Atypical Protein Kinase \( \alpha \) (PKC\( \alpha \)) Promotes Metastasis of Esophageal Squamous Cell Carcinoma by Enhancing Resistance to Anoikis via PKC\( \alpha \)-SKP2-AKT Pathway

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Abstract

Protein kinase \( \alpha \) (PKC\( \alpha \)) is an atypical PKC isoform and participates in multiple aspects of the transformed phenotype in human cancer cells. We previously reported that frequent amplification and overexpression of PKC\( \alpha \) were correlated with lymph node metastasis in primary esophageal squamous cell carcinomas (ESCC). In the present study, short interfering RNA–mediated silencing of PKC\( \alpha \) revealed that this enzyme was required for cell migration, invasion, and resistance to anoikis. In vivo experiments showed that PKC\( \alpha \) suppression decreased tumor growth in esophageal cancer xenografts and lung metastases in nude mice. At the molecular level, knockdown of PKC\( \alpha \) in suspended ESCC cells caused a decrease in S-phase kinase-associated protein 2 (SKP2) that had been reported to promote resistance to anoikis via the PI3K/AKT pathway. AKT phosphorylation was abolished after PKC\( \alpha \) suppression, but AKT activation could be refreshed by PKC\( \alpha \) upregulation, suggesting that PKC\( \alpha \) enhanced cell resistance to anoikis via the PKC\( \alpha \)-SKP2-PI3K/AKT pathway. Addition of the proteasome inhibitor MG132 prevented the decrease of SKP2 in PKC\( \alpha \) silenced cells, and polyubiquitin-SKP2 was elevated after PKC\( \alpha \) depletion, showing that PKC\( \alpha \) might regulate the expression of SKP2 through the ubiquitin-proteasome pathway in suspended cells. Furthermore, overexpression of SKP2 in PKC\( \alpha \)-downregulated cells restored cell resistance to anoikis. Most importantly, PKC\( \alpha \) expression significantly correlated with SKP2 in 133 ESCC tissues \((P = 0.031)\). Taken together, our data show that PKC\( \alpha \) promotes tumorigenicity and metastasis of human esophageal cancer and that SKP2 is a candidate downstream effector of PKC\( \alpha \) signaling in ESCC. Mol Cancer Res; 9(4); 390–402.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common human malignancies. The prognosis of ESCC patients has not significantly improved, with an average 5-year survival rate of 10% to 20% (1). Therefore, exploring the mechanisms involved in the development and progression of this disease is the key to effective treatment.

The protein kinase C (PKC) family consists of at least 12 distinct lipid-regulated protein-serine/threonine kinases that are involved in multiple intracellular pathways and play pivotal roles in regulating cell proliferation, differentiation, and survival (2). The PKC isoenzymes are subdivided into 3 main classes: the cPKCs (conventional PKCs; PKC\( \alpha \), \( \beta I \), \( \beta II \), and \( \gamma \)), the nPKCs (novel PKCs; PKC\( \beta II \), \( \epsilon \), \( \eta \), and \( \theta \)) and the aPKCs (atypical PKCs; PKC\( \xi \) and \( \lambda \)). Atypical protein kinase C type 1 (PKC\( \alpha \)) has been implicated in the establishment of cell polarity and cell survival (3). PKC\( \alpha \) activity can be regulated by PI3K through PDK1 phosphorylation (4) and protein–protein interactions with upstream effectors, such as Ras (5). PKC\( \alpha \) is reported to be amplified and overexpressed in several human cancers, and its overexpression is correlated with poor prognosis in lung, ovarian, and pancreatic cancer (6–10). We recently demonstrated that PKC\( \alpha \) was frequently amplified and overexpressed in primary ESCC tumors. Moreover, the amplification and overexpression of PKC\( \alpha \) were correlated with lymph node metastasis, which indicates that PKC\( \alpha \) might play a role in ESCC progression (9). However, the molecular mechanisms underlying the role of PKC\( \alpha \) in ESCC remain unclear.

S-phase kinase-associated protein 2 (SKP2) acts as a substrate-specific factor for the Skp1-Cul1-F-box (SCF) complex that regulates the destruction of multiple important proteins, including p27, p21, Rb, and p53 (11–13). Elevated SKP2 expression is frequently observed in many tumors, such as breast and prostate carcinomas (14, 15).
Our recent investigation revealed that SKP2 was amplified and overexpressed in primary ESCC tumors (16). Current studies have shown that Cdh1 is a critical E3 ligase that targets SKP2 for ubiquitylation and degradation (17, 18). However, the molecular mechanisms underlying elevated SKP2 expression have not been fully explored.

