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CRKL promotes proliferation, migration, invasion of laryngeal squamous cell carcinoma

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Abstract. – OBJECTIVE: This study aimed to investigate the expression and role of CT10 regulated kinase like (CRKL) in human laryngeal squamous cell carcinoma (LSCC) progression.

PATIENTS AND METHODS: Seventy-four laryngeal cancer cases were detected by the immunohistochemistry S-P method and the results were analyzed. RNA interference was used to downregulate the expression of CRKL in Hep-2 cells. The silencing efficiency was detected by real-time PCR and Western blotting. The cell proliferation, migration, and invasion after transfection were detected by MTT, wound healing assay, transwell invasion assay, and apoptosis assay. Western blot was conducted to determine the function of CRKL/epithelial-mesenchymal transition (EMT) signaling pathway.

RESULTS: The expression of CRKL was higher in LSCC tissues. Patients with higher CRKL expression were correlated with lymph node metastasis and postoperative survival rates. CRKL promoted proliferation, migration, and invasion of Hep-2 cells in vitro.

CONCLUSIONS: These findings suggested that CRKL gene silencing significantly inhibited the proliferation, migration, invasion, and EMT signaling pathway of Hep-2 cells. CRKL is considered to be a new target for the treatment of LSCC.

Key Words:
CRKL, Laryngeal squamous cell carcinoma, EMT, Proliferation, Cell migration, Cell invasion.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is the common histological type of laryngeal malignancy, accounting for 95% of all laryngeal cancers[1]. According to the latest epidemiological survey, there were about 13,360 new laryngeal cancer cases in the United States, and about 3360 deaths in 2017[2]. In 2015, China's cancer statistics reported approximately 26,400 new cases and about 14,500 deaths[3].

The v-crk oncogene has been isolated from chicken retrovirus CT1O. CT10 regulated kinase (CRK) adaptor proteins consist of two alternatively spliced isoforms that contain SH2 and SH3 modular domains[4]. V-Crk avian sarcoma virus CT10 oncogene homolog-like (CRKL) belongs to the CRK family of adapter proteins, and has distinct biological roles, including cell proliferation, adhesion, and migration[5,6]. Additionally, CRKL can also regulate the activity of Ras, JNK, Stat5, and other signaling pathways[7,8]. Pathophysiologically, CRK is overexpressed in a wide array of human cancers, whereby the level of expression often correlates with tumor grade and inversely segregates with overall patient survival[9]. Several previous studies have confirmed the expression and action of CRK and CRK in laryngeal squamous carcinoma, but the same action of CRKL has not been confirmed.

Although the expression of CRKL has been demonstrated in some tumor tissues and cell lines, it has not been reported in solid tumors, especially in laryngeal squamous cell carcinoma. Hence, in this study, the expression of CRKL was investigated using immunohistochemistry. It was observed that the CRKL protein was overexpressed in human laryngeal carcinoma tissues, and subsequently promoted proliferation, migration, and invasion of Hep-2 cells.

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Patients and Methods

Study Subjects and Patient Tissue Samples

This retrospective cross-sectional study included 74 LSCC cases that received treatment between January 2006 and December 2012 at the Affiliated Hospital of Guizhou Medical University (Guiyang, China). As control, 22 normal laryngeal mucosal tissues were isolated more than 2 cm away from the tumors in surgically resected tissues from patients that underwent total laryngectomy. The tissues were archived, fixed in formalin, and embedded in paraffin. This investigation was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University. Clinical and histopathological data were obtained from the medical records. LSCC was diagnosed *via* histopathological examination, and the tumor stage was determined according to the tumor node metastasis (TNM) classification published by the Union for International Cancer Control (UICC). The clinical characteristics of these patients, including sex, age, differentiation grade, T-stage, and lymph node metastasis were recorded. The five-year survival rates are obtained by phone. Two of them lost to follow-up.

