

PKC α is a Potentially Useful Marker for Planning Individualized Radiotherapy for Nasopharyngeal Carcinoma

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Purpose: To examine the expression of protein kinase C alpha (PKC α) in nasopharyngeal carcinoma (NPC) and determine its relationship to the radio-sensitivity of NPC in order to evaluate its potential as a molecular marker for the guidance of individualized radiation therapy for NPC.

Materials and Methods: PKC α expression levels were detected in tumor samples from patients and in NPC cell lines with varying degrees of radio-sensitivity. A survival analysis was performed to analyze the association of PKC α expression with the 5-year overall survival (OS), progression-free survival (PFS), locoregional recurrence-free survival (LRFS), and distant metastasis-free survival (DMFS) in patients. In vitro and in vivo experiments using NPC cell lines were performed to study the effects of down-regulation of PKC α by short hairpin RNA treatment on the radio-sensitivity of NPC.

Results: PKC α expression was up-regulated in the well-differentiated NPC tissues of patients and in the more radio-resistant NPC cell lines. Moreover, high PKC α expression was associated with a worse 5-year PFS and LRFS of patients. shRNA-mediated knockdown of PKC α led to an increase in the sensitivity of NPC cells to radiation therapy, both in vitro as cultured cells and in vivo as tumor xenografts.

Conclusion: The elevated expression of PKC α in NPC and its association with patient PFS indicates that PKC α is a potential molecular marker for guiding precision radiotherapy in NPC patients. Also, the increased radiosensitivity of NPC cells after loss of PKC α identifies PKC α as a promising therapeutic target for enhancing the radio-sensitivity of NPC.

Keywords: PKC α , marker, individualized radiotherapy, nasopharyngeal carcinoma

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy in the mucous epithelium of the nasopharyngeal tissues, and its prevalence shows a distinct geographic distribution. It is primarily endemic in Southeast Asia and the southern provinces of China, with a much higher incidence in these areas than in any other country or region in the world.¹ Radiation therapy has been the first-line standard treatment for NPC,² with some patients receiving intensity modulated radiotherapy (IMRT) and others receiving chemoradiotherapy. Although the overall survival (OS) of NPC patients has improved with advances in these combination radiotherapy treatments, for patients in whom these treatments fail to achieve local control, retreatment is often ineffective and causes serious complications. Because it is currently impossible to accurately predict the efficacy of radiation therapy for individuals, oncologists do not

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have a clear strategy for personalizing the treatment approach for individual NPC patients and, thus, are left to apply similar treatment regimens in all NPC cases. Therefore, research is urgently needed to identify markers that indicate which cases are likely to be more resistant to radiation therapy, as this information would allow for the planning of more individualized treatments.³

Protein kinase C (PKC) is a kinase that is widely expressed in human tissues, and PKC- α (PKC α), a subtype of PKC, has been closely associated with cancer cell proliferation, differentiation, and apoptosis.⁴ We previously explored the relationship between PKC α and local invasion of NPC and found that the PKC α expression level correlates with the 2-year progression-free survival (PFS) and overall survival (OS) of NPC patients.⁵ In the present study, we extended this research in NPC patients, investigating PKC α expression in NPC specimens and analyzing the correlation of PKC α expression with the 5-year survival of NPC patients. To better understand the observed correlation, we evaluated the expression of PKC α in NPC cell lines with varying degrees of radio-resistance and studying the effects of PKC α knockout on their response to radiation treatment both as cultured cells *in vitro* and as tumor xenografts *in vivo*. Together, the results from our analysis of patient specimens and our *in vitro* and *in vivo* experiments provide the evidence that PKC α represents a potential marker of NPC radio-sensitivity as well as a potential target for increasing the radio-sensitivity of NPC.

Materials and Methods

NPC Clinical Samples

NPC samples were collected from a total of 97 patients at initial diagnosis in the Oncology Department of Xiangya Hospital of Central South University between 2010 and 2015. The ethical committee of Xiangya Hospital approved this study (No: 201703513). Use of tissue samples, data collection and review of medical records were performed in accordance with the ethical standards of the committee and the 1964 Declaration of Helsinki and its later amendments. Because of the retrospective nature of our study and lack of patient identification, informed consent was waived for all participants involved in this study. The inclusion criteria included nasopharyngeal carcinoma diagnosed for the first time by biopsy and treated with radical intensity-modulated conformal radiotherapy. Patients were excluded according to the following criteria:

performance status ≥ 2 on the Eastern Cooperative Oncology Group (ECOG) scale, distant metastasis (stage IVb), previous malignancy or other type of tumor; unstable cardiac disease; and current lactation or pregnancy. The nasopharyngeal tumors were paraffin-embedded and formalin-fixed. The samples were collected from untreated patients along with clinical follow-up data on the initial diagnosis. The histological classifications followed the World Health Organization (WHO) classification system: type I, keratinizing carcinomas; type II, differentiated subtype of non-keratinizing carcinoma; and type III, undifferentiated subtype of non-keratinizing carcinoma.

