Clinicopathological features and prognostic implications of Raf kinase inhibitor protein downregulation in tongue squamous cell carcinoma

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Abstract. Raf kinase inhibitor protein (RKIP) is recognized as a suppressor of metastasis, and the downregulation of RKIP is associated with aggressive events and a poor outcome in a variety of solid tumors. However, the clinical relevance of RKIP expression in tongue squamous cell carcinoma (TSCC) remains unclear. In the present study, the expression of RKIP in 85 pairs of TSCC and corresponding adjacent non-cancerous tissues, 30 matched metastatic lesions from the cervical lymph nodes and 32 oral leukoplakia samples were assessed using immunohistochemical methods. The association between RKIP expression and clinicopathological features was then evaluated. Kaplan-Meier survival analysis and Cox proportional hazards model were used to estimate the effect of RKIP expression on the survival time of patients with TSCC. The results revealed that RKIP expression was dramatically downregulated in TSCC, and to an even greater extent in metastatic lesions. RKIP downregulation was significantly associated with the presence of lymphatic metastasis and the clinical stage of TSCC. Furthermore, patients with low RKIP expression demonstrated a significantly shorter overall survival time. Multivariate analysis indicated that RKIP expression may be an independent prognostic factor in TSCC. In conclusion, the present findings indicate that the lack of RKIP expression is of clinical significance and may serve as a prognostic biomarker in TSCC.

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Introduction

Tongue squamous cell carcinoma (TSCC) is the most common type of oral cancer. According to data released by the American Cancer Society, an estimated 13,590 novel TSCC cases were predicted to occur in the USA in 2014, accounting for one-third of all oral cavity and pharynx cancers (1). TSCC tends to demonstrate more aggressive behavior, including a high frequency of local invasion and regional lymph node metastasis (2). Consequently, the mortality rate for TSCC continues to be high, with a typical overall five-year survival rate of <50% (3). In order to delineate the clinical behavior of TSCC and to personalize therapy, the identification of novel biomarkers for tumor aggressiveness is required.

Raf kinase inhibitor protein (RKIP) is a member of the phosphatidylethanolamine-binding protein family. RKIP was original identified as a physiological inhibitor of the Raf/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathway (4,5) and appears to be implicated in a variety of intracellular signaling pathways (6-8) that control cell proliferation (9), differentiation (10), adhesion (11) and epithelial-mesenchymal transition (12). RKIP has also been implicated in tumor progression. It was suggested that RKIP acts as a metastasis suppressor in a variety of malignancies, including prostate (13), breast (14), hepatocellular (15), colorectal (16) and gastric cancers (17). These previous findings prompted the elucidation of the clinicopathological features and implications of RKIP expression in patients with TSCC. Therefore, the present study investigated the protein levels of RKIP in non-cancerous and tumor tissues, and assessed the role of RKIP in the clinical outcome of the patients with TSCC.

Table I. Association between RKIP expression and clinicopathological features in patients with tongue squamous cell carcinoma.

Clinicopathological features	Patients, n	RKIP expression, n (%)		P-value
		High	Low	
Gender				
Male	51	19 (37.3)	32 (62.7)	0.153
Female	34	18 (52.9)	16 (47.1)	
Age, years				
≥55	40	16 (40.0)	24 (60.0)	0.536
<55	45	21 (46.7)	24 (53.3)	
Differentiation				
Well	49	23 (46.9)	26 (53.1)	0.460
Moderately or poorly	36	14 (38.9)	22 (61.1)	
pT stage				
T1-2	66	31 (47.0)	35 (53.0)	0.233
T3-4	19	6 (31.6)	13 (68.4)	
pN stage				
N0	55	29 (52.7)	26 (47.3)	0.021
N+	30	8 (26.7)	22 (73.3)	
pTNM stage				
I-II	45	25 (55.6)	20 (44.4)	0.018
III-IV	40	12 (30.0)	28 (70.0)	

[a]P-value of χ^2 test is shown. pTNM stage, pathological tumor-node-metastasis stage; RKIP, Raf kinase inhibitor protein.

