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Original Article

Correlation of RKIP, STAT3 and cyclin D1 expression in pathogenesis of gastric cancer

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Abstract: RKIP is proposed as a new metastasis suppressor. Our recent study showed that RKIP inhibits malignant phenotypes of gastric cancer cells. However, the underlying mechanism of RKIP function in gastric cancer is unclear. This study aimed to investigate the correlation of RKIP, STAT3 and cyclin D1 expression in the tumorigenesis of gastric cancer. RKIP, STAT3 and cyclin D1 proteins were detected by immunohistochemistry in tissues of gastric ulcer (n = 27), gastric adenomatous polyp (n = 7), intestinal metaplasia (n = 26), dysplasia (n = 40), gastric carcinoma (n = 169) and metastatic lymph node (n = 36). RKIP, STAT3 and cyclin D1 mRNA levels were analyzed by RT-PCR in SGC7901 cells. We found that RKIP protein expression was significantly decreased in advanced gastric cancer and metastatic lymph node tissues while cyclin D1 and STAT3 protein expression was markedly increased in severe dysplasia, gastric cancer and metastatic lymph node tissue ($P < 0.01$). RKIP expression in gastric cancer was negatively correlated with the invasion, TNM stage and lymphoid node metastasis ($P < 0.01$), while cyclin D1 and STAT3 expression was positively correlated with histological differentiation and lymphoid node metastasis ($P < 0.01$). RKIP protein level was negatively correlated with cyclin D1 and STAT3 protein level, while cyclin D1 protein level was positively correlated with STAT3 protein level in gastric cancer samples. Moreover, reconstitution of RKIP in SGC7901 gastric cancer cells led to reduced cyclin D1 and STAT3 mRNA levels. In conclusion, these data suggest that RKIP inhibits gastric cancer metastasis via the downregulation of its downstream target genes STAT3 and cyclin D1.

Keywords: Gastric cancer, raf kinase inhibitor protein (RKIP), STAT3, cyclin D1

Introduction

Gastric cancer is a common malignancy with high incidence worldwide. The early symptom of gastric cancer is not obvious, and most patients have been found at a clinical late stage, with the overall 5-year survival rate of only 30% [1]. The pathogenesis of GC has not been fully understood, and the identification of new tumor markers and therapeutic targets for gastric cancer diagnosis and prognosis has important clinical significance for the prevention and treatment of gastric cancer.

Raf kinase inhibitor protein (RKIP) is a small molecule cytoplasmic protein involved in the regulation of several signal transduction pathways such as Raf-1-MAPK, NF-κB and G protein signaling pathways, and plays important role in cell growth, proliferation, differentiation, apoptosis and other physiological processes [2-4]. Recent studies suggest that RKIP is a new metastasis suppressor that could inhibit the metastasis of prostate cancer, breast cancer and melanoma [5-7]. In vitro experiments indicated that RKIP is a prognostic molecular markers and new targets for cancer therapy [8-10].

The signal transducer and activator of transcription3 (STAT3) and cyclin D1 are important regulatory factors that modulate tumor cell proliferation, apoptosis, invasion, metastasis and immune escape [11, 12]. Interestingly, several studies indicate that STAT3 and cyclin D1 are downstream targets of RKIP because RKIP could regulate the expression and function of STAT3 and cyclin D1 [13, 14]. Our recent study showed that RKIP inhibits the malignant phenotypes of gastric cancer cells [15]. However, the underlying mechanism of RKIP function in gastric cancer is unclear. Therefore, in this study we collected clinical samples of gastric cancer and gastric cancer cell lines to investigate the correlation of RKIP, STAT3 and cyclin D1 expression in the tumorigenesis of gastric cancer.

