Effect of Raf kinase inhibitor protein expression on malignant biological behavior and progression of colorectal cancer

YUN WANG[1,2*], LING-YUN WANG[3*], FEN FENG[4], YANG ZHAO[5], MA-YAN HUANG[1,6], QIONG SHAO[1,7], CUI CHEN[8], HUI SHENG[1,9], DONG-LIANG CHEN[1,2], ZHAO-LEI ZENG[1,9], RUI-HUA XU[1,2] and YU-HONG LI[1,2]

[1]State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center, Collaborative Innovation Center for Cancer Medicine; [2]Department of Medical Oncology, Sun Yat-sen University Cancer Center; [3]Department of Gastroenterology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou; [4]Department of Oncology, The First People's Hospital of Foshan City, Foshan, Guangdong; [5]Department of Anesthesiology, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou; Departments of [6]Pathology, and [7]Molecular Diagnostics, Sun Yat-sen University Cancer Center; [8]Department of Oncology, The First Affiliated Hospital, Sun Yat-sen University; [9]Department of Experimental Research, Sun Yat-sen University Cancer Center, Guangzhou, P.R. China

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Abstract. The Raf kinase inhibitor protein (RKIP) is a novel metastasis suppressor. RKIP was previously found to have low expression in a colorectal cancer (CRC) patient cohort by immunohistochemistry. However, the role of RKIP in CRC remains undetermined. In the present study, immunohistochemistry was performed to compare RKIP expression between 129 paired stage II CRC and adjacent non-tumorous tissues. The correlations between clinical parameters, prognosis and RKIP expression were evaluated. To investigate the effect of RKIP on proliferation and metastasis, RKIP was overexpressed and knocked down in colon cancer cell lines. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Transwell and wound-healing assays were performed. Murine models were established to confirm the influence of RKIP on malignant tumor phenotypes *in vivo*. Our results showed that RKIP expression was significantly decreased in the CRC tissues compared to the adjacent non-cancerous tissues ($p<0.001$) and was correlated with the risk of relapse in stage II CRC ($p<0.05$). Overexpression of RKIP suppressed HCT116 cell metastasis *in vitro* and *in vivo*, whereas knockdown of RKIP expression in SW480 cells and its murine model increased metastatic ability ($p<0.05$). No effect of RKIP on cell proliferation in CRC was observed. These data suggest that RKIP is an important metastasis-suppressor gene in CRC. The re-expression of RKIP could be a potential therapeutic target for antimetastatic strategies for CRC.

Correspondence to: Professor Yu-Hong Li, State Key Laboratory of Oncology in South China and Department of Medical Oncology, Sun Yat-sen University Cancer Center, Collaborative Innovation Center for Cancer Medicine, 651 Dongfeng Road East, Guangzhou 510060, P.R. China
E-mail: liyh@sysucc.org.cn

*Contributed equally

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Introduction

Colorectal cancer (CRC) is the sixth most common malignancy and the fifth leading cause of cancer-related death in China (1). Although a majority of patients with CRC can be cured by surgery, approximately 50% of these patients eventually develop metastasis and succumb to the disease (2). Even at identical stages, the incidence of metastasis varies among patients (3,4), which demonstrates the heterogeneity of these tumors. The underlying molecular mechanism of metastasis is an intricate process, which is still unclear. Thus, defining new metastasis-related biomarkers is an important goal towards prognostic evaluation and targeted therapy.

Raf kinase inhibitor protein (RKIP) is a highly evolutionarily conserved protein of the phosphatidylethanolamine-binding protein family (5), which is ubiquitously expressed in various tissues and organisms in many mammals including human beings (6,7). Human RKIP is a 23 kDa protein that is encoded by a 1,434 bp long mRNA transcribed from a gene located at chromosome 12q24.23 (6,7). It was first designated as RKIP in 1999 due to its negative regulation of mitogen-activated protein kinase (MAPK) signaling through Raf-1 binding (8). Recent data indicate that loss or down-regulation of RKIP expression could be associated with poor prognosis and distant metastasis in certain types of human cancers such as breast (9), prostate (10), ovarian cancer (11) and others (9-16). In CRC, Al-Mulla *et al* first indicated that loss of RKIP expression may be involved in the metastatic process of CRC through immunohistochemistry (IHC) in 269 patients (13). In our studies, the results corroborated the potential prognostic value of RKIP in the distant metastasis of CRC (17-19). However, most of these studies were performed in Western cohorts by histological IHC detection. The role of

RKIP in CRC remains underdetermined. Furthermore, RKIP serves as only a metastatic suppressor without any impact on the tumorigenic phenotype in breast and prostate cancer (9,10); however, it is both a metastatic and tumorigenesis suppressor in ovarian cancer (11). To further illuminate the role of RKIP in CRC, we explored the association between RKIP expression with clinical characteristics and prognosis of CRC, and we further investigated the effect of RKIP on the metastatic and proliferative properties of human colon cancer cells *in vitro* and *in vivo*.

Materials and methods

Patients and tissue specimens. Paraffin-embedded tissue samples were sectioned for IHC from primary tumors and adjacent non-cancerous tissues. The tissues were obtained from 129 randomly selected stage II CRC patients who underwent surgery at the Sun Yat-sen University Cancer Center (Guangzhou, China) from January 1998 to December 2002. The CRC patients were histopathologically and clinically diagnosed with CRC (T3/4N0 M0, stage II) according to the American Joint Committee on Cancer (AJCC) TNM staging system. The clinical information was collected from unprocessed medical files and pathological reports. The study was carried out with the approval of the Ethics Committee of the Sun Yat-sen University Cancer Institutional Board, and prior written informed consent was obtained from all of the patients involved.

