

Reduced RKIP expression levels are associated with frequent non-small cell lung cancer metastasis and STAT3 phosphorylation and activation

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Abstract. The current study examined the role of Raf kinase inhibitor protein (RKIP) in non-small cell lung cancer (NSCLC) metastasis. A total of 100 patients with NSCLC were recruited following pathological diagnosis in the First Affiliated Hospital of Bengbu Medical College. The patients were classified and statistically analyzed according to their clinicopathological characteristics and tumor-node-metastasis stage. Paired tumor tissue and adjacent non-tumor tissue samples were subject to pathological diagnosis and western blot analysis. Transient transfection and lentivirus particle vector-mediated RKIP overexpression, small interfering RNA-mediated silencing, Transwell assays and immunocytochemistry methods were employed to elucidate the role and underlying mechanisms of RKIP and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway in NSCLC metastasis. Furthermore, in order to examine the *in vivo* effects of RKIP, recombinant lentivirus particles containing the RKIP gene were administrated in a mouse NSCLC tumor model via tail vein injection. The results revealed reduced RKIP expression levels in NSCLC tissue compared with corresponding non-cancer tissue. Additionally, RKIP expression levels were inversely associated with NSCLC intra-lung, lymph node and long-distance metastasis. The results also indicated that RKIP was able to block STAT3 activation via phosphorylation and inhibit NSCLC-cell metastasis *in vitro*. Furthermore, RKIP

knockdown was able to promote STAT3 phosphorylation and cell metastasis in NSCLC cell lines. During *in vivo* experiments, RKIP overexpression was able to suppress xenograft tumor metastasis in nude mice. Therefore, RKIP may be an important factor in cancer cell metastasis in patients with NSCLC, and RKIP may inhibit NSCLC-cell invasion by blocking the activation of the JAK/STAT3 signaling pathway.

Introduction

Lung cancer has increased in incidence in the developing world since 2008, from 1.8 to 2 million per year, and this trend is predicted to continue in the future (1). Non-small cell lung cancer (NSCLC) is among the most malignant types of cancer, and has a high mortality rate (2). Approximately 80-90% of lung cancer cases are of NSCLC, and 50-70% of these patients are diagnosed at the advanced stage of disease (1,3). The survival time of patients with lung cancer is 5 years in 10-15% of cases, with the remaining exhibiting poorer survival times (1). Therefore, the early prevention of NSCLC metastasis is a key factor in lung cancer prevention and the development of novel therapeutic agents. A number of studies have investigated the mechanisms underlying NSCLC metastasis (4,5); however, the exact mechanisms underlying this process require further elucidation.

Raf kinase inhibitor protein (RKIP) is a small evolutionarily conserved protein, which was initially identified to function as a physiological inhibitor of the Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway (6). RKIP inhibits the interaction between RAF1 and MEK, thereby preventing RAF-mediated MEK phosphorylation, which is required for signal propagation (7). RKIP is widely expressed in normal human tissues, indicating that it may function in a variety of physiological processes (8). Previous studies have suggested that RKIP may be a suppressor of cancer metastasis, as its loss of or reduced expression levels are highly associated with cancer malignancy and aggressiveness (9,10). Overexpression of RKIP protein has a limited impact on the proliferation of prostate cancer cell proliferation and angiogenesis in mouse models; however, cancer cell invasion and metastasis is significantly

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reduced upon RKIP overexpression (11), indicating its potential tumor suppressor role in cancer metastasis. In a previous study, RKIP mRNA expression levels were demonstrated to be significantly downregulated in NSCLC, and lower mRNA levels were correlated with poorer differentiation and advanced tumor-node-metastasis stage in patients with NSCLC (12). However, the clinical significance of RKIP and its associations with metastasis in NSCLC, as well as its downstream mechanisms and targets, remain to be established.