In the present study, we found that PKCι promoted ESCC cell migration, invasion, resistance to anoikis, growth in soft agar, and tumor growth and lung metastasis in vivo. Mechanistically, we showed that PKCι promoted resistance to anoikis through the SKP2-PI3K/AKT pathway and that PKCι regulated the expression of anoikis-related SKP2 via the ubiquitin-proteasome pathway.

Materials and Methods

Tissue specimens

Fresh tissues containing ESCCs and adjacent histologically normal epithelia were procured from surgical resection specimens collected by the Department of Pathology in the Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. Primary tumor regions and histologically normal esophageal mucosa from the same patients were separated by experienced pathologists and immediately stored at −70°C until use. No treatment was received by any patients before surgery, and all patients signed separate informed consent forms for sample collection.

Plasmid construction and small interfering RNA synthesis

The full-length coding regions of human PKCι and SKP2 were amplified from total cDNA of normal esophageal tissues using the following primers: PKCι-forward: 5'-CGCGGATCC CGCCACCATGCCGACCCAGAGG-3' and reverse: 5'-TTCTGCAGACATCAAAAGAG-3'; and SKP2-forward: 5'-GGGATCC CGCCAGACCCAGAGG-3' and reverse: 5'-CTTTCTGGGTGTTCTGGATTCTC-3'. The resultant PCR products were cloned into the pcDNA 3.1/myc-His A plasmid (Invitrogen). Correct construction was confirmed by DNA sequencing. Duplex PKCι short interfering RNA (siRNA)-1 targeting 5'-AAGCTTGGATAATACCTTAAACA-3' (2095–2113 in NM_002740 of PKCι mRNA; ref. 19), siRNA-2 targeting 5'-TTCTTGGACATCAAAAGAG-3' (1286–1304 in NM_002740 of PKCι mRNA; ref. 20) and a nonsilencing siRNA (5'-TTCTTCGAACGTGTCACGT-3') were chemically synthesized (GeneChem) for transient transfection. A PKCι siRNA-1, 80 nmol/L PKCι siRNA-2 or 40 nmol/L nonsilencing siRNA were used, and cells were harvested 48 hours after transfection. For stable transfections, cells were selected using G418 (200 μg/mL), and clones were isolated by serial dilution. Two PKCι RNAi clones (P4 and P8) and 2 scramble sequence RNAi clones (S1 and S2) were chosen for the subsequent experiments.

RNA extraction and reverse transcription PCR

Total RNA was isolated using Trizol reagent (Life Technologies) according to the manufacturer’s instructions. Five micrograms of total RNA were used to synthesize the first strand of cDNA using 200 units/μL SuperScript II RT (Invitrogen). The primer sequences used for PKCι PCR were forward: 5'-GATGCGTGGTGTGATTGAGG-3' and reverse: 5'-TCACTGTGAGGTGTTGAGGC-3'; and the primer sequences used for SKP2 were forward: 5'-CTTTCTGGGTGTTCTGGATTCTC-3' and reverse: 5'-GATTAGGTTGTGAGGTAGTCG-3'. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a loading control. The PCR program was as follows: denaturation at 95°C for 4 minutes; 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 40 seconds; followed by a 72°C elongation step for 8 minutes; 10 μL of each PCR product were resolved by 2% (w/v) agarose gel electrophoresis.

Western blot analysis

Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), blocked and probed with antibodies against PKCι (1:2,000; BD PharMingen), β-actin (1:5,000; Sigma), AKT (1:1,000; Santa Cruz Biotechnology), p-AKT (Ser473; 1:1,000; Cell signaling technology), AKT1 (1:1,000; Cell signaling technology), Erk1 (1:1,000; Santa Cruz Biotechnology), p-Erk (Tyr204; 1:400; Santa Cruz Biotechnology), SKP2 (1:1,000; Santa Cruz Biotechnology), and ubiquitin (1:500; Santa Cruz Biotechnology). After washing, blots were incubated with horseradish peroxidase–conjugated secondary antibodies and visualized by super ECL detection reagent (Applygen).

Haptotactic migration assays and Matrigel chemoinvasion assays

For the haptotactic cell migration assay, 1 × 10⁵ PKCι siRNA cells, nonsilencing siRNA cells, and parental EC9706 cells were seeded on a fibronectin-coated polycarbonate membrane insert in a Transwell apparatus (Costar). RPMI 1640 containing 20% FBS was added to the lower chamber. After incubation for 20 hours at 37°C in a CO₂ incubator, the insert was washed with PBS, and cells on the top surface of the insert were removed by wiping with a cotton swab. For the Matrigel chemoinvasion assay, the procedure was similar to the haptotactic cell migration assay, except that the Transwell membrane was coated with 300 ng/μL Matrigel (BD Biosciences), and the cells were incubated for 36 hours at 37°C. Cells that migrated to the
bottom surface of the insert were fixed with methanol, stained with 0.4% crystal violet and subjected to microscopic inspection. Cells were counted based on random 5 field digital images at \( \times 200 \).

