**Original Research Article**

# Phosphatidylethanolamine binding protein 1 enhances sensitivity of gastric cancer cell to 5-fluorouracil via inhibition of cell proliferation, migration and invasion

**Mengxin Lin[1-3], Xiaoyan Lin[1-3], Xiaobing Huang[1-3], Qing Liu[1-3], Riping Wu[1-3], Xinli Wang[1-3], Dongta Zhong[1-3]\***

[1]Department of Medical Oncology, Fujian Medical University Union Hospital, [2]Fujian Key Laboratory of Translational Cancer Medicine, [3]Fujian Medical University Stem Cell Research Institute, Fuzhou 350001, Fujian, PR China

*For correspondence: **Email:** qvxib1@163.com

## Abstract

*Purpose:* To determine the association between phosphatidylethanolamine binding protein 1, which is an Raf kinase inhibitor protein (RKIP), and 5-fluorouracil (5-FU) via analysis of the association between RKIP and clinical responses in individuals treated using fluorouracil-based chemotherapy.
*Methods:* Human gastric cancer cell lines MGC-803 and SGC-7901 were used in this study. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptosis and migration were determined with flow cytometry and Transwell chamber assays, respectively. The mRNA and protein expressions of apoptosis-related factors were assayed using real-time polymerase chain reaction (RT-PCR) and Western blotting, respectively, while the expression of RKIP was determined by immunohistochemical staining.
*Results:* Chemotherapeutic drug (5-FU) treatment induced low RKIP expression levels in tumorigenic GC cells, thereby sensitizing the cells to apoptosis (8.57 vs 1.25 %, p < 0.01). The highest RKIP level correlated well with initiation of apoptosis (4.20 vs 1.25 %, p < 0.01). Following in vitro downregulation of RKIP, there was increase in the viability and proliferation of RKIP-inhibited cells over time, and these changes were linked to alterations in cell cycle phases and increased optical density in MTT proliferation assay (1.55 vs 1.18, p < 0.01). In vitro Transwell assay measurement revealed an association between RKIP downregulation and enhancement of cell migration potential (652 vs 436, p < 0.01). Ectopic RKIP expression restored the apoptotic sensitivity of resistant cells (14.30 vs 1.36 %, p < 0.01). This sensitization was annulled by upregulation of survival routes. Reduction of RKIP by expression of antisense and siRNA conferred resistance on cancer cells sensitive to 5-FU-mediated apoptosis (6.88 vs 2.13 %, p < 0.01).
*Conclusion:* Thus, RKIP is a promising therapeutic strategy for improving the efficacy of clinically relevant chemotherapeutic drugs for GC.

*Keywords:* Gastric cancer, Raf kinase inhibitor protein, Cell proliferation, Invasion, Apoptosis, Chemotherapy, Phosphatidylethanolamine binding protein 1

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## INTRODUCTION

Gastric cancer (GC) is the second most prevalent cancer all over the globe. China is among several Asian nations with a high incidence of GC and high level of mortality from the disease. Although the incidence and mortality associated with GC have been declining steadily, prognosis in several cases is bad due to late diagnosis and metastasis.

The Raf kinase inhibitor protein (RKIP) is a globular protein with molecular weight of 20-25 kDa, and it belongs to the PEBP family made up of over 400 members [3]. It (RKIP) is phosphatidylethanolamine-binding protein in bovine brain. Studies have shown that RKIP usually binds to Raf-1 and blocks Raf-1-induced MEK phosphorylation [4,5]. In addition, RKIP regulates signaling routes and influences several processes in cells [6]. Moreover, RKIP exerts anti-angiogenic, anti-intravasating, anti-extravasating and anti-metastatic effects on tumors [7,8]. However, not much is known about the molecular mechanisms involved in the RKIP-induced inhibition of tumor metastasis.

