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PRKD3 promotes malignant progression of OSCC by downregulating KLF16 expression

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Abstract. – OBJECTIVE: We aimed to clarify the molecular mechanism of how PRKD3 promotes the malignant progression of oral squamous cell carcinoma (OSCC).

PATIENTS AND METHODS: 62 cases of OSCC tissues and normal adjacent ones which were further confirmed by a qualified pathologist were collected from patients in the Department of Pathology and Stomatology of our hospital. PRKD3 expression in the above tissue samples was studied by quantitative real-time polymerase chain reaction (qRT-PCR) analysis, and its relationship with clinicopathological characteristics of these OSCC patients was analyzed. Meanwhile, a PRKD3 knockdown expression model was constructed in OSCC cell lines for cell functional experiments. The relationship between PRKD3 and KLF16 was elucidated through bioinformatics and Luciferase reporter gene experiments.

RESULTS: Our data showed that PRKD3 expression in OSCC specimens was remarkably higher than that in adjacent ones. PRKD3 expression showed positive association with the incidence of distant metastasis, but not with other clinical indicators such as gender, age, tumor stage or lymph node metastasis incidence. Patients with high PRKD3 expression had lower overall survival compared to those with low expression. In addition, OSCC cells migration ability and invasiveness were remarkably attenuated after PRKD3 was knocked down. Bioinformatics and Luciferase assay revealed that PRKD3 could directly bind to KLF16 and Western blot suggested that KLF16 was upregulated after PRKD3 was knocked down. In addition, knocking down KLF16 reversed the inhibitory effect of PRKD3 knockdown on invasiveness and metastasis of OSCC cells.

CONCLUSIONS: The highly-expressed PRKD3, remarkably associated with metastasis incidence and poor prognosis of OSCC patients, may accelerate the malignant progression of OSCC through modulating KLF16 expression.

Key Words:
PRKD3, KLF16, Oral squamous cell carcinoma, Malignant progression.

Introduction

Oral squamous cell carcinoma (OSCC), one of the major diseases endangering human health, refers to the squamous cell carcinoma of the oral mucosa. It is the most common malignant tumor of the oral cavity, accounting for about 90% of the malignant tumors of the oral cavity[1-3]. Worldwide, there are about 405,000 new cases of OSCC and about 211,000 deaths every year, and the 5-year survival rate is less than 60%[1,2]. The location of OSCC is often limited and are close to important organs, such as the brain, important blood vessels in the neck, and the upper respiratory tract, etc., which seriously restricts the surgical scope; meanwhile, the large maxillofacial defects and functional defects caused by surgery directly affect the quality of life of patients[4,5]. At present, the clinical treatment of OSCC is still not very satisfactory, the main reasons are as follows. First, there is no specific and effective treatment for oral squamous cell carcinoma. Second, current pathological diagnostic techniques cannot accurately determine the biological characteristics of OSCC, resulting in different prognosis of patients with the same clinical stage for the same treatment[6-8]. Therefore, it is of great significance for the early diagnosis, treatment and prognosis judgment of OSCC to deeply explore the molecular mechanism of the occurrence and development of OSCC and find the markers of gene diagnosis and therapeutic targets[9,10].

PRKD is a serine/threonine protein kinase, belonging to the calcium/calmodulo kinase

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(CaMK) superfamily[11,12], and there are three members of the family, PRKD1, PRKD2 and PRKD3[12]. PRKDs possess numerous molecular targets and play a pivotal role in various cell biological functions, such as cell migration, cell proliferation, protein transport, epithelial interstitiation, angiogenesis and gene transcriptional regulation[13,14]. However, its dysregulation will affect the normal behavior of cells and eventually produce carcinogenesis, which is why abnormal expression of PRKDs has been found in many tumors[14,15]. The function of PRKDs is tissue-specific, serving as a tumor suppressor or oncogene in different tissues[15]. PRKD1 is absent in some aggressive types of OSCC, while PRKD3, which acts as an oncogene, is upregulated, making PRKDs an effective target for the treatment of OSCC[16,17]. Additionally, inhibition of PRKD3 or treatment with PRKDs broad-spectrum inhibitors can be used as an effective treatment for certain types of OSCC[17]. However, until now, little is known about the substrates regulated by PRKDs, and few studies have[17] been conducted on the signal transduction network of PRKDs. Therefore, we explored the signal transduction network of PRKDs and the key nodes in the network in OSCC through multiple omics, so as to provide corresponding measures and potentially effective treatment methods for the treatment of OSCC.