**Adhesion assay**

PKC\(_i\) siRNA cells, nonsilencing siRNA cells, and parental EC9706 cells were plated on 100 ng/mL Matrigel-coated 96-well plates at a density of 5 \( \times 10^5 \) per well. The cells were incubated for 20, 40, 60, and 90 minutes at 37°C, respectively, in a CO\(_2\) incubator. Nonattached cells were removed by 3 washings with PBS. Attached cells were analyzed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega) assay, according to the user’s manual.

**Assessment of anoikis and treatment of inhibitor**

Cells were cultured in dishes coated with PolyHEMA (Sigma), as described previously (22). Six-well polyHEMA plates were made by applying 1.5 mL of a 10 mg/mL solution of polyhydroxyethylmethacrylate in ethanol onto the plate and drying in the tissue culture hood. Cells were transiently transfected for 48 hours, trypsinized, and plated onto polyHEMA plates. Cells were harvested to assay for apoptosis using an Annexin V-FITC apoptosis detection kit (Sigma) and analyzed using flow cytometry after 24 hours of growth in suspension. Attached cells were treated for 24 hours with 20 \( \mu\text{mol/L} \) PI3K inhibitor LY294002 [2-[4-(morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; Calbiochem] at 24 hours posttransfection of PKC\(_i\) siRNA. Detached cells were kept in suspension with 20 \( \mu\text{mol/L} \) of the PI3K inhibitor LY294002 for 24 hours. To investigate whether SKP2 degradation was proteasome-dependent, detached cells were kept in suspension with 10 \( \mu\text{mol/L} \) of the proteasome inhibitor MG-132 (Sigma) for 24 hours.

**Soft agar assay for colony formation**

Colony formation assay in soft agar was based on a modified method (23). Cells transfected with PKC\(_i\) RNAi or scramble sequence RNAi were plated in 6-well plates at a density of 500 cells per well. Three independent wells were examined per clone. The cells were incubated at 37°C in 5% CO\(_2\). After 3 weeks in culture, colonies were stained with 0.2% p-iodonitrotetrazolium violet (Sigma) and counted.

**Xenograft assays in nude mice**

Mixtures of stably transfected P4 and P8 or S1 and S2 clones were injected into 4- to 6-week-old female athymic nu/nu nude mice (Vital River). For each group, 6 mice were injected subcutaneously (s.c.) with 1 \( \times 10^6 \) cells per animal. The mice were sacrificed 4 weeks after injection and examined for s.c. tumor growth. Immunohistochemistry was performed on 5-\(\mu\text{m}\) sections of paraffin-embedded tissues in the lung. The tissues were fixed in Bouin’s solution, embedded in paraffin, cut into 5-\(\mu\text{m}\) sections, and stained with hematoxylin and eosin (H&E).

**In vivo ubiquitination assay**

Cells on 10-cm plates were transfected with PKC\(_i\) siRNA-1 or nonsilencing siRNA. After being transiently transfected for 24 hours, cells were trypsinized, plated onto polyHEMA plates, and treated with MG132 for 24 hours. Then cells from each plate were collected into 2 aliquots. One aliquot (3 mg of cell lysate) was used for each immunoprecipitation reaction with 5 \( \mu\)g SKP2 antibody. Immunocomplexes were pulled down as described previously (24), resolved by SDS-PAGE, and immunoblotted for the presence of the polyubiquitin-SKP2 protein complex using anti-ubiquitin antibody. Another aliquot was used for conventional Western blotting to confirm PKC\(_i\) expression knockdown and for a loading control.

**Tissue microarray and immunohistochemical staining**

A total of 133 formalin-fixed, paraffin-embedded ESCCs and corresponding normal epithelia were placed on the tissue microarray. For each case, normal epithelia were repeated 3 times and cancer tissues were repeated 5 times. Immunohistochemical analysis was performed as mentioned before. PKC\(_i\) and SKP2 expression scores were determined according to our previously published papers (9, 16).

**Statistical analysis**

The differences in results between groups were compared using ANOVA or Student’s \( t \) test. Data were expressed as mean \( \pm \) SD. The correlation between PKC\(_i\) and SKP2 expression levels was analyzed using the Spearman correlation test. All statistical analyses were performed with the SPSS 13.0 Statistical program for Windows. \( P \) values less than 0.05 were considered significant.

