

## Original Article

# Increased expression of RACK1 promotes cell proliferation and indicates a poor prognosis in patients with laryngeal squamous cell carcinoma

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**Abstract:** Objective: The receptor for activated protein kinase C (RACK1) has been implicated in the development and progression of several human malignancies, but its role in human laryngeal squamous cell carcinoma (LSCC) has not been investigated. Methods: We performed quantitative real-time PCR (qRT-PCR) and western blot to analyze the expression of RACK1 in LSCC tissues and their matched adjacent non-tumor tissues. Immunohistochemistry (IHC) was carried out to analyze the expression levels of RACK1 in 72 paraffin-embedded LSCC tissues. The correlation between RACK1 expression and clinicopathological features was assessed by Chi-square analysis. The survival data of LSCC patients was analyzed by the Kaplan-Meier and log rank tests. Results: Expression levels of RACK1 mRNA and protein were increased in LSCC tissues than that in adjacent non-tumor tissues ( $P < 0.05$ ). RACK1 expression was significantly correlated with T classification, clinical stage and lymph nodes metastasis ( $P < 0.05$ ). LSCC patients with high RACK1 expression exhibited shorter survival time compared to those with low RACK1 expression ( $P < 0.05$ ). Moreover, multivariate analysis further demonstrated that upregulated expression of RACK1 was an independent prognostic factor for LSCC patients ( $P < 0.05$ ). Conclusions: RACK1 play an important role in the progression of LSCC, and present as a useful prognostic marker and a potential therapeutic target for LSCC patients.

**Keywords:** Laryngeal squamous cell carcinoma, RACK1, immunohistochemistry, prognosis

## Introduction

Laryngeal malignancies are the second most common cancers of the head and neck, and more than 95% of cases worldwide are diagnosed as laryngeal squamous cell carcinoma (LSCC) [1, 2]. Now, the main treatment for LSCC is resection, post-operative radiotherapy and chemotherapy as the adjuvant therapies [3]. Although patients with LSCC benefit from multiple treatments, the outcomes for these patients have remained unsatisfactory during the last few decades [4]. Most patients diagnosed with advanced stage laryngeal cancer die of recurrence and/or metastasis [5]. Therefore, new diagnostic and therapeutic targets for LSCC are urgently needed.

The receptor for activated protein kinase C (RACK1), also known as GNB2L1, is a 36-kilo-

dalton cytosolic protein [6]. It was first reported as an anchoring protein with 7 WD40 (Trp-Asp) repeats [7]. RACK1 has been identified to interact with abundant signaling molecules including protein kinase C (PKC), PER1 and Src [8-10]. It is regarded as a platform in various signal transduction pathways. As to the role of RACK1 in cancers, it plays crucial roles in cell division, invasion and migration [11, 12]. However, the function of RACK1 in LSCC has not been investigated.

In the present study, RACK1 expression in LSCC tissues at mRNA and protein levels was detected, and corresponding adjacent non-tumor tissues were used as contrast. Furthermore, the relationships between RACK1 expression and clinicopathological features were analyzed. Finally, we evaluate the value of RACK1 as a prognostic biomarker for LSCC patients.

## RACK1 increased in LSCC

**Table 1.** Correlation between RACK1 protein expression and different clinicopathological features in LSCC patients

Variable	Number	RACK1 expression		P value
		Low	High	
Age (years)				0.144
<60	35	12	23	
≥60	37	19	18	
Gender				0.921
Male	33	14	19	
Female	39	17	22	
Smoking				0.923
No	26	11	15	
Yes	46	20	26	
Differentiation				0.331
Well	23	8	15	
Moderately/poorly	49	23	26	
T classification				0.007
T1-2	38	22	16	
T3-4	34	9	25	
Clinical stage				0.009
I-II	25	16	9	
III-IV	47	15	32	
Lymph nodes metastasis				0.002
No	51	28	23	
Yes	21	3	18	