Signal transducer and activator of transcription 3 (STAT3) is activated by various growth factors, cytokines and oncogenic proteins, as well as being constitutively activated in various types of malignancy (13). A previous study reported that STAT3 functions downstream of the Raf/ERK signaling pathway, and is associated with cancer metastasis (14). However, whether RKIP participates in the phosphorylation and activation of STAT3 during NSCLC cell metastasis remains to be established. A previous study reported that increased RKIP level and Raf/ERK/STAT3 pathway activation were found in metastatic NSCLC patients (15). The current study examined the role of RKIP and STAT3 phosphorylation in NSCLC, based on clinical sample analysis and *in vitro* and *in vivo* experiments.

Materials and methods

Cell lines and reagents. The A549 and NCI-H1299 human NSCLC cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) at 37°C in a humidified atmosphere containing 5% CO₂. Antibodies against RKIP (cat. no. 13006), STAT3 (cat. no. 9139), phosphorylated (p)STAT3 (cat. no. 9145), ERK (cat. no. 9102) and pERK (cat. no. 4370) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and β-actin (cat. no. 7210) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Patient tissue samples and pathological diagnosis. Between March 2015 and August 2015, 100 patients with NSCLC were recruited for the present study at The First Affiliated Hospital of Bengbu Medical College (Bengbu, China). All patients underwent curative surgical resection and none had received preoperative chemotherapy or radiotherapy. All patients were diagnosed by pathological methods. The current study was performed with the approval of the Bengbu Medical College Ethics Committee, and informed consent was obtained from all patients that were recruited.

Immunohistochemical staining. Immunohistochemical staining for RKIP and phosphorylated (p)STAT3 was performed according to previous protocol (16). Briefly, antigen retrieval was performed by boiling tissue samples in an autoclave for 20 min in 1X antigen retrieval Citra solution (BioGenex, Fremont, CA, USA). The tissue samples were fixed with 4% formaldehyde at room temperature for 24 h. After

fixation, samples were sectioned at a thickness of 5 µm for further analysis. Sections were blocked with PBS containing 5% goat serum (Sigma-Aldrich; Merck Millipore) and 0.1% Triton X-100, followed by incubation with anti-RKIP antibody (dilution, 1:200) or pSTAT3 antibody (dilution, 1:500) at 4°C in a humidified chamber for 12 h. Following washes with PBS, the tissue samples were incubated at room temperature with horseradish peroxidase (HRP)-labeled secondary antibody (cat. no. 8114, 1:1,000 dilution; Cell Signaling Technology, Inc.) for 2 h and visualized with a 3,3'-diaminobenzidine kit (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were observed and recorded using a Leica DM3000 microscope.

Western blotting. siRNA transfected cells were lysed in ice-cold lysis buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 30 min, supplemented with protease and phosphatase inhibitors (Sigma-Aldrich; Merck Millipore). Following centrifugation for 15 min at 12,000 × g at 4°C, the supernatant was collected and the protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Inc.). Total protein (50 µg/lane) was separated by 10% SDS-PAGE and transferred to Hybond-C nitrocellulose membranes (GE Healthcare Life Sciences, Chalfont, UK). The membranes were incubated with primary antibodies RKIP, pSTAT3 or β-actin (1:2,000) overnight at 4°C, respectively, and subsequently probed with HRP-conjugated secondary antibodies (cat. no. 8114; 1:2,500 dilution; Cell Signaling Technology, Inc.) for 1 h at room temperature. Chemiluminescent signals were developed using LumiGLO[®] reagent (Cell Signaling Technology, Inc.) and detected using the ChemiDoc[™] XRS+ gel documentation system (Bio-Rad Laboratories, Inc.). In grayscale quantification, Photoshop version 8.0 (Adobe Systems, Inc., San Jose, CA, USA) was used, setting actin grayscale as control.