IHC. The corresponding tissue blocks were cut into 5 μm thick sections. Hematoxylin and eosin (H&E) sections were used for analyzing the tumor location. IHC was performed with a rabbit-RKIP antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to standard procedures (20). Human benign prostate tissues were used as a positive control. For the negative control, tissue slides were incubated with PBST instead of the rabbit-RKIP antibody. In the present study, a semi-quantitative estimation was made by both the extent and intensity of immunoreactivity: the percentage of staining was defined as follows: ≤5%=0, >5 to ≤25%=1, >25 to ≤50%=2, and >50%=3. The scores for intensity were evaluated as 0 for negative staining; 1 for weak staining; 2 for moderate staining; and 3 for strong staining. Finally, the indexed sum was acquired by the addition of the intensity grade and the percentage of the staining area. Slides were scored by two independent pathologists who were blinded to the patient data. Discrepancies were resolved by consensus after reevaluation of the slides. A score of 4.5 was suggested as a cut-off for dichotomizing RKIP levels for the prognosis of TTP and overall survival (OS) according to ROC curves. If the final score was <4.5, the tumor was considered to have low expression; otherwise, the tumor was considered to have high expression.

Cell culture and transfection. Human colon cancer cell lines (HT-29, HCT116, SW480 and LoVo) were obtained from the American Type Culture Collection and were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified chamber with 5% CO_2 at 37˚C. For stable overexpression of endogenous RKIP, the coding sequence of RKIP was amplified and subcloned into the LV5 (pGCMV/GFP+Puro) vector, a lentivirus purchased from GenePharma Biotech (Shanghai, China), according to the manufacturer's instructions. HCT116 cells were then transfected with the overexpressing RKIP lentivirus or negative control lentiviral vectors and named HCT116/RKIP and HCT116/vector cells, respectively. To generate stable RKIP-knockdown cells, an annealed short interfering RNA (siRNA) for RKIP was inserted into the pGPU6/GFP/Neo vector (GenePharma Biotech) according to the manufacturer's instructions to obtain pGPU6/GFP/Neo containing RKIP targeting short hairpin RNA (pGPU6/GFP/Neo-shRKIP). The target sequence for effective knockdown of RKIP expression was 5'-CCC ACC CAG GTT AAG AAT A-3'. pGPU6/GFP/Neo-shRKIP or empty pGPU6/GFP/Neo vectors, which served as negative controls, were transfected into SW480 cells named SW480/sh-RKIP and SW480/vector, respectively. All stable clonal cells generated were selected and cultured according to the manufacturer's instructions for further studies. Western blot assays were used to detect the expression of RKIP in all stable cell lines as described below.

Quantitative real-time RT-PCR. Total RNA was isolated from the tissues or cell lines using TRIzol (Invitrogen). Then, 2 μg of total RNA was reverse-transcribed into cDNA in a 10 μl reaction solution for real-time PCR (RT-PCR) using GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) as directed by the manufacturer. The programmed parameters for RT-PCR were as follows: heating at 95˚C for 10 min to activate AmpliTaq Gold polymerase, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing and extension at 60˚C for 60 sec. The expression levels of β-actin were used as an endogenous control to ensure equal loading of the samples. Invitrogen synthesized both oligonucleotide primers for RKIP and β-actin (Shanghai, China). The primer sequences used were as follows: RKIP forward, 5'-CAA TGA CAT CAG TGG CAC AGT C-3' and RKIP reverse, 5'-CAC AAG TCA TCC CAC TCG GCC TG-3'; β-actin forward, 5'-TGG ATC AGC AAG CAG GAG TA-3' and β-actin reverse, 5'-TCG GCC ACA TTG TGA ACT TT-3'. The relative mRNA expression of RKIP was normalized against the internal control and analyzed by the $2^{-\Delta\Delta ct}$ method. The experiment was performed in triplicate and repeated three times.

Western blot analysis. Western blot analyses were performed using a standard protocol (21). Next, 24 μg of protein extracts was subjected to 12% SDS-PAGE gel electrophoresis. A rabbit anti-RKIP antibody (1:50; Santa Cruz Biotechnology) and a rabbit anti-GAPDH antibody (1:5,000; Cell Signaling Technology, Danvers, MA, USA) were used for analysis according to the manufacturer's instructions. RKIP protein levels were normalized to the total GAPDH levels on the same membrane, which were visualized using an enhanced chemiluminescence ECL detection system (KeyGen Biotech, Nanjing, China).

MTT assay. Cell proliferation was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HCT116/vector and HCT116/RKIP cells were

plated at a density of 2,000 cells/well, and SW480/vector and SW480/sh-RKIP cells were plated at 4,000 cells/well. The absorbance value of each sample was read at 570 nm using a microplate reader. All experiments were performed in octuplicate to calculate the average result.

Wound-healing assay. To determine the effect of RKIP on cell mobility, a scratch test was performed. Monolayer cells were scrape-wounded using standard micropipette tips. The vertical distance of the inner face of the denuded zone was measured using a fluorescence inverted microscope at 0, 12, 24 and 36 h. All experiments were performed in triplicate.

Transwell migration and invasion assays. To better evaluate the invasive and migratory potentials of the cells, 24-well Transwell chambers (8 μm pores) from BD Biosciences were used. For the migration assay, the tumor cells were resuspended at a density of $6x10^5$ cells in 200 μl serum-free medium and transferred to the top chamber of each insert without matrix gel. Then, 500 μl of serum was added to the lower compartment as the chemotactic factor. After incubation for 16 h, the non-migrating cells on the upper side were removed, and the cells that migrated to the undersurface were fixed and dyed with 0.1% crystal violet. In parallel, the invasion Transwell assay was performed as mentioned above with the inclusion of Matrigel mix pre-coated on the inserts and cultured for 24 h. The number of migrating or invading cells was microscopically quantified by counting five independent fields. The final results were compared using the mean of triplicate assays (22).