### Materials and methods

#### Patients and tissue samples

Pairs of LSCC tumor and adjacent non-tumour tissues used in this study were from 72 patients who underwent partial or total laryngectomy in the Department of Otolaryngology-Head and Neck Surgery, The First People's Hospital of Shangqiu between 2006 and 2008. No patients received any anticancer treatments before admission. After surgery, the specimens were directly snap-frozen in liquid nitrogen, and stored at -80°C for extraction of RNA and total protein. The other part of specimens were fixed in buffered formalin for 48 h, embedded in paraffin, and sectioned into 4 µm slides for immunohistochemistry detection. The study was approved by the Research Ethics Committee of The First People's Hospital of Shangqiu. Informed consent was obtained from all patients. The main clinical and pathological variables of all patients were described in **Table 1**.

#### Quantitative Real-time PCR

Total RNA from the tissues were extracted using the Trizol reagent (Takara) according to the manufacturer's instructions. 2 µg RNA from each sample was used for cDNA synthesis. To amplify the spliced form of human RACK1, the primers were as follows: 5'-GATTCTGGAAAT-ATTGACTCTT-3' and 5'-AACTGGGCCCTTCTGG-GTAGAC-3'. For GAPDH, an internal control, the primers were 5'-AGAAGGCTGGGGCTCATTG-3' and 5'-AGGGGCCATCCACAGTCTTC-3'. The amplification conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles at 94°C for 30 s, and 60°C for 45 s. mRNA levels were expressed as relative quantification values and calculated by  $2^{-\Delta\Delta CT}$  method. All assays were performed in triplicate, and the mean value was used for the analysis.

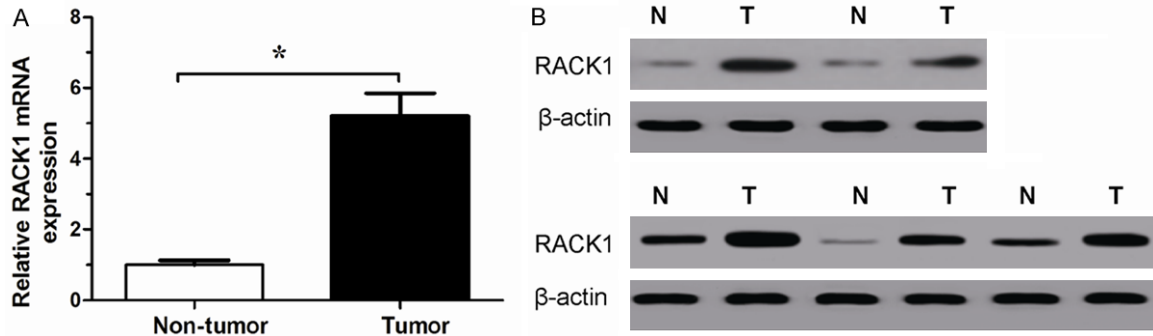
#### Western blot

The total protein was extracted from tissues lysed in RIPA buffer with protease inhibitors. Protein concentration was quantified by bicinchoninic acid protein assay kit (Beyotime). Equal amounts of proteins were separated by 12% SDS-PAGE and then transferred into PVDF membranes (Millipore). After blocking in 5% fat-free milk in TBS, the blotting membranes were incubated with primary antibody (Abcam) at 4°C overnight. After washing, they were incubated with HRP conjugated goat anti-rabbit IgG (Biotech) for 1 h at room temperature. Bands were finally visualized by employing the enhanced chemiluminescence (ECL).