**Results**

**RNAi of PKC\(_i\) in EC9706 cells**

EC9706 cells were used to explore the role of PKC\(_i\) in ESCC in the current study because they are an ESCC cell line with PKC\(_i\) overexpression (data not shown) and high metastatic ability. Two duplex PKC\(_i\) siRNAs, targeting sequences in the 3’UTR and the coding region of the PKC\(_i\) gene, respectively, PKC\(_i\) siRNA-1 and siRNA-2, were chemically synthesized for transient downregulation of PKC\(_i\) expression. After transient transfection for 48 hours,
PKC\textsubscript{i}iRNA-2 could both effectively suppress the expression of PKC\textsubscript{i} by Western blot analysis. A, representative photos of a haptotactic migration assay and a matrigel chemoinvasion assay in PKC\textsubscript{i}iRNA, nonsilencing siRNA, and parental EC9706 cells. B, Western blot analysis of PKC\textsubscript{i} expression in PKC\textsubscript{i}siRNA, nonsilencing siRNA, and parental cells after transient transfection. C, statistical plots of migration and matrigel chemoinvasion assay. The number of PKC\textsubscript{i}siRNA cells that transversed the Transwell membranes in the haptotactic migration assay and the matrigel chemoinvasion assay was significantly decreased, compared with nonsilencing siRNA and the parental cells. Column, mean of 3 individual experiments; Bars, SD; *, \textit{P} < 0.05.

Western blot analysis showed that PKC\textsubscript{i}siRNA-1 and siRNA-2 could both effectively suppress the expression of PKC\textsubscript{i} (Fig. 1B). Double-stranded oligonucleotides, synthesized according to PKC\textsubscript{i}siRNA-2, were inserted into PGCSI.U6.neo.GFP shRNA expression vectors. As shown in Figure 3A, PKC\textsubscript{i} expression was suppressed in P4 and P8 clones. In this study, we refer to PKC\textsubscript{i} stably downregulation clones as PKC\textsubscript{i}RNAi. Parallel experiments were performed using the scramble sequence RNAi clones (S1 and S2). Stably transfected clones were used for \textit{in vitro} assays and colony-formation assays.

**PKC\textsubscript{i} depletion decreased the motility of ESCC cells**

Our previous results showed that elevated expression of PKC\textsubscript{i} was correlated with lymph node metastasis in ESCC (9). Metastasis is a complicated procedure that involves many steps. Migration, invasion, and adhesion are all key components of metastasis, so we examined whether PKC\textsubscript{i} is involved in these stages. Haptotactic cell migration assays showed that after PKC\textsubscript{i} depletion, fewer cells migrated to the bottom of the chamber, compared with non-silencing and parental cells (Fig. 1A top and 1C). The invasive potential of the PKC\textsubscript{i}siRNA cells, as assessed by measuring the ability of cells to transverse a reconstituted basement membrane of Matrigel, was reduced when compared with that of the control cells (Fig. 1A, lower panel and 1C, bottom). Western blot analysis confirmed the suppression of PKC\textsubscript{i} expression in PKC\textsubscript{i}siRNA-transfected cells (Fig. 1B). These experiments were repeated 3 times with similar results.

Because the PKC\textsubscript{i}-induced increase in migration could be the result of an increase in the adhesion of tumor cells to the substrate, we evaluated the adhesive abilities of 3 groups of cells by measuring the number of cells attached to Matrigel. No significant differences were detected by MTS assays among the groups (Supplementary Fig. S1). Thus, knockdown of PKC\textsubscript{i} expression drastically suppressed the mobility of EC9706 cells \textit{in vitro}.

**PKC\textsubscript{i} knockdown promoted cell anoikis via inactivation of the PI3K/AKT pathway**

We subsequently analyzed whether PKC\textsubscript{i} expression affected cell-cycle progression or apoptosis. Flow cytometric results showed that PKC\textsubscript{i} downregulation had no effect on these phenotypes (Supplementary Fig. S2 and S3). We investigated apoptosis of cells after detachment, because PKC\textsubscript{i} gene amplification and overexpression are associated with lymphoid metastasis (9) and anoikis is one type of apoptosis involved in tumor metastasis (25). The percentage of PKC\textsubscript{i}siRNA apoptotic cells was approximately 2-fold higher than the control and parental cells by Annexin V-FITC/propidium iodide staining (Fig. 2B, C). To confirm that the results of the siRNA experiments described before were due to the inhibition of PKC\textsubscript{i} expression, we expressed human PKC\textsubscript{i} as a transgene in PKC\textsubscript{i}siRNA cells. In this experiment, PKC\textsubscript{i}siRNA-1 was used to knock down PKC\textsubscript{i} expression because it targets the 3\textquotesingle UTR of endogenous human PKC\textsubscript{i}mRNA, which makes it possible to reconstitute PKC\textsubscript{i} expression in PKC\textsubscript{i}siRNA-1 cells using an
exogenous human PKCι expression construct that lacks the 3’UTR targeting sequence. When a plasmid encoding the PKCι gene was transfected into PKCι siRNA-1 cells, reexpression of PKCι rescued the ability of PKCι siRNA-1 cells to inhibit anoikis (Fig. 2E, F, G), demonstrating a specific requirement for PKCι in the anoikis-resistance of EC9706 cells.