Immunohistochemistry

Surgically excised tumor specimens were fixed with 10% neutral formalin, embedded in paraffin, and 4-μm-thick sections were prepared. They were deparaffinized by fresh xylene, followed by rehydration in graded alcohol. Subsequently, the sections were treated with peroxide block for 15 min at room temperature to block endogenous peroxidase, followed by antigen retrieval for 15 min in a pressure cooker. The sections were incubated with power block for 15 min and then with ready to use primary antipodoplanin monoclonal antibody (clone D2-40; Autostainer Plus, Dako, Glostrup, Denmark) for 40 min. After that, the sections were incubated with diaminobenzidine (DAB) chromogen for 5-10 min. Finally, slides were washed and hematoxylin was used as a counterstain. Staining of the cell nucleus and/or cytoplasm for laryngeal squamous cell was considered positive expression. Two independent blinded investigators examined all the tumor slides randomly. Five views were examined per slide, and 100 cells per view were observed. The intensity of CRKL staining was scored as 1 (no signal), 2 (light brown), 3 (brown), and 4 (dark brown). The percentage scores were assigned as 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The scores of each tumor sample were multiplied to obtain a final score of 0-8. Tumor samples scoring 4-8 were considered to exhibit CRKL overexpression.

Cell Culture and Transfection

The LSCC Hep-2 cell line (CHI Scientific, Inc., Maynard, MA, USA) was grown in minima essential medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C and 5% CO_2. For transfections, 2×10^5 Hep-2 cells were seeded in 6- well plates and transfected with 100 nmol/L miRNAs with Lipofectamine RNAiMAX transfection reagent (Hua Lianke biotechnology co. LTD, Wuhan, China) according to the manufacturer's protocol. The siRNAs targeting CRKL (Hua Lianke biotechnology co. LTD, Wuhan, China) were sense: 5-GGUUCCCACCUUACUACAU-3 and antisense: 5-AGAAGGUCAUCGCAACGAA-3 and a scrambled siRNA was used as a negative control.

Western Blot

Proteins were isolated from the transfected cell lines using radioimmunoprecipitation assay (RIPA) lysis buffer (Bioswamp, Shanghai, China) and quantified using a bicinchoninic acid (BCA) protein quantification kit (Bioswamp, Shanghai, China). The proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Thereafter, the membranes were sealed with 5% fat-free milk and incubated with specific antibodies against CRKL (1:200, Abcam, Cambridge, MA, USA), GAPDH (1:1000, Cell Signaling co., Danvers, MA, USA), overnight at 4°C. The horseradish peroxidase (HRP)-labeled secondary antibody was used to couple the primary antibodies at room temperature for 1 h. Blots were visualized and quantified using chemiluminescence (Beyotime, Shanghai, China) and gel imaging system (Tanon Co., Shanghai, China).

RNA Isolation and Real-Time RT-PCR

High-purity Total RNA Rapid Extraction Kit (TaKaRa, Otsu, Shiga, Japan) was used to extract the total RNA from cell samples. Complementary deoxyribose nucleic acids (cDNAs) were compound according to the Reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). The fol-

lowing primers were employed, CRKL forward: 5'-CGCTCAACCTCAGACCAC-3', CRKL reverse: 5'-CACCAACCTCTAATGCCA-3'; β-actin forward: 5'-ACACTGTGCCCATCTACG-3', β-actin reverse: 5'-TGTCACGCACGATTTCC-3'. The reaction condition of real-time PCR was as follows: 3 min at 95°C, followed by 39 cycles containing 5 s at 65°C, 50 s at 65°C. Real-time PCR was conducted using fluorescence quantitative instrument (Bio-Rad, Hercules, CA, USA).