Small interfering (si)RNA interference. The experiment was performed according to previous protocol (17). Briefly, 3 × 10⁵ A549 and NCI-H1299 cells/2 ml were seeded in 6-well plates 1 day prior to transfection. 10 µM siRNAs (cat. no. sc-36430; Santa Cruz Biotechnology, Inc.) were transfected into the cells with the aid of FuGENE[®] transfection reagent (Promega Corporation, Madison, WI, USA). Three days later, cells were collected using lysis assay buffer, and incubated on ice for 30 min with protease inhibitors cocktail (Sigma-Aldrich; Merck Millipore).

Transwell assay. The migration of NCI-H1299 cells *in vitro* was determined using Transwell assays (pore size, 8-µm; Corning Incorporated, Corning, NY, USA). Briefly, cell-free DMEM (0.8 ml) with or without human interleukin-6 (10 ng/ml; BD Biosciences, San Jose, CA, USA) was placed in the lower chamber. Here, IL-6 was used as chemoattractant to induce cells migration from upper chamber to the lower plate. Microglial suspension (0.1 ml; 5 × 10⁴ cells/well) was placed in the upper chamber and the plates were incubated for 24 h at 37°C. The inserts were subsequently removed and the cells on the upper surface were removed with cotton pads. Cells on the lower surface were air dried at room temperature and stained with hematoxylin and eosin (H&E) method after 15 min fixation at room temperature. Migration ability was quantified and

compared by counting the number of migrated cells in the lower chamber. Five random fields at x40 magnification were counted for each condition under a phase-contrast microscope. Each experiment was repeated three times. Results were presented as the number of cells counted per field.

Lentivirus construction and infection. Lentivirus particles containing the RKIP gene were constructed using the pLenti7.3/V5-DEST™ Gateway® Vector kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The lentiviral vectors were co-transfected with virus packing vectors to form mature lentivirus particles in 293FT cells (Thermo Fisher Scientific, Inc.). The viral supernatant was harvested to determine the viral titer as previously described (18), and added to the A549 and NCI-H1299 cells for target gene expression.

Xenograft metastatic in vivo study. The current animal experiment was approved by the Bengbu Medical College Ethic Committee (Bengbu, China) in 2015. A total of 15 nude mice were randomly divided into 2 groups. Nude mice (6 weeks old, 11 male and 4 female, weight 15-18 g) were purchased from the Animal Center of Bengbu Medical College (Bengbu, China) and kept in the Animal Center for SPF level feeding at 25°C, with a 12 h day/night cycle. Group one (six mice) received parenteral injection via tail vein with A549 cells as controls. Group two (nine mice) were injected with pLenti7.3-RKIP infected and overexpressed stable cells, with ≥90% positively expressing RKIP, using a modified tail vein injection transplantation model from a previous study (19). The transduction efficiency was determined by green fluorescent protein-positive counting under a fluorescent microscope. Briefly, 5- to 6-week-old BALB/c nude mice were injected with 1×10^6 A549 cells per mouse through the tail vein. At six weeks following injection, mice were examined using Xenogen IVIS imaging (PerkinElmer, Inc., Waltham, MA, USA) to detect tumor metastasis status. Subsequently, the mice were sacrificed using the cervical dislocation method. Lung tissue was collected for analysis and the tumor number and volume within the lung was measured. One part of tumor tissue was fixed for H&E staining analysis, and the remaining was stored in a liquid nitrogen tank for subsequent studies.

Statistical analysis. Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Continuous variables were expressed as the mean ± standard error and analyzed using the Student's *t*-test (two-tailed), U test, χ^2 test and Fisher's exact test. Spearman's rank correlation analysis was performed to determine the correlation between two rank-order variables. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

RKIP expression levels are reduced in NSCLC cancerous tissues. Following obtaining ethical approval and informed patient consent, 100 patients with NSCLC were recruited to the present study. The clinicopathological information of patients is summarized Table I. There were 21 cases of TNM stage I/II and 79 cases of stage III/IV NSCLC. The variation

in levels of RKIP protein expression between these two groups was statistically significant ($P = 0.008$), with reduced RKIP expression levels in patients with late-stage NSCLC. A total of 82 patients had lymph node metastasis, whereas 18 were negative for metastasis. Of these, 47 (57.32%) patients with metastasis exhibited low RKIP expression levels and 9 (50.0%) of the patients without metastasis had high RKIP expression levels, a difference that was statistically significant ($P = 0.001$). When NSCLC distant metastasis was compared, 63 patients (65.08%) had developed distant metastasis and exhibited lower levels of RKIP expression, whereas 27 patients (72.97%) had intra-lung metastasis with significantly higher levels of RKIP expression ($P = 0.018$).

