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Clinical significance of raf-1 kinase inhibitor protein in oral squamous cell carcinoma and its role in cell metastasis

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Abstract. – OBJECTIVE: To detect the expression pattern of raf-1 kinase inhibitor protein (RKIP) in oral squamous cell carcinoma (OSCC) samples and to explore its clinical significance in OSCC metastasis.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot assay were conducted to detect the expression of RKIP in OSCC tissues and cells. The relationship between RKIP expression and OSCC clinicopathological characteristics was statistically analyzed. Transwell assay, wound healing assay, and Western blot were used to detect the influence of RKIP on the metastasis ability of OSCC cells.

RESULTS: RKIP was significantly downregulated in OSCC samples. Low expression of RKIP predicted high incidence of metastasis in OSCC patients. *In vitro* experiments demonstrated that overexpression of RKIP could significantly inhibit invasion and migration abilities of OSCC cells.

CONCLUSIONS: RKIP was a novel factor involved in OSCC progression, which was a potential biomarker and therapeutic target for the patients.

Key Words:
Oral squamous cell carcinoma (OSCC), Raf-1 kinase inhibitor protein (RKIP), Invasion and migration, Epithelial-mesenchymal transition (EMT).

Introduction

Oral squamous cell carcinoma (OSCC) is the most common oral malignant tumor. Its incidence increases each year, seriously threatening human health[1]. Comprehensive therapy of surgery combined with chemoradiotherapy is generally adopted for OSCC patients at present. Great progresses have been made in the diagnosis and treatment of OSCC in recent years. However, its 5-year survival rate is still low. About 1/3 patients eventually and inevitably suffer relapse and metastasis. The poor prognosis in OSCC patients can be attributed to the strong local invasion and metastasis capacities and a high malignant degree[1,2]. Therefore, further exploring the molecular mechanisms of invasion and metastasis in OSCC and finding out the best therapeutic targets are reliable ways to improve the prognosis of OSCC.

Raf-1 kinase inhibitor protein (RKIP) is a conserved protein and also a member of the phosphatidyl ethanolamine-binding protein (PEBP) family[3]. It shows a close interaction with the Raf-1/MER/ERK pathway. The RKIP gene is located on human chromosome 12q24.23 and transcribed from 4 exons, with a messenger ribonucleic acid (mRNA) of 1,507 nucleotides in length. It plays a certain role in the pathophysiological process of cell membrane biosynthesis and nerve growth and apoptosis[4,5]. RKIP interferes in the Raf-1-MEK1/2-ERK1/2 pathway and represses the signal transduction of NF-κB and G protein-coupled receptor kinases[6,7]. In addition, abnormally expressed RKIP is associated with the development, progression, invasion and migration of many tumors[8-11]. The correlations of RKIP with clinicopathological features and prognosis in many tumors have been studied. However, studies on its role in OSCC are rare. Therefore, in this study, the possible role of RKIP in OSCC was explored in clinical sample analyses and *in vitro* experiments.

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Patients and Methods

Tissue Specimens

The research was approved by the Ethics Committee, and informed consent was obtained from patients or their family members. A total of 52 paraffin embedded OSCC sections were tissues in microarray which were obtained from Shanghai Xinchao Biotechnology Co., Ltd. The selection of patients was based on the Guideline proposed by the Union for International Cancer Control (UICC). The patients enrolled were diagnosed with primary OSCC, received no chemoradiotherapy before surgery, and underwent expanded resection of primary lesions and neck dissection (unilateral or supraomohyoid neck dissection). The clinical stage and pathological grade of these patients were determined according to the Union for International Cancer Control (UICC) TNM staging criteria (UICC2002) and the WHO pathological classification standard, respectively. Specimens were rapidly placed in cryogenic vials, marked with sample information, cryopreserved in liquid nitrogen, and then, transferred into a nitrogen canister until RNA extraction.

Cell Culture and Transfection

The OSCC cell line Tca8113 and the normal oral mucosal keratinocyte cell line HOK were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) in an incubator at 37°C. The medium was replaced regularly for passage according to cell growth. Then, the cells were transferred into a 6-well plate and transfected with LV-RKIP (100 pmol) and LV-NC (negative control) using Lipofectamine™ 2000 and OPTI-MEMI (Invitrogen, Carlsbad, CA, USA) when 60% of them were fused. After 48 h, the total RNA was extracted for later use.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNAs were extracted from tissue samples or transfected cell samples by TRIzol method (Invitrogen, Carlsbad, CA, USA) and quantified using a Nano-Drop2000 microspectrophotometer. RNA was purified using the DNase I. The reverse transcription reaction was conducted using a reverse transcription kit according to the instructions of the kit. The template strand complementary deoxyribose nucleic acid (cDNA) was subjected to qRT-PCR. CT value was recorded for calculating the relative expression level of RKIP by the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-GCACCGTCAAGGCTGAGAAC-3', and reverse: 5'-TGGTGAAGACGCCAGTGGA-3'; RKIP: forward: 5'-CAATGACATCAGCAGTGGCACAGTC-3', and reverse: 5'-CACAAGTCATCCCACTCGGCCTG-3'.