Clinical Staging Method, Image Assessment, and Treatment

Patients' computed tomography (CT) and magnetic resonance imaging (MRI) scans were independently reviewed with the Varian Eclipse Plan System by two radiation oncologists. Stage was determined by comprehensively evaluating the patients' clinical manifestations, physical examination results, and CT or MRI scans according to Seventh Edition of the American Joint Committee on Cancer (AJCC) clinical staging standard. Measurable disease was defined by the RECIST criteria. According to the No. 50 and 62 documents of the ICRU, all patients were treated by IMRT, with prescribed doses as follows: primary tumor, 70.62–73.92 Gy (2.14–2.24 Gy/33 fractions); high-risk lymph nodes, 69.96 Gy (2.12 Gy/33 fractions); intermediate-risk sites, 59.4–61.05 Gy (1.8–1.85 Gy/33 fractions), and low-risk sites, 50.4–51.8 Gy (1.8–1.85 Gy/28 fractions). The radiation was delivered by a linear accelerator (Varian, USA) with five daily fractions per week. Paclitaxel/docetaxel and/or platinum were used as neo-adjuvant, concurrent, and adjuvant chemotherapy. OS was defined as the duration from the first day of initial diagnosis to the date of death from any cause or the last follow-up on August 31, 2020. Progression-free survival (PFS) and locoregional recurrence-free survival (LRFS), and distant metastasis-free survival (DMFS) were defined as the interval from the first day of initial diagnosis to confirmed disease progression, local recurrence, and distant metastasis, respectively. Progressive disease was defined as at least a 20% increase in the sum of the diameters of target lesions (an absolute increase of at least 5 mm) or the appearance of one or more new lesions. Stable disease was defined as neither sufficient shrinkage

to qualify as partial response nor sufficient growth to qualify as progressive disease.⁶

Immunohistochemical Staining

All xenograft samples and NPC tissue blocks were fixed and embedded in paraffin according to standard procedures. Following de-paraffinization, sections were subjected to heat-mediated antigen retrieval in 0.01 mol/L citrate buffer (pH 6.0). After cooling to room temperature, the samples were treated with 1% methanol/30% H₂O₂ to block endogenous peroxidase activity. Nonspecific protein binding was blocked using 5% bovine serum albumin (BSA) solution. Next, sections were rinsed with phosphate-buffered saline (PBS) and incubated with anti-PKCa (Santa Cruz Biotechnology, 1:40) overnight at 4° C. The next day, the sections were rinsed with PBS at room temperature and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, UK) for 30 min. Then, after 3–5 min of washing, the sections were incubated with 3,3'-diaminobenzidine (DAB) for visualization. Finally, the samples were rinsed with distilled H₂O and counterstained with hematoxylin.

The scores for PKCa-reactive NPC cells in patient samples were determined by evaluating the staining degree and percentage of positive cells. For cases of positive cytoplasmic staining, PKCa scores were assigned as follows: 0, absence of expression or expression in <10% of NPC-positive cells; 1, staining in 10–49% of NPC-positive cells; and 2, staining in ≥50% of NPC-positive cells. The immunohistochemical staining results were independently evaluated by two pathologists.

Cell Culture and Cell Line Generation

The NPC cell lines CNE1, CNE2, and HK1 were donated by the Cancer Research Institute of Central South University.^{7–9} The use of the cell lines was approved by the ethics committee of Xiangya Hospital, and the CNE1 and CNE2 cells were authenticated by STR profile before the study. A radio-resistant cell line, denoted as CNE2-resistant (CR) cells, was established via exposure of parental CNE2 cells to gradually increasing doses of irradiation.^{10,11} The final surviving CNE2 cells were verified with significant radiation-resistant characteristics and defined as radioresistant CNE2 cells (CR).¹⁰ The radiation was delivered with a Varian linear accelerator (2100EX, San Francisco, CA, USA). The cell culture flask was covered with a 1.5-cm layer of compensation glue during irradiation. In addition, a 6-MeV

electron beam was chosen, and the radiation dose rate was 300 cGy/min. All cell lines were cultured at 37°C in 5% CO₂ and grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (Invitrogen, USA). Cells in the exponential growth phase were used in the experiments.

Plasmid Construction and Transfection

To knockdown PKCa, two target sequences were selected: the human PKCa target 1 (5-GGGATCGAACAACAAGGAATG-3) and target 2 (5-GCGTCCTGTTGTATGAATGC-3).¹² To generate PKCa short hairpin (sh)RNA, the coding sequences for the shRNAs were cloned using the Lenti-X™ shRNA Expression System (Clontech). Then 293T cells were co-transfected with lentiviral vectors, envelope plasmids, and packaging plasmids for 24 h before collection of the lentiviral supernatant. The CR and CNE1 cells were transduced with control or shPKCa lentiviral particles for 48 h and then cultured for 72 h with 2 µg/mL puromycin for selection. The efficacy of transfection was verified by Western blot analysis.

