siRNA and ACHN-DN plasmid (2.5 μg of plasmid DNA) transfections were both performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). Lipofectamine® 2000 was used for 12 μl in a 2-ml transfection system. After 24 h of transfection, the cells were replaced by RPMI-1640 basal medium for 24 h and were then processed in two parts. In the first part the cells were harvested following siRNA transfection and RPMI-1640 basal medium treatment for 24 h; In the second part the cells were continuously treated with G418 (600 μg/ml) for selecting stable clones for a period of 14 days. The medium was changed every 48 h and colonies of G418-resistant cells were selected.

Samples. In total, 20 patients (aged 31-79 years; 10 men and 10 women) who were diagnosed with primary kidney carcinoma were selected from the Department of Urology in the First Hospital of China Medical University (Shenyang, China) between December 2011 and July 2013. Seven cases had left RCC and thirteen cases had right RCC. The diagnosis was confirmed by pathological examination, and the histological subtype was identified as RCC. In addition, none of the patients accepted chemotherapy or radiation therapy treatments. Samples of the normal control kidney tissue were collected from each patient with a distance of >3 cm from the tumor. A part of tumor tissues and the corresponding normal tissues were quickly frozen in -80˚C for protein extraction and the remainder was perfused with phosphate-buffered saline (PBS) and fixed with 4% formalin overnight for paraffin embedment and immunohistochemical staining.

Quantitative polymerase chain reaction (qPCR) analysis. RNAs were extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and were then quantified using a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Reagents were purchased from Takara Bio, Inc. (Otsu, Japan) and were used to detect the expression of PKCα. The reaction was 20 μl in total and a LightCycler 480 qPCR system (Roche Diagnostics, Basel, Switzerland) was used for the reaction. The conditions were the following: 50˚C for 2 min, 95˚C for 5 min, 45 cycles of 95˚C for 40 sec and 55˚C for 30 sec. Data were analyzed using the $2^{-\Delta\Delta ct}$ method and mRNA expression was normalized against β-actin RNA. The primer sequences for β-actin were as follows: Sense, 5'-CTCCATCCTGGCCTCGCTGT-3' and anti-sense, 5'-GCTGTCACCTTCACCGTTCC-3'. In addition, the PKCα mRNA expression was detected using the following primers: Sense, 5'-GGAACCACAAGCAGTATT-3' and anti-sense, 5'-GTCCTTCTGAATCCAACAT-3'. The PKCβ sequences were: Sense, 5'-AAATGCTCCCTCAACCCT-3' and anti-sense, 5'-TCAAATCCCAATCCCAAA-3' and the PKCγ sequences were: sense, 5'-GCGGCTGGAACGATTGGA-3' and anti-sense, 5'-TGGCGGCGGGTGAGATTAC-3'.

Western blotting. The purchased cells were lysed in the culture flask using RIPA buffer containing the protease inhibitor phenylmethanesulfonyl fluoride, then centrifuged at 13,200 x *g* for 30 min. Total cellular proteins were extracted from kidney tissues using a protein extraction buffer RIPA containing protease inhibitors. Equal quantities of protein (50 μg cells and 100 μg tissues) extracts were subjected to 10 % SDS-PAGE electrophoresis at 220 V and the resolved proteins were transferred onto polyvinylidene fluoride membranes. The membranes were then blocked for 60 min in a 37˚C temperature-controlled shaking table, and subsequently incubated with the following primary antibodies: Polyclonal mouse anti-PKCα (1:1,000), polyclonal rabbit anti-PARP-1 (1:1,000), polyclonal rabbit anti-caspase-3 (1:1,000), polyclonal rabbit anti-caspase-9 (1:1,000) and anti-GAPDH (1:2,000) overnight at 4˚C. The following day, Tris-buffered saline and Tween-20 was used to remove unbound antibodies and then incorporated with the secondary antibody diluted at 1:2,000 for 60 min in a 37˚C temperature-controlled shaking table. The bands were then visualized by chemiluminescence using the EC3 Imaging System (UVP LLC, Cambridge, UK).