Cell Migration and Invasion

Cells were resuspended in serum-free Dulbecco's Modified Eagle's Medium (DMEM) medium and then diluted to 1×10^5 cells/ml. The cells were transferred into the transwell chambers, where 0.5 ml cell suspension was added to each well, and 0.75 mL DMEM containing 10% FBS cultivate was added to the lower 24 hole plate and incubated at 37°C for 24 h. Then, the cells were immobilized with 4% polyformaldehyde for 10 min at room temperature. The cells in the upper chambers were removed by a cotton swab. After that, the chambers were washed twice with phosphate-buffered saline (PBS), cells were stained with 0.1% crystal violet for 5 min, and then the chambers were washed thrice with PBS. The number of cells was counted from five different fields with a microscope.

Cell Viability Assay

The effect of CRKL on Hep-2 cell proliferation was determined with Cell-Titer 96 Aqueous MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay kit (Bioswamp, Shanghai, China) according to the manufacturer's protocol. Hep-2 cells at different time points (24, 48, and 72 h) were incubated with 20 μL MTT for 4 h at 37°C. Then, the supernatant was carefully aspirated in the dark and 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The cells were constantly shaken for 10 min and then the absorbance was measured at 490 nm. All the experiment was repeated thrice.

Apoptosis Assays

Apoptosis assays were conducted by using AnnexinV-FITC (fluorescein isothiocyanate) apoptosis Detection Kit I (Bioswamp, Shanghai, China). Briefly, the cells were gathered and resuspended in 200 μL binding buffer. Then, 5 μl Annexin V-FITC was added and mixed well, followed by incubation with 5 μl propidium iodide at 4°C away from light for 30 min. Cells were then immediately detected with a flow cytometry (Beckman Coulter, Miami, FL, USA).

Wound Healing Assay

Cells were seeded in 6-well plates and pretreated with 1 μg/mL mitomycin C for 1 h when the cell density reached 80%-90%. Straight "scratches" were generated at the bottom of the plates with 200 μL pipette tips. The cellular debris was washed away and the cells were cultured with serum-free DMEM medium at 37°C in 5% CO_2. The images were captured at 0 h, 24 h, and 48 h with an inverted phase-contrast microscope (Motic Group Co., Ltd., Xiamen, China). The migration distances were measured using IPWIN60 software (Media Cybernetics, Inc, Rockville, MD, USA).

Statistical Analysis

All statistical analyses were conducted using Statistical Product and Service Solutions (SPSS) software 13.0 (SPSS, Inc., Chicago, IL, USA). All experiments were repeated thrice. The data are summarized as means±SD (standard deviation). The differences in CRKL expression between LSCC and mucosa samples were analyzed by Chi-square test. The differences of GC clinicopathologic features in LSCC were analyzed by Chi-square test. All the differences between multiple means were evaluated by two-tailed Student's *t*-test. The relationship between CRKL, MMP-2, MMP-9, Ki67, β-catenin, and cyclin D expression was analyzed by Chi-square test. $p<0.05$ was considered as statistically significant

Results

The immunohistochemistry results demonstrated that CRKL was significantly over-expressed in LSCC. The CRKL protein was mainly expressed in the cytoplasm of LSCC (Figure 1). There are significant differences between the tissues of LSCC and laryngeal mucosal tissues ($C^2=4.878$, $p=0.026$; Table I). These results suggested that the CRKL levels were not correlated with age, cell differentiation, and T stage. However, higher CRKL levels were correlated with cervical lymph node metastasis and positive survival rate (Table II).

Establishment of Hep-2 Cell Model With CRKL Silencing

To address the function of CRKL in laryngeal cancer, we established a stable CRKL-silenced