Colony Formation and Radio-Sensitivity Assays

Cells were seeded in six-well plates and subjected to irradiation (IR) treatment for 24 h with a dose range 0–6 Gy delivered with the Varian linear accelerator (2100EX, San Francisco, California, USA). Our preliminary experiment showed that 6 Gy is an appropriate dose for further experiments. The medium was changed every 5 days after radiation, and after 10–12 days, the cells were washed three times at room temperature, fixed in paraformaldehyde for 15 min, and stained with crystal violet for 10 min. After washing with de-ionised water, the plates were left to air dry. For calculation of colony formation, clones with more than 50 cells were counted as colonies. Analysis was performed using the linear quadratic model of GraphPad software.

Flow Cytometry

Cells were seeded in six-well plates, irradiated with 2Gy 16h after seeding, and harvested at 48h. Then 1 × 10⁴ cells were treated by Annexin V-FITC Apoptosis Detection Kit (Beyotime, China), and apoptosis was analyzed by flow cytometry (Guava easyCyte, Millipore, Germany).

Preparation of Proteins and Western Blotting

For protein extraction, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer for 30 min on ice. Protein concentrations were then measured with BCA protein assay kits (Thermo-Scientific, USA). Protein samples (40–50 µg) were prepared and separated by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto 0.22- and 0.45-µm polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were immersed in Tris-buffered saline with Tween 20 (TBST) containing 5% non-fat dry milk, shaken at room temperature for 1 h, and then incubated for 1 h at room temperature in solutions of the following primary antibodies at 4°C for 16–18 h: anti-PKCα (1:500, Santa Cruz Biotechnology, USA) and anti-tubulin (1:1000, Santa Cruz Biotechnology). After washing with TBST, the blots were incubated with the secondary antibodies (1:1000 Santa Cruz Biotechnology) for 1 h at room temperature. Finally, the protein bands were visualised with a Thermo Scientific-Pierce Enhanced Chemiluminescence (ELC) substrate detection system.

NPC Xenograft Model

The animal experiments were conducted according to the Guidelines of the Animal Handling and Care in Medical Research Committee of Central South University and were approved by the Animal Ethics Committee of Central South University (No: 201703512). Six- to eight-week-old female nude mice obtained from Vital River Laboratories (Beijing, the original breeding pairs were purchased from Charles River) were used to establish the human NPC xenograft model. After stable transduction with PKCα shRNA or shControl lentivirus, 5×10^6 CR cells were inoculated into the left flanks of the mice to form the NPC xenografts. Tumor volume was measured every 2 days from the third day after subcutaneous inoculation. Irradiation treatment was applied once the tumor volume reached 60–80 mm³. Radiation was delivered at 2 Gy per fraction every other day for three fractions. When the tumor volume reached a maximum of 1500 mm³, the mice were euthanized, and the xenografts were harvested for further analyses.¹³

Apoptosis in xenografts was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining using the ApopTag® Fluorescein In-Situ Apoptosis Detection Kit (Merck Millipore, Germany) after preparation of sections for staining as described above for human NPC specimens. The fluorescence intensity was analysed by ImageJ (National Institutes of Health, USA).

Statistical Analysis

All in vitro and in vivo experiments were performed at least three times. The statistical analyses were conducted using SPSS 23.0 software (SPSS, Inc., USA). For comparison of data between two groups, Student's *t* test or chi-square test was used for in vitro data. Spearman rank correlation coefficients were used to determine the correlations of PKCα and histological classification. Survival was estimated using the Kaplan–Meier method, and the results were compared with the Log rank test. *P* values <0.05 were considered statistically significant. All statistical tests were two-sided.

Results

Expression of PKCα is Up-Regulated in Well-Differentiated NPC

Tumor samples were obtained from 97 patients with histologically diagnosed NPC. The patients ranged in age from 22–72 years and had WHO types I, II, or III NPC (stages II to IVa; Table 1). All patients exhibited a performance status of less than 2 on the Eastern Cooperative Oncology Group (ECOG) scale.

It is generally accepted in radiation oncology that poorly differentiated malignant tumours tend to be more sensitive to radiation therapy. As WHO type I is rare in NPC, we detected and analyzed PKCα expression in NPC specimens including WHO type II and III, considering WHO type II NPC to be well differentiated and WHO type III NPC to be poorly differentiated. Based on the expression level score derived from immunohistochemical staining analysis of PKCα (Figure 1A), a rank correlation analysis revealed that PKCα was differentially expressed

Table 1 Characteristics of NPC Patients Whose Samples Were Used for Immunohistochemical Staining of PKCα

| Characteristic | | n |
|-----------------|-------------------------------|----|
| Age | ≥47 yrs | 49 |
| | <47 yrs | 48 |
| Sex | Male | 66 |
| | Female | 31 |
| Clinical stage | II | 3 |
| | III | 35 |
| | IVa | 59 |
| Histologic type | WHO I | 1 |
| | WHO II | 26 |
| | WHO III | 68 |
| | Not evaluated by WHO criteria | 2 |