Table 1. Expression of RKIP, STAT3 and cyclin D1 in different lesions of gastric mucosae

Tissue type	Case	RKIP positive (%)	STAT3 positive (%)	cyclin D1 positive (%)
Gastric ulcer	27	23 (85.2)	6 (22.2)	4 (14.8)
Adenomatous polyp	7	5 (71.4)	1 (14.3)	1 (14.3)
Intestinal metaplasia	26	18 (69.2)	6 (23.1)	8 (30.8)
Moderate dysplasia	17	13 (76.5)	4 (23.5)	6 (35.3)
Severe dysplasia	23	16 (69.6)	12 (52.2)	10 (43.5)
Early gastric cancer	48	32 (66.7)	32 (66.7)*	30 (62.5)*
Advanced gastric cancer	121	36 (29.8)*	86 (71.1)*	89 (73.6)*
Metastatic lymph node	36	1 (2.8)*,#	23 (63.9)*	19 (52.8)*

*$P < 0.05$, #$P < 0.01$ compared to gastric ulcer.

Materials and methods

Clinical samples

Paraffin-embedded tissue specimens, including 7 cases of gastric adenomatous polyp diameter greater than 2 cm, 27 cases of gastric ulcer, 40 cases of dysplasia (including 17 cases of moderate dysplasia and 23 cases severe dysplasia), 26 cases of intestinal metaplasia, 48 cases of early gastric cancer, 121 cases of advanced gastric cancer and 36 cases of gastric cancer metastasis lymphoid tissues, were taken from Department of Pathology at Xiangya Hospital, stained by hematoxylin-eosin (HE) staining, and evlauated by two experienced pathologists. All patients with gastric cancer were diagnosed without radiotherapy or chemotherapy. The protocols were approved by The Ethics Committee of Xiangya Hospital.

Cell lines

Human gastric cancer SGC7901 cell line was purchased from Key Laboratory of Cancer Proteomics of Ministry of Health, Central South University, and cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C in 5% CO_2. Stable cell lines pcDNA3.1 (+)/SGC7901 and pcDNA3.1 (+)-RKIP/SGC7901 were described previously [15].

Immunohistochemical staining

Tumor tissues and normal tissues were fixed in 40 g/L paraformaldehyde and paraffin wax-embedded. Subsequently, 4 mm-thick serial sections of the tissues were cut. The sections were washed carefully with 0.01 M phosphate buffered saline (PBS) three times (10 min each), and then blocked with 2% goat serum in 0.01 M PBS containing 0.3% Triton X-100 (PBS-X) for 1 h at room temperature (RT). The sections were incubated at 4°C overnight with antibody against RKIP, cyclin D1, and STAT3 (Santa Cruz Biotechnology). Afterwards, the slides were subjected to immunohistochemical staining using PV-6001/6002 kit (Zhongshan Golden Bridge Biotechnology, Beijing, China). After visualizing the reaction with the DAB chromogen, the slides were counterstained with haematoxylin and observed under microscope. In negative control experiments, the primary antibodies were replaced with PBS.

Quantitative real-time PCR

Total RNA was extracted from the cells using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed using 2 ug RNA using Reverse Transcription System (Progema, USA). Real-time PCR was performed using SYBR Premix Ex Taq (Perfect Real Time) kit (TaKaRa, Japan). The primers were as follows: RKIP, 5'-ATAGACCCACCAGCATTTCG-3' and 5'-ACTGTGCCACTGCTGATGTC-3', cyclin D1, 5'-CCTGTCCTACTACCGCCTCA-3' and 5'-CACCTCCTCCTCCTCCTCTT-3', STAT3, 5'-TGCTGGTGACTGGATAGCAG-3' and 5'-CTCCTTGGAAGGTGCTGAAG-3', GAPDH, 5'-CCACCCATGGCAAATTCCATGGCA-3' and 5'-GGTGGACCTGACCTGCCGTCTAGA-3'.

Statistical analysis

The data were expressed as mean (X) ± standard deviation (S). Statistical analysis was performed using SPSS17.0 software (SPSS, Inc., Chicago, IL, USA) and $P < 0.05$ was considered significant.