Histological sections of cancer and non-cancer tissues were stained with anti-RKIP or anti-pSTAT3 antibodies. Negative or weak staining of RKIP was observed in the majority of cancerous tissues compared with intense staining in non-cancerous tissues (Fig. 1A). However, intense pSTAT3 staining was observed in cancerous tissues (Fig. 1A). Bar chart quantification of RKIP and pSTAT3 positive staining revealed a significant difference between cancerous and non-cancerous tissues (Fig. 1B; $P = 0.00256$; paired *t*-test). Furthermore, patients with long distance metastasis exhibited lower RKIP expression levels compared with patients with within-lung metastasis ($P = 0.018$; Table I).

Reduced RKIP expression levels are associated with NSCLC intra-lung or long distance metastasis. Tissues obtained from patients that were lymph node metastasis-positive and -negative were examined using western blotting. The results revealed that patients that were positive for lymph node metastasis had lower levels of RKIP protein expression, as compared with tissues from patients that were negative for metastasis (Fig. 2A). The grayscale density of each band was evaluated using Photoshop software, and calculated in Prism 5.0 software. The results revealed that tissue from patients with lymph node metastasis had significantly lower levels of RKIP expression compared with that of patients without metastasis (Fig. 2B; $P = 0.0013$; χ^2 test). This finding indicated that NSCLC metastasis may be associated with RKIP expression.

RKIP blocks IL-6-induced phosphorylation-mediated STAT3 activation and inhibits NSCLC cells metastasis. In order to elucidate whether RKIP overexpression is able to inhibit STAT3 phosphorylation in NSCLC cells, the RKIP gene was constructed into pLenti6.3/V5-DEST lentiviral vectors. Initially, the infective efficiency of the constructed RKIP lentivirus vector was assessed by examining GFP fluorescence, indicating that ≥90% of the cancer cells were infected with viral vectors (Fig. 3A), and western blot analysis also demonstrated RKIP overexpression (data not shown). The western blotting results revealed that overexpression of RKIP significantly suppressed IL-6-dependent ERK and STAT3 phosphorylation (Fig. 3B, $P = 0.028$). Upon overexpression of RKIP through Lenti7.3-RKIP infection, the Transwell invasive ability of NSCLC cells decreased (Fig. 3C, $P = 0.034$). Therefore, the results demonstrated that RKIP overexpression was able to inhibit STAT3 phosphorylation and cell migration in NSCLC cell lines.

Table I. Clinicopathological characteristics of patients with NSCLC and RKIP expression levels.

Clinical characteristics	Number of cases	RKIP expression levels			P-value
		Low (%)	Moderate (%)	High (%)	
Gender					
Male	76	40 (52.63)	23 (30.26)	13 (17.11)	0.341
Female	24	9 (37.50)	11 (45.83)	4 (16.67)	
Age					
≥60	56	18 (32.14)	23 (41.07)	15 (26.79)	0.452
<60	44	19 (43.18)	13 (29.55)	12 (27.27)	
TNM stage					
I/II	21	12 (57.14)	7 (33.33)	2 (9.52)	0.008
III/IV	79	41 (51.90)	23 (29.11)	15 (18.99)	
Lymph node metastasis					
Positive	82	47 (57.32)	22 (26.83)	13 (15.85)	0.001
Negative	18	5 (27.78)	4 (22.22)	9 (50.00)	
Distant metastasis					
Within-lung	37	3 (8.11)	7 (18.92)	27 (72.97)	0.018
Long distance	63	41 (65.08)	17 (26.98)	5 (7.94)	

RKIP, Raf kinase inhibitor protein; NSCLC, non-small cell lung cancer; TNM, tumor node metastasis.