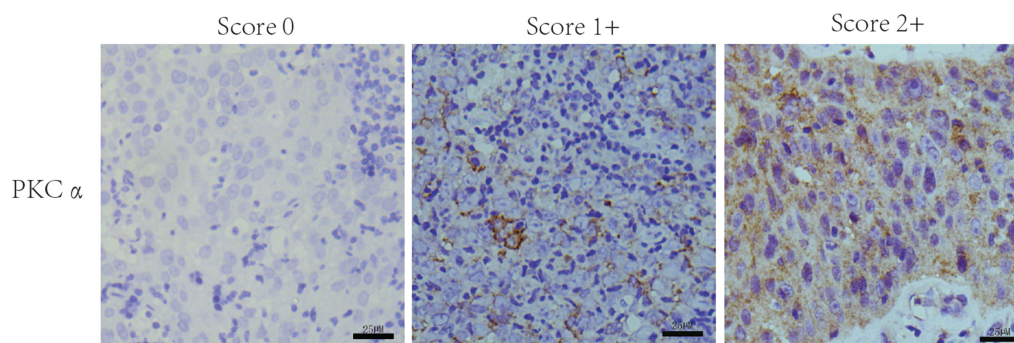


Figure 1 Expression of PKC α in NPC tissues. Representative images of PKC α staining in pathological specimens of NPC. The criteria for scores of 0 (left column), 1+ (middle column) and 2+ (right column) are described in the Materials and Methods (scale bar = 25 μ m).

between the WHO II and WHO III groups (Spearman correlation coefficient 0.290; $p=0.005$; Table 2).

PKC α Expression is Associated with 5-Year Progression-Free Survival and LRFS in NPC Patients

To further confirm whether PKC α expression is related to the survival outcomes of radiation therapy for NPC, we divided the NPC cases into a PKC α expression-positive group and PKC expression-negative group, according to pathological scores for PKC α staining (0, negative; and score 1 and 2, positive). From a survival analysis based on the 5-year follow-up, the OS rates of the PKC α -negative and PKC α -positive groups were 97.2% and 86.5%, respectively ($p=0.084$; Figure 2A). The PFS rate of the PKC α -negative group was significantly higher than that of the of the PKC α -positive group (91.7% vs 77.0%, $p=0.045$; Figure 2B). The LRFS rate of the PKC α -negative was also significantly higher than that of the PKC α -positive group (94.4% vs 80.3%, $p=0.043$; Figure 2C). Finally, the DMFS rates of the PKC α -negative and PKC α -positive groups did not differ significantly (94.4% vs 85.2%, $p=0.139$; Figure 2D).

PKC α Expression is Up-Regulated in Radiation-Insensitive NPC Cell Lines

Given that radiation therapy is the primary treatment for NPC and our results above suggest that PKC α expression

is related to patients' PFS, we examined the level of PKC α expression in NPC cell lines that exhibit varying degrees of radio-sensitivity. First, to test radio-sensitivity, colony formation by NPC cell lines was assessed after irradiation at 0 or 6 Gy (Figure 3A). Then PKC α expression in these cell lines was detected by Western blotting. CR cells, which are considered largely radio-resistant (Figure S1A and B), showed significantly greater PKC α expression compared with CNE2 cells. Also CNE1 cells, a cell line with rather poor response to radiation, also showed elevated PKC α expression. In sharp contrast, in the radiation-sensitive cell line HK1, PKC α expression was extremely low (Figure 3B).

Reduced PKC α Expression Increases the Radio-Sensitivity of NPC Cells in vitro

To investigate whether PKC α regulates the radio-sensitivity of NPC cells, we performed loss-of-function assays using two independent shRNAs targeting PKC α . As shown in Figure 4A and B, shRNA treatment using two target sequences sh#1 and sh#2 resulted in successful down-regulation of PKC α in both the CR and CNE1 cell lines. We then examined the survival fractions of CR and CNE1 cells after 6 Gy of irradiation with or without PKC α knockdown. As shown in Figure 4C and D, knockdown of PKC α significantly increased the radio-sensitivity of CR and CNE1 cells compared with control cells.

Table 2 Correlation Between the Intensity of PKC α Expression in Nasopharyngeal Carcinoma (NPC) and Histological Classification

| | PKC α - | PKC α 1+ | PKC α 2+ | Spearman's Rank Correlation Coefficient | p-value |
|---------|----------------|-----------------|-----------------|---|---------|
| WHO II | 6 | 5 | 15 | 0.290 | 0.005** |
| WHO III | 30 | 22 | 16 | | |

Note: ** $p<0.01$.