We apply quantitative real-time polymerase chain reaction (qRT-PCR) technique to detect PRKD3 mRNA expression in 62 cases of OSCC tissues and discusses its association with certain clinical pathological characteristics of these OSCC patients including tumor TNM (tumor node metastasis) stage, metastasis incidence, patient' age, gender, so as to illustrate the role of PRKD3 in the progression of OSCC and thus provide a new train of thought and theoretical basis for early diagnosis, gene therapy and prognosis prediction of this cancer.

Patients and Methods

Patients and OSCC Samples

A total of 62 cases of pathologically confirmed OSCC tissue samples and corresponding clinical and pathological data were collected from the Department of Pathology and Stomatology of our hospital. All patients did not accept radiotherapy, chemotherapy or any biological treatment before surgery. This investigation complies with the Helsinki Declaration Clinical Practice Guidelines. Exclusion criteria: patients complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure. The clinical TNM staging of tumors was based on the International Union Against Cancer (UICC) standard. This study was approved by the Ethics Committee of The First People's Hospital of Lianyungang, and the signed written informed consents were obtained from all participants before the study.

Cell Lines and Reagents

Human OSCC cells (Fadu, SCC-25, CAL-27, Tca8113) and one normal human oral epithelial cell (Hs 680.Tg) purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured with Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C in an incubator with 5% CO_2.

Transfection

Lentiviral transfection was performed with sh-PRKD3 (GenePharma, Shanghai, China) when cell density reached to 35%-50% according to the manufacturer's instructions. These cells were harvested 48 hours later for these experiments, including qRT-PCR, Western Blot, cell function experiment and so on.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in a 96-well plate (2×10^3 cells/well) with 100 uL culture medium. CCK-8 test (Dojindo Molecular Technologies, Kumamoto, Japan) was performed based on the manufacturer's protocol. The absorbance value at 450 nm of each well was measured by a microplate reader.

Transwell Cell Migration Assay

Transwell chambers (Millipore, Billerica, MA, USA) were inserted by using a 24-well plate according to the manufacturer's instructions. 3×10^5 cells suspended in 200 µL of serum-free medium was applied in the upper layer of the chamber with 600 µL of medium in the bottom. After 48-h incubation, migratory cells in the bottom were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 10 random selected fields per sample.

Cell Wound Healing Test

After 48 h of transfection, cells were digested, centrifuged and resuspended in medium without FBS to adjust the density to 5 x 10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well), and the confluency of the cells reached 90% or more the next day. After the stroke, cells were rinsed gently with phosphate-buffered saline (PBS) for 2-3 times and observed again after incubation in low-concentration serum medium for 24 h.

QRT-PCR

TRIzol method (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from tissue samples. Complementary deoxyribose nucleic acid (cDNA) synthesis was accomplished using Prime Script reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). qRT-PCR was performed according to the instructions of SYBR® Premix Ex Taq ™ (TaKaRa, Otsu, Shiga, Japan) kit, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal reference. Primers used in qPCR reaction: PRKD3 forward: 5'-GAGCCTGCCACTGCTAACTA-3'; reverse: 5'-GTCCTCATTTTCATTCTGGGGG-3'. KLF16 forward: 5'-GGACGCACACAGGGGAAC-3'; reverse: 5'-CACAGAGGGCAGGAGAAGC-3'. GAPDH forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3'; reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Cells were lysed, shaken on ice for 30 minutes, and centrifuged at 14,000 × g for 15 minutes at 4°C. Immunoblotting was carried out using primary antibodies against PRKD3 (1:1500), KLF16 (1:1500), and labeled secondary antibody (1:1000). The intensity of protein expression was determined using alpha SP image analysis software.

Luciferase Assay

A sequence of 300 base pairs upstream and downstream of the 3′-untranslated region (3′-UTR) conservative binding site of PRKD3 and the target gene KLF16 was synthesized, and amplified using PCR technology, and the PCR product was purified after amplification. After preparing the reagents in the Dual-Luciferase reporter gene detection system, the Luciferase luminescence value was detected. Then, stop solution was added and the *Renilla* luminescence value was measured.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 program (IBM Corp., Armonk, NY, USA) was used for processing. The data were presented as X ± SD (standard deviation). $p<0.05$ was considered statistically significant. Continuous variables were analyzed by *t*-test, categorical variables were analyzed by χ^2-test or Fisher's exact probability method. Kaplan-Meier method was used to evaluate the prognosis survival time of patients, and the differences between different curves were compared using Log-rank test.

