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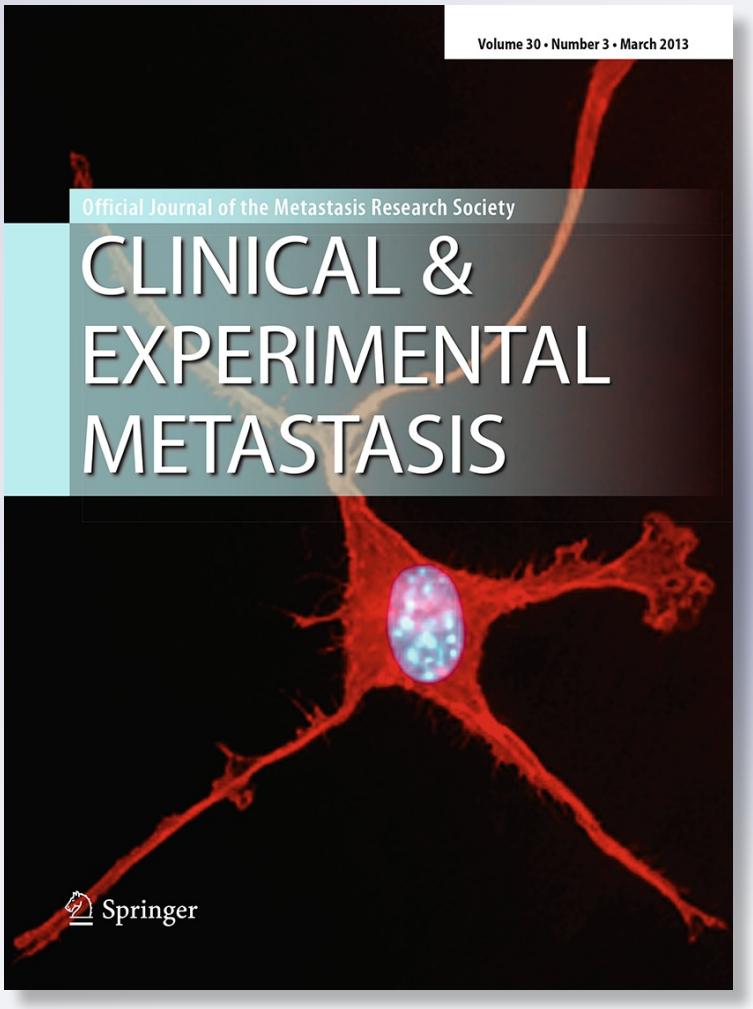

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RESEARCH PAPER

Aberrant methylation and loss expression of RKIP is associated with tumor progression and poor prognosis in gastric cardia adenocarcinoma

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Abstract Raf kinase inhibitory protein (RKIP) has been identified as a member of a novel class of molecules which implicated in cancer progression and suppress the metastatic spread of tumors. The aim of this study was to investigate the promoter methylation and expression of RKIP, determine the prognostic significance of RKIP in gastric cardia adenocarcinoma (GCA). MSP approach and immunohistochemistry methods were used respectively to examine methylation status and protein expression of RKIP in GCA tissues. The frequency of RKIP methylation in GCA tumor tissues (62.1 %) was significantly higher than that in corresponding normal tissues (4.1 %) and was associated with TNM stage, histological differentiation, depth of invasion, LN metastasis, distant metastasis or recurrence, and upper gastrointestinal cancers (UGIC) family history. Positive staining of RKIP in GCA tumor tissues (34.5 %) was significantly decreased than that in corresponding normal tissues (84.1 %) and was associated with RKIP methylation. RKIP may act as a tumor suppressor gene in GCA by regulation of the Raf-1/MEK/ERK signaling pathway. GCA patients in stage III and IV, with positive UGIC family history, and hypermethylation and down-expression of RKIP were most likely to develop metastatic disease and also showed the worse survival. RKIP methylation in GCA was an independent prognostic marker for survival using multivariate Cox regression analysis ($P = 0.04$). In all, aberrant hypermethylation of RKIP may be one of the mechanisms that lead to loss or down expression of the gene in GCA especially in individuals with UGIC family history. Additionally, hypermethylation and loss of RKIP expression may be used as a marker to predict clinical outcome of GCA.