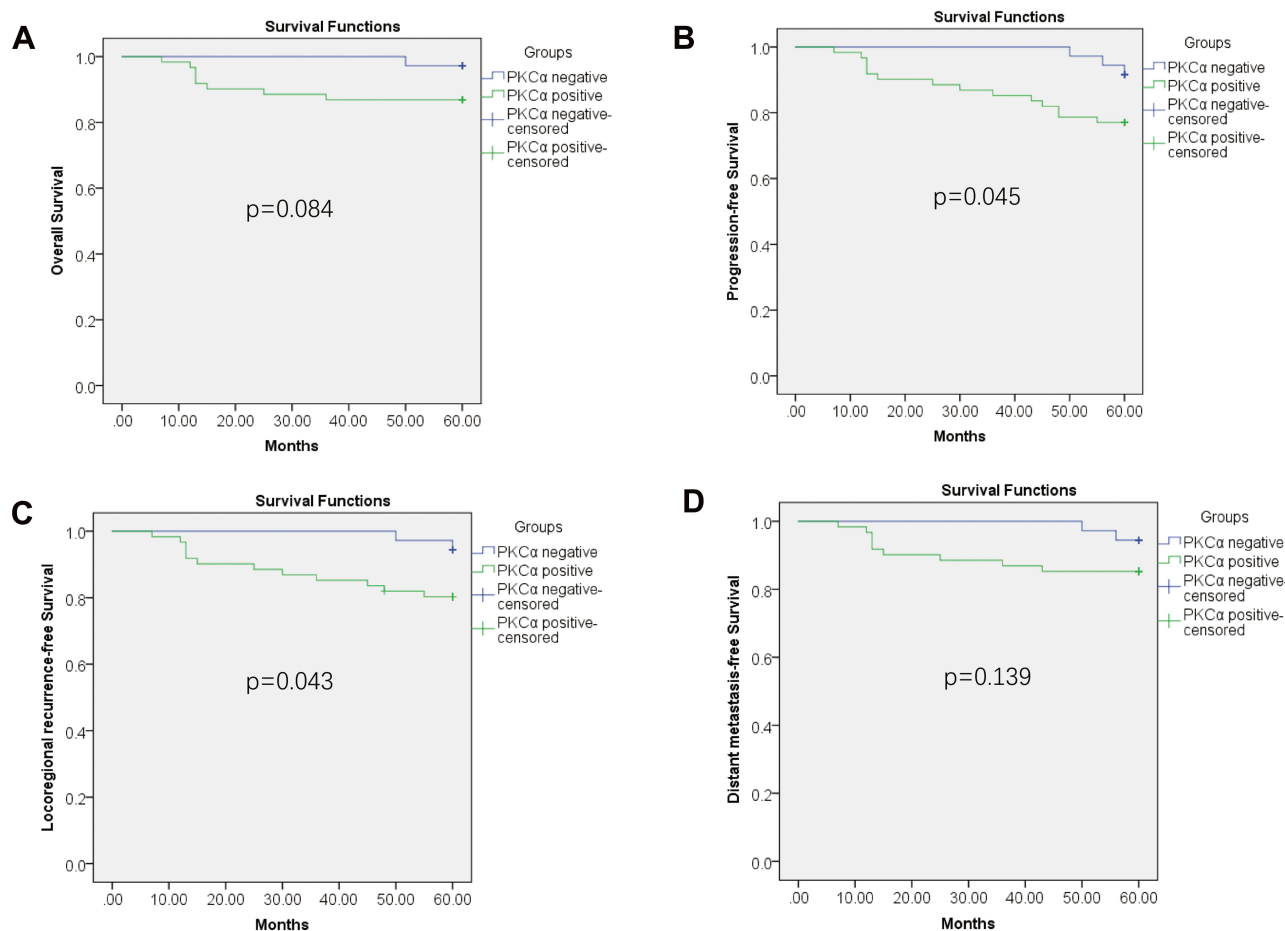


Figure 2 Association of PKC α expression with survival among NPC patients. (A) Comparison of 5-year OS between NPC patients in the PKC α -negative and -positive groups. (B) Comparison of 5-year PFS between NPC patients in the PKC α -negative and -positive groups. (C) Comparison of 5-year LRFS between NPC patients in the PKC α -negative and -positive groups. (D) Comparison of 5-year DMFS between NPC patients in the PKC α -negative and -positive groups.

Reduced PKC α Expression Increases the Radio-Sensitivity of CR Xenografts in vivo

Next, we established a mouse NPC xenograft model using CR cells to investigate whether knockdown of PKC α could enhance NPC sensitivity to radiation in vivo. After subcutaneous inoculation of shPKC α - or scramble control-treated CR cells into nude mice, the two types of NPC cells formed tumors well. From day 7 to day 11 (5 days), we treated mice three times, once every other day, with 2-Gy fractions. Consistent with the results in vitro, tumor growth in the shPKC α group was significantly inhibited in response to radiation treatment, compared with that in the control groups (Figures 5A, B and S2). Immunohistochemical staining of harvested xenografts confirmed down-regulation of PKC α in the shPKC α -treated group (Figure 5C). Additionally, more apoptotic tumor cells were observed in the xenografts from the shPKC α group after radiation treatment (Figure 5D and

E). Together, these results indicate that down-regulation of PKC α can increase the sensitivity of CR cells within NPC xenografts to radiation therapy.