Results

PRKD3 Is Highly Expressed In OSCC

PRKD3 expression in 62 patients with OSCC detected by qPCR was remarkably higher than that in corresponding adjacent normal specimens (Figure 1A). We divided above OSCC tissue specimens into high PRKD3 expression and low expression group (Table I), and detected a positive correlation between PRKD3 level and the incidence of distant metastasis, suggesting that overexpression of PRKD3 may be relevant to the malignant degree of OSCC (Figure 1B). However, no significant difference was detectable between PRKD3 level and patients' gender, age, T stage or lymph node metastasis. Figure 1C shows that patients in high PRKD3 expression group had lower survival rate compared to patients in low expression group (Figure 1C). In addition, *in vitro* cell experiments also indicated that PRKD3 was remarkably overexpressed in OSCC cell lines in comparison to the normal human oral horizontal cell (Hs 680.Tg) (Figure 1D).

Knockdown of PRKD3 Suppresses the Invasiveness of OSCC Cells

In OSCC cell lines Fadu and CAL-27, transfection of two sets of PRKD3 lentiviral knockdown vectors with different sequences effectively downregulated PRKD3 protein expression (Figure 2A). Subsequently, transwell experiments and cell wound healing assay revealed that knockdown of PRKD3 remarkably inhibited the migration (Figure 2B) and crawling ability (Figure 2C) of OSCC cells.

KLF16 Is the Target of PRKD3

To further clarify by which PRKD3 accelerates the malignant progression of OSCC, we performed bioinformatics analysis and found a potential re-

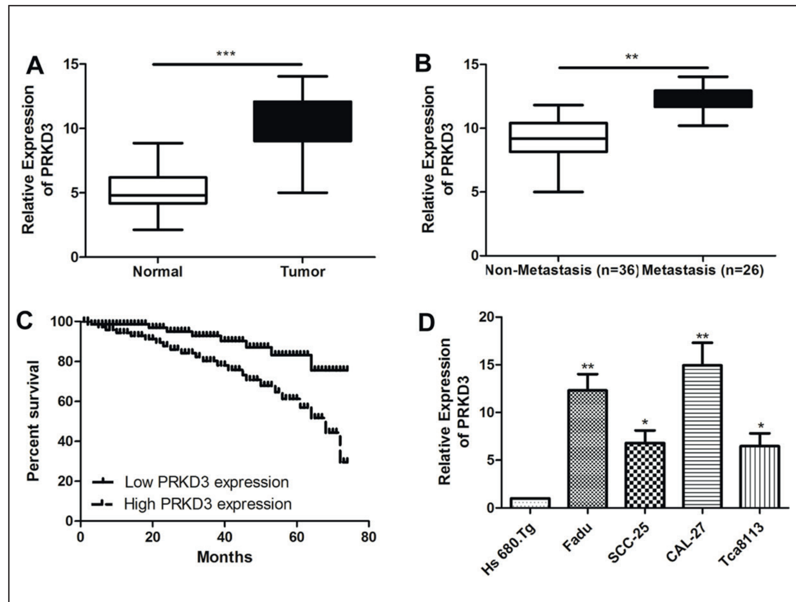

Figure 1. PRKD3 is highly expressed in oral squamous cell carcinoma tissues and cell lines. **A,** qRT-PCR was used to detect the expression of PRKD3 in tumor tissues and adjacent tissues of oral squamous cell carcinoma. **B,** qRT-PCR was used to detect the expression level of PRKD3 in patients with oral squamous cell carcinoma with or without distant metastasis. **C,** Kaplan Meier survival curve of patients with oral squamous cell carcinoma based on PRKD3 expression; the prognosis of patients with high expression was significantly worse than that with low expression. **D,** qRT-PCR was used to detect the expression level of PRKD3 in oral squamous cell carcinoma cell lines. Data are average ± SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

lationship between KLF16 and PRKD3. KLF16 was remarkably upregulated in OSCC cells after knockdown of PRKD3, measured by Western blot assay (Figure 3A). In addition, the results of Luciferase assay showed that overexpression of KLF16 remarkably attenuated the Luciferase activity of wild-type PRKD3 vectors ($p<0.05$), without reducing that of mutant vectors or empty vectors, further proving that PRKD3 can be targeted by KLF16 through this binding site (Figure 3B). Meanwhile, a significant reduction in KLF16 expression was found in above-mentioned OSCC tissue samples compared to the normal ones (Figure 3C), and a negative correlation between PRKD3 expression and KLF16 in OSCC tissues was shown in Figure 3D.