In vivo proliferation and metastasis assays. Female BABL/c athymic nude mice aged 5 weeks were purchased from the Animal Center of Guangdong Province (Guangzhou, China) and maintained under specific pathogen-free conditions. For the *in vivo* proliferation assays, a total of 10^6 cells of HCT116/RKIP or SW480/sh-RKIP were injected subcutaneously into the left dorsal flanks of nude mice and the corresponding negative control cells were injected into the right (n=6). An algorithm, volume = length x width x length x 0.5236 (23), was used to calculate the tumor volume every 4 days. Four weeks after injection, the animals were sacrificed and the tumors were weighed. To investigate the effect of RKIP on metastasis, a concentration of $2x10^7$ cells/ml of the HCT116/RKIP, HCT116/vector, SW480/sh-RKIP and SW480/vector cells was injected into the tail veins of mice. Six weeks after injection, the mice were sacrificed, and the lungs and livers were dissected out and embedded in paraffin. The animal tissue blocks were then cut into 4 μm sections consecutively for further staining with H&E. The micrometastases in the lungs and livers were examined and counted by pathologists who had no prior knowledge of the mouse groups (24). All of the *in vivo* experiments were conducted in strict accordance with the National Institutes of Health guidelines. The protocol was approved by the Ethics Committee of Animal Experiments of the Sun Yat-sen University Cancer Center.

Statistical analysis. The associations between RKIP expression levels and clinical characteristics were evaluated using the Chi-square analysis. Survival curves were drawn using the Kaplan-Meier method and assessed by the log-rank test.

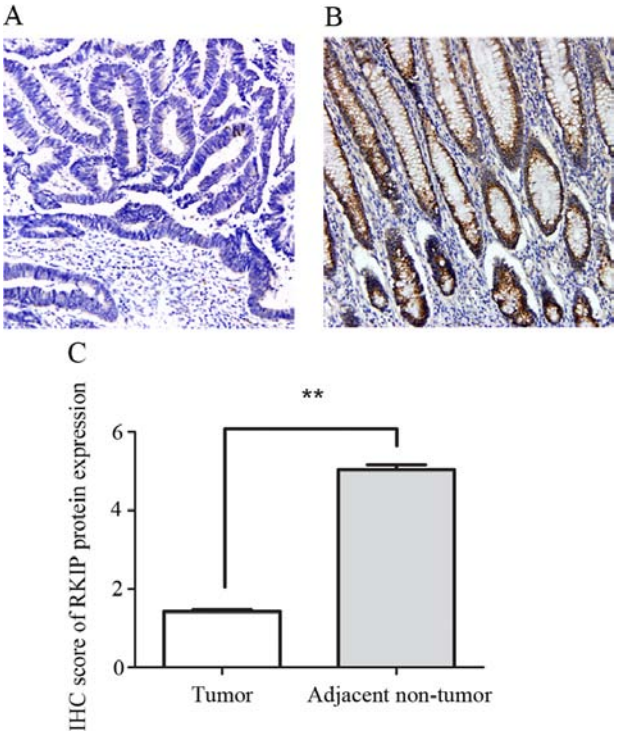

Figure 1. Comparison of RKIP expression in tissues by IHC. (A and B) Representative immunohistochemical images of tumor and adjacent non-tumor tissues, respectively. Magnification, x200. (C) RKIP expression was significantly lower in tumor tissues than adjacent non-tumorous tissues as detected by IHC. Error bars represent the standard error of the mean; $^{**}p<0.01$. RKIP, Raf kinase inhibitor protein; IHC, immunohistochemistry.

Disease-free survival (DFS) was defined as the interval between the operation date to the date of metastasis or recurrence and OS was computed from the date of surgery to the date of death, or the last follow-up. Univariate and multivariate analyses were performed using the Cox proportional hazards regression model. A Student's t-test was used to analyze the single comparison between two means. All tests were two-sided and considered to be significant with p-values of <0.05. Data are expressed as the mean ± standard error mean (SEM) unless otherwise stated. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 13.

Results

Loss of RKIP expression in CRC and its correlation with prognosis. In order to investigate the expression pattern of RKIP in human CRC, IHC was performed to detect RKIP in 129 cases of primary CRC tumor samples as well as 127 cases of adjacent non-cancerous tissues. RKIP staining had a predominantly cytoplasmic and membrane-associated distribution and was rarely nuclear (Fig. 1A and B). The average IHC score in 129 primary CRC tumor samples was 3.95, which was significantly lower than that in the 127 adjacent non-cancerous tissues (average IHC score was 5.04, p<0.001) (Fig. 1C). Moreover, 74 of the 129 primary CRC tumor samples (57.4%) had low RKIP protein expression. In contrast, in the adjacent non-cancerous tissues, only 35 of 127 cases (27.7%) had low RKIP expression staining (p<0.001; Table I). These results indicate that

Table I. Comparison of RKIP expression between tumor and adjacent non-tumor tissues.

		RKIP expression		
Tissue type	N	Low, n (%)	High, n (%)	P-value
T	129	74 (57.4)	55 (42.6)	<0.001[a]
ANT	127	35 (27.6)	92 (72.40	

T, tumor tissue; ANT, adjacent non-tumor tissue. RKIP, Raf kinase inhibitor protein. [a]evaluated by Pearson's Chi-square test.

RKIP expression was significantly downregulated in CRC in comparison to the adjacent non-cancerous tissues.

Among the 129 cases of primary stage II CRC, 74 cases of primary CRC tumor samples had low RKIP protein expression, and reduced RKIP expression was found to have a significant correlation with tubular adenocarcinoma (p=0.015; Table II). No statistically significant association was found between RKIP expression and other clinicopathological variables including gender, age, location of primary mass, tumor size, depth of invasion and tumor differentiation (Table II).