Several signaling pathways are negatively modulated by RKIP. However, the precise pathways or effectors involved have not yet been identified. Thus, the identification of the signaling pathways and elucidation of the effector genes regulated by RKIP will not only enhance knowledge of the mechanism of suppression of metastasis, but will also be beneficial for inhibition of metastasis in the clinics. In this study, based on previous findings, the effect of RKIP on malignancy of GC and sensitivity to chemotherapy were investigated. Therefore, the expressions of RKIP protein in GC cells and normal cells were assayed, and the effects of RKIP suppression on the malignancy of GC and sensitivity to chemotherapy were determined. Specifically, the study was designed to determine if there is an association between RKIP expression and clinical response in GC cases subjected to fluorouracil-based chemotherapy.

## EXPERIMENTAL

### Cell lines, culturing and transfection

Human GC cell lines MGC-803 and SGC-7901 were obtained from Cell Bank of Chinese Academy of Sciences. They were maintained in RPMI-1640 having 10 % FBS and 1 % penicillin-streptomycin at 37 $^\circ$C in a 5 % $CO_2$ humidified incubator. The culture-related chemicals were bought from Hyclone, while 5-FU was bought from Sigma-Aldrich.

### Overexpression plasmid and shRNA

The RKIP shRNA targeting open reading frame of RKIP was 5′-CGAGCAGCTGTCTGGGAA GTA-3′. An unrelated 19-nt sequence (5′-TTCTCCGAACGTGTCACGT-3′) was used as shRNA-negative control. The coding sequence of RKIP was cloned into pcDNA3.1 plasmid by Clonetech. The negative control used was pcDNA3.1. In the overexpression process, $2 \times 10^6$ cells from each cell line were plated separately and cultured overnight. The serum-free medium was changed, followed by addition of siRNA or plasmid with lipofectamine 3000 (Invitrogen). After incubation for 6 h, the medium was replaced with 10 % FBS. After culturing for 48 h, the efficiency of inhibition or overexpression was measured with RT-PCR and Western blotting.

### MTT assay

In cell proliferation assay, each cell line was plated at a density of 2000 cells/well in triplicates in 96-well plates and were subjected to incubation for 2 days at 37°C in a 5 % $CO_2$ humidified chamber, followed by addition of 10 µL of MTT (10mg/mL) to each well, and incubation for 2 h at 37°C. Thereafter, the MTT was discarded, and 100 uL of dimethyl sulphoxide (Sigma) was added to every well, so as to solubilize the formazan crystals formed. The absorbance of formazan solution was read at 595 nm in a microplate reader (Thermo Fisher).

### Protein extraction and Western blot assay

Cells and tissues were lysed in 50 mM radioimmunoprecipitation assay buffer containing 150 mM sodium chloride, 1% NP-40, protease inhibitor, 0.5 % Na deoxycholic acid and 1 mM PMSF. The protein content of the lysate was measured using BCA protein assay kit. Equal protein levels were separated via SDS-PAGE, and trans-blotted onto PVDF membrane using semi-dry transfer. The membrane was blocked by incubation using 5 % skimmed milk for 60 min, and was thereafter incubated overnight with the following primary antibodies: RKIP (ab76582, Abcam); GAPDH (ab8245, Abcam); Bax (ab77566, Abcam); caspase-3 (ab32042, Abcam); Bcl-2 (ab32124, Abcam), and RKIP (ab76582, Abcam). Following rinsing, the membrane was treated with the 2° antibodies goat anti-mouse IgG HRP (m21001) and goat anti-rabbit IgG HRP (m21002) at room temperature for 60 min. Thereafter, ECL and Western blot detection system (GE Lifescience) were used to measure bound antibodies.

## RNA extraction and RT-PCR

Total RNA extraction was done using TRIzol reagent. First-strand complementary miRNA was produced from RNA with PrimeScript RT master Mix Perfect Real Time. The RT-PCR was done using SYBR green (Takara, Dalian, China) on Applied Biosystem Stepone Plus RT-PCR system, with GAPDH as loading control. Table 1 shows the sequences of the primers used.

## Flow cytometry

Cell apoptosis was measured flow cytometrically. Samples for cell cycle were harvested via tryptic digestion and rinsed two times in PBS. After addition of Annexin V-FITC and PI in the dark, the samples were allowed to stand at laboratory temperature for 15 min, followed with washing twice with binding buffer.