KLF16 Reverses the Effect of PRKD3 Expression on the Migration of OSCC Cells

To further verify the targeting effect of PRKD3 on KLF16, OSCC cell lines were co-transfected with PRKD3 and KLF16 knockdown vectors. The results of Western blot assay showed that the co-transfection with PRKD3 and KLF16 knockdown vectors could increase the reduced

Table I. Association of PRKD3 expression with clinicopathologic characteristics of oral squamous cell carcinoma.

Parameters	No. of cases	PRKD3 expression Low (%)	PRKD3 expression High (%)	*p*-value
Age (years)				0.436
< 60	25	16	9	
≥ 60	37	20	17	
Gender				0.065
Male	30	21	9	
Female	32	15	17	
T stage				0.200
T1-T2	50	31	19	
T3-T4	12	5	7	
Lymph node metastasis				0.121
No	38	25	13	
Yes	24	11	13	
Distance metastasis				0.008
No	36	26	10	
Yes	26	10	16	

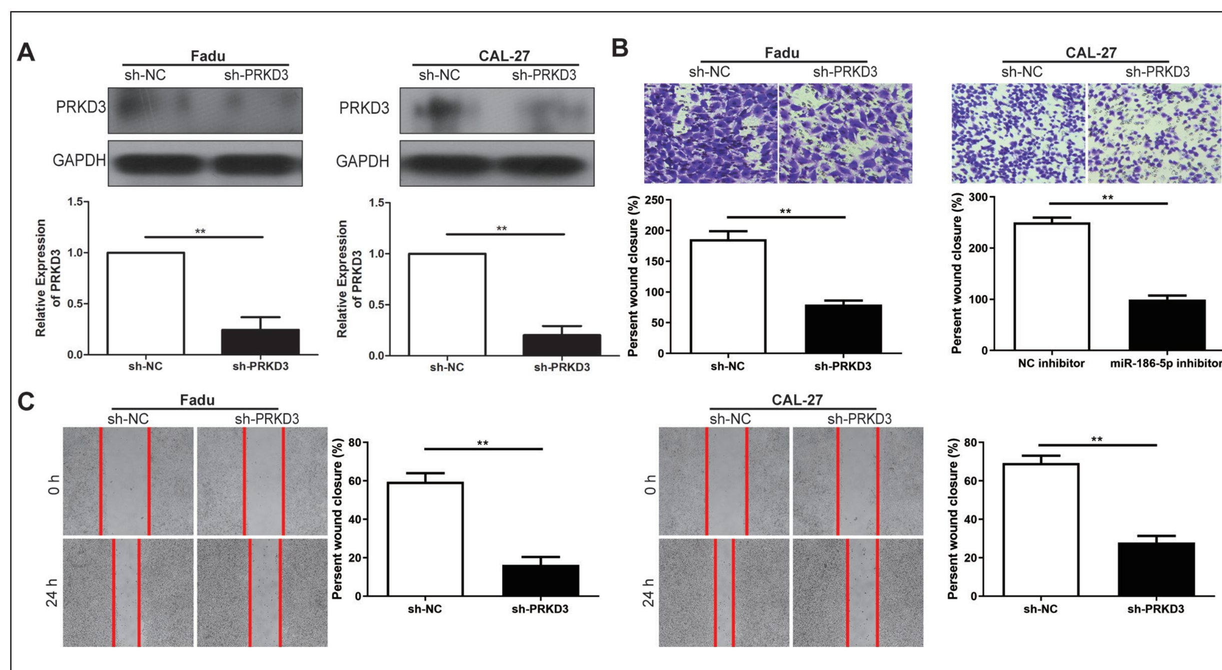

Figure 2. Knockdown of PRKD3 can inhibit the invasion and migration ability of oral squamous cell carcinoma cells. **A,** Western blot and qRT-PCR experiments verified the interference efficiency of PRKD3 after transfection of PRKD3 knockdown vector in oral squamous cell carcinoma cell lines Fadu and CAL-27. **B,** Transwell migration assay was used to detect the migration ability of oral squamous cell carcinoma cells after transfection of PRKD3 knockdown vector in oral squamous cell carcinoma cell lines Fadu and CAL-27 (magnification: 40 ×). **C,** Cell wound healing assay was used to examine the crawling ability of oral squamous cell carcinoma cells after transfection of PRKD3 knockdown vector in oral squamous cell carcinoma cell lines Fadu and CAL-27 (magnification: 40 ×). Data are average ± SD, ** $p<0.01$.