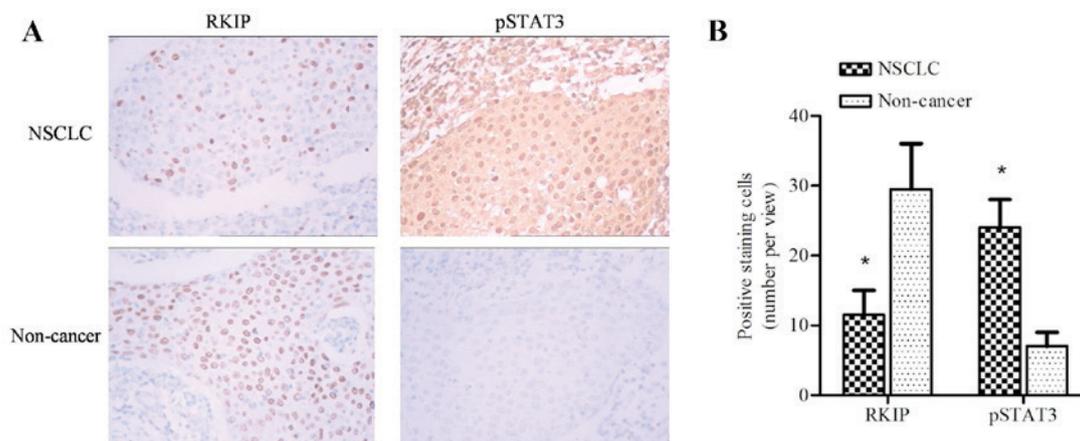


Figure 1. (A) Paired cancer and non-cancer tissues from patients with NSCLC were stained with anti-RKIP or anti-pSTAT3 antibody. (B) Counting of RKIP and pSTAT3-positively stained cells under phase-contrast microscopy are presented in bar charts produced using Prism 5.0 software ($P < 0.05$; paired *t*-test; error bars represent the mean + standard deviation). NSCLC, non-small cell lung cancer; pSTAT3, phosphorylated signal transducer and activator of transcription 3; RKIP, Raf kinase inhibitor protein.

RKIP knockdown promotes STAT3 phosphorylation and NSCLC-cell metastasis. A549 and NCI-H1299 NSCLC cells were seeded into 6-well plates 24 h prior to RKIP siRNA interference. Transfection reagent-mediated siRNA interference was administered to cancer cells. At 48 h following incubation, cells were collected and analyzed. Western blot results revealed that STAT3 phosphorylation was decreased in the presence of IL-6 stimulation in the two transfected cell lines, compared with the control group (Fig. 4A and B).

RKIP overexpression suppressed xenograft tumor metastasis in nude mice. The *in vivo* study was designed to mimic the progressive growth of lung metastatic tumors through tail

vein injection of infected or control A549 NSCLC cells at a density of 1×10^6 cells/mouse. At 6 weeks following transplantation, bioluminescence was utilized to conduct the lung cancer cell-derived tissue metastasis assay. Fluorescence signals from mice were detected using the Xenogen IVIS imaging system. The results indicated that the RKIP overexpression group had lower numbers of metastatic tumors within the lung or the whole body, compared with the control group (Fig. 5A; $P = 0.0063$; Fisher's exact test). Hematoxylin and eosin staining was performed on mouse tissues, revealing that the lungs exhibited reduced tumor mass in the RKIP overexpression group, compared with the control group (Fig. 5B). The experimental results indicated the overexpression of