## Assay of cell migration

This was carried out using a Transwell chamber membrane with 8.0 μm pores in 24-well plates (Millipore). $2 \times 10^4$ cells in 100 uL serum-deficit medium were seeded onto the upper part of the Transwell chamber, while the lower chamber contained 1 mL 10 % FBS medium. After culturing for 8 h, the medium was discarded, and the non-migrating cells were wiped off with cotton bud, while migrative cells on the lower chamber were stained with 0.1 % crystal violet.

## Immunohistochemistry

Tissue sections (4-μm thick) were subjected to standard immunochemical staining procedures. The stained sections were examined (10 fields per section) for positive nuclear staining at x 400 amplification, followed by scoring. The % tumor-positive cells was classified viz: < 10 % = 0; 10 – 30 % = 1; 31 – 50 % = 2, and > 50 % =3. Total scores of 0 - 1 and ≥ 2 were considered as negative and positive, respectively.

## Statistical analysis

The results are presented as mean ± SD. (SPSS 17.0). Statistical analysis was done using *t*-test with SPSS 17.0. Values of $p < 0.05$ were assumed as indicative of significant differences.

# RESULTS

## Downregulation of RKIP

Table 1 shows that there was strong positive expression of RKIP in 85.0 % (68/80) of para-tumor tissue specimens, while the corresponding value in GC tissue was only 18.75 % (15/80) ($p < 0.001$). Figure 1 A presents the results of staining for GC and non-GC tissues. The original data in TCGA revealed higher expression of RKIP in GC than in non-cancerous tissues (Figure 1 B and C). These findings suggest that RKIP might be associated with the progression of GC.

## RKIP inhibited cell proliferation and enhanced chemosensitivity to 5-FU

To elucidate the role of RKIP in GC, MGC-803 and SGC-7901 cell lines were used to conduct further functional studies. In the first step, RKIP, sh-RKIP or NC was transfected into GC cells in order to unravel the gain- or loss-of-function effect on GC cell proliferation. Western blot and RT-PCR assays indicated that the transfection efficiency of RKIP increased 3 to 4 times, while the transfection efficiency of sh-RKIP decreased 2 to 3 times, when compared to control cells (Figure 2 A – F).

**Table 1:** Sequences of the primers used in the experiment

| Gene | forward | Reverse |
|------|---------|---------|
| RKIP | CAGCAGGAAGGATCCCAAATA | CACATAATCGGAGAGGACTGTG |
| Bax | GGAGCTGCAGAGGATGATTG | AGTTGAAGTTGCCGTCAGAA |
| Bcl-2 | GGAGGATTGTGGCCTTCTTT | GTTCAGGTACTCAGTCATCCAC |
| Caspase-3 | GCAGGACACCACAATGAGTA | GCCTAGATCCCTGAGCTTTG |
| GAPDH | GGTGTGAACCATGAGAAGTATGA | GAGTCCTTCCACGATACCAAAG |

The $2^{-\Delta\Delta Ct}$ procedure was used for calculation of mRNA levels, relative to GAPDH

**Table 2:** RKIP expression profiles in GC and para-carcinoma tissues

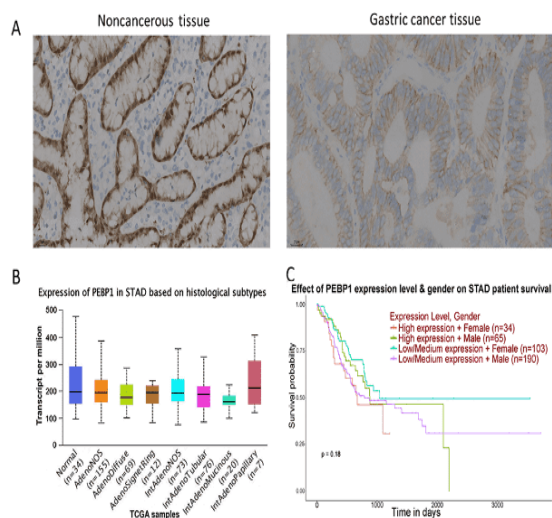| Tissue | Staining category | | | | |
|--------|------|------|------|------|---------|
| | *0* | *1+* | *2+* | *3+* | *P*-value |
| Gastric cancer (n=80) | 40 (50) | 25 (31.25) | 15 (18.75) | 0 (0) | < 0.001 |
| Para-carcinoma (n=80) | 6 (7.50) | 6 (7.50) | 46 (57.50) | 22 (27.50) | |