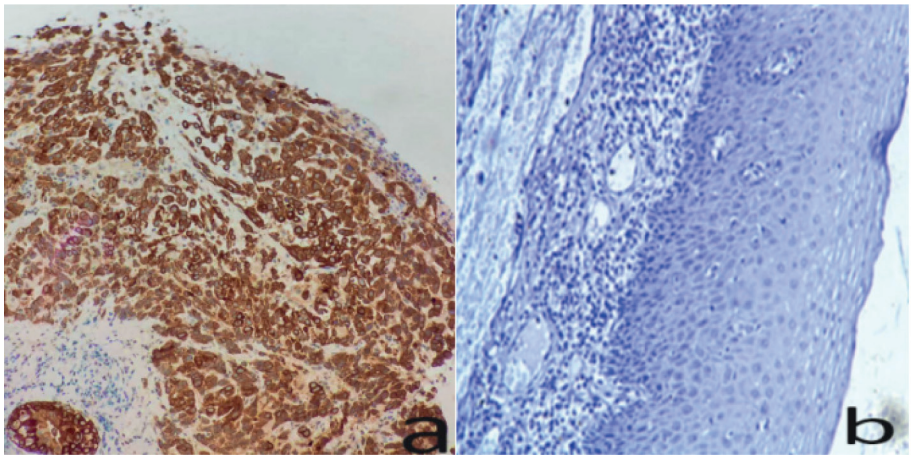

Figure 1. The expression of CRKL in LSCC and laryngeal mucosal tissues (SP×200%). **a,** laryngeal squamous cell carcinoma tissues. **b,** laryngeal mucosal tissues. Magnification, 200x.

cell model in Hep-2 cells and validated its knockdown efficiency through Western blotting and real-time RT-PCR. As shown in Figure 2A and 2B, the expression of CRKL was dramatically down-regulated in CRKL-silenced Hep-2 cells compared with that of mock and negative control.

Effect of CRKL Silencing on Proliferation and Apoptosis of Hep-2 Cells

We explored the effect of CRKL on proliferation of Hep-2 cells. As shown in Figure 3A, MTT assay revealed that silencing of CRKL significantly inhibited the proliferation of Hep-2 cells compared with negative control group and mock group. Similarly, the effect of CRKL on apoptosis of Hep-2 cells was also explored. As shown in Figure 3B, silencing of CRKL significantly induced apoptosis of Hep-2 cells.

Effects of CRKL Silencing on Migration and Invasion of Hep-2 Cells

The effects of CRKL on migration and invasion of Hep-2 cells were investigated. As shown in Figure 4A, the results of wound healing assay demonstrated that knockdown of CRKL evidently weakened the migratory ability of Hep-2 cells compared to that of negative control group and mock group. Moreover, the invasion ability of Hep-2 cells was also inhibited after CRKL knockdown compared with that of negative control group and mock group (Figure 4B).

CRKL Regulates Malignant Behavior of Tumors by Epithelial to Mesenchymal Transition (EMT) Signaling Pathway

By detecting the expression of EMT-related proteins using Western blot analysis, we investigated CRKL mediation of EMT signaling pathway of Hep-2 cells. As shown in Figure 5, MMP-2, MMP-9, Ki67, β-catenin, and cyclin D expression levels were significantly declined with downregulation of CRKL. These results suggested that CRKL regulated the malignant behavior of LSCCs by *in vitro* by activating the EMT signaling pathway.

Discussion

In the present study, CRKL was over-expressed in LSCC tissues compared to laryngeal mucosal tissues. CRKL levels were not correlated with age, cell differentiation, T stage, and survival rate. However, higher CRKL levels were correlat-

Table I. The positive expression rates of CRKL in laryngeal squamous cell carcinoma (LSCC) and normal laryngeal mucosa (control).

Group	LSCC (n=74)	Normal laryngeal mucosa (n = 22)	X^2	*p*-value
CRKL	48/74 (64.9%)	2/22 (9.1%)	4.878	0.026

Table II. The correlation between expression characteristic of CRKL in LSCC specimens and LSCC GC clinicopathologic features.