Molecular mechanisms of anoikis resistance have been described concerning several signaling pathways in different cell types. Our previous studies indicated that PI3K/AKT and MEK/Erk are 2 critical pathways mediating the anoikis of ESCC cells (16, 26, 27). In this study, we examined the potential effects of PKCι on the activation of PI3K/AKT and MEK/Erk pathways. After PKCι depletion and cell suspension, p-AKT expression was downregulated (Fig. 2D), and no significant differences in p-Erk expression were observed in the 3 groups. Furthermore, p-AKT expression was suppressed no matter whether PKCι-silenced cells
were attached, detached, or replated. In addition, forced transient PKC\(\iota\) expression in PKC\(\iota\) siRNA-1 cells could activate AKT phosphorylation in all 3 conditions, demonstrating a specific requirement for PKC\(\iota\) in AKT activation (Fig. 5C, D, E).

**Knockdown of PKC\(\iota\) inhibited colony formation of ESCC cells in soft agar and s.c. tumor growth in nude mice**

To investigate the effect of PKC\(\iota\) expression on tumor growth, we examined in vitro cell growth rate using MTS cell proliferation assays. No significant changes in growth rate were observed between PKC\(\iota\) RNAi and scramble sequence RNAi cells (Fig. 3B). By contrast, depletion of PKC\(\iota\) significantly blocked the ability of EC9706 cells to grow as anchorage-independent colonies in soft agar (Fig. 3C, D). Tumor formation assays in nude mice were performed to investigate the effect of PKC\(\iota\) expression on tumor growth in vivo. PKC\(\iota\) RNAi and scramble sequence RNAi cells were inoculated into 6 nude mice in each group. Both groups developed tumors, but the tumors formed from the scramble sequence RNAi cells grew faster than did...
the PKC\textit{\textsubscript{\textalpha}} RNAi tumors. The mice were sacrificed 4 weeks after injection. Tumors formed from scramble sequence RNAi cells were significantly heavier than tumors formed from PKC\textit{\textalpha} RNAi cells \(((P < 0.05); \text{Fig. } 3E, F)\). Immunohistochemical staining confirmed the presence of PKC\textit{\textalpha} expression in the formalin-fixed, paraffin-embedded sections of the s.c. tumors (Fig. 3G).

### PKC\textit{\textalpha} inhibition decreased lung metastases in nude mice

Given that increased cell migration and suppression of anoikis both contribute to the metastatic potential of cancer cells, we tested whether inhibition of PKC\textit{\textalpha} would affect the ability of EC9706 cells to metastasize. PKC\textit{\textalpha} RNAi and scramble sequence RNAi cells were introduced via tail vein into 8 nude mice from each group. Eleven weeks after injection, the animals were sacrificed and examined for the presence of lung metastases. In the scramble sequence RNAi group, 2 mice developed visually observable lung nodules and another 2 mice developed small tumor nodules that could be observed only under the microscope in H&E sections of the lungs. However, no tumor nodules were found either visibly or under the microscope in H&E sections of mouse lungs in the PKC\textit{\textalpha} RNAi group. Representative lungs and their corresponding H&E staining are shown in Figure 4A. The average number of lung metastases of the mice injected with PKC\textit{\textalpha} RNAi cells was significantly lower than that of mice injected with scramble sequence RNAi cells \((P < 0.05); \text{Fig. } 4B)\). These results suggest that PKC\textit{\textalpha} expression promotes tumor metastases \textit{in vivo}.

### PKC\textit{\textalpha} regulated resistance to anoikis through SKP2

Our previous study suggested that SKP2 was also implicated in PI3K/AKT-mediated anoikis resistance in EC9706 cells (16). Therefore, we hypothesized that there may be a regulating relationship between SKP2 and PKC\textit{\textalpha}. Western blot analysis in this study indicated that SKP2 expression did not change in adherent cells after PKC\textit{\textalpha} knockdown (Fig. 5A), but was markedly decreased in detached cells (Fig. 5B). To verify that PKC\textit{\textalpha} directly controlled SKP2 expression, we transfected vectors encoding the PKC\textit{\textalpha} gene into PKC\textit{\textalpha} siRNA-1 cells, and transfected cells were placed in attached, detached, and replated states. Expression of exogenous PKC\textit{\textalpha} restored intracellular level of the SKP2 protein only in the detached cells (Fig. 5C, D, E), which confirmed that PKC\textit{\textalpha} could only regulate SKP2 expression in detached ESCC cells. When plasmds encoding the SKP2 gene were transfected into PKC\textit{\textalpha} siRNA-1 cells, elevated SKP2 expression rescued the ability of PKC\textit{\textalpha} siRNA-1 cells to inhibit anoikis. These data revealed a novel required role for PKC\textit{\textalpha}-SKP2 signaling in ESCC cells anoikis-resistance (Fig. 5F, G).