Western Blots Analysis

After lysis with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China), the protein was quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China), followed by denaturation and centrifugation. Then, the supernatant was collected, and the protein was loaded. Thereafter, Western blotting was routinely performed. The expression of target proteins was expressed as the ratio of the gray value of target band to the gray value of GAPDH.

Cell Invasion and Migration

Transfected cells were inoculated into a 6-well plate. Until the cell confluence was about 90%, a 200 μL sterile pipettor was utilized to make scratches, with the tip perpendicular to the 6-well plate. After that, the cells were washed with phosphate-buffered saline (PBS) and photographed as 0 h. Cells were cultured in serum-free medium for 24 h and photographed for calculating wound closure percentage.

In migration assay, a transwell chamber was used. Serum-free medium was added to the upper chamber, and complete medium containing 10% FBS was added to the bottom chamber. Next, the transwell chamber was incubated for 24 h. Afterward, the medium in the chambers was aspirated, and the chamber was washed with PBS. Then, the cells in the upper chamber were wiped off with cotton swabs, and those in the bottom were fixed with 4% paraformaldehyde for 30 min, washed with PBS for 3 times, and stained with crystal violet for 30 min. Migratory cells were photographed using a microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test, followed by post-hoc test (Least Significant Difference). Chi-square test was performed for

the association between RKIP level and clinicopathological parameters of OSCC patients. The influence of RKIP on cell function was evaluated using the *t*-test. *p*-values < 0.05 were considered statistically significant.

Results

The Expression of RKIP In Clinical Tissues and the Relationship of RKIP With Clinicopathological Features

In this study, qRT-PCR was performed to detect the expression level of RKIP in 52 pairs of OSCC tissues and adjacent normal tissues which were obtained from Shanghai Xinchao biotechnology Co.,Ltd. The results showed that the expression level of RKIP in OSCC tissues was significantly lower than that in para-cancer tissues (Figure 1A). According to the median expression level of RKIP, the patients were divided into RKIP high-expression group and low-expression group. The association between RKIP expression and clinicopathological features of patients was analyzed (Table I). The expression level of RKIP was associated with tumor grade, lymph node metastasis, and TNM stage of OSCC.

The Expression of RKIP In Cell Lines and the Effects of RKIP on the Metastasis Ability of Cells

In cell function experiments, we first detected the expression level of RKIP in OSCC cell line Tca8113 and the normal oral mucosal keratinocyte cell line HOK. Consistent with the expression pattern in clinical samples, RKIP was downregulated in OSCC cells (Figure 1B). To further study the effect of RKIP on the invasion and migration ability of OSCC cells *in vitro*, the RKIP over-expression cell model was constructed by transfecting LV-RKIP into Tca8113 cells. Its transfection efficiency was verified by detecting mRNA and protein levels by qRT-PCR and Western blot, respectively. As shown in Figure 2A-2B, transfection of LV-RKIP could effectively increase RKIP expression in OSCC cells.

To explore the possible mechanism of RKIP on regulating invasion and migration in OSCC, the elative levels of epithelial-mesenchymal transition (EMT)-associated markers were examined. The results showed that after LV-RKIP transfection, the epithelial molecular marker (E-cadherin) was significantly upregulated, while mesenchymal molecular markers (N-cadherin and vimentin) were downregulated, suggesting that RKIP affected the EMT in OSCC cells (Figure 2C-2D). EMT occurs in the first step of tumor metastasis, and it is also the key link for tumor invasion and metastasis. Many signaling pathways and cytokines are involved in the regulation of EMT. This study first revealed that RKIP inhibited EMT in OSCC, suggesting that RKIP might be a very important target for anti-metastasis therapy.