Discussion

It is generally accepted that PKC functions as a carcinogenic promoter;¹⁴ however, decreased activity and expression levels of several subtypes of PKC also have been found in many malignant tumors.¹⁵ These seemingly conflicting findings reveal the complexity of the cellular functions of PKC and its subtypes in cancer development.¹⁶

Studies have also reported that PKC expression is related to therapeutic resistance in cancer cases,¹⁷ and PKC α expression has been suggested to be a predictor of tumor recurrence after treatment.^{18,19} In primary human fibroblasts, PKC signaling was shown to prevent irradiation-induced apoptosis.²⁰ Furthermore, in 2017 Scott et al proposed a gene expression-based radiation-

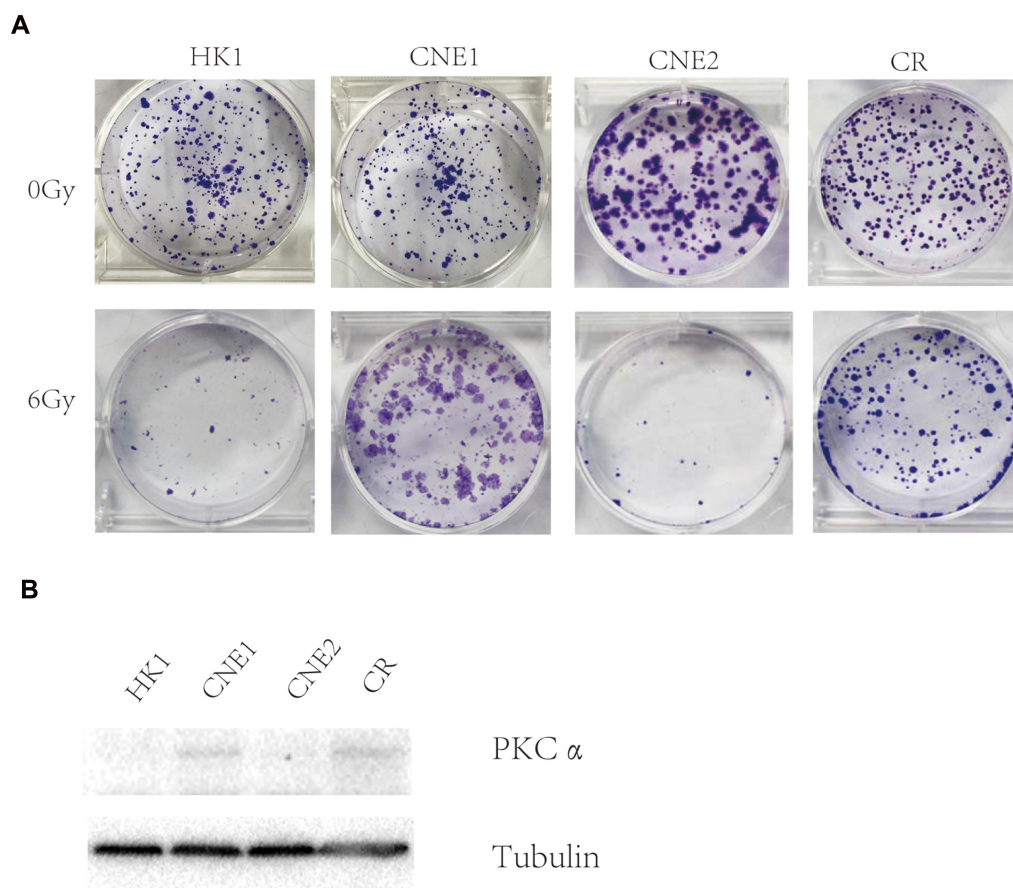


Figure 3 PKC α expression in NPC cell lines. **(A)** Clonogenic assay of HK1, CNE2, CNE1 and CR cells after exposure to 0 and 6 Gy radiation ($n=3$; $P<0.05$). **(B)** Western blotting analysis of PKC α expression in HK1, CNE1, CNE2 and CR cells, Tubulin was used as a loading control.

sensitivity index that includes PKC β expression for planning an individualized radiation dose for NPC.²¹ These studies indicate that the PKC signaling pathway is involved in the regulation of resistance to therapy and, specifically, radio-resistance. Therefore, the results of our present study confirm the link between PKC α expression and radio-resistance and provide evidence for the potential value of PKC α as a molecular marker for guiding individualized radiation therapy for NPC. As a marker in NPC, high PKC α expression would indicate the need for a relatively high dose of radiation therapy to improve the likelihood of local disease control and thereby improve patients' survival. It is foreseeable that under the guidance of individualized PKC α levels, radiation oncologists will be able to make radiation treatment plans more individualized. At the same time, comprehensive treatment plans including chemotherapy could be optimized to avoid excessive treatment. In addition, in view of the results of the present study,

the significance of PKC α expression in the WHO classification deserves further exploration.

The main types of NPC are WHO type II and III, and WHO type I NPC, which is also defined as keratinizing carcinomas, is extremely rare. In our study, PKC α expression in WHO type II cases was higher than that in type III cases, which is consistent with previous conclusion that PKC is related to the differentiation of normal cells and malignant tumors.^{22–24} However, the NPC-specific mechanism of PKC α in the regulation of cancer cell differentiation requires further investigation.