Immunohistochemical staining. Fresh kidney tissue samples obtained from the patients (~1.5x1.5x0.2 cm) were perfused with PBS and fixed with 4% formalin overnight. The next day, tissues were embedded in paraffin and then 4-μm sections were placed on glass slides. Afterwards, antigen retrieval was performed for 2.5 min. Next, an immunohistochemical kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) was used, then the slides were incubated with primary antibody (monoclonal mouse anti-PKCα; 1:100; sc-8393; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. Subsequently, 3,3'-diaminobenzidine staining was applied for 3 min then rinsed out immediately. In addition, hematoxylin staining solution was used for nuclear counterstaining, and the dyes were rinsed well under running water for >3 h. Finally, the slides were mounted and analyzed using an upright metallurgical microscope (IX71S8F-3; Olympus Corporation, Tokyo, Japan).

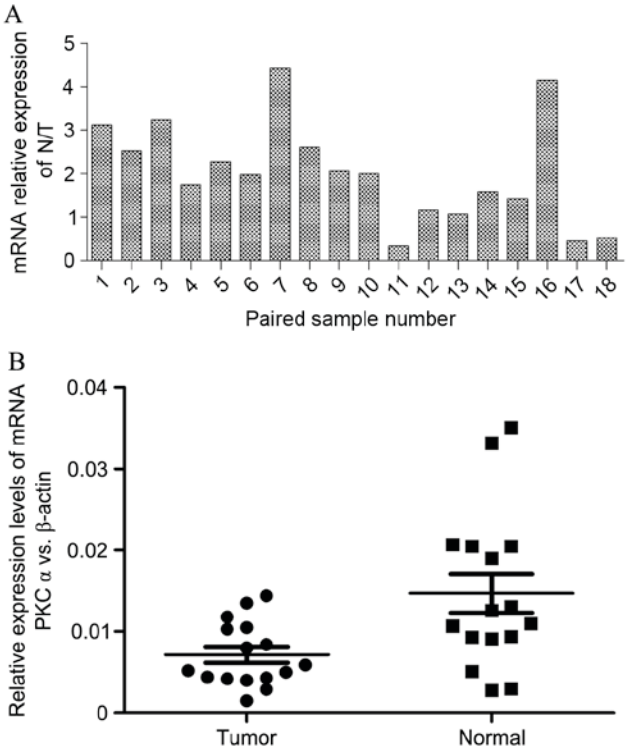

Figure 1. (A) Fifteen cases displayed downregulated expression of protein kinase α in renal clear cell carcinoma tissues, and number 11, 17 and 18 were considered to be outliers (P=0.001). (B) PKCα had a lower expression in renal cell carcinoma tissues after abandoning pairs of N/T but no statistical significance was observed (P=0.07). PKCα, protein kinase C α; N/T, normal/tumor tissues.