Materials and methods

Patients and tissue samples. All tissue samples were obtained from the Affiliated Hospital of Stomatology, Sun Yat-Sen University (Guangzhou, China) between January 2007 and December 2012. In total, 85 pairs of paraffin-embedded tissue samples from patients with TSCC, consisting of TSCC tissue and adjacent non-cancerous tissues, were assessed. An additional 32 oral leukoplakia lesions were also analyzed (Table I). In patients that demonstrated lymph node involvement (n=30), the corresponding lymph node metastases were also examined. The specimens were obtained from the patients subsequent to radical surgery, with informed consent being obtained from all patients for the use of the surgically-resected specimens for research purposes, according to the guidelines for research on human tissues and samples set by the Institution Review Board of Sun Yat-Sen University. No patients received any form of adjuvant therapy prior to surgery. All histopathological diagnoses were based on the Union for International Cancer Control or the 2002 American Joint Committee on Cancer criteria (18), and the diagnoses were reviewed by two experienced pathologists.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were subjected to immunohistochemical analysis. The 4-μm tissue sections were routinely dewaxed, rehydrated and blocked using 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) at 95˚C for 20 min. To reduce non-specific binding, the slides were blocked with 10% goat serum for 30 min at room temperature. The tissue sections were incubated in a humidified chamber at 4˚C overnight with a primary polyclonal IgG rabbit anti-human RKIP antibody (dilution, 1:100; catalog no. sc-28837; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Subsequent to washing three times in phosphate-buffered saline (PBS), the slides were incubated with horseradish peroxidase and visualized with diaminobenzidine (DAKO Deutschland GmbH, Hamburg, Germany), and the slides were counterstained with hematoxylin. To confirm the specificity of the immunostaining, a negative control was included in each run by substituting PBS for the primary antibody.

Evaluation of immunohistochemical staining. Five random fields of each section were viewed under a light microscope (Axioskop 40; Zeiss, Oberkochen, Germany) at x400 magnification. The sections were independently examined and scored by three investigators, who were blind to the clinical features and outcomes of the cases. The expression of RKIP was scored by multiplication of the average signal intensity, which was scored on a scale of 0-3, and percentage of positively stained tumor cells, which was scored on a scale of 0-4, as previously described (18). The final immunoreactive score reported is the average of the scores from the three investigators. Receiver operating characteristic (ROC) analysis resulted in tissues with a final score >4 being classified as demonstrating high RKIP expression (sensitivity, 81.5%; specificity, 93.7%). The tissues that obtained a score ≤4 were classified as demonstrating low RKIP expression.