To further investigate the prognostic significance of RKIP expression, DFS and OS analyses were performed in these 129 CRC cases using Kaplan-Meier analysis with a log-rank test (Fig. 2). At the end of the follow-up time, 17.8% of the cases (23/129) were observed to have metastasis and/or disease recurrence. Patients with low RKIP expression had a shorter DFS time than those with high RKIP expression (median DFS 125 vs. 143 months; p=0.032; Fig. 2A). Additionally, the T3 stage population (106 cases) was separately analyzed to evaluate the prognostic effect of RKIP by excluding the 23 T4 stage cases. A good correlation between the low RKIP expression and relapse independent of T stage (median DFS 106 vs. 126 months; p=0.049; Fig. 2B). Furthermore, univariate and multivariate analyses indicated that RKIP expression was an independent prognostic factor for CRC relapse (Table III). However the relationship between RKIP expression and overall survival was not statistically significant both in the entire studied population (median OS 125 vs. 107 months; p=0.058; Fig. 2C) and in the T3 stage population (median OS 126 vs. 108 months; p=0.065; Fig. 2D).

Effects of RKIP expression on proliferation and metastasis of CRC in vitro. We detected both RKIP mRNA and protein levels by qPCR and western blotting in four CRC cell lines (HT29, Lovo, SW480 and HCT116) and in one normal colonic tissue. RKIP was found to be reduced in all these CRC cell lines compared to the normal tissue (Fig. 3A and B). To further investigate the influence of RKIP on malignant phenotypes in CRC *in vitro*, the CRC cell line HCT116 which had the relatively lowest RKIP expression level, was chosen for reconstituting RKIP expression. The CRC SW480 cells which had the relatively highest RKIP expression level were chosen for RKIP shRNA knockdown experiment. The effects of exogenous RKIP overexpression in HCT116 cells and RKIP knockdown in SW480 cells were confirmed by western blotting (Fig. 3C).

Table II. Correlation between the RKIP level and the clinicopathological features of stage II CRC patients.

		RKIP expression		
Features	N	Low, n (%)	High, n (%)	P-value
Gender				
Male	75	43 (57.3)	32 (42.7)	0.993[b]
Female	54	31 (57.4)	23 (42.6)	
Age (years)				
≤60	73	45 (61.6)	28 (38.4)	0.262[b]
>60	56	29 (51.8)	27 (48.2)	
Location				
Colon	69	36 (52.1)	33 (47.8)	0.136[b]
Rectum	60	38 (63.3)	22 (36.7)	
Tumor size (cm)				
≤5	69	44 (63.8)	25 (36.2)	0.115[b]
>5	60	30 (50.0)	30 (50.0)	
T classification				
T3	106	59 (55.7)	47 (44.3)	0.401[b]
T4	23	15 (65.2)	8 (34.8)	
Histology				
TA	118	72 (32.2)	46 (39.0)	**0.015**[a]
MA	11	2 (8.3)	9 (75.0)	
Pathological differentiation				
Poor	19	10 (61.0)	9 (47.4)	0.651[b]
Moderate/well	110	64 (58.2)	46 (41.8)	
Preoperative CEA (ng/ml)				
≤5	102	58 (56.9)	44 (43.1)	0.981[b]
>5	21	12 (57.1)	9 (40.9)	
Preoperative LDH				
Normal	112	63 (56.3)	49 (43.8)	0.783[b]
Elevated	15	9 (60.0)	6 (40.0)	

TA, tubular adenocarcinoma; MA, mucinous adenocarcinoma; RKIP, Raf kinase inhibitor protein; CRC, colorectal cancer; CEA, carcinoembryonic antigen; LDH, lactate dehydrogenase. [a]Evaluated by the Chi-square test with continuity correlation; [b]unless otherwise noted, evaluated by the Pearson's Chi-square test.

Cell motility was determined using migration and invasion assays. As shown in Fig. 4A and B, the restoration of RKIP expression inhibited HCT116/RKIP cells from penetrating the polycarbonate membrane and a collagen matrix by an average of 48.5 and 45.6%, respectively, compared to the HCT116/vector cells. Similar results were observed in the SW480 cell line in which RKIP knockdown significantly increased cell migration and invasion (Fig. 4C and D). Moreover, RKIP suppression of cell motility was confirmed by a wound healing assay. At 32 h, wound closure in the HCT116/RKIP cells was modest compared to the HCT116/vector cells (26.7±6.1 vs. 65.1±4.0%; p<0.001). Conversely,