Values are presented as number (%)

**Figure 1:** Representative expression profiles of RKIP in GC tissues. (A) Immunohistochemical staining of RKIP in gastric cancer tissues and surrounding non-cancerous tissue samples. Scale bar = 20 μm. (B) RNA expression levels of RKIP gene in STAD. (C) Kaplan-Meier curves for overall survival, as stratified with RKIP expression in STAD

To determine the influence of RKIP on the malignant behavior of the GC cell lines, *in vitro* motility assays were carried out. Results from MTT assay indicated that combined treatment with RKIP+5-FU markedly suppressed the proliferation of the two cell lines, when compared with the control. This situation was reversed by shRKIP+5-FU where FU markedly suppressed the proliferative potential of the GC cells, when compared with the RKIP knockdown plasmid (Figure 3 A – D). Thus, RKIP blocked the proliferation of the GC cells and enhanced their sensitivity to 5-FU.

## A combination of RKIP and 5-FU promote apoptosis of GC cells

Based on the results of MTT assay, flow cytometry was used to assess the apoptotic influence of RKIP on GC cell lines. Double staining of infected MGC-803 cells with Annexin V-FITC and PI showed obvious increases in apoptosis in RKIP-overexpressing cells, when compared with control cells, while transfection with RKIP inhibitor decreased the population of apoptotic MGC-803 cells. When 5-FU was added, there was an enhancement in proportion apoptotic cells, relative to non-chemotherapy drug group. Thus, RKIP promoted apoptosis GC cell line MGC-803, and enhanced the sensitivity of the cells to 5-FU (Figure 4 A and B). Similar results were obtained with SGC-7901 cell line (Figure 5 A and B).
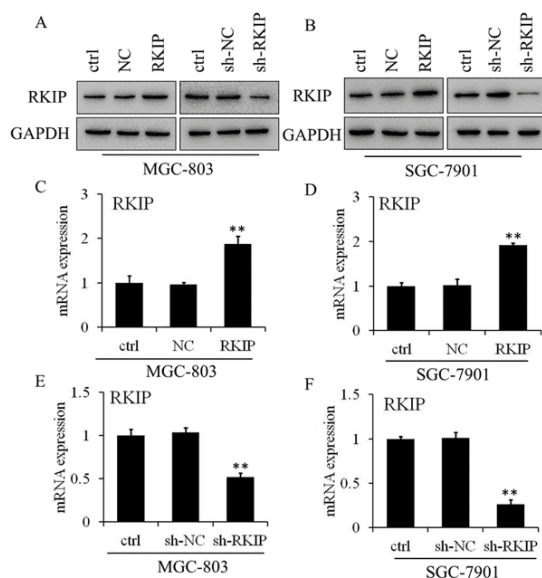