### PKC\textit{\textalpha} regulated SKP2 protein expression through the ubiquitin-proteasome pathway

RT-PCR results showed that SKP2 mRNA level was not changed in detached cells with decreased PKC\textit{\textalpha}. However, the addition of the proteasome inhibitor MG132 could prevent the decreased SKP2 protein level in detached cells after PKC\textit{\textalpha} depletion (Fig. 6A). To further support these results, immunoprecipitation experiments with anti-SKP2 antibody were utilized, followed by immunoblotting with anti-ubiquitin antibody. In the presence of MG132, the ubiquitin was observed in the SKP2 immunocomplex, and its amount was substantially increased in the PKC\textit{\textalpha} knockdown group (Fig. 6B, top). Western blot analysis (Fig. 6B, bottom) confirmed PKC\textit{\textalpha} knockdown and ubiquitination assay loading control.

The aforementioned results indicate that PKC\textit{\textalpha} activated AKT phosphorylation and enhanced the intracellular level of SKP2 protein in detached ESCC cells. In addition, recent reports have suggested that the PI3K/AKT pathway regulates SKP2 expression level by one or more unknown mechanisms (28–30). Based on these data, we hypothesized that PKC\textit{\textalpha} might regulate SKP2 expression through AKT signaling. To investigate this, we treated EC9706 cells with the PI3K inhibitor LY294002. As shown in Figure 6C and D, the loss of p-AKT revealed robust inhibition of PI3K

![Figure 4](image_url). Depression of PKC\textit{\textalpha} decreases lung metastases in nude mice. A, each mouse had 1.5 x 10\textsuperscript{6} cells injected via tail vein, and the mice were sacrificed 11 weeks after injection. Mouse lungs were then photographed after Bouin’s fixation (top), and the sections of the lungs were stained with H&E (bottom). B, number of lung metastases of the 8 mice 11 weeks postinjection in each group.
activity. However, inhibition of PI3K activity did not result in decreased of SKP2 protein expression either in attached or detached cells, demonstrating that the PI3K/AKT pathway did not regulate SKP2 expression in our experiment. Interestingly, we found decreased PKC\(\text{\textalpha}\) protein level concomitant with the loss of phosphorylation by AKT.

**Figure 5.** PKC\(\text{\textalpha}\) regulated resistance to anoikis through SKP2 in ESCC cells. A, after transient transfection with PKC\(\text{\textalpha}\) siRNA and nonsilencing siRNA, SKP2 expression was unchanged when PKC\(\text{\textalpha}\) expression was repressed in attached EC9706 cells. B However, after transient transfection for 48 hours and detachment growth for 24 hours, SKP2 expression was decreased when PKC\(\text{\textalpha}\) expression was repressed. C, Western blot analysis of cell lysates of PKC\(\text{\textalpha}\)-forced transient expression PKC\(\text{\textalpha}\) siRNA-1 cells and vector-transfected nonsilencing siRNA and PKC\(\text{\textalpha}\) siRNA-1 cells for SKP2, PKC\(\text{\textalpha}\), p-AKT on Ser473, and \(\beta\)-actin in attached cells. D, Western blot analysis of cell lysates of PKC\(\text{\textalpha}\)-forced transient expression PKC\(\text{\textalpha}\) siRNA-1 cells and vector-transfected nonsilencing siRNA and PKC\(\text{\textalpha}\) siRNA-1 cells for SKP2, PKC\(\text{\textalpha}\), p-AKT on Ser473, and \(\beta\)-actin in detached cells. After transient transfection, cells were cultured on polyHEMA-coated dishes for 24 hours. E, Western blot analysis of cell lysates of PKC\(\text{\textalpha}\)-forced transient expression PKC\(\text{\textalpha}\) siRNA-1 cells and vector-transfected nonsilencing siRNA and PKC\(\text{\textalpha}\) siRNA-1 cells for SKP2, PKC\(\text{\textalpha}\), p-AKT on Ser473, AKT, and \(\beta\)-actin in replated cells. After transient transfection and growth in suspension, cells were replated on normal 6-well dishes for 24 hours. F, Western blot analysis of cell lysates of SKP2-forced transient expression PKC\(\text{\textalpha}\) siRNA-1 cells and vector-transfected nonsilencing siRNA and PKC\(\text{\textalpha}\) siRNA-1 cells for SKP2, PKC\(\text{\textalpha}\), p-AKT on Ser473, AKT, and \(\beta\)-actin in detached cells. After transient transfection, cells were cultured on polyHEMA-coated dishes for 24 hours. G, forced transient expression of SKP2 in PKC\(\text{\textalpha}\) siRNA-1 cells led to a decrease of apoptotic cells in an anoikis assay. Columns, mean of individual experiments; Bars, SD; *, P < 0.05, mean of 3 individual experiments.