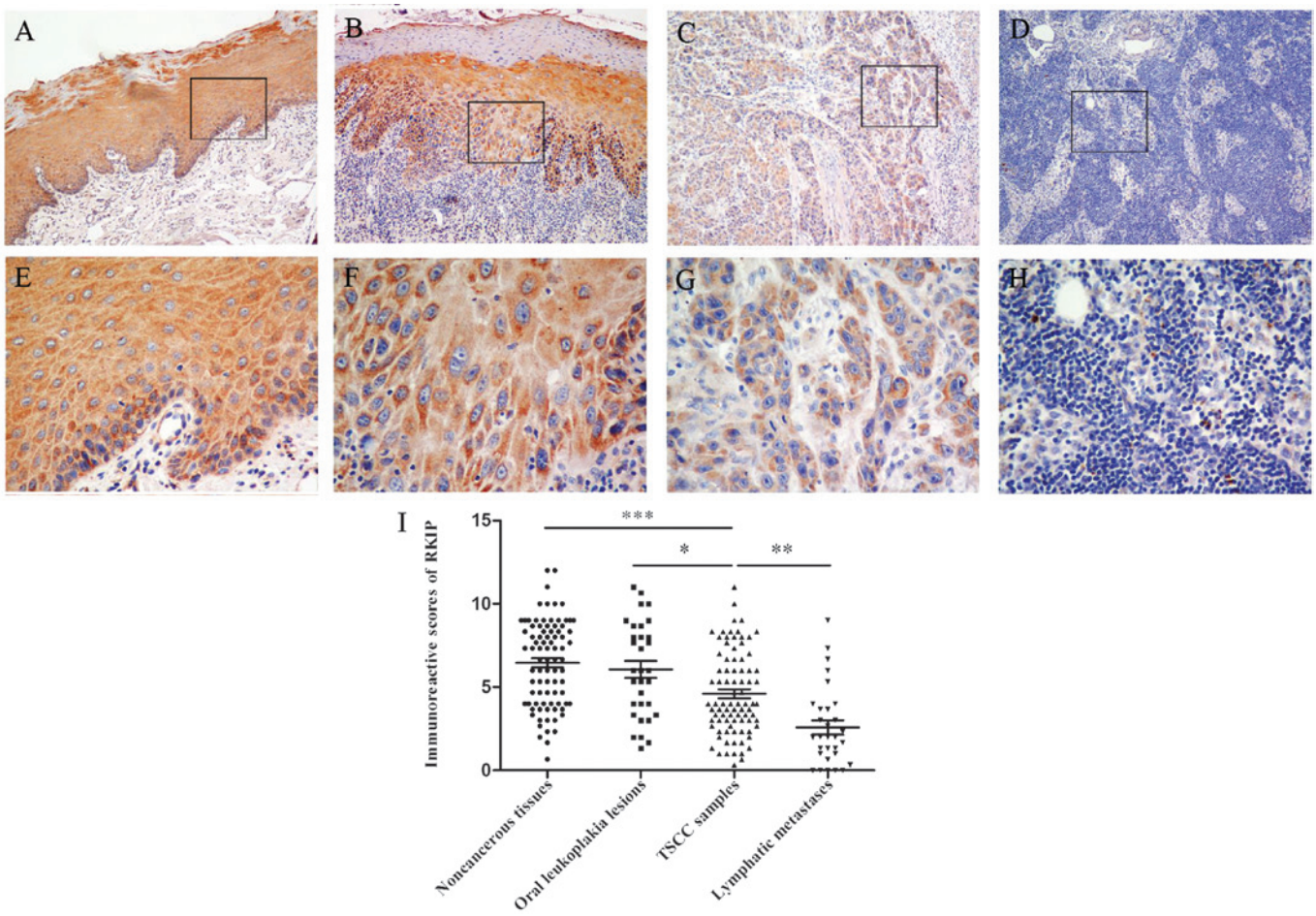

Figure 1. Expression of RKIP in TSCC tissues. (A) Strong RKIP staining in non-cancerous tongue tissue adjacent to TSCC. (B) Positive RKIP staining in oral leukoplakia sample. (C) Moderate RKIP staining in a TSCC tissue sample. (D) Absence of RKIP staining in matched metastatic lesions. (A-D) Magnification, x100. (E-H) Increased magnification of the rectangles in A-D, respectively. Magnification, x200. (I) A vertical scatter plot was depicted to demonstrate the relative expression level of RKIP in various tissues. RKIP, Raf kinase inhibitor protein; TSCC, tongue squamous cell carcinoma.

Statistical analysis. All statistical analyses were performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The association between RKIP expression and clinicopathological parameters was determined using the χ^2 test. Survival was evaluated using the Kaplan-Meier method, and the statistical significance of the differences between the survival of patients was examined using the Log-rank test. Analysis of the association between the survival time and the clinicopathological variables was performed by univariate and multivariate analyses using a Cox regression. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The clinicopathological characteristics of the patients are summarized in Table I. The patients consisted of 51 males and 34 females, with a median age of 54 years (range, 22-82-years). Post-operative pathological tumor-node-metastasis (pTNM) staging evaluation revealed stage I disease in 15 patients, stage II disease in 30 patients, stage III disease in 22 patients and stage IV disease in 18 patients. The histopathological diagnoses consisted of well-differentiated TSCC in 49 patients (57.6%), moderately differentiated TSCC in 30 patients (35.3%) and poorly differentiated TSCC in 6 patients (7.1%). The median follow-up time was 39 months (range, 4-83 months) and 37.6% (32/85) of patients had succumbed to TSCC at the time of the final follow-up.

Pattern of RKIP expression in TSCC and association with clinicopathological parameters. In non-cancerous tissues adjacent to TSCC and oral leukoplakia lesions, RKIP was detected in almost all the layers of the epithelia, and was mainly expressed in the cytoplasm. In TSCC samples, RKIP was also predominantly detected in the cytoplasm, and sporadic nuclear staining was also found. Overall, 43.5% (37/85) of the primary tumor tissues demonstrated high RKIP expression, while 71.8% (61/85) of the corresponding adjacent non-cancerous tissues and 65.6% (21/32) of the oral leukoplakia lesions demonstrated high RKIP expression (Table II). There was no statistically significant difference between RKIP expression in adjacent non-cancerous tissues and oral leukoplakia lesions (P=0.518). However, a significant decrease in RKIP expression was noted in TSCC samples compared with either adjacent non-cancerous tissues (P=0.000) or oral leukoplakia lesions (P=0.033).

In order to evaluate the role of RKIP in TSCC, the association between RKIP expression and any of the clinicopathological parameters was investigated (Table I). The results

Table II. RKIP expression level in TSCCs and related tissues.