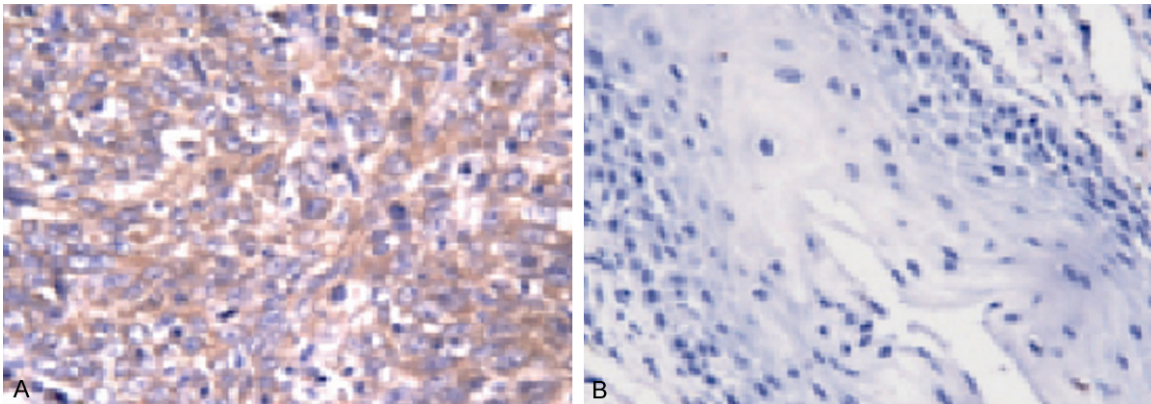
#### Immunohistochemistry

All samples were fixed in 10% formaldehyde solution, embedded in paraffin blocks, cut in 4 µm thick sections, and mounted on glass slides. Each slide was dewaxed in xylene and rehydrated in grade alcohol, followed by boiling in 10 mmol/L of citrate buffer (pH 6.0) for antigen retrieval. After inhibition of endogenous peroxidase activities for 30 min with methanol containing 0.3% H<sub>2</sub>O<sub>2</sub>, the sections were blocked with 2% bovine serum albumin for 30 min and incubated overnight at 4°C with primary antibody (Abcam). After washing thrice with PBS, the slides were incubated with horseradish peroxidase conjugated rabbit anti-mouse IgG for 30 min, followed by reaction with diaminobenzidine and counterstaining with Mayer/

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**Figure 1.** The RACK1 expression levels in LSCC tissues. A. Expression of RACK1 mRNA in 5 matched pairs of LSCC tissues and adjacent non-tumor tissues were examined by qRT-PCR. B. Expression of RACK1 protein in 5 matched pairs of LSCC tissues (T) and adjacent non-tumor tissues (N) were examined by Western blot. Results are expressed as mean  $\pm$  SD for three replicate determination. \* $P < 0.05$ .



**Figure 2.** Immunohistochemical staining for RACK1 in LSCC tissues and adjacent non-tumor tissues. Positive expression of RACK1 was mainly localized in the cytoplasm of tumor cells. Negative expression of RACK1 protein in primary LSCC specimens (A) and adjacent non-tumor tissues (B).

hematoxylin. Negative control was done by omission of the primary antibody and substituting it with nonspecific mouse IgG.

### Evaluation of immunohistochemical staining

Immunohistochemical (IHC) staining was evaluated independently by two pathologists. The level of RACK1 staining was based on the intensity of staining and the proportion of positively stained cancer cells. The following staining scores were applied: Intensity [0 (no staining), 1 (weak staining), 2 (moderate staining), 3 (strong staining)]; the proportion positive tumor cells [0 ( $\leq 5\%$  positive tumor cells), 1 (6%-25% positive tumor cells), 2 (26%-50% positive tumor cells), 3 (51%-75% positive tumor cells) and 4 ( $\geq 76\%$  positive tumor cells)]. The sum of staining intensity score and percentage of positive tumor cells score used as the final staining

score (ranges: 0-7) for RACK1. The expression of RACK1 was divided into low expression (scored 0-3) and high expression (scored 4-7). There was no discrepancy in overall interpretation of immunohistochemistry results between the two pathologists.

### Statistics

All data were presented as mean  $\pm$  SD and analyzed by using SPSS 18.0. The Chi-square test was used to analyze the correlations between RACK1 expression and clinicopathological features in LSCC patients. Kaplan-Meier method and log-rank test were applied to examine the overall survival. Univariate and multivariate analyses of variables were conducted using Cox proportional hazards regression model.  $P$  values less than 0.05 were considered significant.