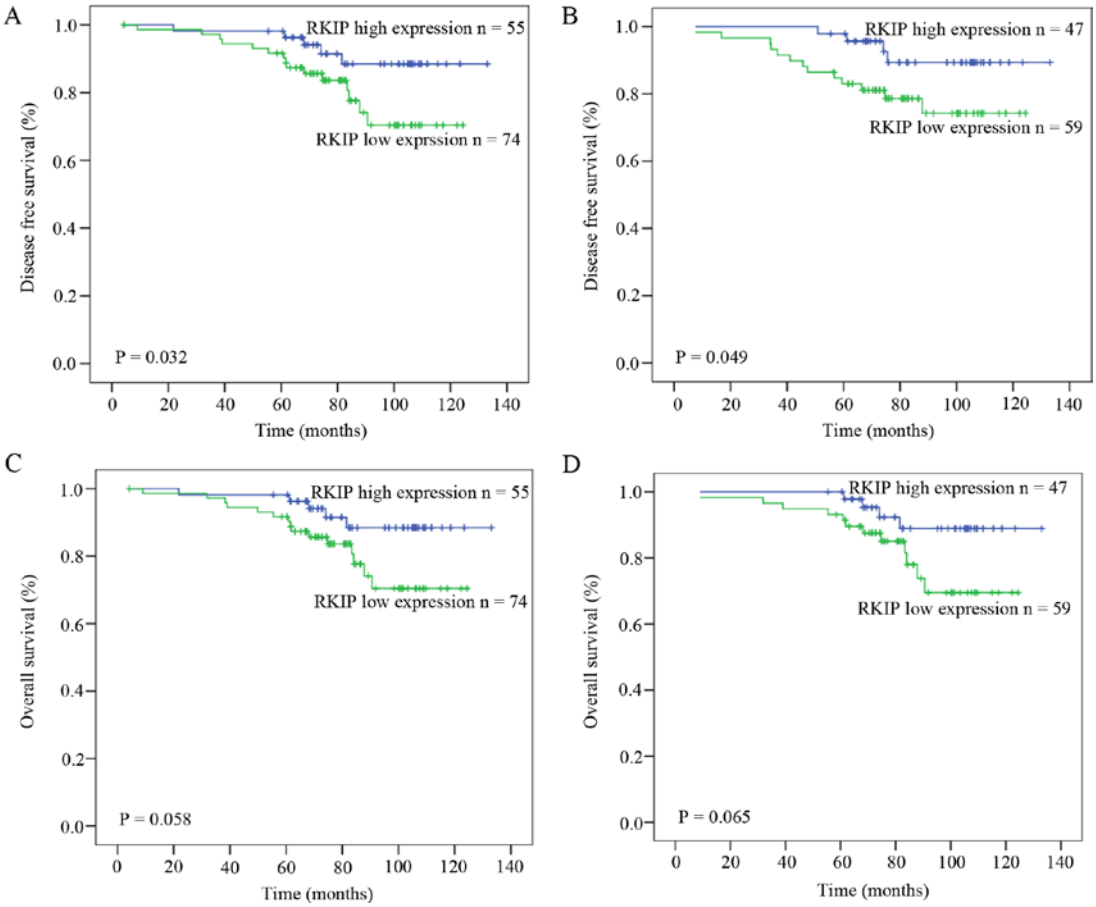

Figure 2. Effect of RKIP expression on prognosis. (A and C) Kaplan-Meier analyses of DFS and OS based on RKIP expression in all patients, respectively. (B and D) Kaplan-Meier curves of DFS and OS for T3 stage patients divided according to low RKIP expression vs. high RKIP expression, respectively. RKIP, Raf kinase inhibitor protein; DFS, disease-free survival; OS, overall survival.

Table III. Univariate and multivariate analyses of various prognostic parameters for metastasis or relapse in stage II CRC patients.

	Univariate analysis		Multivariate analysis	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years (≤60/>60)	1.024 (0.990-1.059)	0.170	0.791 (0.286-2.185)	0.650
Location (colon/rectum)	1.568 (0.687-33.580)	0.285	1.265 (0.454-3.520)	0.653
Tumor size (≤5/>5 cm)	0.873 (0.383-1.991)	0.747	1.077 (0.397-2.920)	0.884
Histology (TA/MA)	0.458 (0.062-3.396)	0.445	0.000 (0.000-)	0.982
Pathological differentiation (P/M/W)	1.061 (0.450-2.498)	0.893	0.535 (0.188-1.518)	0.240
T classification (T3/T4)	1.600 (0.590-4.339)	0.356	1.971 (0.575-6.749)	0.280
PBO (yes/no)	1.074 (0.363-3.176)	0.897	2.258 (0.626-8.139)	0.213
LN (<12/≥12)	0.335 (0.078-1.439)	0.142	0.174 (0.035-0.876)	**0.034**
Adjuvant chemotherapy (yes/no)	0.935 (0.218-4.003)	0.928	0.610 (0.105-3.537)	0.581
Preoperative CEA (≤5/>5 ng/ml)	2.789 (1.125-6.914)	**0.027**	3.471 (1.258-9.580)	**0.016**
Preoperative LDH (normal/elevated)	1.861 (0.625-5.543)	0.265	2.276 (0.607-8.540)	0.223
RKIP expression (low/high)	0.333 (0.123-0.8970	**0.030**	0.265 (0.082-0.861)	**0.027**

HR, hazard ratio; CI, confidence interval; TA, tubular adenocarcinoma; MA, mucinous adenocarcinoma; P, poor; M, moderate; W, well; PBO, preoperative bowel obstruction; LN, number of lymph nodes at biopsy. CRC, colorectal cancer; CEA, carcinoembryonic antigen; RKIP, Raf kinase inhibitor protein.

the distance closure of the SW480/vector cells was much shorter than that of the SW480/sh-RKIP cells (47.7±4.1 vs. 70.2±2.3%; p<0.001; Fig. 4E and F). Next, the effect of RKIP on proliferation of cancer cells was evaluated using the MTT