**Figure 2:** Efficacy of RKIP knockdown or overexpression by lentivirus. Western blotting analysis of efficiency of RKIP knockdown or overexpression by lentivirus in the GC cells (A and B). Efficiency of RKIP knockdown or overexpression by lentivirus in MGC-803 and SGC-7901 cell lines (C-F). ctrl: control; LV-NC: negative control; LV-RKIP: overexpression. **$P < 0.01$, compared to LV-NC.
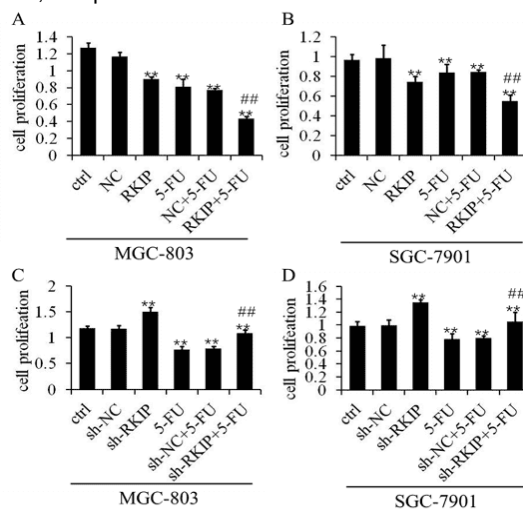


**Figure 3:** RKIP inhibited cell proliferation and enhanced sensitivity to 5-FU. (A) Upregulation of RKIP inhibited the proliferation of GC cell line MGC-803 when treated with 5-FU. The proliferation curves were obtained from MTT assay using absorbance readings at 592 nm. (ctrl: control; LV-NC: negative control; LV-RKIP overexpression). **$P < 0.01$, vs LV-NC, ## $P < 0.01$, compared to NC+5-FU). (B) Downregulation of RKIP promoted proliferation of MGC-803 cells when exposed to 5-FU. (C) Upregulation of RKIP inhibited the proliferative capacity of GC cell line SGC-7901 when exposed to 5-FU. (D) Downregulation of RKIP promoted the proliferation of SGC-7901 cells when exposed to 5-FU
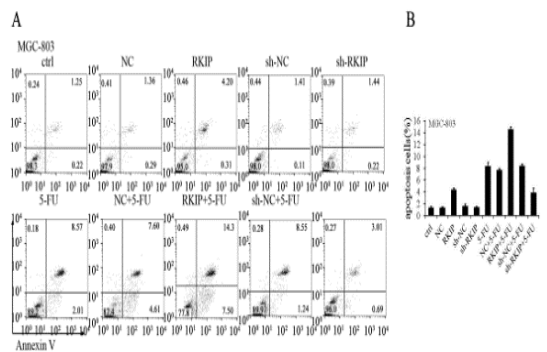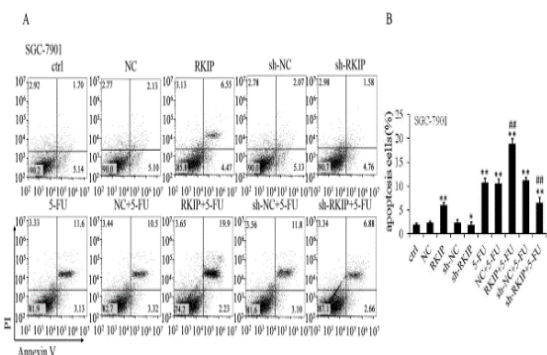
**Figure 4:** Combination of RKIP and 5-FU promoted apoptosis of GC cells. (A-B) Flow cytometric results of percentage apoptosis in MGC-803 cells. (ctrl: control; LV-NC: negative control; LV-RKIP overexpression). **P* < 0.01, vs LV-NC, LV-sh-RKIP: RKIP knockdown; **p* < 0.01, vs LV-sh-NC. ## *P* < 0.01, compared to NC+5-FU or sh-NC+5-FU.



**Figure 5:** Combination of RKIP and 5-FU promoted apoptosis of GC cells. (A-B) Flow cytometric results of percentage apoptosis in SGC-7901 cells. (ctrl: control; LV-NC: negative control; LV-RKIP overexpression). **P* < 0.01, vs LV-NC, LV-sh-RKIP: RKIP knockdown; **p* < 0.01, vs LV-sh-NC. ## *P* < 0.01, vs NC+5-FU or sh-NC+5-FU.