expression of PRKD3 induced by sh-PRKD3, as well as decrease the expression of KLF16, both in Fadu and CAL-27 cell lines (Figure 4A). Subsequently, we observed by transwell experiments that simultaneous downregulation of above two proteins reversed the inhibitory effect of sh-PRKD3 on OSCC cell migration capacity (Figure 4B).

Discussion

OSCC, one kind of squamous cell carcinoma of oral mucosa, is the most common malignant tumor in the oral cavity, accounting for about 90% of the malignant tumors[1-3]. The occurrence and progression of tumors are a process involving multi-genes, multi-factors and multi-stages, among which tumor cells evade immune surveillance and killing through certain mechanisms is an important link[18]. Existing domestic and foreign clinical and animal experiments have clarified some of the mechanisms of tumor immune evasion, such as the secretion of immunosuppressive molecules and the inhibition of tumor cell apoptosis or the induction of immune cell apoptosis, the blocking, hiding and isolation of tumor antigens, and weakened immunogenicity of tumor surface antigens[18]. OSCC, like other malignant tumors, are not only associated with cell proliferation and metastasis caused by activation of oncogene and inactivation of tumor suppressor gene, but also relevant to the reduction of cell apoptosis[4-7].

Studies[14-16] have confirmed the characteristic expression of PRKD3 in different types of tumor tissues and peripheral blood. PRKD3 is reported to be involved in tumor etiology, pathology, clinical classification and staging, tumor hormone secretion, tumor drug resistance and prognosis[11,14]. It can also be engaged in multiple cancer-related events, such as cell cycle, apoptosis, cell migration, angiogenesis, cancer metabolism, cancer immunity, and so on[12-15]. According to the analysis of protein phosphate profile, interacting proteome, and transcriptome data, it was found that cell cycle, cell movement-related pathways, adhesive connections,