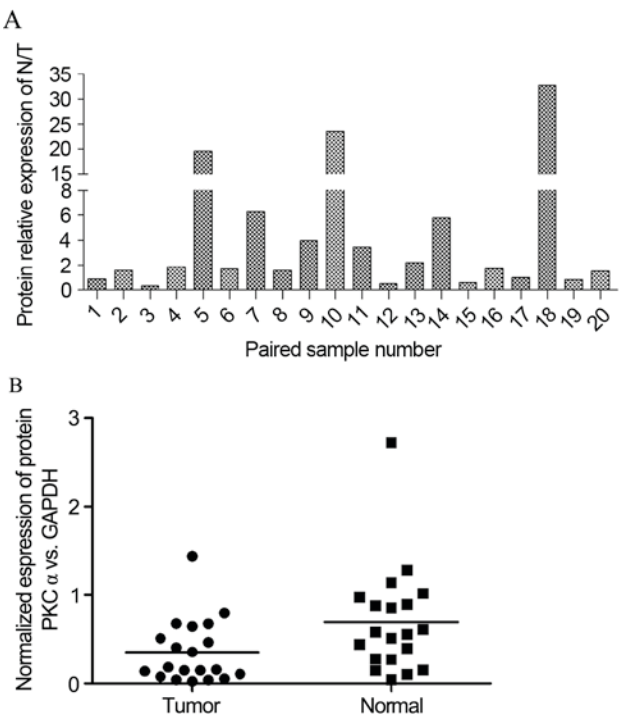

Figure 2. (A) Western blotting was used to measure the PKCα protein in 20 pairs of kidney tumor tissues and their matched normal tissues. Normal tissues had a higher expression than the kidney tumor tissues (P=0.006). (B) Expression of PKCα in kidney N/T was assessed by western blotting (unmatched pairs). PKCα exhibited a higher expression in normal tissues compared with the kidney tumor tissues (P=0.016). PKCα, protein kinase Cα; N/T, normal/tumor tissues.

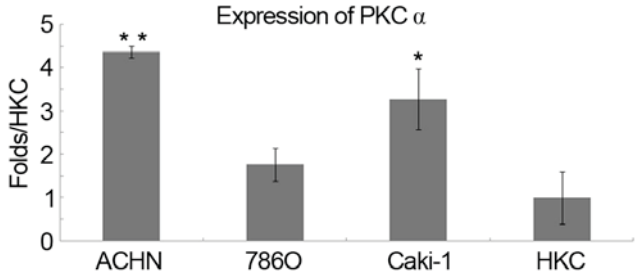

Figure 3. PKCα mRNA expression in four kidney cancer cell lines (ACHN, 786-O, Caki-1 and HKC) was assayed using quantitative polymerase chain reaction. β-actin was used as an internal control. PKCα expression was significantly greater in ACHN and Caki-1 cells than in the non-malignant cells, HKC. HKC expression was used as the basis for comparison against the other cell lines. ACHN vs. HKC, **P=0.004; Caki-1 vs. HKC, *P=0.033; 786-O vs. HKC, P=0.116. PKCα, protein kinase C α.

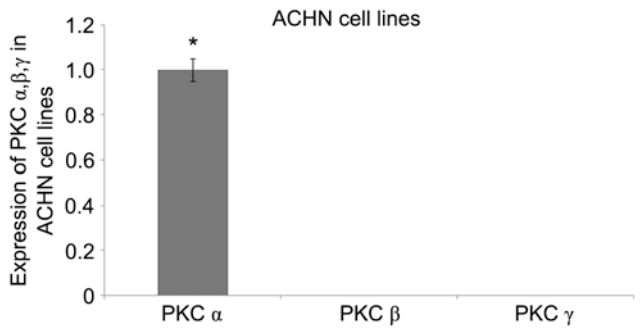

Figure 4. Expression of PKCα in ACHN cells was much higher than the other two PKC classical types, PKCβ and PKCγ (*P<0.01). PKC, protein kinase C.

Flow cytometry analysis. After 24 h with the PKCα inhibitors, the cells were washed with PBS and detached with 0.25% trypsin. Subsequently, the cells were centrifuged at 1,200 x *g* for 5 min and the supernatant solutions discarded. Next, cells were washed twice with PBS, and the mixture was centrifuged at 1,200 x *g* for 5 min. Afterwards, the cell mass was resuspended in 400 μl PBS and detected with a Annexin V-fluorescein isothiocyanate/propidium iodide kit (Beyotime Institute of Biotechnology, Haimen, China) using a flow cytometer (FACSCalibur Flow Cytometer; BD Pharmingen, San Diego, CA, USA) and following the manufacturer's instructions. Q2 represented late stage apoptotic and Q4 represented early apoptotic cells. In addition, Q2+Q4 was used to detect apoptosis.

Scratch wound healing assay. The migration capacities of ACHN cells and the PKCα-DN-expressing cell line ACHN-DN were investigated. The two types of cells were plated at a density of $1x10^5$ cells/well in 24-well plates. Cells were then incubated in RPMI-1640 medium containing 10% FBS for 24 h to 80% confluence, then a scratch was performed using a 200 μl pipette tip, and the cells were grown for 24 h in serum-free medium. The scratch spaces were then analyzed using an inverted microscope.