Results

RKIP, STAT3 and cyclin D1 expression in gastric cancer clinical samples

Immunohistochemical analysis of RKIP, STAT3 and cyclin D1 expression in gastric clinical sam-

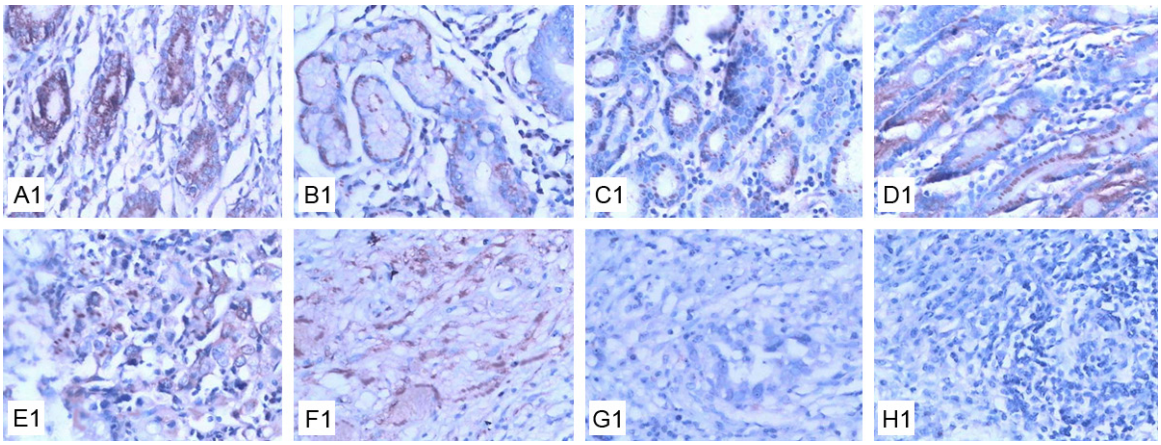

Figure 1. Expression of RKIP protein in different lesion of gastric mucosae. RKIP staining was positive in gastic ulcer (A1), adenomatous polyp (B1), moderate dysplasia and intestinal metaplasia (C1), severe dysplasia (D1), early gastric cancer (E1) and moderately differentiated adenocarcinoma (F1), but was negative in poorly differentiated adenocarcinoma (G1) and metastatic lymph node tissues (H1). Magnification: ×400.

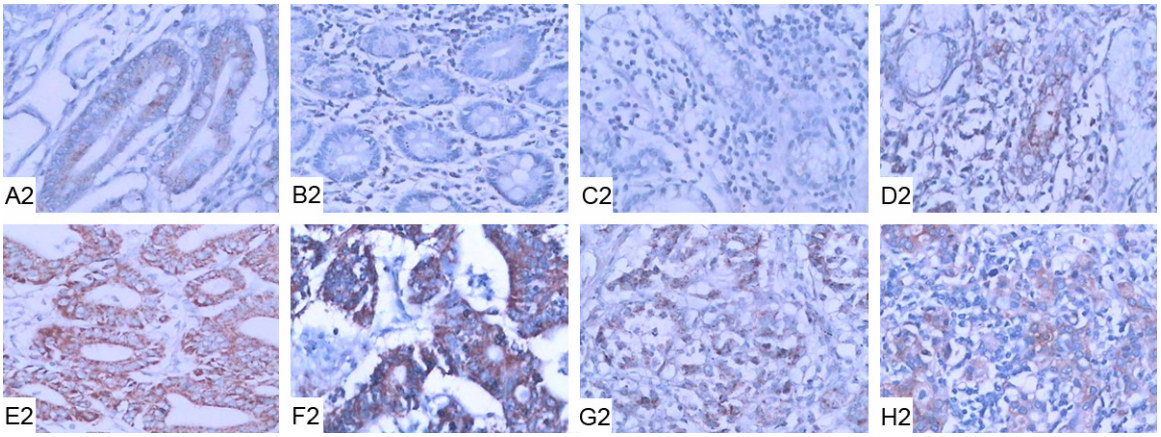

Figure 2. Expression of STAT3 protein in different lesion of gastric mucosae. STAT3 staining was weakly positive in gastic ulcer (A2) and intestinal metaplasia (D2), was negative in adenomatous polyp (B2) and moderate dysplasia (C2), but was positive in severe dysplasia (E2), early gastric cancer (F2), poorly differentiated adenocarcinoma (G2) and metastatic lymph node tissues (H2). Magnification: ×400.