A limitation of this study was the inability to determine the relationship between the expression of PKC α and the immunohistochemical markers of differentiation in order to understand more clearly whether PKC α is related to the differentiation of nasopharyngeal carcinoma. In addition, the apoptosis inhibition pathway related to PKC α has not been clarified and is worthy of further exploration.

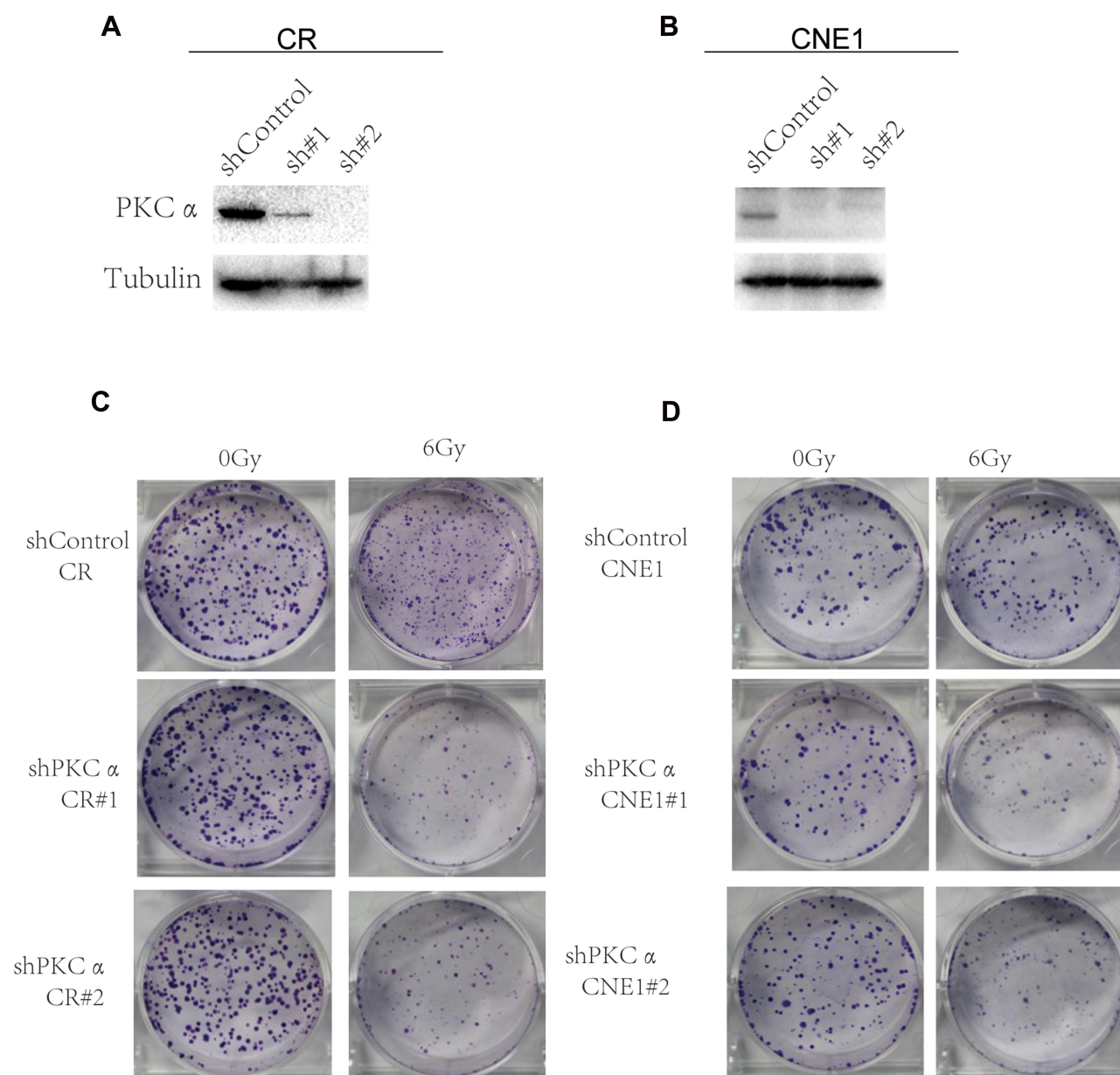


Figure 4 Inhibition of PKC α sensitizes NPC cells to radiation in vitro. **(A)** Western blotting of PKC α expression in CR cells after treatment with shControl, shPKC α #1, or shPKC α #2. **(B)** Western blotting of PKC α expression in CNE1 cells after treatment with shControl, shPKC α #1, or shPKC α #2. **(C)** Clonogenic assay for CR cells treated with shControl or shPKC α and later exposed to 0 or 6 Gy radiation (n=3; P<0.05). **(D)** Clonogenic assay for CNE1 cells treated with shControl or shPKC α and later exposed to 0 or 6 Gy radiation (n=3; P<0.05).