Statistical analysis. Statistical analysis was performed using SPSS for windows 13.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA). In addition, the independent and the

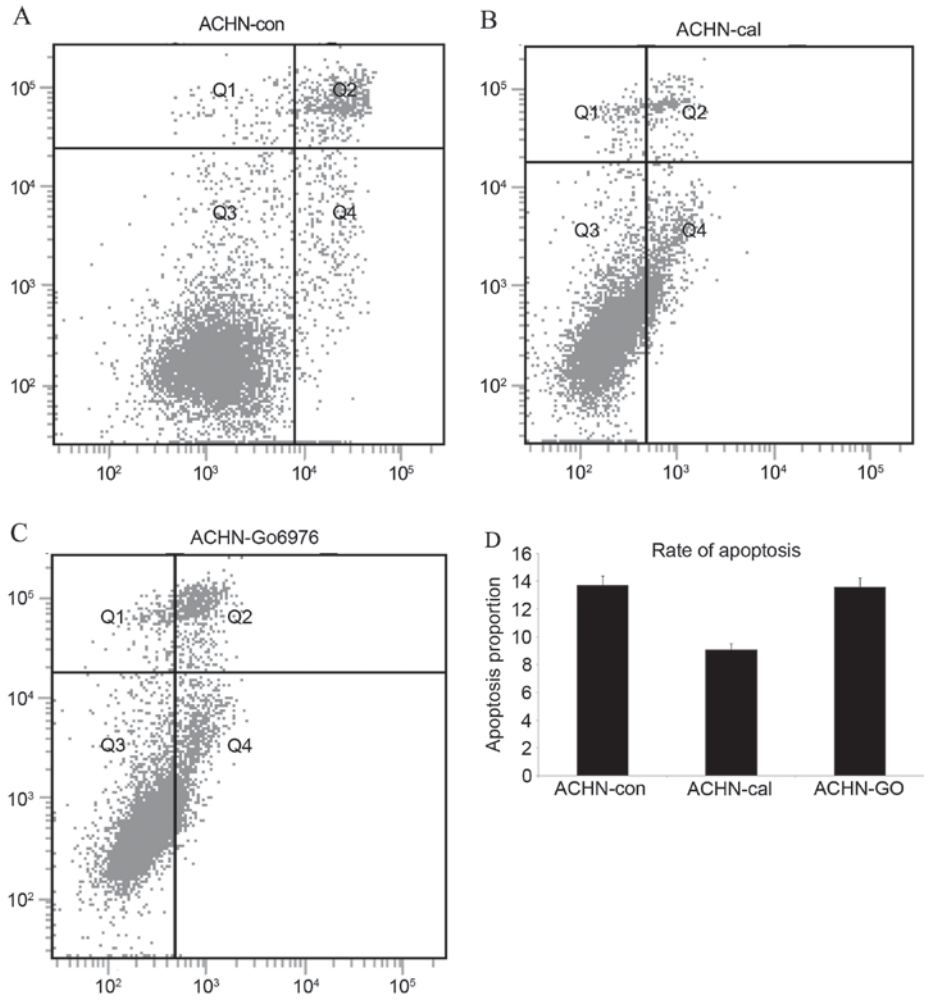

Figure 5. Inhibition of PKCα did not promote the apoptosis of kidney cancer cells. (A-D) The effect of the two inhibitors on the apoptosis of human kidney cancer cells was investigated by flow cytometry. Apoptosis rates were determined in ACHN cells lines following treatment with calphostin C and GO6976. In this chart, 'cal' represented calphostin C, vs. 'con', which represented the control. ACHN-con, vs. ACHN-Cal: P=0.24. ACHN-con, vs. ACHN-GO6976: P=0.98. The apoptosis rate was not different after the two drugs were added. PKCα, protein kinase C α.