Keywords RKIP · Methylation · Expression · Gastric cardia adenocarcinoma

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Introduction

Raf kinase inhibitory protein (RKIP), also termed as phosphatidylethanonolamine binding protein (PEBP), is distinct from other known proteins and its function is still being elucidated [1]. In addition to binding phospholipids [2], RKIP has been reported to bind nucleotides and opioids [3], and regulate growth and differentiation in a variety of species [4]. RKIP is now well established to function via MAP kinase (MAPK) signal transduction as a regulator of the spindle assembly checkpoint. In mammalian cells, RKIP functions as an inhibitor of the MAPK signaling module comprised of Raf-1/MEK/ERK1,2 [5]. RKIP inhibits Raf-1 activation by preventing phosphorylation of key regulatory sites on Raf-1 [6]. Upon growth factor stimulation of cells, RKIP is phosphorylated at S153 by protein kinase C (PKC) causing its dissociation from Raf-1 [7]. The released, phosphorylated RKIP binds and inhibits GRK2, potentiating G protein-coupled receptor (GPCR) signaling [8]. Thus, RKIP functions as an environmental sensor or "switch" that flips from down regulating the amplitude and dose response of Raf-1-mediated ERK activation and resultant DNA synthesis to ameliorating GPCR down regulation.

A growing body of evidence suggests that RKIP is a novel candidate gene on the expanding list of metastasis suppressors [9–13]. A link between RKIP and cancer was

first established in prostate cancer, with RKIP showing reduced expression in prostate cancer cells and the lowest expression levels in metastatic cells, and restoration of RKIP expression in metastatic prostate cancer cells inhibited invasiveness of the cells in vitro and in vivo in spontaneous lung metastasis but not the growth of the primary tumor in a murine model [9]. From then on, experiments from several laboratories have demonstrated that malignant melanomas [10], breast cancer [11], insulinomas [12], and colorectal cancer [13] frequently displayed a marked decrease in RKIP expression, and this decrease correlates with the extent of metastatic disease. Loss of RKIP was also found to be associated with higher stage and/or recurrence of cancer, the presence of vascular invasion and metastasis in cancer, and worse survival time, suggesting that RKIP has potential as a molecular determinant of tumor metastasis and may, therefore, serve as a prognostic marker [14–18]. Further analysis showed that high methylation of the promoter region of RKIP gene results in the decreased expression of RKIP in colorectal cancer [19].

Gastric cancer is one of the most frequent cancers in the world especially in less developed regions. In China, based on two national mortality surveys conducted in 1970s and 1990s, there is an obvious clustering of geographical distribution of gastric cancer in the country, with the high mortality being mostly located in rural areas, especially in Gansu, Henan, Hebei, and Shanxi Provinces [20, 21]. Gastric cardia adenocarcinoma (GCA), which was formerly registered as gastric cancer or esophageal cancer, has been diagnosed independently in very recent years, due to the improvement in early endoscopic screening and pathologic diagnosis. China is a country with high incidence regions of GCA, especially in Taihang mountain of North China. Interestingly, the district which showed higher incidence of GCA also showed higher incidence of esophageal squamous cell carcinoma (ESCC) and epidemiological investigation has demonstrated that the influence of these factors such as diet habit, *Helicobacter pylori* (HP) infection on the occurrence and development of GCA and gastric cancer were different, however, GCA showed some common points on the occurrence when compared with ESCC [22]. A series of epidemiological studies have also demonstrated that a variable proportion of ESCC and GCA cases strongly point to an upper gastrointestinal cancers (UGIC) family history especially in the district of Taihang mountain of North China. The exact mechanisms of the occurrence of GCA still remain unclear for the moment. Exogenous factors including nutrition deficiency, unhealthy living habits, consumption of alcohol and tobacco, pathogenic infections are generally considered as the risk factors for developing GCA in China [23, 24], however, only a subset of individuals exposed to the above listed exogenous risk factors would develop GCA, suggesting that multiple genetic and epigenetic events may contribute to the progression of GCA.