Tissue type	Patients, n	RKIP expression, n (%)	
		High	Low
Adjacent non-cancerous tissue	85	61 (71.8)	24 (28.2)
TSCC tissue	85	37 (43.5)	48 (56.5)
Oral leukoplakia lesions	32	21 (65.6)	11 (34.4)
Lymph node metastases	30	5 (16.7)	25 (83.3)

RKIP, Raf kinase inhibitor protein; TSCC, tongue squamous cell carcinoma.

Table III. Cox proportional hazards model analysis of variables affecting survival in patients with tongue squamous cell carcinoma.

Variable	Comparison	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P-value	HR (95% CI)	P-value
Gender	Male vs. female	0.693 (0.333-1.445)	0.328		
Age	≥55 years vs. <55 years	1.104 (0.549-2.219)	0.781		
Differentiation	Well vs. moderately or poorly	1.727 (0.858-3.475)	0.126		
pT stage	T1-2 vs. T3-4	0.811 (0.350-1.890)	0.625		
pN stage	N^0 vs. N^+	2.261 (1.127-4.539)	0.022	1.512 (0.503-4.546)	0.462
pTNM stage	I-II vs. III-IV	2.135 (1.041-4.380)	0.038	1.286 (0.412-4.011)	0.665
RKIP expression	High vs. low	3.015 (1.299-6.999)	0.010	2.567 (1.083-6.083)	0.032

pTNM stage, pathological tumor-node-metastasis stage; RKIP, Raf kinase inhibitor protein; HR, hazard ratio; CI, confidence interval.

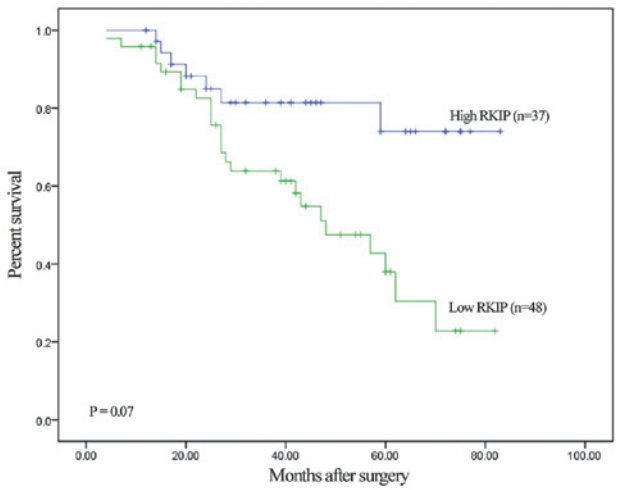

Figure 2. Keplan-Meier survival analysis for patients with TSCC. The P-value was determined using the log-rank test. Survival time was calculated based on the date of surgery and the last follow-up. Comparison of the overall survival between TSCC patients with low RKIP and high RKIP expression tumors. RKIP, Raf kinase inhibitor protein; TSCC, tongue squamous cell carcinoma.

revealed that RKIP expression was significantly associated with the pN stage (P=0.021) and pTNM stage (P=0.018). No significant association was identified between RKIP expression and gender (P=0.153), age (P=0.536), tumor differentiation (P=0.460) and pT stage (P=0.233).

Loss of RKIP in lymph node metastases. RKIP expression was considerably less frequent in patients with lymph node involvement (26.7%; 8/30) compared with patients without node involvement (52.7%; 29/55). In addition, RKIP expression was less frequent in the corresponding lymph node metastases (16.7%; 5/30) compared with the primary TSCC tissues (P=0.009). Notably, 5 of the metastases demonstrating low RKIP expression completely lacked RKIP expression, obtaining an immunoreactive score of 0 (Fig. 1D and H).

Association between RKIP expression and survival in TSCC patients. To investigate the association between RKIP expression and the clinical outcome of TSCC patients, the association between the survival status and RKIP expression of patients was analyzed. The results revealed that patients possessing a TSCC tumor with low RKIP expression demonstrated a significantly worse prognosis compared with patients that possessed a tumor with high RKIP expression (P=0.007; Fig. 2). The mean survival time of patients with low RKIP expression (n=37) was 44.1 months, while the mean survival time of the patients with high RKIP expression (n=48) was 30.3 months.