Parameters	CRKL (+) (n=74)	X^2	*p*-value
Age (Y):		0.113	0.737
≥60	25/36 (69.4)		
<60	25/38 (65.8)		
T stage:		0.967	0.326
T1-T2	29/40 (72.5)		
T2-T3	16/34 (47.1)		
Differentiation:		0.793	0.373
High	32/53 (60.4)		
Moderate and low	15/21 (71.4)		
Lymph node metastasis		6.024	0.014
Yes	16/18 (88.9)		
No	32/56 (57.1)		
Postoperative survival rates		0.00	0.476
≥5 years	37/58 (63.8)		
<5 years	10/12 (83.3)		

Establishment of Hep-2 cell model with CRKL silencing.

ed with cervical lymph node metastasis. Then, we continued to explore the effect of CRKL on malignant phenotype of LSCC Hep-2 cells. Our results indicated that knockdown of CRKL in Hep-2 cells inhibited cell proliferation, migration, and invasion. CRKL, a member of the CRK family,

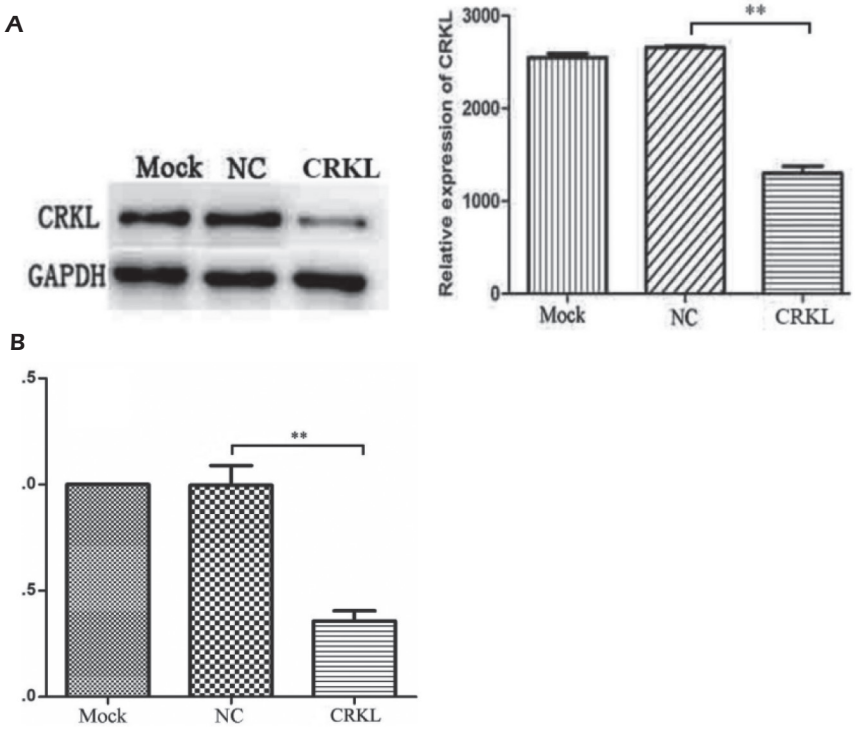

Figure 2. CRKL-silenced the Hep-2 cells expression. **A,** Representative Western blot and quantification of CRKL protein expression in CRKL-silenced Hep-2 cells. There are no differences between NC groups and mock group (t=14.659, 17.212, $^{**}p$<0.01). But significant differences between control group and NC/mock group were observed (t=1.308, p>0.05). **B,** Analysis of CRKL transcription level in CRKL-silenced Hep-2 cells by RT-PCR. The mRNA levels of CRKL in the cell line were significantly lower than that in negative control and mock groups (t=13.300, 6.145; $^{**}p$<0.01). There are no differences between NC group and mock group (t=0.033, p>0.05). NC: negative control.