ples was shown in **Table 1** and **Figures 1-3**. Statistical analysis showed that RKIP expression in the samples of gastric ulcer, gastric precancerous polyps, dysplasia, precancerous lesions of intestinal metaplasia showed no significant difference (χ^2 = 2.385, P > 0.05). In addition, RKIP expression in the samples of early gastric cancer and precancerous lesions showed no significant difference (χ^2 = 6.371, P > 0.05). However, RKIP expression in advanced gastric carcinoma and metastatic lymph nodes was significantly lower than in benign precancerous diseases, precancerous lesions and early gastric cancer (χ^2 = 47.717, P < 0.01), STAT3 and Cyclin D1 protein expression showed no significant difference in the samples of gastric ulcer, gastric polyps, moderate dysplasia and intestinal metaplasia (χ^2 = 2.682 and 3.483, P > 0.05). Compared with the above samples, STAT3 and Cyclin D1 expression in the samples of severe dysplasia, gastric cancer, advanced gastric cancer and metastatic lymph node tissue was significantly higher (χ^2 = 4.836-25.782, P < 0.05).

Correlation of RKIP, STAT3, cyclin D1 expression and clinical characteristics of gastric cancer

The correlation of RKIP, STAT3, cyclin D1 expression and clinicopathological parameters of gastric cancer was shown in **Table 2**. The results

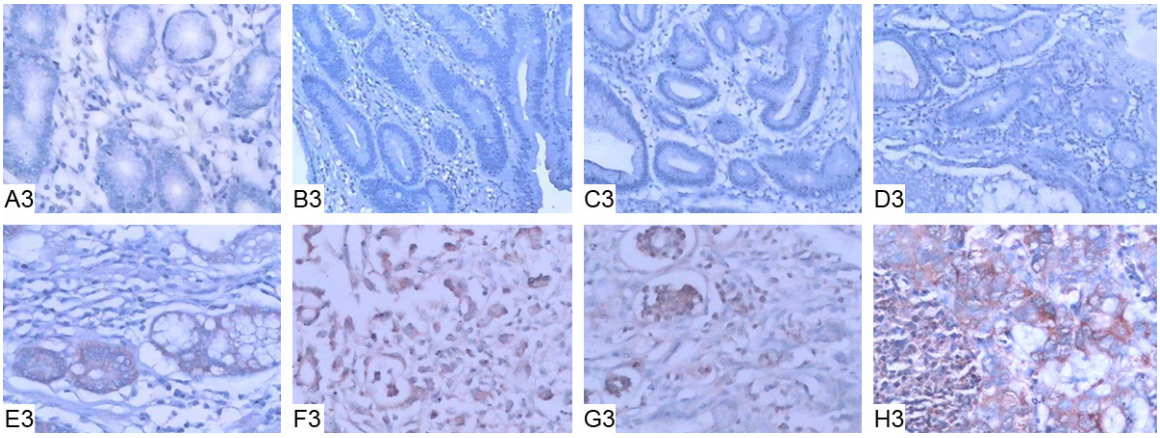

Figure 3. Expression of cyclin D1 protein in different lesion of gastric mucosae. Cyclin D1 staining was negative in gastic ulcer (A3), adenomatous polyp (B3), intestinal metaplasia (C3) and moderate dysplasia (D3), was positive in severe dysplasia (E3), early gastric cancer (F3), poorly differentiated adenocarcinoma (G3) and metastatic lymph node tissues (H3). Magnification, ×400.