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**Table 2.** Univariate and multivariate analyses of prognostic factors in LSCC patients

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age (years)	1.169	0.592-1.718	0.327			
≥60 vs <60						
Gender	1.329	0.628-2.124	0.166			
Male vs Female						
Smoking	2.426	0.718-4.913	0.078			
Yes vs No						
Differentiation	1.821	0.694-3.163	0.102			
Moderately/poorly vs Well						
T classification	2.173	1.212-5.332	0.014	1.863	1.118-4.957	0.009
T3-4 vs T3-4						
Clinical stage	2.507	1.418-8.275	0.026	2.148	1.287-7.179	0.015
III-IV vs I-II						
Lymph nodes metastasis	3.226	1.775-7.972	0.007	2.946	1.575-7.042	0.003
Yes vs No						
RACK1	2.304	1.328-8.317	<0.001	2.073	1.209-7.873	0.002
High vs Low						

HR: Hazard ratio; CI: confidence interval.

### Results

#### *RACK1 protein expression in LSCC tissues*

We examined RACK1 mRNA and protein expression in 5 pairs of LSCC tissues and the adjacent non-tumor tissues by qRT-PCR and Western blot. As shown in **Figure 1**, both RACK1 mRNA (**Figure 1A**) and protein (**Figure 1B**) was higher in LSCC tissues than that in the adjacent non-tumor tissues ( $P<0.05$ ). We further examined the expression of RACK1 protein in 72 paraffin-embedded LSCC samples and adjacent non-tumor tissues by IHC analysis. Our data showed that RACK1 protein was markedly increased in the LSCC tissues compared to adjacent non-tumor tissues (**Figure 2**). Moreover, the IHC data showed that RACK1 was mainly localized in the cytoplasm of carcinoma cells (**Figure 2**). Those data suggested that RACK1 may take part in the genesis of LSCC.

#### *Correlation of RACK1 expression with clinicopathological features*

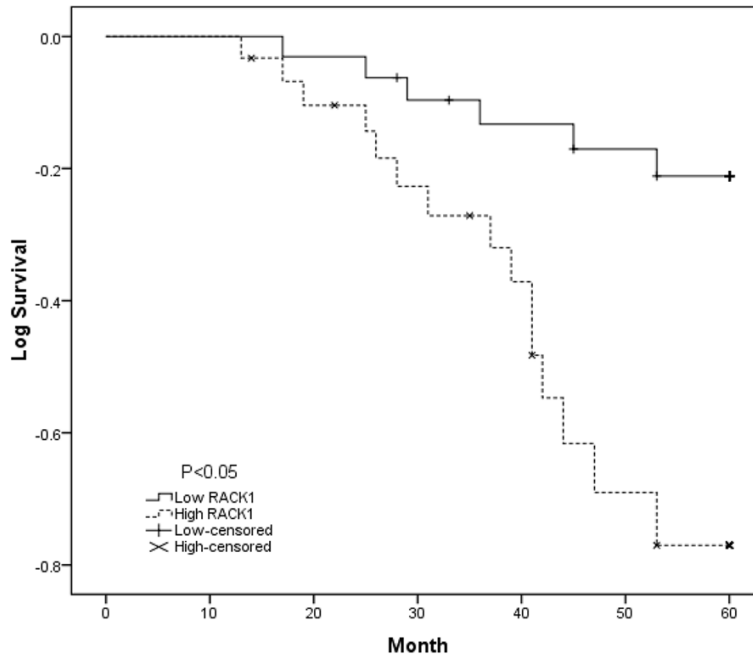
The IHC staining data showed that a significant increased of RACK1 in LSCC tissues compared with adjacent non-tumor tissues. 41 of the LSCC cases exhibited high expression of RACK1 (scored 4-7), whereas 31 had low expression of RACK1 (scored 0-3). The relationship between RACK1 expression and clinicopathological fea-

tures were summarized in **Table 2**. The results showed that RACK1 expression was significantly correlated with T classification, clinical stage and lymph nodes metastasis ( $P<0.05$ ). However, no significant association was observed between the expression of RACK1 and other clinicopathological features, including age, gender, smoking, and differentiation ( $P>0.05$ ).