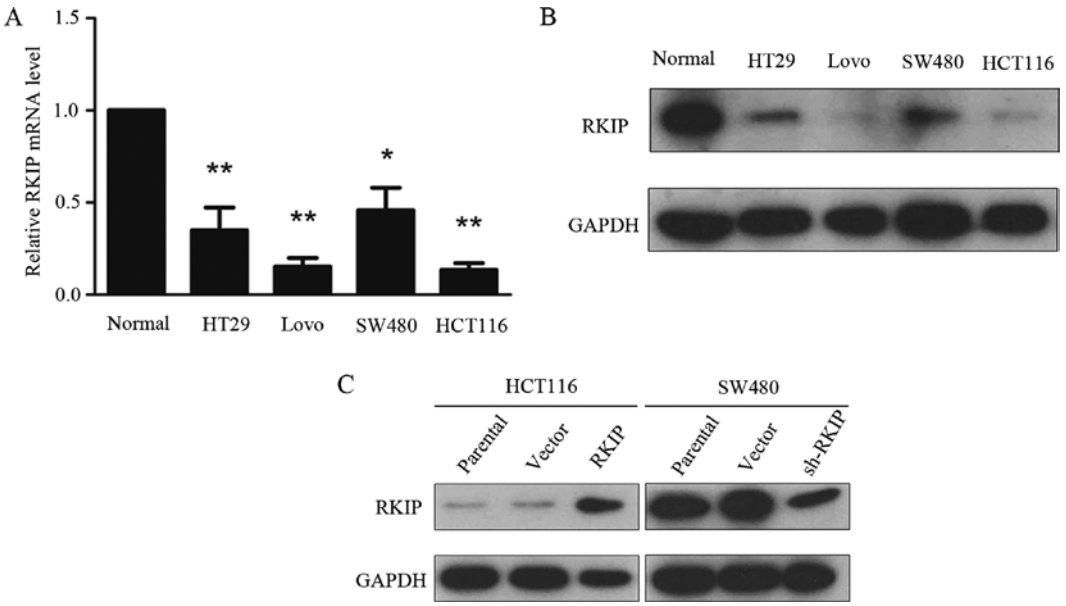

Figure 3. Reduction of RKIP mRNA and protein in the cancer cell lines. (A and B) RKIP mRNA and protein levels were detected in colon cancer cell lines (HT29, Lovo, SW480 and HCT116) and normal colonic tissue by qPCR (A; normalized to β-actin) and western blotting (B; normalized to GAPDH). Error bars represent the standard error of the mean; *p<0.05; **p<0.01. (C) The level of RKIP protein was increased after overexpression in the HCT116 cells and decreased after knockdown in the SW480 cells. RKIP, Raf kinase inhibitor protein.

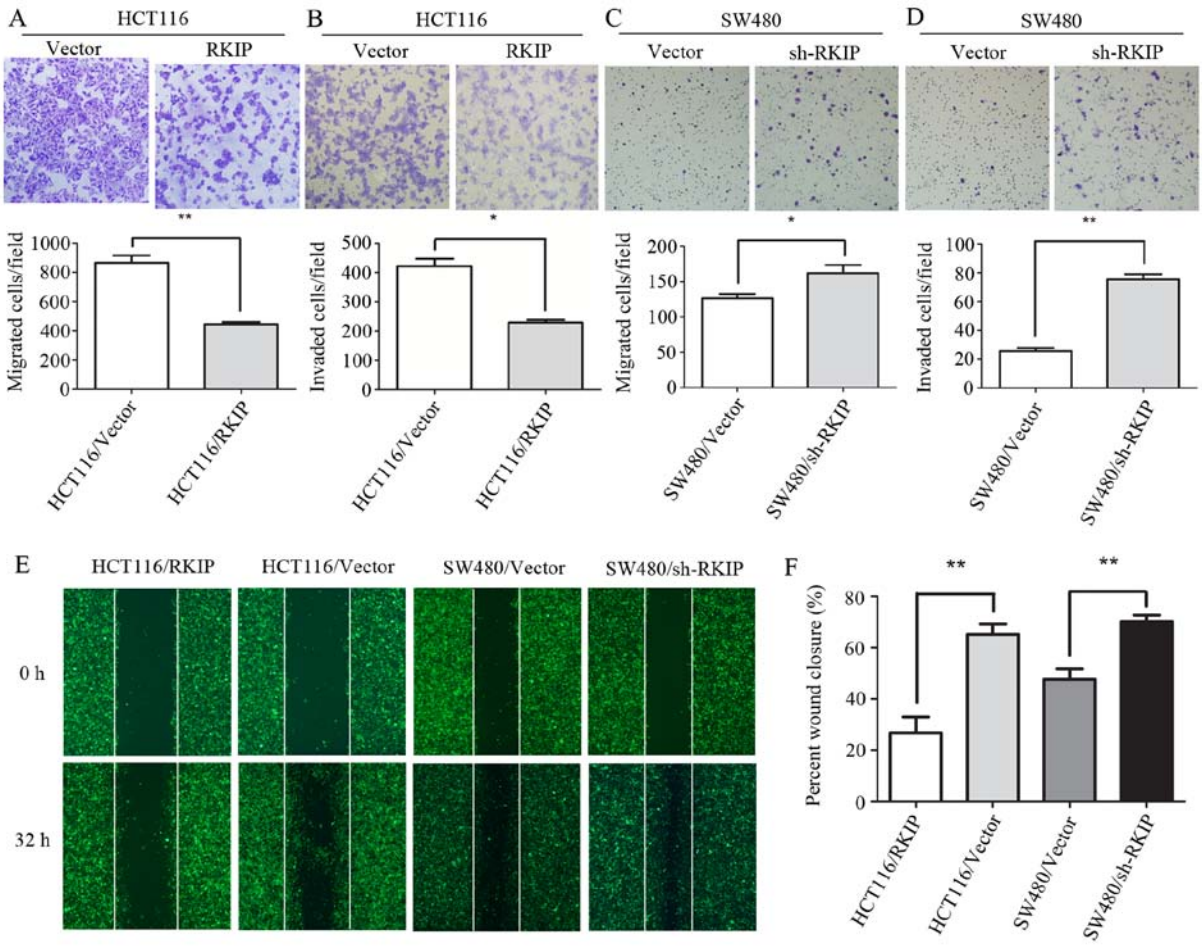

Figure 4. Effect of RKIP expression on *in vitro* malignant phenotypes. (A and C) Migration Transwell assay. Restoration of RKIP impaired the migratory ability of HCT116 cells (A), whereas knockdown of RKIP enhanced the migratory ability of SW480 cells (C). (B and D) Overexpression of RKIP reduced cell invasion in HCT116 cells (B), whereas knockdown of RKIP promoted this ability in SW480 cells (D) as demonstrated by invasion Transwell assays. (E and F) The wound healing assay was measured under a fluorescence inverted microscope at 36 h, indicating that the HCT116/RKIP cells had reduced wound healing and conversely, SW480/sh-RKIP had increased wound healing ability, compared to the control cells. Bars, ± SEM; *p<0.05; **p<0.01. RKIP, Raf kinase inhibitor protein.