Table 2. Correlation of RKIP, STAT and cyclin D1 expression with clinicopathological characteristics of gastric cancer

Characteristics	Case	RKIP positive positive N (%)	*P*	STAT3 positive N (%)	*P*	cyclin D1 positive N (%)	*P*
Age (years)							
≤55	104	43 (41.3%)		74 (72.1%)		74 (71.2%)	
>55	65	25 (38.5%)	0.179	44 (67.7%)	0.633	45 (69.2%)	0.790
Sex							
Male	125	50 (40.0%)		88 (70.4%)		88 (70.4%)	
Female	44	18 (40.9%)	0.947	30 (68.2%)	0.783	31 (70.5%)	0.995
Lauren type							
Intestinal type	101	42 (41.6%)		58 (57.4%)		73 (72.3%)	
Diffuse type	68	26 (38.2%)	0.530	60 (88.2%)	0.000	46 (67.6%)	0.518
Infiltration degree							
Mucosa and submucosa	48	33 (68.8%)		31 (64.5%)		22 (45.8%)	
Muscularis or serosa	68	24 (35.3%)		47 (69.1%)	0.486	54 (79.4%)	0.000
Penetrating serosa layer	53	11 (20.8%)	< 0.01	40 (75.5%)		43 (81.1%)	
Lymphatic metastasis							
N_{1-3}	97	22 (22.7%)		83 (85.6%)		86 (88.5%)	
N_0	65	46 (70.8%)	< 0.01	35 (53.8%)	0.000	33 (50.8%)	0.000
Tumor differentiation							
Well	14	6 (42.9%))		6 (42.9%)		7 (50.0%)	
Moderately	31	13 (41.9%)		21 (67.7%)	0.013	18 (58.1%)	0.033
Poorly	124	49 (39.5%)	0.736	97 (78.2%)		94 (75.8%)	
TNM stage							
I	48	33 (68.3%)		30 (62.5%)		25 (52.1%)	
II	23	11 (47.8%)		16 (69.6%)	0.398	16 (69.6%)	0.003
III/IV	98	24 (24.5%)	< 0.01	72 (73.5%)		78 (79.6%)	

showed that RKIP, STAT3, cyclin D1 protein expression was not correlated with the age and gender of gastric cancer patients. RKIP expression level was negatively correlated with the

Table 3. The correlation of RKIP and cyclin D1 or STAT3 protein expression in gastric cancer

	cyclin D1		*P*	STAT3		*P*
	Positive (%)	Negative (%)		Positive (%)	Negative (%)	
RKIP positive	13 (14.4)	17 (18.9)	< 0.01*	11 (12.2)	19 (21.1)	< 0.01#
RKIP negative	50 (55.6)	10 (11.1)		57 (63.3)	3 (3.3)	

*r = -0.411, **P* < 0.01, RKIP *vs.* cyclin D1, #r = -0.640, #*P* < 0.01, RKIP *vs.* STAT3.

Table 4. The correlation of cyclin D1 and STAT3 protein expression in gastric cancer

	cyclin D1		*P*
	Positive (%)	Negative (%)	
STAT3 positive	53 (58.9)	15 (16.7)	< 0.01
STAT3 negative	10 (11.1)	12 (13.3)	

r = 0.305, *P* < 0.01, cyclin D1 *vs.* STAT3.

depth of invasion, TNM stage and lymph node metastasis, but not correlated with tumor differentiation and Lauren classification. Cyclin D1 expression was positively correlated with tumor differentiation, Lauren classification and lymph node metastasis, but not with the depth of invasion and TNM stage. STAT3 protein expression was positively correlated with the depth of invasion, TNM stage, lymph node metastasis and tumor differentiation, but not with Lauren type of gastric cancer.