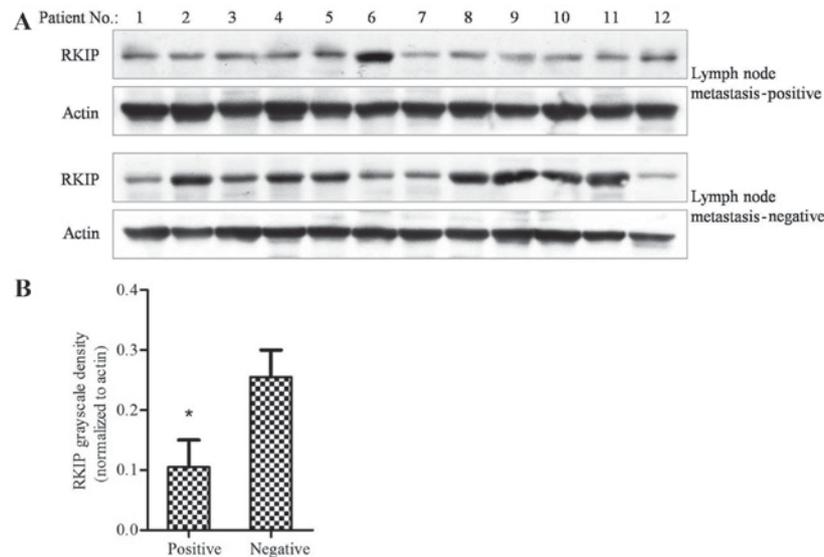


Figure 2. (A) Western blot analysis of RKIP expression levels in tissue from lymph node metastasis-positive or lymph node metastasis-negative patients with NSCLC. (B) The grayscale density of RKIP protein expression was evaluated using Photoshop software. The comparison of RKIP expression levels between these two sub-groups was analyzed using the Mann-Whitney U-test ($P < 0.05$; error bars represent the mean + standard deviation). NSCLC, non-small cell lung cancer; RKIP, Raf kinase inhibitor protein.