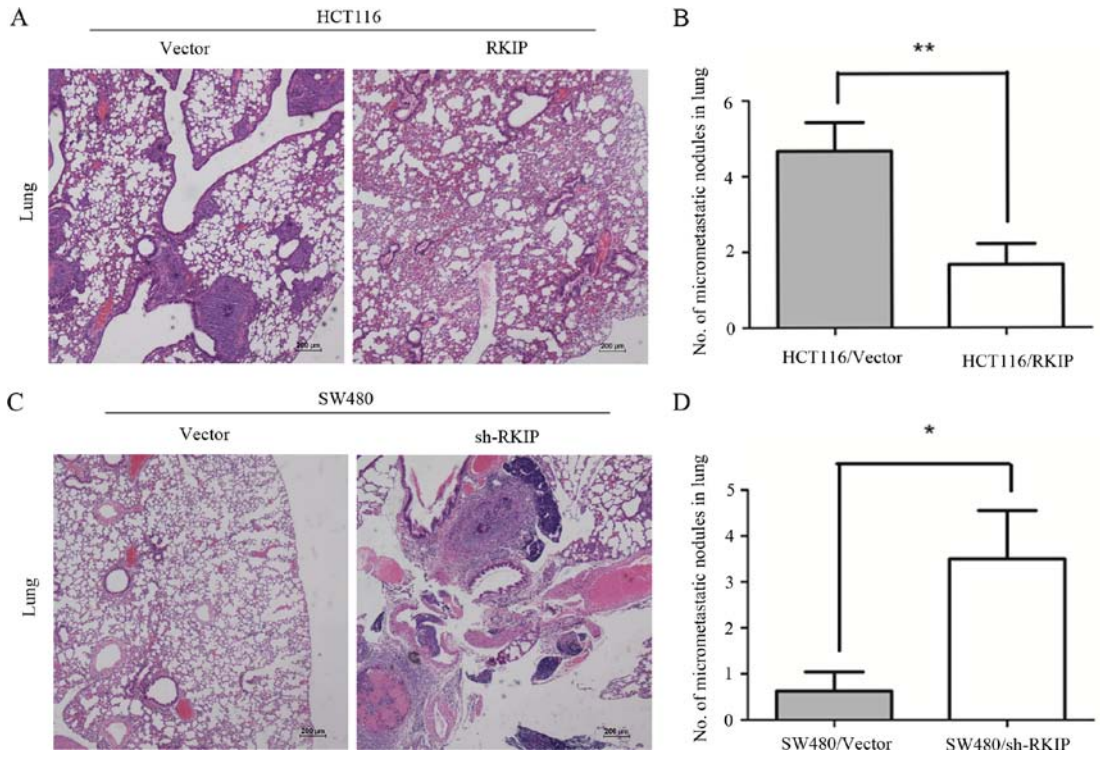

Figure 5. RKIP inhibits the metastasis of colorectal cancer cells in murine models. (A and B) The average number of lung micrometastases in mice injected with HCT116/RKIP cells was less than those injected with HCT116/vector cells. Magnification, x40. (C and D) The average number of pulmonary micrometastases in mice injected with SW480/vector cells was less than those injected with SW480/sh-RKIP cells. Magnification, x40. Bars, ± SEM; $^{*}p<0.05$; $^{**}p<0.01$. RKIP, Raf kinase inhibitor protein.

assay in the transfected cells. The cell proliferation curve indicated that neither exogenous overexpression nor reduction of RKIP had generated statistically significant differences in cell proliferative rates (p>0.05).

Effect of RKIP expression on CRC proliferation and metastasis in vivo. To further verify the inhibitory effect of RKIP on metastasis *in vivo*, the two paired transfected cell lines (HCT116/RKIP and HCT116/vector, SW480/sh-RKIP and SW480/vector) were injected into the tail veins of nude mice. After 6 weeks, the nude mice were sacrificed to measure lung and liver metastases. Only mice injected with the HCT116/vector cells had observed macrometastases when compared to the HCT116/RKIP group, including 66.7% (4/6 mice) pulmonary, 16.7% (1/6 mice) hepatic, 50% (3/6 mice) subcutaneous and 16.7% (1/6 mice) muscle macrometastases, indicating that the overexpression of RKIP markedly decreased the incidence of metastasis. Furthermore, the lung and liver micrometastases obtained from the four groups were examined and counted under a microscope. The average number of lung micrometastases in mice injected with the HCT116/RKIP cells was 64.3% less than that in mice injected with the HCT116/vector cells (p=0.010; Fig. 5A and B). The average number of pulmonary micrometastases in mice injected with the SW480/vector cells was 79.6% less than that in those injected with the SW480/siRKIP cells (p=0.039; Fig. 5C and D), which confirmed the role of RKIP in metastasis suppression *in vivo* in CRC. However, there were no statistically significant differences in the comparison of the average number of liver micrometastases between the two groups (p>0.05). To explore the effect of RKIP on tumorigenesis *in vivo*, the reconstructed cells and their control cells were injected into the left and right dorsal flanks of nude mice, respectively, as described in Materials and methods. The tumor growth curve was generated by measuring tumor volume over time, and the data showed no statistically significant difference between the reconstructed cells and their control cell groups. RKIP neither suppressed nor promoted tumor growth (Fig. 6).