Invasion and migration ability are the basis of tumor cells to metastasize[12]. In transwell assay, transfection of LV-RKIP could significantly reduce the invasion ability of Tca8113 cells (Figure 3). Similar results were obtained from the wound

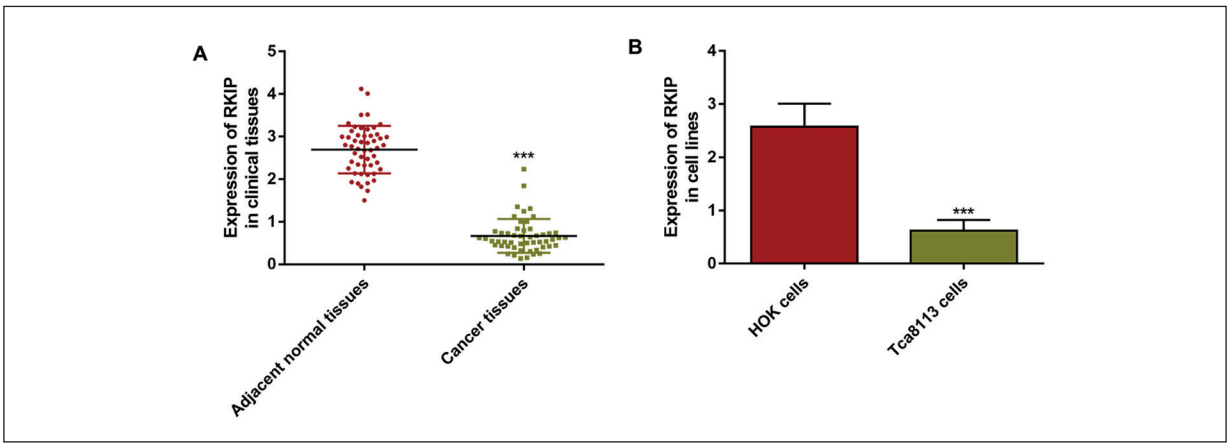

Figure 1. A, Expressions of RKIP were measured in OSCC and para-cancer tissues by qRT-PCR. (***p<0.001). **B,** Expressions of RKIP were measured in OSCC cell line (Tca8113) and normal oral mucosal keratinocyte cell line (HOK) by qRT-PCR. (***p<0.001).

Table I. The association between RKIP expression level and clinicopathological characteristics of OSCC.

Parameters		Total	RKIP level		p
			Low	High	
Gender	Female	23	13	10	0.577
	Male	29	13	16	
Age	≥ 60	39	17	22	0.748
	< 60	13	7	6	
Smoking	Non-smoker	25	10	15	0.399
	Smoker	27	15	12	
Tumor site	Tongue	20	11	9	0.468
	Non-tongue	32	13	19	
Grade	Well/moderate	36	11	25	0.001
	Poor	16	14	2	
T stage	T1-T2	34	15	19	0.787
	T3-T4	18	8	10	
Lymph node metastasis	No	34	16	18	0.025
	Yes	18	15	3	
TNM stage	I-II	31	10	21	0.002
	III-IV	21	17	4	

healing assay (Figure 3), suggesting that RKIP could weaken the ability of tumor cells to migrate.

Discussion

Oral cancer is the 6th most common tumor in the world[13]. Due to the particularity of the oral, maxillofacial anatomy, and the malignant biological behaviors of OSCC, severe challenges are faced in the treatment of oral cancer. Surgery or radiotherapy achieves a good therapeutic effect on early-stage OSCC[14]. However, most OSCC patients tend to have cervical lymph node metastasis or distant metastasis at the time of diagnosis. Surgery combined radiation or chemotherapy could provisionally delay the progression of stage III/IV OSCC but cannot improve their long-term survival rate. As a result, OSCC gradually becomes a major public health issue.