Correlation between RKIP, STAT3, cyclin D1 expression in gastric cancer

In gastric cancer, RKIP expression was negatively correlated with STAT3 protein and cyclin D1 protein expression (*P* < 0.01, **Table 3**), while cyclin D1 expression was positively correlation with STAT3 expression (*P* < 0.01, **Table 4**).

RKIP, STAT3, cyclin D1 mRNA levels in gastric cancer cells

To confirm the correlation of RKIP, STAT3, and cyclin D1 expression, we used SGC7901 cells stably overexpressing RKIP. RT-PCR analysis showed that compared to SGC7901 group and pcDNA3.1 (+)/SGC7901 group, PKIP mRNA level was significantly higher while STAT3 and cyclin D1 mRNA levels were significantly lower in pcDNA3.1 (+) -RKIP/SGC7901 group (**Figure 4**). These data indicate that RKIP mRNA expression is negatively correlated with STAT3 protein and cyclin D1 mRNA expression.

Discussion

Recent data suggest the role of RKIP as a potential metastasis suppressor. Our in vitro experiments showed that RKIP gene transfection inhibited SGC7901 gastric cancer cell growth and migration, increased apoptosis and delyed G0-G1 to S phase transition [15]. Notably, RKIP expression in intestinal-type gastric cancer was significantly lower and RKIP was proposed as an independent prognostic factor for intestinal gastric cancer [13]. Furthermore, immunohistochemical analysis showed that RKIP expression level was the highest in non-neoplastic gastric tissue, low in primary gastric cancer tissue, and the lowest in metastatic gastric cancer tissue, suggesting that RKIP may play a role in the tumorigenesis and metastasis of gastric cancer [16].

In this study we further characterized the expression of RKIP in normal gastric tissue, atrophic gastritis and gastric cancer tissues. Our results indicated high RKIP protein expression in clinical smaples of gastric ulcer, gastric adenomatous polyps, moderate dysplasia, and severe dysplasia, while in samples of advanced gastric cancer, particularly with lymph node metastasis of primary gastric cancer tissues and metastatic lymph tissue RKIP protein expression level was significantly reduced or absent, suggesting that RKIP downregulation may be not involved in the early gastric epithelial carcinogenesis process, but contribute to the progression of gastric cancer and lymph node metastasis. Further clinical and pathological analysis revealed that RKIP protein expression was negatively correlated with the depth of invasion, TNM stage and lymph node metastasis. Our data are consistent with previous studies reporting thst RKIP is downregulated in late events of gastric carcinogenesis [16-18].

As a Raf kinase inhibitor protein, RKIP plays important role in regulating MARK signaling pathway cascade. STAT3 and cyclin D1 are important components of cell signaling pathways such as Ras/Raf/ERK pathway. Upon the activation of Ras/Raf/ERK pathway transcrip-