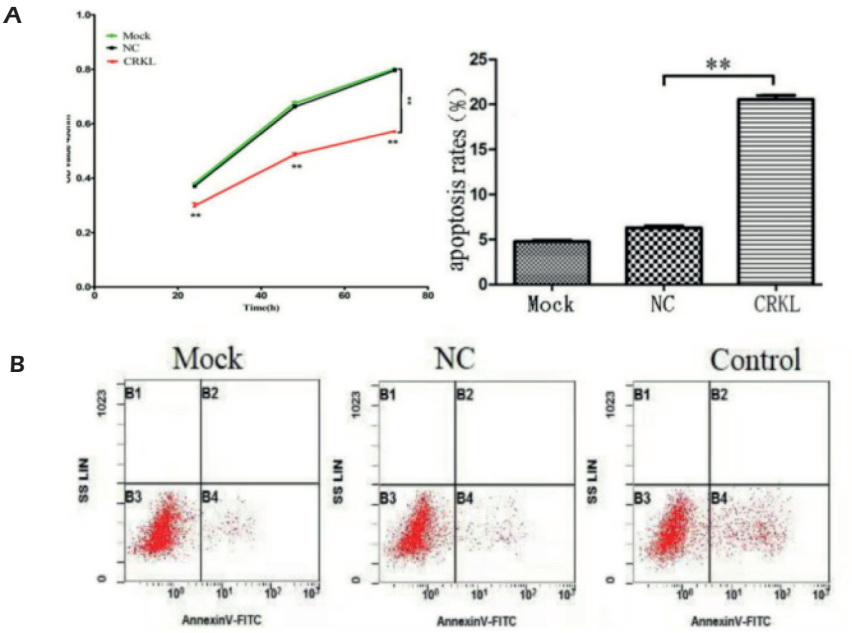

Figure 3. Knockdown of CRKL inhibited proliferation and apoptosis of Hep-2 cells. **A,** Cell proliferation was evaluated through MTT assay in Hep-2 cells after knockdown of CRKL. Hep-2 cells with CRKL knockdown showed a decrease in cell proliferation compared with NC and mock group (**p<0.01). **B,** Cell apoptosis was evaluated through flow cytometry analysis. The results showed significant differences between Hep-2 cells with CRKL knockdown and negative group, mock group (t=33.136, 28.697, **p<0.01). There are no differences between NC groups and mock group (t=1.698, p>0.05). NC: negative control.