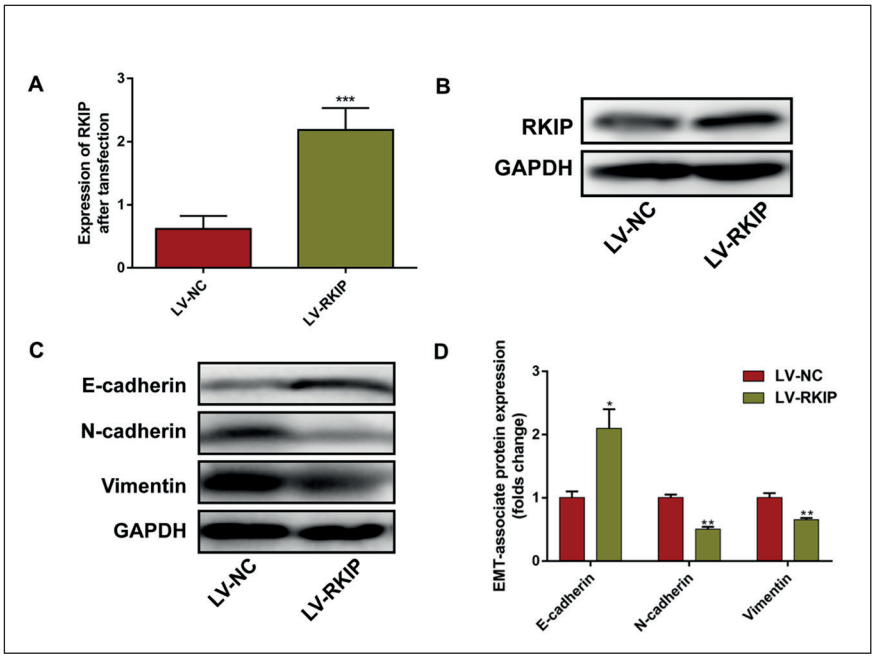

Figure 2. A-B, Expression of RKIP after transfection of LV-RKIP or LV-NC were measured by qRT-PCR and Western blot. (***p<0.001). **C-D,** EMT-associated protein expressions detected by Western blot assay. (*p<0.05, **p<0.01).

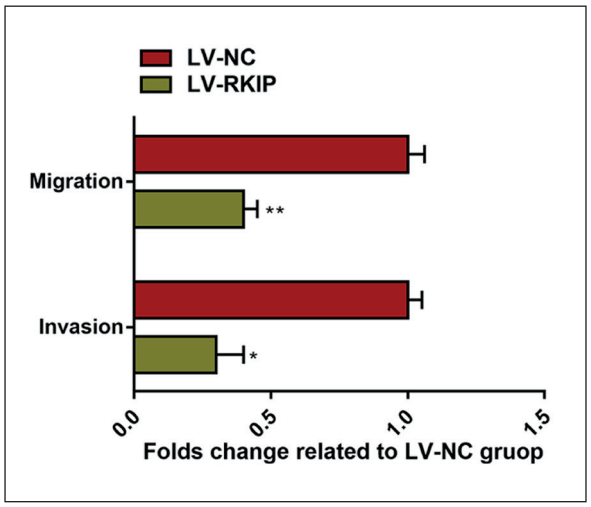

Figure 3. Cell invasion and migration detected using transwell and wound healing assay and detected by microscope (magnification ×200). (*p<0.05, **p<0.01).

The invasion and migration of tumors are markers of their malignancy and directly related to the prognosis, which are affected by many factors. Studying the metastasis mechanism of tumors at the molecular level could find out therapeutic targets, providing a theoretical basis for the early intervention and targeted therapy of tumors. The exact mechanism of RKIP in tumor metastasis remains unclear. The inhibitory role of RKIP in tumor invasion and migration of tumors has been identified by suppressing tumor angiogenesis and intravascular invasion. Recent research suggested that RKIP regulates the expressions of matrix metalloproteinases (MMPs) to repress the migration and invasion of tumors. Beshir et al[15] revealed that RKIP negatively regulates some MMPs, especially MMP-1 and MMP-2, thus impeding the spread of tumors. Furthermore, Beach et al[16] found that the expression of Snail, a vital gene involved in EMT, is negatively regulated by RKIP in prostate cancer cells.

EMT is a physiological phenomenon in the development of multicellular biological embryos and a basic biological event maintaining the balance of living tissues. In addition, EMT plays a crucial role in the invasion and metastasis of multi-type tumors[17-20]. As a molecular event in the development and progression of epithelial-derived tumors, EMT is mainly manifested as a biological process that involves a transition from epithelial phenotypes of cells to mesenchymal phenotype through specific procedures. Major characteristics of EMT are the downregulated E-cadherin and upregulated Vimentin and N-cadherin. EMT weakens cell-cell adhesion, resulting in increased mobility and invasion.

In our *in vitro* study, Tca8113 cells were transfected with LV-RKIP to upregulate RKIP. The experimental results showed that compared with those in the control group, the expression of E-cadherin in Tca8113 cells was upregulated, while Vimentin and N-cadherin were downregulated after the overexpression of RKIP. It is suggested that RKIP induced EMT in OSCC. Subsequently, both wound healing and transwell assay showed that the overexpression of RKIP weakened the migration and invasion capacities of Tca8113 cells than those of controls. It is indicated that overexpression of RKIP could limit the invasion and migration of OSCC cells, consistent with our previous findings.

Conclusions

Shortly, RKIP affected the metastasis of OSCC, and it might become a new diagnostic index and therapeutic target for OSCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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