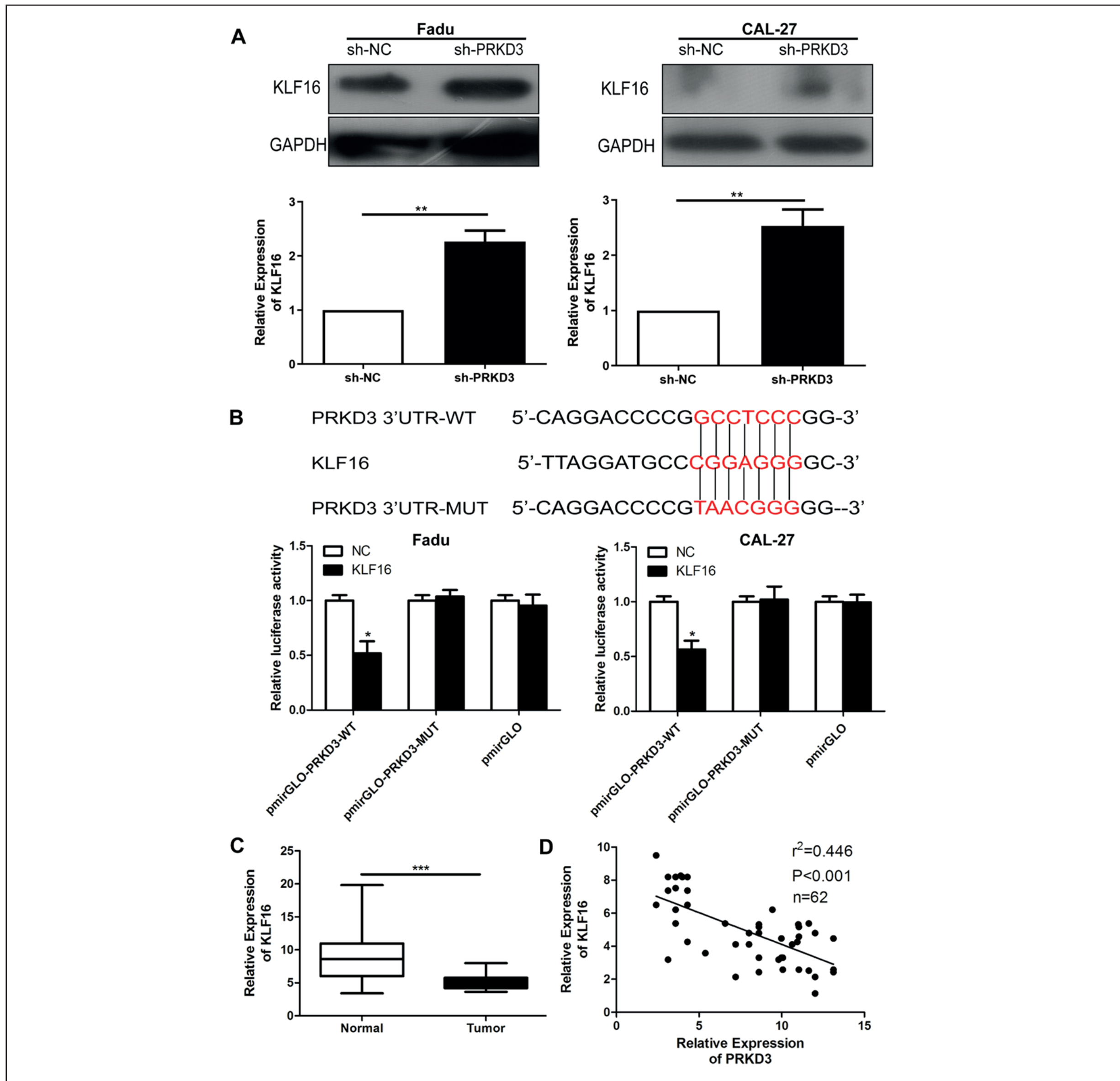

Figure 3. PRKD3 directly targets KLF16. **A,** Western blot and qRT-PCR verified the expression levels of KLF16 protein and mRNA after transfection of PRKD3 knockdown vector in oral squamous cell carcinoma cell lines Fadu and CAL-27. **B,** Dual Luciferase reporter experiment verified the direct targeting of PRKD3 to KLF16. **C,** qRT-PCR was used to detect the expression of KLF16 in tumor tissues and adjacent tissues of oral squamous cell carcinoma. **D,** The expression of PRKD3 and KLF16 was significantly negatively correlated in oral squamous cell carcinoma. Data are average ± SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

and actin cytoskeleton regulation continue to appear in the PRKDs regulatory mechanism network, indicating that the expression of PRKD3 can affect the activity of PRKDs, and significantly promotes the metastatic ability of cancer cells[15-17]. Therefore, this objective of this study was firstly to elucidate the oncogenic role of PRKD3 in the progression of OSCC, as well as its specific mechanism. This research detected a highly expressed PRKD3 both in OSCC tissues and cell lines. Meanwhile, clinicopathological data analysis revealed that the overexpression of PRKD3 was positively correlated with distant metastasis incidence and poor prognosis of OSCC patients, suggesting that PRKD3 may serve as an oncogene in this cancer. In addition, we detected that knockdown of PRKD3 could accelerate the migration of OSCC cells