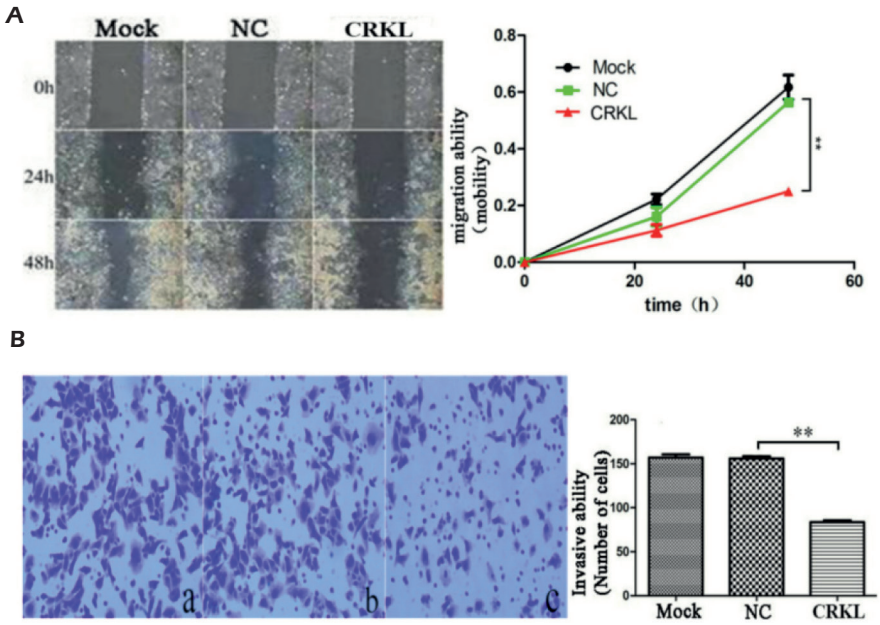

Figure 4. Knockdown of CRKL inhibited the migration and invasion of Hep-2 cells. **A,** Migratory ability of Hep-2 cells was evaluated by scratch assay following CRKL knockdown (Magnification, 10x). At 48 h, the migration distance of cells in the control group was significantly lower than that in the mock control group and the negative control group (t=8.954, 16.578, **p<0.01). **B,** Transwell assay was carried out to assess the invasive ability of Hep-2 cells after down-regulation of CRKL (Magnification, 40x). The number of transmembrane cells in the control group (85±5.03) was significantly lower than that in the mock group (157±6.24) and the negative control group (156±4.58). The results are presented as means ± SD and two-tailed t-test (t=15.476, 17.982, **p<0.01). There are no differences between NC groups and mock group (t=0.224, p>0.05). NC: negative control.

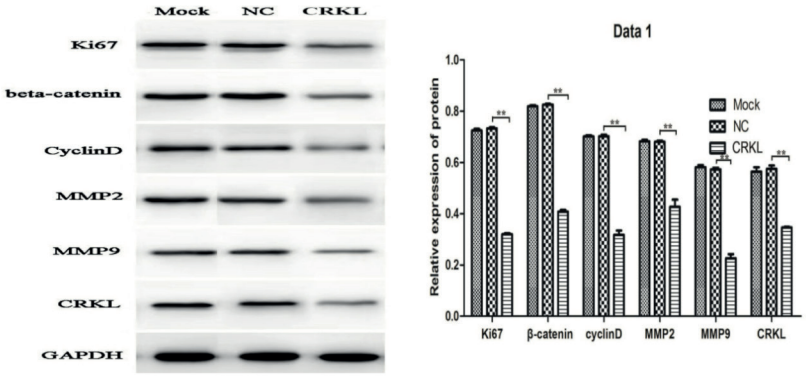

Figure 5. Expression levels of MMP-2, MMP-9, Ki67, β-catenin, and cyclin D in CRKL-silenced Hep-2 cells or control cells were detected through Western blotting. The results showed that the expression of EMT core protein in control group was down-regulated with CRKL silencing. $^{**}p<0.01$.

promotes progression of several other malignancies. Fathers et al[10] showed that CRK adaptor proteins act as key signaling integrators for breast tumorigenesis. Lin et al[11] demonstrated that CRKL promotes lung cancer cell invasion through ERK-MMP9 pathway. Wang et al[12] showed that overexpression of CRKL was correlated with poor prognosis and cell proliferation in non-small cell lung cancer. Zhao et al[13] demonstrated that CRKL protein overexpression enhanced cell proliferation and invasion in breast cancer. Fu et al[14] showed that CRKL protein overexpression enhanced cell proliferation and invasion in pancreatic cancer.

The functions of EMT/CRKL signaling pathway in human LSCC were determined by analyzing the correlation between EMT core protein expression and CRKL. The results demonstrated that silencing of CRKL significantly down-regulated the expression of Ki67, *β-catenin,* cyclin D, MMP2, and MMP9 in Hep-2 cells. EMT pathway is a highly conservative signaling pathway in biological evolution, and plays an important role in various cancers, especially in invasion and metastasis[15]. The EMT/CRKL signaling pathway plays an important role in various cancers, such as lung cancer[16], pancreatic ductal adenocarcinoma[17], and glioblastoma[18]. Matsumoto et al[19] found that CRK protein was over-expressed in lung cancer, with lower E-cadherin expression, and was associated with poor prognosis. Matsumoto et al[19] showed that CRK was over-expressed in bladder cancer, and induced EMT through HGF/c-Met/Crk signaling pathway to progress cancer cell migration and invasion. The knockout of CRK in bladder cancer cells was associated with declined EMT activity. In conclusion, CRKL promotes the proliferation, migration, and invasion of LSCC by promoting the occurrence of EMT.

Conclusions

In summary, these results showed that CRKL was over-expressed in LSCC tissues. CRKL over-expression promoted human LSCC proliferation, migration, and invasive ability. CRKL is expected to be a new target for the treatment of LSCC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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