**Correlation between PKC\(\text{\textalpha}\) and SKP2 expression in ESCC tissues**

To determine the relationship between PKC\(\text{\textalpha}\) and SKP2 in tumor tissues, we analyzed their protein expression using an immunohistochemical approach combined with tissue microarray. Immunohistochemistry results showed that of
133 specimens tested, all normal epithelia had negative or weak PKC\(\iota\) and SKP2 expression, whereas 50 (37.6%) of the tumors had more intense staining of PKC\(\iota\), and 40 (30.1%) exhibited high SKP2 expression than normal tissues. PKC\(\iota\) expression was significantly correlated with SKP2 expression in the tumors (\(P = 0.031\), Table 1 and Fig. 7).

**Discussion**

We previously reported that both frequent amplification and overexpression of PKC\(\iota\) were associated with lymph node metastasis in ESCC, suggesting that PKC\(\iota\) might play a key role in the progression of the disease (9). To evaluate this possibility, we first assessed PKC\(\iota\) protein expression in
a panel of ESCC cell lines. We chose EC9706 cells for the PKC\(\alpha\) functional studies because they were shown to have high PKC\(\alpha\) expression (data not shown) and are frequently used in ESCC metastasis research (16, 26, 27).

Previous studies suggested that PKC\(\alpha\) was involved in nicotine-activated migration and invasion of H1299 human non–small-cell lung cancer cells and promoted glioblastoma cell motility and invasion by repression of RhoB expression (31, 32). To explore the effect of PKC\(\alpha\) on ESCC cell migration and invasion, we performed transient transfections with 2 PKC\(\alpha\)-targeted siRNAs in EC9706 cells. Both PKC\(\alpha\) siRNAs significantly suppressed PKC\(\alpha\) expression and induced both inhibition of cell migration and invasion of ESCC cells.

Anoikis, one type of apoptotic cell death due to the loss of cell adhesion, is critical for the regulation of tissue homeostasis in tissue remodeling, development, fibrosis, and tumor metastasis (33). Here, we showed that decreased PKC\(\alpha\) expression was associated with an increase in anoikis in ESCC cells and that reexpression of PKC\(\alpha\) could reinstate anoikis resistance in PKC\(\alpha\)-silenced cells. We postulate that PKC\(\alpha\) overexpression may help malignant cells survive in an anchorage-independent manner, leading to metastasis in ESCC patients. Molecular mechanisms of anoikis resistance have been described concerning several signaling pathways in different cell types. Our previous studies indicated that PI3K/AKT and MEK/Erk are 2 critical pathways mediating the anoikis of ESCC cells (16). We therefore tested the hypothesis that PKC\(\alpha\) might regulate SKP2 expression through AKT signaling. Previous reports and our data, we hypothesized that PKC\(\alpha\) could activate AKT phosphorylation. Based on previous reports and our data, we hypothesized that PKC\(\alpha\) might regulate SKP2 expression through AKT signaling. In this study, we used siRNAs targeting PKC\(\alpha\) for functional studies because they were shown to have high PKC\(\alpha\) expression (data not shown) and are frequently used in ESCC metastasis research (16, 26, 27).

Moreover, elevation of SKP2 can reestablish cellular resistance to anoikis in ESCC cells with PKC\(\alpha\) knockdown. Taken together, these results suggest that in detached ESCC cells, PKC\(\alpha\) overexpression elevates the expression of SKP2 protein, promotes activation of the PI3K/AKT pathway, and increases resistance to anoikis in ESCC. PKC\(\alpha\) expression was significantly correlated with SKP2 in 133 ESCCs (\(P = 0.031\)), which confirmed the regulation between PKC\(\alpha\) and SKP2 in vivo. To our knowledge, this is the first report that PKC\(\alpha\) is involved in cell anoikis through SKP2 regulation, which extends our understanding of the roles of PKC\(\alpha\) and SKP2 in tumour metastasis.