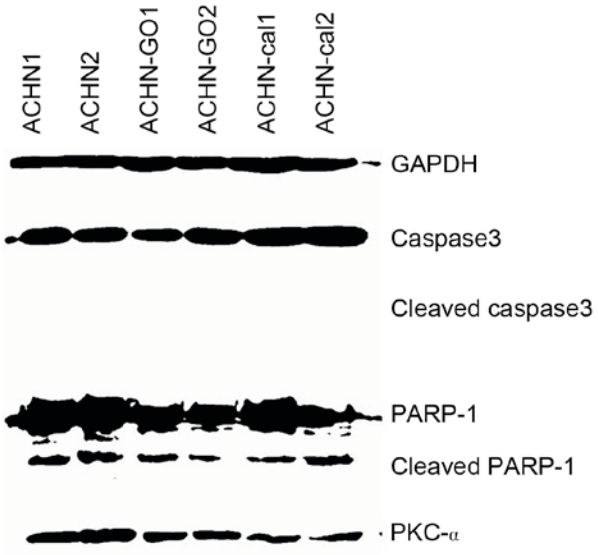

Figure 6. Apoptosis-regulated proteins had no apparent changes after the addition of the two inhibitors of PKCα-calphostin C and GO6976. In the chart, 'GO' represented GO6976; 'cal' represented calphostin C. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PARP-1, poly-ADP-ribose polymerase 1; PKCα, protein kinase C α.

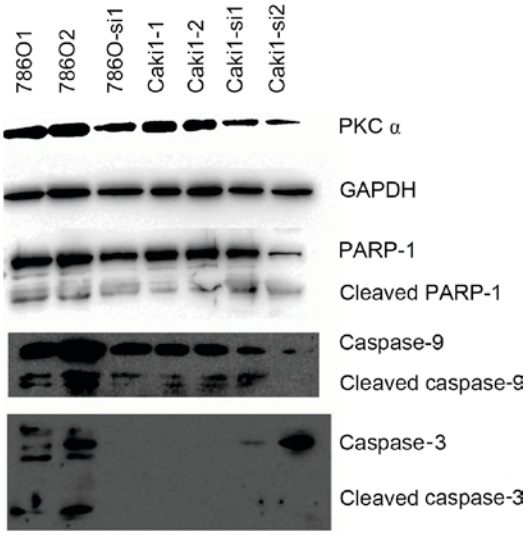

Figure 7. 786O-si and Caki1-si both represented the PKCα knockdown cells. Cleaved proteins of caspase-3, -9 and PARP-1 were all absent from the transfected cells, which suggested that PKCα has no impact on suppressing apoptosis in kidney carcinoma. PKCα, protein kinase C α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PARP-1, poly-ADP-ribose polymerase 1.

paired t-test were used. P<0.05 was used to indicate a statistically significant difference.

Results

PKCα expression was frequently decreased in human kidney cancer tissues. In total, 18 pairs of cancer tissue and their corresponding normal samples were selected. The results of Fig. 1A indicated that PKCα was significantly downregulated in the majority of kidney carcinoma (15 out of 16) using qPCR, where numbers 17 and 18 were considered to be outliers based on statistical algorithms. Afterwards, the pairs were disrupted to compare normal tissues and tumor tissues, and a high expression rate of PKCα in kidney normal tissues was identified (Fig. 1B). In addition, the results of western blotting revealed that the levels of PKCα were evidently decreased in ~80% kidney tumors (16 in 20) (Fig. 2A). However, kidney cancer tissues still show a lower expression than before, after the pairs were separated (Fig. 2B).

qPCR results demonstrated that the expression of PKCα was upregulated in metastatic cancer cell lines. The expression of PKCα was examined by qPCR in renal tubular epithelial HKC cells, in two types of metastatic cancer cell lines and in the human RCC cell line, 786-O. The results indicated that PKCα was significantly upregulated in ACHN (P=0.004) and Caki-1 (P=0.033) cells compared with the HKC cells (Fig. 3). However, proteins associated with metastasis require further studies in the two cell lines.