To further assess whether RKIP expression is a prognostic parameter in patients with TSCC, regression analysis using a Cox proportional hazards model was performed. The covariate

parameters included several clinicopathological variables in addition to RKIP, as reported in Table III. In univariate analysis, low RKIP expression, lymph node involvement and pTNM stage III or IV lesions demonstrated a significantly increased hazard ratio (HR) for poor prognosis. In addition, multivariate analysis was performed using variables that demonstrated a significantly increased HR in univariate analysis. The results of multivariate analysis revealed that RKIP expression was the only independent prognostic predictor of TSCC outcome (P=0.032; Table III). These results strongly indicated that the downregulated RKIP expression in TSCC patients is closely associated with a poor prognosis.

Discussion

RKIP plays an important role in cell growth (9), mitosis (19), motility (20) and apoptosis (21) by regulating multiple intracellular signaling pathways, including the Raf/MEK/ERK pathway (4,5), nuclear factor-κB pathway (6), G-protein coupled receptor signaling cascade (7,8) and glycogen synthase kinase-3β/β-catenin pathway (22). Previous studies have revealed that RKIP acts as a tumor suppressor and suppressor of metastasis in a variety of malignancies (13-17). However, little is known about the function of RKIP in oral cancer, and particularly in TSCC. The present study observed that RKIP was intensively expressed in non-cancerous and pre-cancerous tongue tissues. This result is consistent with a previous study that reported a high rate (85.7%) of RKIP immunostaining in normal tissue of the head and neck, consisting of tissues from the tongue, lip, mouth, larynx and pharynx (16). By contrast, the present study found that RKIP expression is markedly reduced in human TSCC tissues. Notably, the level of RKIP expression was found to be even lower in the matched tissues from lymph node metastasis. Loss of RKIP expression was also found to be significantly associated with the presence of lymphatic metastasis and advanced clinical stage. This finding is consistent with the reported association between the reduction of RKIP and tumor progression and metastasis in numerous other cancers. However, the present study found no significant association between RKIP expression and tumor size or histological grade in patients with TSCC. These results indicate that although RKIP affects progression and metastasis, it does not have an influence on the tumorigenic properties of TSCC. Similarly, previous studies have reported that RKIP expression is not associated with tumor size or histological grade in breast cancer (14). In addition, the restoration of RKIP expression has been reported to suppress invasion and lung metastasis in prostate cancer, but not tumor growth (13). Thus, the present results suggest that RKIP may function as a suppressor of metastasis in TSCC.

Furthermore, the loss of RKIP expression is associated with a poor prognosis in prostate (23), gastric (24), pancreatic (25), and bladder cancers (26) and glioma (27). In the present study, well-established prognostic markers, such as lymph node metastasis and an advanced pTNM stage, were significantly associated with patient survival time. The present study revealed that reduced cytoplasmic expression of RKIP, which was found in 56.5% of the tissues, is significantly associated with a shorter overall survival time in patients with TSCC. In addition, a trend was observed between the loss of RKIP expression and the presence of metastasis. Accordingly, patients with positive lymphatic metastases demonstrated relatively lower RKIP expression compared with the patients without metastases. Although the mechanistic basis for these subset differences is not known, the identification of these differences may aid in future tailored therapy and early prediction of the survival time of patients with TSCC. Additional studies with larger cohorts are required to validate the role of RKIP as a prognostic marker in TSCC.

To the best of our knowledge, the current study is the first study that has analyzed RKIP expression in TSCC to be reported in the literature. The expression level of RKIP was also assessed in the metastatic lymph node lesions of TSCC. The present results revealed that loss of RKIP expression was significantly associated with tumor progression and metastasis. Despite the importance of RKIP in tumor progression and the development of metastasis, the mechanism of RKIP downregulation remains largely unknown. Previous studies have investigated the CpG methylation status of the RKIP promoter in human cancers as a possible mechanism for the downregulation (28,29), but the results are inconsistent and may not completely explain the loss of RKIP expression in malignancies. Future studies evaluating the possible mechanisms of RKIP downregulation in TSCC may be required.

The present study reports that RKIP expression is lost during TSCC progression, and in particular, is absent in lymph node metastases. The current study presents evidence that the loss of RKIP expression in TSCC is associated with the clinicopathological characteristics of cancer aggressiveness. Notably, loss of RKIP expression is associated with poor survival time and may act as a potential biomarker of poor prognosis in TSCC patients. Additional studies in larger series and with *in vitro* and *in vivo* models are required to assess the role of RKIP expression in the tumor progression, metastasis and survival of TSCC patients.

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