To explore the possible mechanisms of PKC\(\alpha\) regulation of SKP2 expression, we first determined the SKP2 mRNA level in suspended PKC\(\alpha\)-siRNA cells by RT-PCR and found that the SKP2 mRNA expression was unchanged after PKC\(\alpha\) knockdown. Previous studies had suggested that SKP2 transcription was regulated by several important signaling pathways (29, 34). However, our data showed that PKC\(\alpha\) did not regulate SKP2 at the transcrptional level in ESCC cells. We then postulated that PKC\(\alpha\) might regulate SKP2 expression on the posttranscriptional level. When suspended cells were treated with the proteasome inhibitor MG132, the decrease in SKP2 protein expression after PKC\(\alpha\) depletion disappeared, which strongly suggested that PKC\(\alpha\) might regulate SKP2 expression via proteasomes. To confirm this hypothesis, an in vivo ubiquitination assay was performed. The results indicated that SKP2 degradation initiated by PKC\(\alpha\) depletion was mediated via the ubiquitin-proteasome pathway.

Cdh1 has been revealed as an E3 ligase that promotes SKP2 destruction (17, 18). Several studies have reported on the relationship between the PI3K/AKT pathway and SKP2 (28–30). It has been observed that in 293T cells AKT1 phosphorolates SKP2 at Ser 72, which is required to disrupt the interaction between Cdh1 and SKP2, and as a result prevents SKP2 degradation by the APC-Cdh1 ubiquitin ligase complex (35, 36). In the present study, we found that PKC\(\alpha\) could activate AKT phosphorylation. Based on previous reports and our data, we hypothesized that PKC\(\alpha\) might regulate SKP2 expression through AKT signaling. However, the addition of LY294002 did not change SKP2 protein level either in attached or detached EC9706 cells, indicating that the PI3K/AKT pathway did not regulate

<table>
<thead>
<tr>
<th>PKC(\alpha)</th>
<th>SKP2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative + weak positive</td>
<td>64</td>
<td>19</td>
</tr>
<tr>
<td>Strongly positive</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>93</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 1. Correlation between PKC\(\alpha\) and SKP2 expression in 133 ESCCs

NOTE: Correlation coefficient \(\rho = 0.202, P = 0.031\).
SKP2 expression in our experiments. In addition, coimmu-
noprecipitation analysis showed that AKT1 did not interact
with SKP2 (Supplementary Fig. S4B). Our previous study
found that decreased SKP2 reduced p-AKT expression and
that the PI3K/AKT pathway is the downstream target
of SKP2 (16). Our previous and current data showed that
SKP2 acts upstream of AKT, and PKC\(\theta\) might regulate
SKP2 protein degradation through other molecules but not
PI3K/AKT pathway. Moreover, Mao and colleagues
cotransfected breast cancer BT-549 cells with expression
constructs encoding V5-tagged PKC\(\theta\) and HA-tagged
AKT and then assessed coimmunoprecipitation of the
transfected PKC\(\theta\) with AKT. They observed that exo-
genous PKC\(\theta\) was present at low level in AKT immuno-
precipitates, indicating that PKC\(\theta\) interacts with AKT in
breast cancer cells (37). We did not observe PKC\(\theta\), AKT1
and SKP2 coprecipitation in detached EC9706 cells in the
present study (Supplementary Fig. S4A). These differential
observations in our and other experiments might reflect that
there exist distinct signaling networks in different cell lines.
The detailed mechanism underlying PKC\(\theta\) regulation of
SKP2 through the ubiquitin-proteasome pathway remains
to be elucidated. Interestingly, we found that the addition
of LY294002 also resulted in a decrease in PKC\(\theta\) protein level,
indicating a possible negative feedback loop between PI3K/
AKT and PKC\(\theta\) that may help to maintain the balance
between cell survival and apoptosis.

In conclusion, our results provide evidence that PKC\(\theta\)
promotes tumor metastasis through increased cell migration
ability and anoikis resistance in ESCC. PKC\(\theta\) enhances
resistance to anoikis via the PKC\(\theta\)-SKP2-AKT pathway.
The data suggest that PKC\(\theta\) is a candidate oncogene
involved in the progression of ESCC and that SKP2 is a
downstream effector of PKC\(\theta\) signaling in ESCC.

Figure 7. There is a correlation between PKC\(\theta\) and SKP2
expression in human ESCC. The
tissues were incubated with anti-
PKC\(\theta\) (1:100) or anti-SKP2
antibody (1:200) and visualized by
diaminobenzene (DAB).
Representative photos showed
consistent expression of PKC\(\theta\)
and SKP2 in tumor tissues.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Atypical Protein Kinase C\(\eta\) (PKC\(\eta\)) Promotes Metastasis of Esophageal Squamous Cell Carcinoma by Enhancing Resistance to Anoikis via PKC\(\eta\)-SKP2-AKT Pathway

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