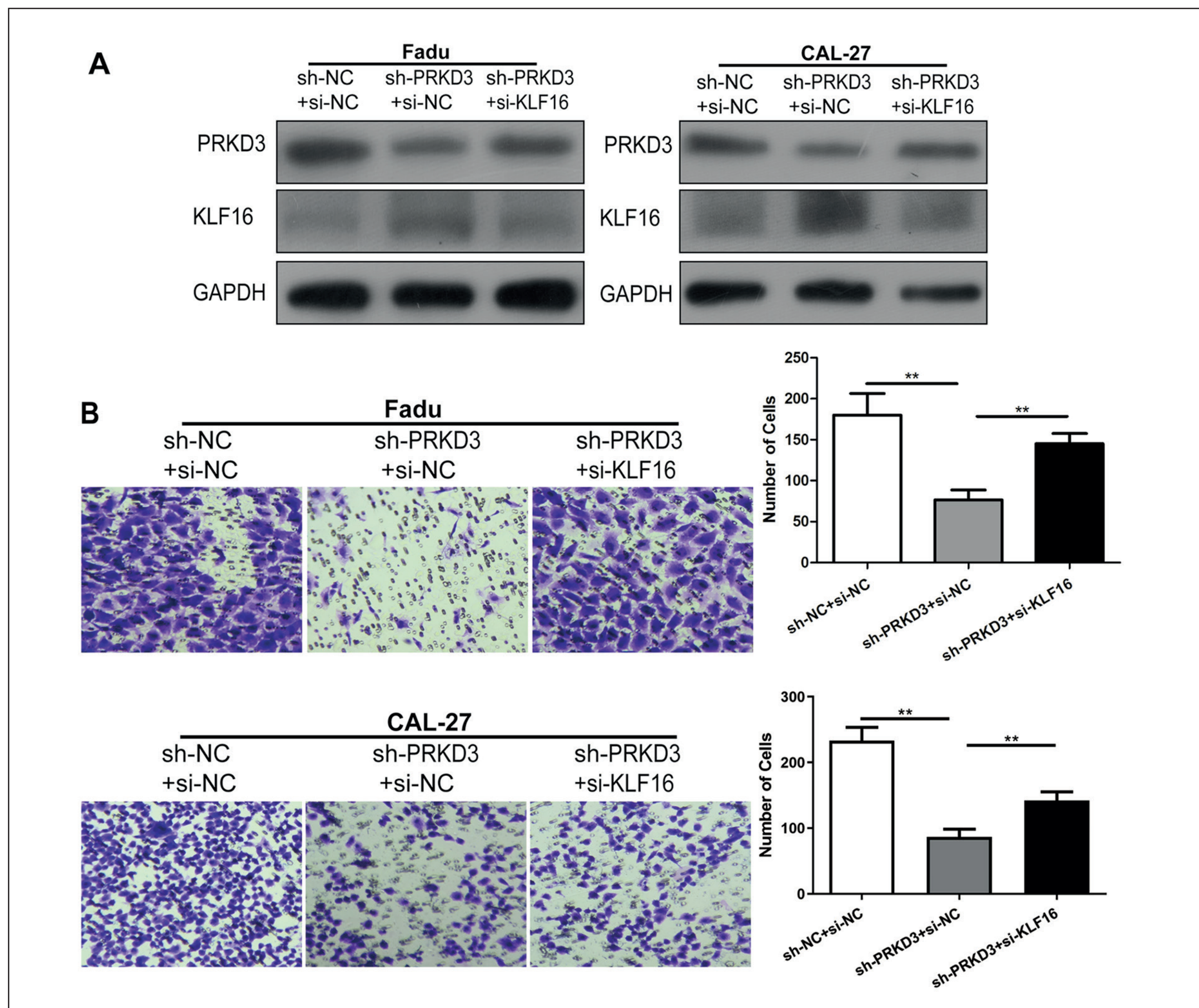

Figure 4. PRKD3 can regulate the expression of KLF16 in oral squamous cell carcinoma cell lines. **A,** Western blot and qRT-PCR were used to detect the expression levels of PRKD3 and KLF16 after transfection of PRKD3 and KLF16 knockdown vectors in oral squamous cell carcinoma cell lines Fadu and CAL-27. **B,** Transwell migration assay was used to detect the migration ability of oral squamous cell carcinoma cells after transfection of PRKD3 and KLF16 knockdown vectors in oral squamous cell carcinoma cell lines Fadu and CAL-27 (magnification: 40 ×). Data are average ± SD, ** $p<0.01$.

by performing transwell and cell wound healing assay. However, the exact mechanism by which it regulates the malignant progression of OSCC remains elusive.

Bioinformatics analysis and Luciferase reporter assay results demonstrated a binding relationship between KLF16 and PRKD3. Meanwhile, either in cells or in tissues, the expression of KLF16 and PRKD3 was found negatively correlated, suggesting that PRKD3 and KLF16 are closely associated with the malignant progression of OSCC. Finally, co-transfection experiments *in vitro* revealed that KLF16 knockdown could up-regulate PRKD3 expression, thus reversing the effect of reduced PRKD3 expression on the migration ability of OSCC. Our findings revealed that KLF16 could reverse the promotive effects of PRKD3 on the metastatic abilities in OSCC cell lines by *in vitro* experiments.

Conclusions

In summary, PRKD3, closely associated with metastasis incidence and poor prognosis of OSCC patients, can accelerate the malignant progression of this cancer through modulating KLF16, which may become a new target for OSCC gene therapy.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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