## RKIP promoted apoptosis via regulation of apoptosis-related factors in GC

Data showed that RKIP exerted tumor-suppressing effect by regulating cell apoptosis of the GC cell lines. The protein expressions of caspase-3 and Bax were downregulated in GC cells when transfected with RKIP plasmid. On the other hand, Bcl-2 expression was increased in RKIP-overexpressing cells, and suppressed in RKIP-inhibited cells. Moreover, 5-FU consistently enhanced the protein expressions of Bax and caspase-3, while inhibiting that of Bcl-2 (Figure 6 and Figure 7). Quantitative RT-PCR (qRT-PCR) was also used to determine the mRNA profiles of Bcl-2, caspase-3 and Bax. It was found that overexpression of RKIP inhibited Bcl-2 expression, while it promoted the protein expressions of Bax and cleaved-caspase-3 in

MGC-803 and SGC-7901 cells. The chemotherapy drug 5-FU promoted mRNA expressions of Bax and caspase-3, while it inhibited mRNA expression of Bcl-2 (Figure 8 and Figure 9).
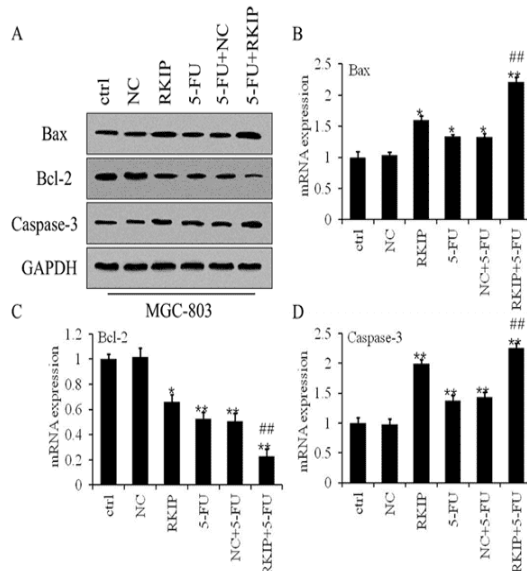


**Figure 6:** RKIP promoted apoptosis of GC cells through regulation of apoptosis-related factors. (A) Protein levels of apoptotic factors in MGC-803 cells, as assayed by immunoblotting. (B - D) mRNA levels of apoptotic genes in MGC-803 cells, as assayed by RT-PCR
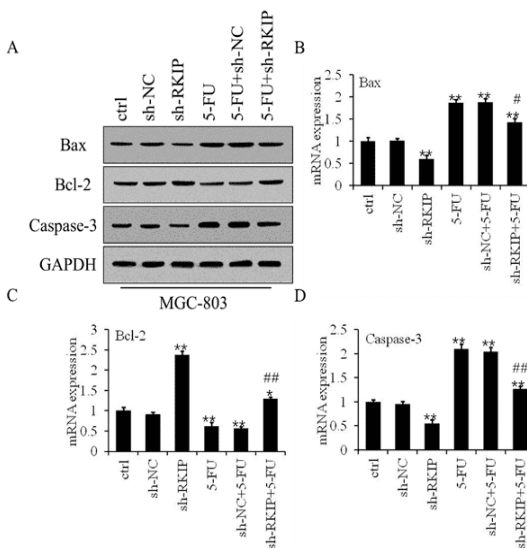