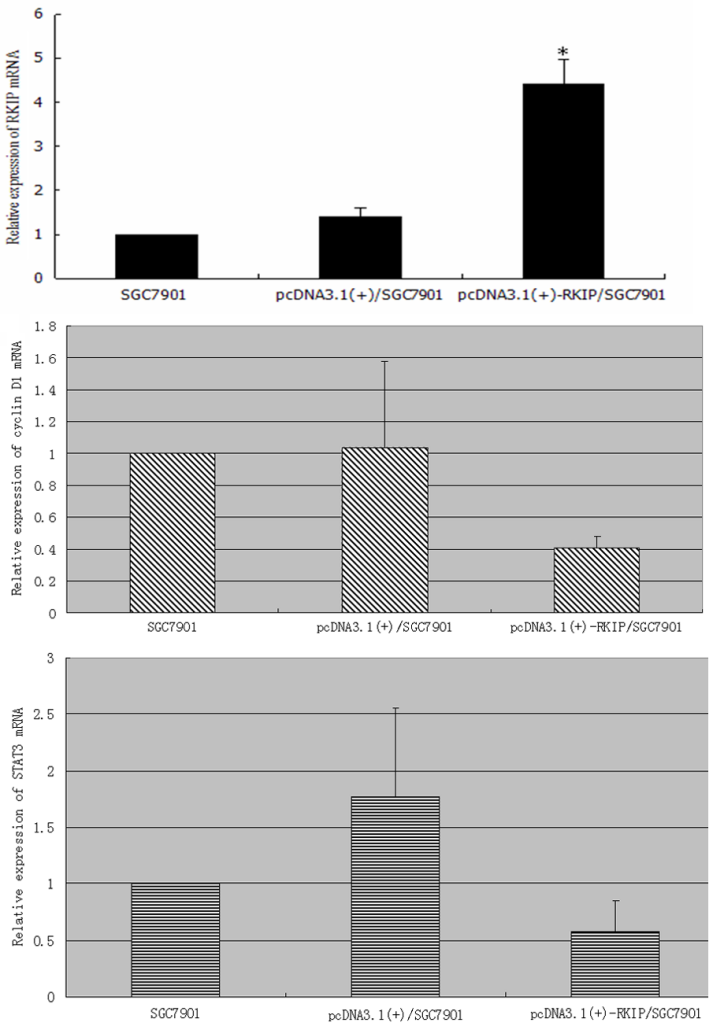

Figure 4. RKIP, cyclin D1 and STAT3 mRNA expression in SGC7901 cell lines. RKIP, cyclin D1 and STAT3 mRNA levels were determined by RT-PCR. Data were expressed as X ± S (n = 3). *$P < 0.01$.

tion factor STAT3 is phosphorylated and activated, which then activates the transcription of downstream targets such as cyclin D1, VEGF, Bcl-xL, leading to the modulation of cell growth, proliferation and differentiation [19, 20]. Since RKIP is proposed as a potential gastric cancer metastasis suppressor, we wondered whether its role in gastric cancer tumorigenesis is related to the regulation of cyclin D1 and STAT3 expression. We performed immunohistochemical staining of RKIP, cyclin D1 and STAT3. The results indicated that RKIP protein expression and STAT3 and cyclin D1 protein expression was negatively correlated, while STAT3 expression was positively correlated with cyclin D1 expression. Furthermore, we used gastric cancer cell line SGC7901 to construct RKIP overexpressing SGC7901 gastric cancer cell lines, RT-PCR analysis confirmed that reconstitution of RKIP expression led to significant downreguation of STAT3 and cyclin D1 mRNA expression levels, consistent with the results of immunohistochemical analysis. These data suggest that RKIP can negatively regulate the expression of its downstream targets STAT3 and cyclin D1, which may contribute to its tumor suppressor function. In support of our speculation, a recent study showed that STAT3 activation is crucial for pre-metastatic niche formation and metastasis of gastric cancer [21]. In addition, RKIP mediated inhibition of tumor metastasis may be related to the regulation of cell cycle proteins (cyclin) expression and regulation of cell cycle checkpoint function. Leslie et al. showed that excessive activation of STAT3 signaling pathway in tumor cells led to significantly increased cyclin D1 mRNA level while mutated cyclin D1 promoter significantly inhibited STAT3-induced cyclin D1 transcription [22]. These reports are consistent with our data and indicate that RKIP inhibits tumor invasion and metastasis via the downregulation of STAT3 and cyclin D1 expression.

Invasion and metastasis of gastric cancer is a complex multi-step pathological process that involves a variety of genes and factors. In the present study based on the analysis of clincal sampels and cell line of gastric cancer, we demosntrated that RKIP is involved in the development and metastasis of gastric cancer. Taken together, we hypothesized that RKIP is a suppressor of gastric cancer metastasis. Therefore, detecting RKIP expression level will help predict metastatic potential of gastric cancer and assess the prognosis of gastric cancer patients. On the other hand, RKIP is a promising target for gene therapy of gastric cancer metastasis.

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Disclosure of conflict of interest

None.

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