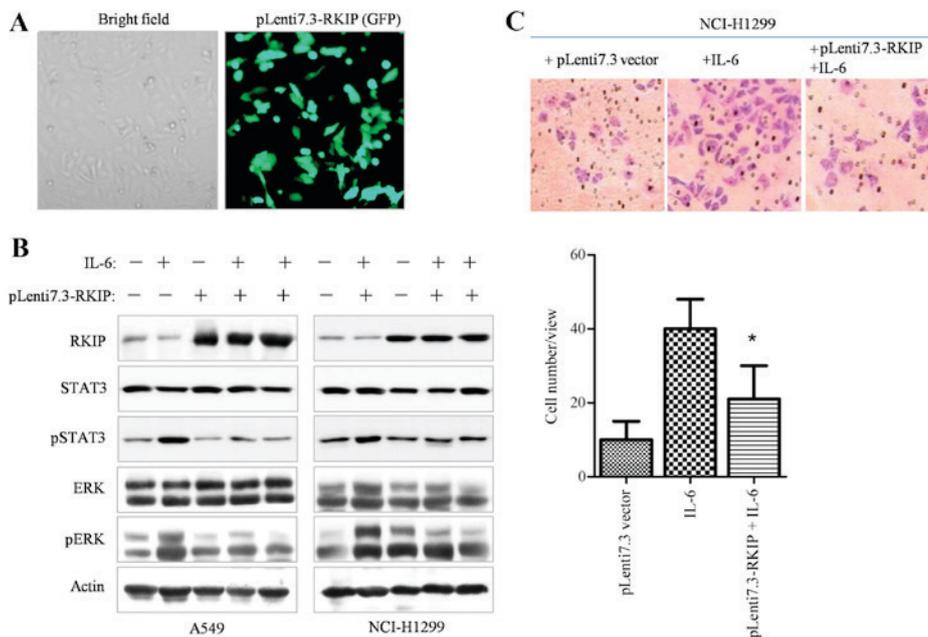


Figure 3. (A) GFP detection indicated that $\geq 90\%$ of NSCLC cells were effectively infected with virus particles. (B) Western blotting results revealed that overexpressed RKIP significantly suppressed ERK and STAT3 phosphorylation in A549 and NCI-H1299 cells. (C) Transwell invasion assay was performed to assess the invasiveness of NSCLC cells infected with blank lentivirus or RKIP-containing lentivirus. Representative images of invaded cells are shown, and 5 random fields were counted per well ($P < 0.05$). GFP, green fluorescent protein; NSCLC, non-small cell lung cancer; ERK, extracellular signal-regulated kinase; pERK, phosphorylated extracellular signal-regulated kinase; STAT3, signal transducer and activator of transcription 3; pSTAT3, phosphorylated signal transducer and activator of transcription 3; IL-6, interleukin 6; RKIP, Raf kinase inhibitor protein.

RKIP may block A549 cancer cell metastasis and growth through the blockade of downstream signaling pathways, including the RAF/ERK/STAT3 pathway.

Discussion

The poor prognosis and shorter survival time of patients with NSCLC are closely associated with tumor cell aggressive

and long distance metastasis (2). The biological behavior of NSCLC cells may also impact the efficacy of therapeutic agents, due to its malignancy (2,20). In the present study, based on the data from 100 patients with NSCLC, negative or weak staining of RKIP was observed in the majority of cancerous tissues, compared with an intense signal in non-cancerous tissues (Fig. 1A). Patients at an increased clinical stage (III/IV) exhibited lower levels of RKIP expression,

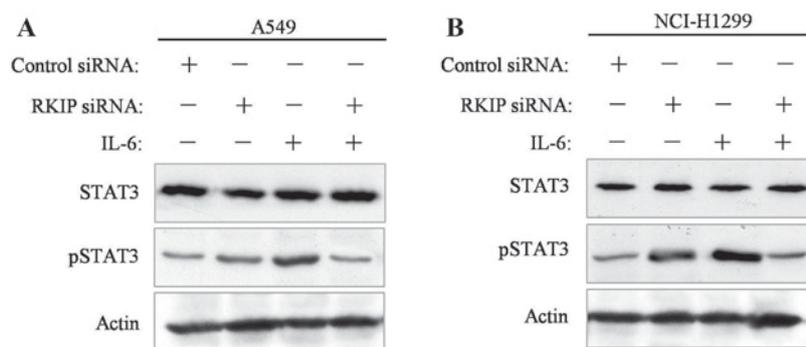


Figure 4. Western blot analysis revealed that siRNA-mediated RKIP silencing blocked IL-6-induced STAT3 phosphorylation in (A) A549 cells and (B) NCI-H1299 cells. RKIP, Raf kinase inhibitor protein; IL-6, interleukin 6; STAT3, signal transducer and activator of transcription; pSTAT3, phosphorylated signal transducer and activator of transcription 3; siRNA, small interfering RNA.