**Figure 7:** Downregulated RKIP inhibited apoptosis of GC cells through regulation of apoptosis-related factors. (A) Protein levels of apoptotic proteins in MGC-803 cells, as assayed with Western blotting. (B - D) mRNA levels of apoptotic genes in MGC-803 cells, as assayed with RT-PCR
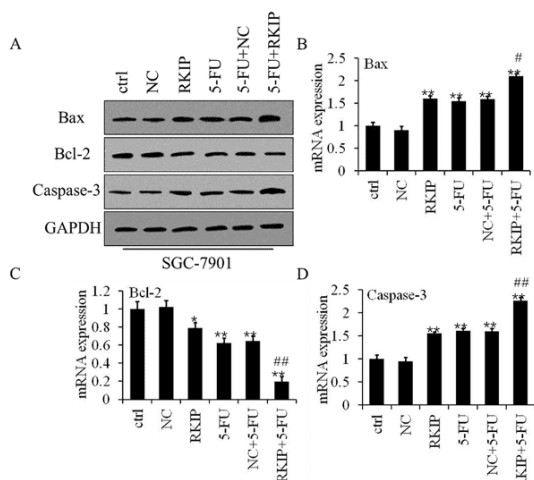
**Figure 8:** RKIP promoted apoptosis of GC cells through regulation of apoptosis-associated factors in SGC-7901. (A) Protein profiles of apoptotic factors in SGC-7901 cells, as assayed with immunoblotting. (B-D) mRNA profiles of apoptotic genes in SGC-7901 cells, as assayed with RT-PCR
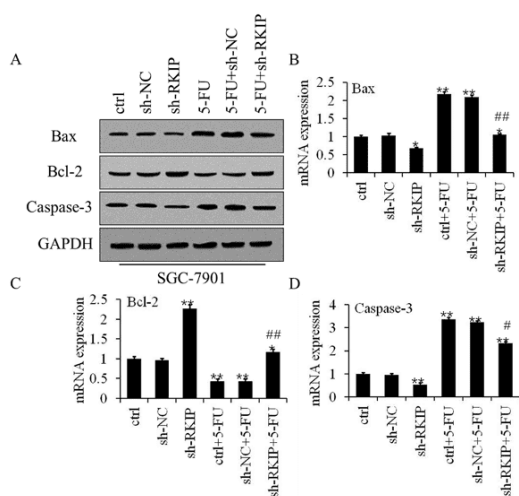


**Figure 9:** Downregulated RKIP inhibited apoptosis of GC cells through regulation of apoptosis-related factors in SGC-7901. (A) Protein profiles of apoptotic factors in SGC-7901 cells, as assayed with Western blotting. (B-D) mRNA expression profiles of apoptotic genes in SGC-7901 cells, as assayed with RT-PCR

## A combination of RKIP and 5-FU inhibited cell migration in GC cells

Ectopic RKIP expression markedly inhibited chemoattractant-mediated migration of MGC-803 and SGC-7901 cells, while RKIP knockdown significantly promoted the migration of these cells. 5-Fluorouracil (5-FU) inhibited the migration of the GC cells. In the presence of 5-FU, overexpression of RKIP inhibited the migration of the GC cells (Figure 10 and Figure

11). Thus, the observed anti-metastatic capacity may be due to targeted control of malignant behavior in the GC cells.
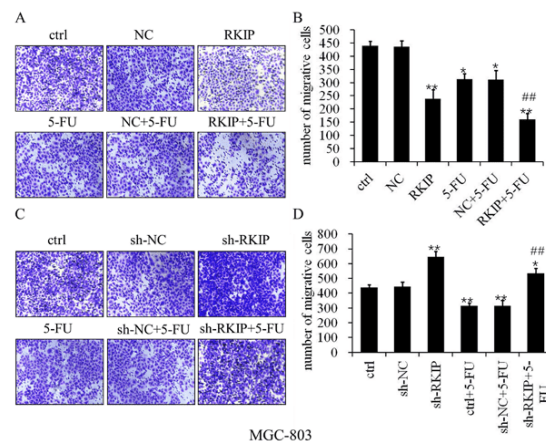


**Figure 10:** RKIP+5-FU suppressed migration of GC cells line MGC-803. A and B: upregulated RKIP and 5-FU suppressed the migration of MGC-803 cells. C and D: downregulated RKIP promoted the migration of MGC-803 cells. Bar graph showing populations of migrated cells in randomly-selected fields
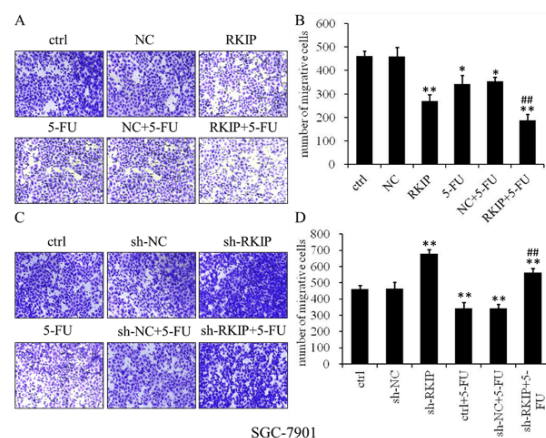