Drugs, siRNA and plasmids were used to decrease PKCα, however, there was no increase in apoptosis. Initially, the expression pattern of the PKC classical types family (PKCα, β and γ) was analyzed in ACHN cells. qPCR analysis revealed that PKCα expression was prominent in the ACHN cell line, whereas the expression levels of PKCβ and PKCγ was too faint to be detected (Fig. 4), indicating that PKCα may mediate specific and vital functions in kidney cancer cells. Calphostin C and GO6976 were inhibitors of the PKC classical types. Since PKCα occupied the main status, the effect of the other two PKC types was considered to be minimal. In addition, the two drugs simply inhibited PKCα. The effects of calphostin C (100 nM) and GO6976 (500 nM) were investigated by flow cytometry and western blotting, and the results demonstrated that PKCα expression was reduced. Furthermore, three types of apoptosis-regulated proteins were also detected (caspase-3, -9 and PARP-1). PKC inhibitors led to a no significant increase in the apoptosis rates of ACHN cells (Fig. 5), and there were no increasing cleaved bands of the apoptosis-regulated proteins (Fig. 6). Based on the lack of increase of apoptosis-regulated protein expression following treatment with the PKC inhibitors in kidney cancer cells, it was speculated that PKCα may be not be an inhibitor of apoptosis. In order to verify this assumption, Lipofectamine 2000 was used to transfect siRNA in order to knockdown PKCα in ACHN and Caki-1 cells. In addition, it was used to get the kinase inactivation plasmid (ACHN-DN) to inactivate PKCα. A successful knockdown of PKCα was confirmed by western blotting. In addition, no significant effects were identified on basal apoptosis-regulated protein expression (Fig. 7). ACHN-DN inactivated PKCα but increased its expression. Meanwhile, the condition of apoptosis-regulated proteins remained the same (Fig. 8). These results suggested that PKCα have not yet been found to have a major impact on suppressing apoptosis in kidney carcinoma.

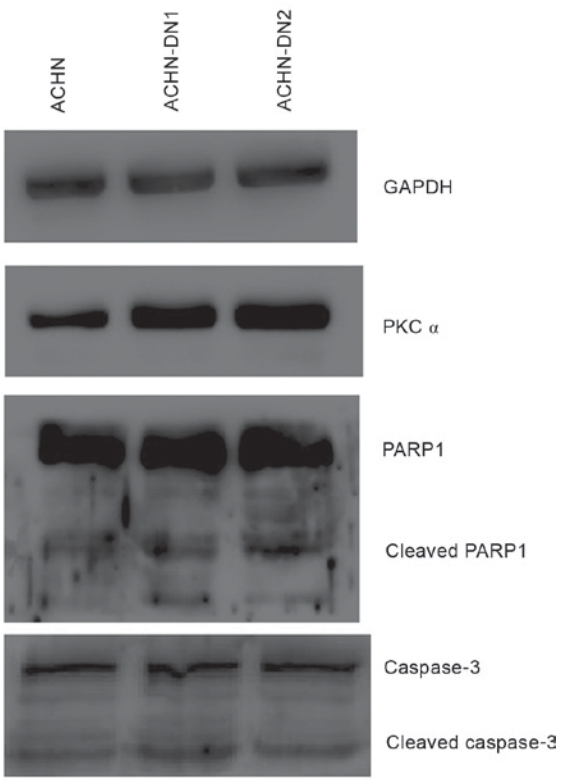

Figure 8. Following inactivation of PKCα kinase in the ACHN cell lines, there was no apoptosis-regulated protein increase. This result proved that when PKCα lost its activity, the viability of kidney cancer cells did not change. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PARP-1, poly-ADP-ribose polymerase 1; PKCα, protein kinase C α.

PKCα protein stained strongly in cytoplasm of normal kidney tubular epithelial cells but not in glomeruli. On account of its higher and more prevalent expression in the kidney, PKCα expression was also investigated by immunohistochemical analysis of RCC and normal sections. It was demonstrated that PKCα protein was expressed in the normal kidney proximal tubular epithelial and distal convoluted tubule cells, and it stained the cytoplasm strongly, whilst being absent or negligible in the glomeruli. In addition, PKCα staining in RCC tissue sections was shown to have a very weak brown staining throughout the cytoplasm and sometimes even negatively stained. Finally, almost all 24 pairs of tissues exhibited similar staining characteristics, where strong staining of PKCα protein was indicated in the cytoplasm of normal kidney tissue and weak staining was observed in RCC tissues (Fig. 9).

Decrease of migration ability following transfection with PKCα-DN. In order to analyze a possible effect of PKCα on kidney cancer cell migration, wound-healing assay was applied. It was revealed that transfection with PKCα-DN significantly inhibited the migration of ACHN cells (Fig. 10).