Conclusions

PKC α expression is increased in NPC tissues as well as in radio-resistant NPC cell lines. Moreover, a significant association was found between PKC α expression and the 5-year PFS and LRFS of NPC patients. Together, the findings of this study indicate that PKC α is a potential molecular marker of radio-resistance in

NPC, and thus, measurement of its expression level may aid in treatment planning for NPC patients. Moreover, our findings that loss of PKC α expression rendered NPC cells more resistant to radiation therapy both in vitro and in vivo suggest that PKC α is also a potential target for enhancing the radio-sensitivity of NPC.

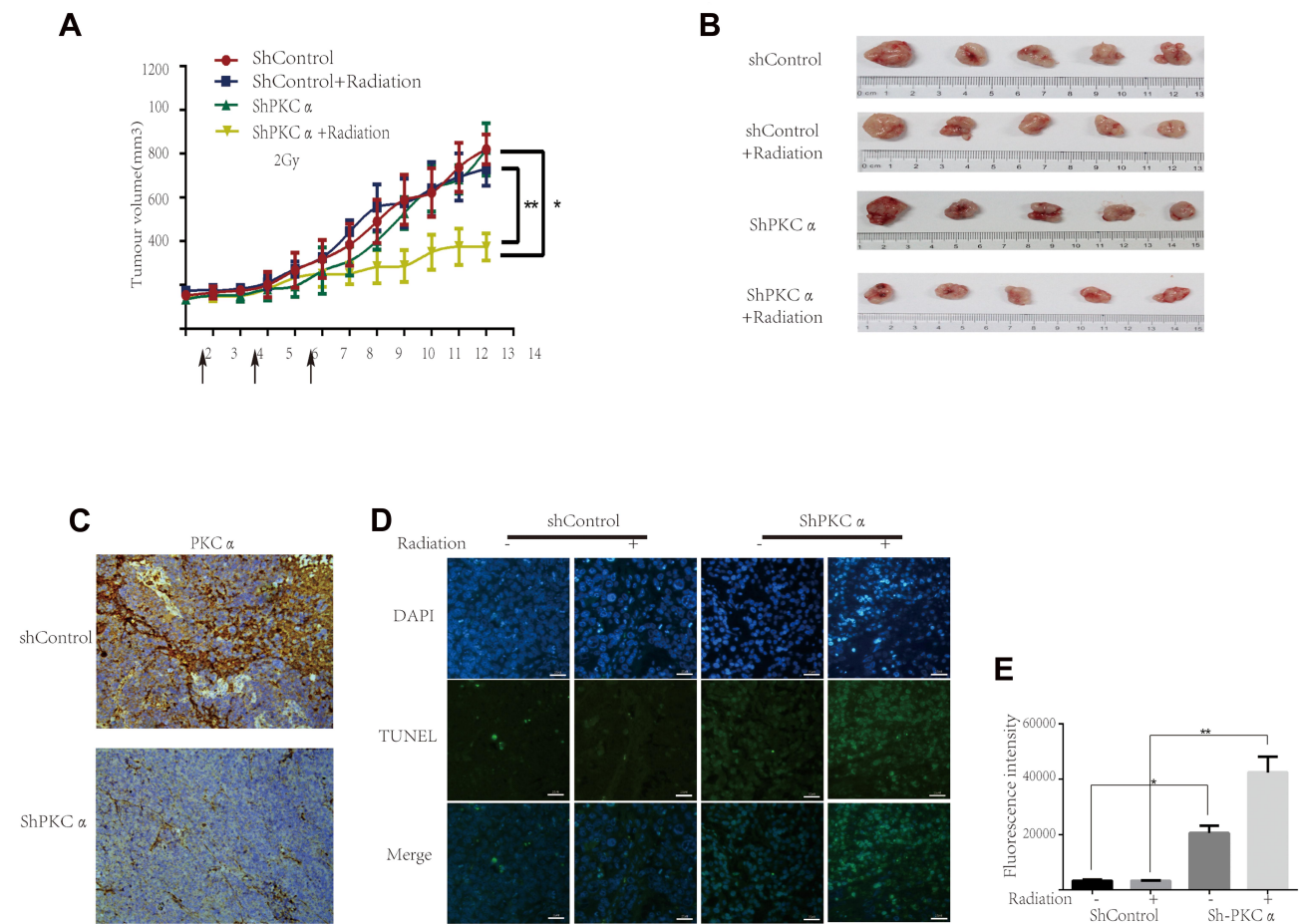


Figure 5 Inhibition of PKC α sensitized NPC xenografts to irradiation in vivo. **(A)** Growth curves of shControl- and shPKC α -CR tumors with or without radiation treatment (2 Gy \times 3 fractions, n=5). **(B)** Nude mouse tumor growth status (n=5). **(C)** Immunohistochemical staining of PKC α in shControl- and shPKC α -CR xenografts (scale bar = 25 μ m). **(D)** Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of apoptotic cells in shControl- or shPKC α -CR xenografts with or without radiation treatment (scale bar = 25 μ m). **(E)** Quantitative analysis of TUNEL staining data in (C). TUNEL-positive cells were counted in at least 8 fields on each slide. *P<0.05, **P<0.01.

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Disclosure

The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

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