**Figure 11:** RKIP+5-FU suppressed migration of GC cells line SGC-7901. A and B: overexpression of RKIP and 5-FU inhibited the migration ability of SGC-7901 cells. C and D: downregulated RKIP promoted the migration ability of SGC-7901 cells. Bar graph showing populations of migrated cells in randomly-selected fields

## DISCUSSION

One vital factor in the prognosis of GC, a disease which accounts for most tumor-associated deaths, is metastatic change in the lymph node. Studies have established that RKIP inhibits metastasis in many types of cancer [12-14]. In this study, the levels of RKIP were directly correlated with cellular tumorigenicity and susceptibility to apoptosis. The MGC-803 and

SGC-7901 cells exert tumorigenicity in nude mice, and they express limited amounts of RKIP, but this may be markedly upregulated with chemotherapy. It has been suggested that sensitization may result from enhancement of death signal pathway [15-17]. On the other hand, RKIP may render tumor cells susceptible to apoptotic changes via suppression of their proliferation, migration and invasion. It is not clear if the sensitization effect of RKIP on proliferation or apoptosis is specific to GC cells.

The results from studies on GC cell lines MGC-803 and SGC-7901 revealed low RKIP concentrations which were not significantly affected when the cells were exposed to DNA impairment. Consistent with the apoptosis-inducing effect of RKIP, there was low level of apoptosis following 2 days of exposure to DNA-impairing drugs at doses that induced aggravated apoptotic changes in MGC-803 cells. However, the anti-tumor agent 5-FU induced apoptosis in MGC-803 and SGC-7901. Therefore, these results are in agreement with the view that RKIP is involved in apoptosis, and they suggest that the expression of RKIP is probably controlled via multiple routes following exposure to apoptotic agents. Moreover, it was shown that normalization of RKIP concentrations in GC cell lines triggered cell proliferation, migration and apoptosis.

This study has demonstrated that RKIP inhibited cell colony formation and invasion of GC cells. These results suggest that downregulation of RKIP might promote the conversion of a normal cell to a tumor cell. Consistent with results from *in vitro* studies on the GC cell lines, it was also shown that the expressions of RKIP were downregulated in the GC cells, when compared with normal cells. These findings are consistent with those obtained recently in a study which identified RKIP as a new and medically-important inhibitor of metastasis in prostate carcinoma. The results of the present study suggest that cancer cell metastasis may be suppressed using drug-induced expression of RKIP, leading to enhancement of apoptosis.

In the present study, it has been demonstrated that chemotherapy-induced rapid upregulation of RKIP-triggered apoptosis in human gastric cancer cells. However, in tumor cells insensitive to DNA-damaging drugs, exposure to 5-FU did not upregulate RKIP expression. In contrast, ectopic expression of RKIP sensitized these cells to apoptotic changes, while RKIP downregulation conferred insensitivity to 5- FU by relieving its suppressive effect on two main survival routes in

tumors. These results indicate that RKIP is a new indicator of apoptosis in cancers.

## CONCLUSION

These results suggest that RKIP suppresses cell proliferative as well as cell migratory and invasive capacity in GC cell lines. Thus, it may be reasonably hypothesized that RKIP may serve as an inhibitor gene in human GC. Thus, it is a new index of prognosis, and a therapeutic target for gastric cancer.

## DECLARATIONS

### *Acknowledgement*

### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Dongta Zhong was involved in the conception and design of the study, manuscript revision, funding support and study supervision. Mengxin Lin contributed to design of the study as well as the experiments, data analysis and manuscript writing. Xiaoyan Lin, Xiaobing Huang, Riping Wu and Xinli Wang contributed to the design of the study and interpretation of the data. All authors read and approved the final manuscript.

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