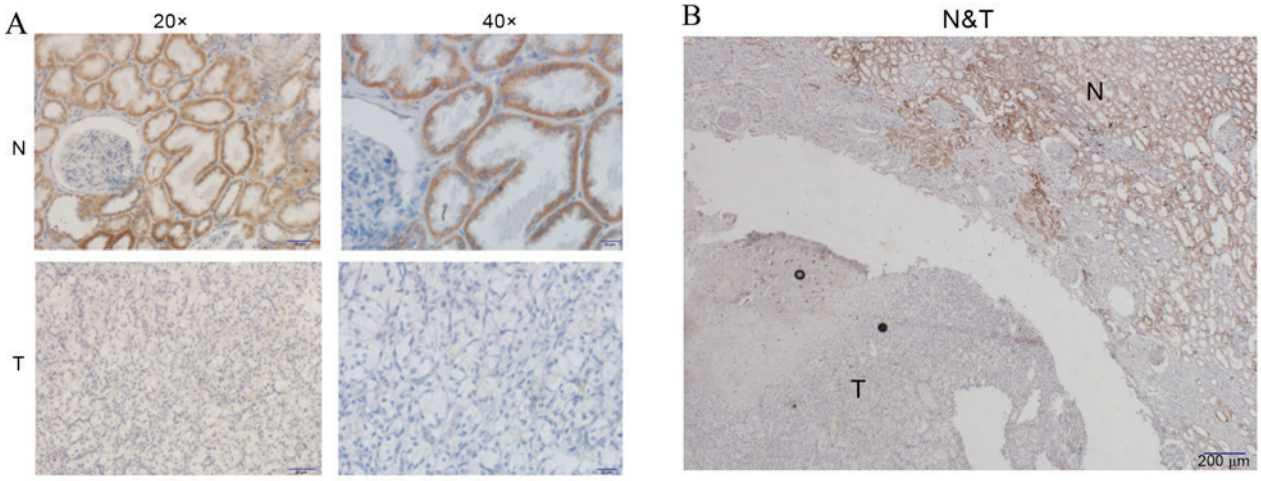

Figure 9. (A) Representative images from immunohistochemical staining showed that protein kinase α was localized in the cytoplasm in normal kidney proximal tubular and distal convoluted tubule cells, with strong brown staining. (B) In addition, renal clear cell carcinoma tissues were almost negatively stained. N, normal tissue; T, tumor tissue.

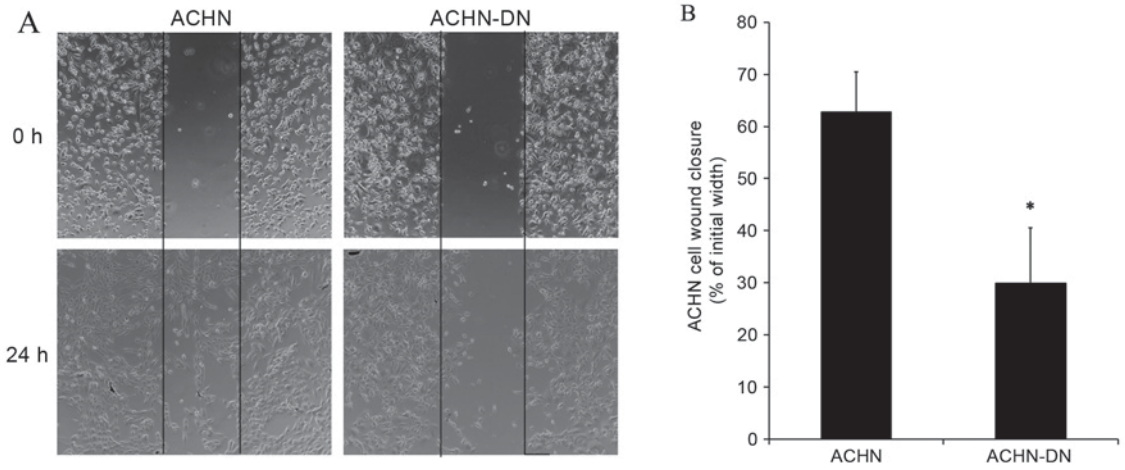

Figure 10. (A) Scratches width was compared in order to verify the capability of migration of ACHN and ACHN-DN cells. (B) The results showed that after inhibiting the kinase activity of protein kinase C α, the cells' healing rate decreased (*P=0.003).