#### *Correlation between RACK1 protein expression and patients' survival*

To explore the relationship between RACK1 expression and LSCC patients' survival, we applied Kaplan-Meier analysis. Among the 72 LSCC patients, 51 patients were alive and 21 were died at the last clinical follow-up. Our results showed that LSCC patients with LSCC that express high level of RACK1 have a shorter overall survival time compared to patients with LSCC that express low level of RACK1 (**Figure 3**,  $P<0.05$ ). These results revealed that high level of RACK1 expression is a bad sign for LSCC patients. To determine whether RACK1 expression could serve as an independent prognostic factor, we examined overall survival (OS) using the Cox proportional hazards regression model. RACK1 expression and several other clinicopathological factors, such as T classification, clinical stage and lymph nodes metastasis were found to be associated with OS by univariate analysis. All variables were further analyzed

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**Figure 3.** Kaplan-Meier survival analysis of overall survival in all patients with LSCC, according to the expression of RACK1 protein (log-rank test,  $P < 0.05$ ).

by multivariate analysis, which revealed that RACK1 expression, T classification, clinical stage, and lymph nodes metastasis were independent significant prognostic factors for OS (Table 2).

### Discussion

The prognosis of LSCC is quite poor, because most patients are diagnosed at an advanced stage or metastasis, when treatments are less effective. Therefore, a better understanding of this disease and finding useful biomarkers to predict the progression may lead to improvement in the diagnosis and treatment of LSCC.

In the present study, our data showed that the expression of RACK1 was significantly higher in LSCC tissues than that in adjacent non-tumor tissues. The RACK1 expression was associated with T classification, clinical stage and lymph nodes metastasis, suggesting that RACK1 can be used as a potential biomarker for LSCC patients with metastasis. Furthermore, we found that LSCC patients with high RACK1 expression had a shorter OS than those with low RACK1 expression. Multivariate analysis revealed that the expression level of RACK1 was an independent prognostic factor for LSCC patients. These data demonstrated that RACK1

might play a critical role in LSCC development and progression and have the potential ability to be a novel prognostic biomarker in LSCC.

It has been previously reported several novel prognostic factors including SOX2 [13], UHRF1 [14], PTK6/Brk [15]. Additionally, differentiation, clinical stage and lymph node metastasis have also been shown to be correlated with outcome of LSCC patients [16]. However, the efficacy of such prognostic markers for clinical application remains unknown. Therefore, further investigation is warranted to discover novel prognostic markers which would be valuable in improving the prognostic efficacy of LSCC patients.

RACK1 is a crucial scaffold and anchoring protein, which plays a vital role in multiple signaling pathways. Recent studies showed that RACK1 dysregulated in several cancers and played an important role in cancer progression. For example, Jin et al. showed that RACK1 was overexpressed and correlated with malignant degrees in colorectal carcinoma patients, which might serve as a specific biomarker and potential antitumor target [17]. Cao et al. reported that RACK1 could promote breast carcinoma migration/metastasis via activation of the RhoA/Rho kinase pathway [18]. Wang et al. indicated that RACK1 could predict poor prognosis and regulate progression of esophageal squamous cell carcinoma through its epithelial-mesenchymal transition [19]. Chen et al. showed that loss of RACK1 promoted metastasis of gastric cancer by inducing a miR-302c/IL8 signaling loop [20]. Our study expanded the tumor oncogenic role of RACK1 in LSCC.

In conclusion, RACK1 was upregulated in LSCC patients, and positively correlated with malignant degrees in LSCC patients, which might serve as a novel biomarker and potential therapeutic target. Further investigations, especially molecular mechanisms of RACK1 in LSCC are warranted.

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**Disclosure of conflict of interest**

None.

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