Discussion

Metastasis contributes to the majority of CRC-related mortalities. Thus, a large number of genes have been identified as metastasis-suppressor genes for the use of clinical predication and treatment strategies. Previous studies suggest that RKIP may be a crucial metastasis-suppressor gene in CRC, based on Western patient cohorts (13,17-19). Our results showed that low RKIP expression significantly predicted the high risk of distant metastases in Chinese CRC stage II disease patients and showed a trend towards poor overall survival, although the results of overall survival were not statistically significant, most likely due to the low sample size and data mining. We also verified the independent negative prognostic value of RKIP in T3N0M0 stage disease. These results are consistent with previous studies. Doyle *et al* indicated that loss of RKIP predicted poor prognosis in Western Dukes' B CRC patients (17). Zlobec *et al* also reported that loss of RKIP endowed node-negative patients with a similar probability of metastasis as node-positive patients with positive RKIP expression (19). Nevertheless, in 74 patients with low RKIP

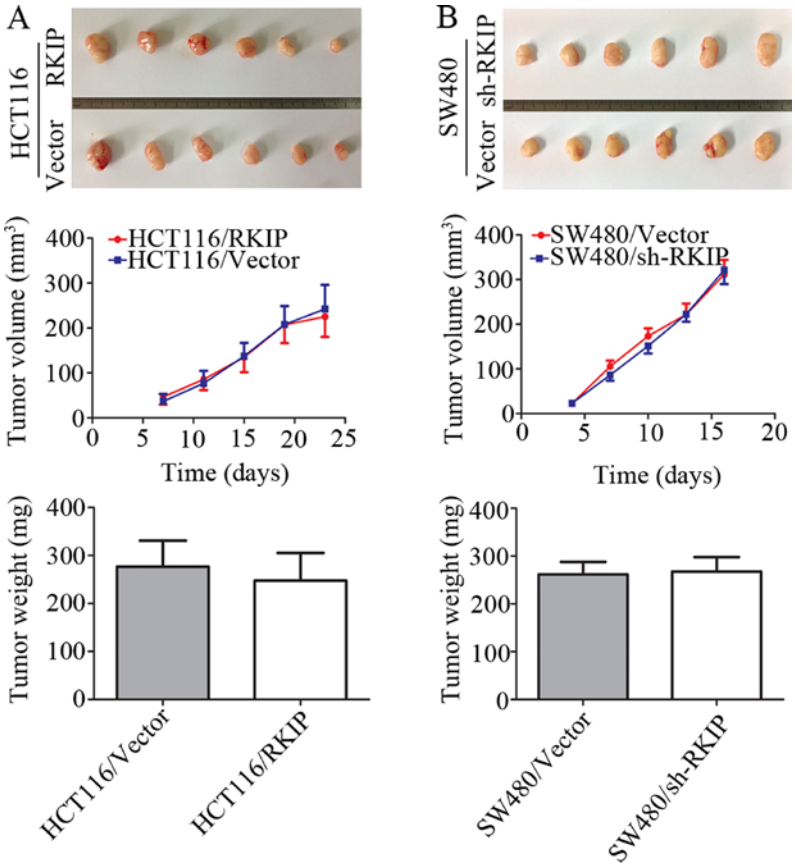

Figure 6. RKIP had no effect on proliferation *in vivo*. (A and B) Images of tumors derived from HCT116/RKIP or HCT116/vector (A), SW480/sh-RKIP or SW480/vector cells (B), after subcutaneous injection into nude mice. There were no statistically significant differences in the tumor growth curve and tumor weight between the two groups ($p>0.05$). RKIP, Raf kinase inhibitor protein.

expression, 77.0% of cases (57/74) were without metastasis or recurrence during the follow-up period, suggesting that a number of factors are likely involved and combined in assessing accurate risk-stratification. In the present study, RKIP expression was found to have a significant correlation with tubular adenocarcinoma; however, this conclusion should be carefully drawn due to a small sample bias.

To the best of our knowledge, most of the previous studies concerning RKIP in CRC were performed in Western cohorts by histological detection (13,17-19). Research focusing on the role of RKIP in CRC tumor biology *in vitro* and *in vivo* is limited. To this end, we overexpressed and knocked down RKIP expression in colon cancer cell lines, demonstrating that the ectopic expression of RKIP inversely affected cell migration and invasion abilities, which are two prerequisite components of the metastasis cascade. To convincingly confirm the inverse association between RKIP expression and metastasis, orthotopic nude mice were sacrificed to set-up a metastatic animal model. This revealed that knockdown of RKIP enhanced *in vivo* metastasis, whereas restoration of RKIP impaired metastasis. Additionally, we demonstrated that RKIP had limited impact on the cell proliferation or the subcutaneous transplanted model in nude mice. These results were in line with previous studies in prostate (10), breast (9) and gastric cancer (15). However, our results were not comparable to studies in epithelial ovarian cancer (11) and insulinoma (25) in which RKIP inhibited cell proliferation. Thus, the regulation of cell proliferation by RKIP is presumably under exquisite regulatory control through different signal transduction pathways in specific types of cancer.

In the present study, we clarified the role of RKIP in metastatic suppression; however, the RKIP-mediated pathological signal cascade that antagonizes CRC metastasis remains unclear. It has been reported that RKIP mediates crosstalk between distinct pathways, including the Raf/MERK/ERK (8), NF-κB (26,27) and G-protein pathways (28,29), and GSK3β signaling (30), which are all involved in pro-metastatic signaling pathways. It is possible that RKIP plays a pivotal role in coordinating more than one metastatic regulatory pathway ultimately suppressing the expression of metastasis-associated genes. To date, E-cadherin (31), matrix metalloproteinases (MMP-2 and MMP-9) (31), signal transducer and activator of transcription 3 (STAT3) (32), basic leucine zipper transcription factor 1 (BACH1) (33), and high mobility group AT-hook 2 (HMGA2) (34) have been shown to be downstream in the mechanism targeting metastatic suppression of RKIP in prostate and breast cancer. Further investigation of the mechanism of RKIP loss and its downstream signal transduction in CRC is warranted.

In conclusion, the present study indicates that reduced RKIP expression is correlated with metastasis and recurrence of disease in stage II CRC patients, as well as migration and invasion in colon cancer cell lines and animal models. RKIP is an important metastasis-suppressor gene in CRC.

Re-expression of RKIP may be a potential therapeutic target for an antimetastasis strategy in CRC.

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