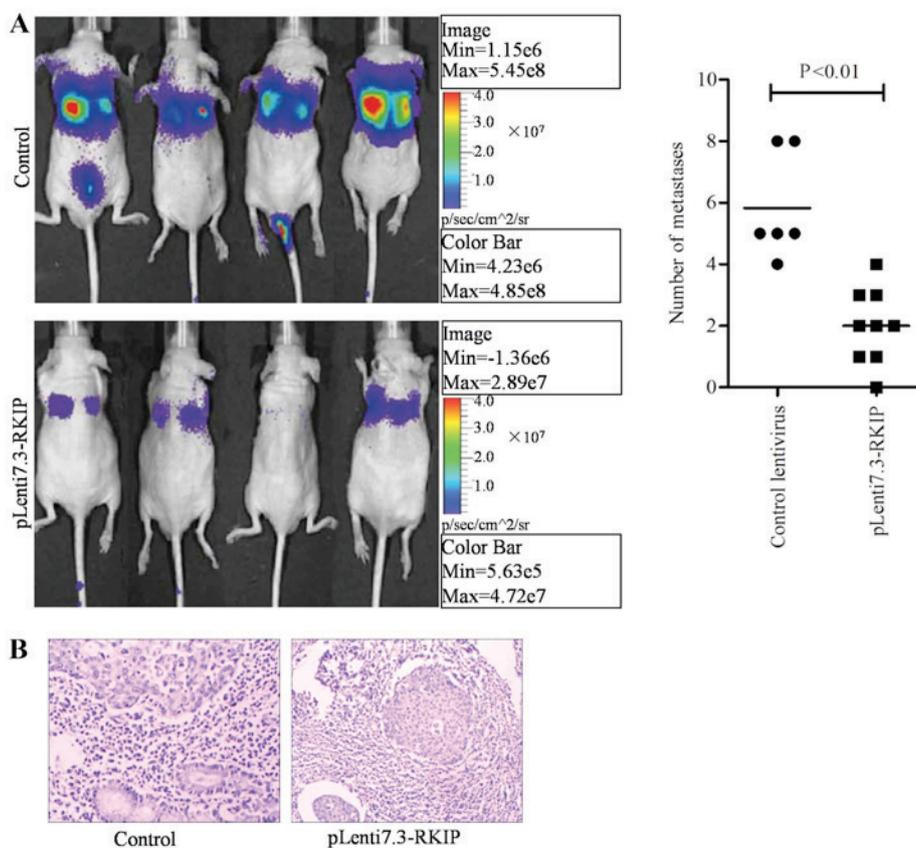


Figure 5. (A) Xenogen IVIS imaging of xenograft-bearing mice, which were injected with normal A549 cells or RKIP-overexpressing A549 cells through the tail vein. Counting of metastasis hotspots from these two groups is presented in the bar chart. (B) Hematoxylin and eosin staining was performed on tumor tissues. RKIP, Raf kinase inhibitor protein.

whereas stage I/II patients had higher levels of RKIP expression and an improved prognosis. In total, 57.32% patients with lymph node metastasis exhibited low levels of RKIP expression, but 50% of lymph node metastasis-negative patients had high levels of RKIP expression. Additionally, the statistical data revealed that 65% of long-distance metastasis patients had low levels of RKIP expression, whereas >72% of patients with within-lung metastasis exhibited high levels of RKIP expression ($P=0.018$, Table I). Taken together, these findings reveal that NSCLC metastasis is inversely correlated with RKIP expression levels. This result is concordant with a previous study examining NSCLC and RKIP expression (12).

A previous study revealed that silenced Raf expression promoted tumor invasive ability, whereas its upregulation suppressed tumor invasive ability (21). Other previous studies have indicated that Raf kinase activated transcriptional activity via activation of the ERK signaling pathway (22,23). RKIP is a negative regulator of the RAS-mitogen-activated protein kinase and ERK signaling pathway (24). A previous study indicated using immunohistochemistry that RKIP expression levels were detectable in all non-cancerous prostate tissues and were decreased to low or undetectable levels in all prostate cancer metastases (25). Similar results have been demonstrated in malignant melanoma, colorectal, breast, thyroid and liver

cancer (26-28). Concordant with the results from previous studies, the current study also revealed that RKIP may be associated with the Raf/MEK/ERK/STAT3 pathway, and clinical data from 100 patients with NSCLC also supported the hypothesis that RKIP expression levels are associated with NSCLC metastasis. Patients with positive metastatic lymph nodes, with intra-lung or long-distance metastasis, had lower RKIP protein levels (Figs. 1 and 2; Table I), and lower RKIP expression levels were observed with increasing TNM stage. Previous findings have demonstrated that pRKIP is a predictive indicator of lung cancer survival (24). Therefore, future studies may further examine the role of RKIP in NSCLC, as well as investigating pRKIP, its kinase and underlying mechanisms in this form of cancer.

In conclusion, the present study suggests an important role for RKIP in NSCLC on the basis of *in vitro* data, *in vivo* metastatic experiments and clinical statistic analysis. The findings strengthen the hypothesis that RKIP suppresses NSCLC cell metastasis through blocking of the Raf/ERK/STAT3 signaling pathway.

Acknowledgements

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