In addition, an altered morphology of ACHN cells was noted following transfection. ACHN cells acquired a more angular shape compared to the untransfected control cells, and the healing rate was subject to statistical analysis.

Discussion

The present study revealed that the PKC family had different biological effects in different types of tumors. The upregulation or downregulation of PKC and their isoenzyme or their biological effect had previously been studied in human tumor samples (13-17). Downregulation of PKC isozymes was associated with the occurrence and progression of several human cancer types. Previous studies had verified that three types of PKC isozymes, α, β and δ, were predominant in the normal bladder epithelium. Furthermore, PKCβ and δ were downregulated in transitional cell carcinomas; however, PKCα increased (18,19).

In the present study, two inhibitors (calphostin C and GO6976) were used to decrease PKCα. As earlier work confirmed, Ca^{2+} spiking can be prevented by the PKC inhibitor calphostin C (20) due to conventional PKCs (α, βI, βII and γ) being activated by Ca^{2+}. Therefore, calphostin C mainly inhibited PKCα, βI, βII and γ. In addition, GO6976 had previously been demonstrated to inhibit PKC isoenzymes α and βI (21-23). In Fig. 4, it was indicated that PKCβ and γ had low expression in the kidney cancer cell line, therefore GO6976 and calphostin C mainly inhibited PKCα. Thus, apoptosis-related proteins were selected to evaluate the impact on apoptosis following the addition of the two inhibitors. It was revealed that the expression of apoptosis-related proteins, caspase-3, -9 and PARP-1, had not changed significantly, and no hydrolysis bands had evidently increased. Although the focus was on siRNA and the PKCα inactive plasmid, there was no increase observed in cell apoptosis. Therefore, PKCα was not considered to have a major impact on suppressing apoptosis in kidney carcinoma.

Based on the results on the detection of tumor tissues, PKCα was found to have a high expression in normal kidney

but decreased in tumor tissues. In other words, PKCα may decrease when normal kidney tissue becomes cancerous. Notably, when we compared one patient's tumor tissues with normal ones, PKCα presented a lower expression in tumors. However, when we disrupted the pairs the result demonstrated no statistical significance. These results indicated that the expression of PKCα varied from person to person; therefore, the most accurate method was to compare tumor tissue pairs with the normal control ones.

Fig. 3 indicated that in two kidney cancer transferred cell lines, ACHN and Caki-1, PKCα was markedly expressed. However, more research is required in order to understand the mechanism and metastasis-related proteins in the two cell lines, and whether they are correlated with PKCα. Previous research affirmed that in retinal pigment epithelium cells, the wound healed more slowly in the siRNA-PKCα compared to the non-siRNA group at the three time points (24). This result suggests that PKCα may be important in cell migration. In the results of the present study, following inactivation of PKCα, cell migration was inhibited which suggested that PKCα may also be important in the ability to move in kidney cancer cell lines.

Immunohistochemical staining revealed that the expression of PKCα protein was detected in the normal kidney proximal tubular epithelial and distal convoluted tubule cells, which were predominantly located in the cytoplasm, and exhibited very strong staining. Meanwhile, in tumor sections, PKCα stained very weakly or not at all. This observation was consistent with the results of western blotting. In conclusion, it was shown that the inhibition of PKCα may not be due to a significant gain in expression of apoptotic proteins, and that PKCα does not function by suppressing apoptosis in kidney cancer cells. In addition, PKCα was decreased in tumor tissues of the kidney. Therefore, down-regulation of PKCα may be an early event in the development of kidney carcinoma, but the mechanisms by which PKCα functioned remained elusive. In conclusion, the results of the present study demonstrated that inhibition of PKCα may not contribute to apoptosis progression in kidney carcinoma.

Acknowledgements

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