Even with the potential importance of RKIP in carcinogenesis, little has been investigated about the role of RKIP in GCA. In the present study, we attempted to detect the relationship of RKIP methylation to a series of pathological parameters in GCA, and investigate the correlation of RKIP methylation and protein expression and its role in patient survival, in order to gain more information on the role of RKIP with regard to the pathogenesis and outcome in GCA.

Materials and methods

Cases and specimens

All study subjects were ethnically homogeneous Han nationality and permanent residents of high incidence regions of GCA in Hebei Province. Tumor and corresponding adjacent normal tissues were obtained from 145 GCA cases, which were all inpatients for surgical treatment in the county hospital of high incidence regions between the years of 2003 and 2005. All subjects were interviewed by professional interviewers for their gender, age, histopathological diagnosis, and UGIC family history. All gastric cardia carcinomas were adenocarcinomas with their epicenters at the gastroesophageal junction, i.e. from 1 cm above until 2 cm below the junction between the end of the tubular esophagus and the beginning of the saccular stomach [25]. Individuals with at least one first-degree relative or at least two second-degree relatives having esophageal/cardia/gastric cancer were defined as having family history of UGIC. Information on clinicopathologic characteristics was available from hospital recordings and pathological diagnosis. Recurrence and survival data were ascertained through the Tumor Registry and hospital chart review.

Affection tissues and corresponding normal tissues were formalin fixed and paraffin embedded. All sample sections were stained in H&E and were examined by two experienced pathologists. The study was approved by the Ethics Committee of Hebei Cancer Institute and informed consent was obtained from all recruited subjects.

RKIP methylation analysis via MSP method

Genomic DNA from tumor and corresponding normal sections were isolated by manual microdissected method from paraffin-embedded tissue slides using a simplified proteinase K digestion method. To examine the DNA methylation patterns, we treated genomic DNA with sodium bisulfite, as described previously [26]. In brief, 2 μg of DNA were denatured with 2 M NaOH at 37 °C for 10 min, followed by incubation with 3 M sodium bisulphite (pH 5.0) at 50 °C for 16 h. Bisulphite treated DNA was then purified (DNA Cleanup Kit; Promega, Madison,

WI, USA), incubated with 3 M NaOH at room temperature for 5 min, precipitated with 10 M ammonium acetate and 100 % ethanol, washed with 70 % ethanol, and resuspended in 20 μl of distilled water.

The methylation status of RKIP was then determined by methylation specific polymerase chain reaction (MSP) as described previously [13]. The primer sequences of the promoter region for the methylated form were 5′-TTTAGCGATATTTTTTGAGATACGA-3′ (sense) and 5′-GCTCCCTAACCTCTAATTAACCG-3′ (antisense) (205 bp), and the primer sequences for the unmethylated form were 5′-TTTAGTGATATTTTTTGAGATATGA-3′ (sense) and 5′-CACTCCCTAACCTCTAATTAACCAA-3′ (antisense) (205 bp). Each step of the MSP utilized a 25 μl reaction volume, 0.5 μl of Taq-DNA-polymerase and 1 μl of DNA template and included 35 cycles at the annealing temperature 52 °C. Genomic DNA, methylated in vitro by CpG methyltransferase (Sss I) following the manufacturer's directions (New England BioLabs, Inc., Beverly, MA), was used as a positive control and water blank was used as a negative control. MSP products were analyzed on 2 % agarose gel with ethidium bromide staining, and were determined to have methylation if a visible band was observed in the methylation reaction (Fig. 1). Reactions were performed in duplicate with 10 % of the samples, in order to ensure the reproducibility and consistency of the results.

RKIP, p-MEK, and p-ERK protein expression via immunohistochemical staining

In order to elucidate the role of RKIP in the Raf-1/MEK/ERK signaling pathway in GCA, protein expression of RKIP and phosphorylation status of MEK and ERK was determined by immunostaining using the avidin–biotin complex immunoperoxidase method, which was performed on parallel histopathological sections from paraffin-embedded tumor section and corresponding normal tissues. Endogenous peroxidase was blocked with 3 % hydrogen peroxide for 10 min, followed by microwave antigen retrieval for 9 min at 98 °C in 10 mM sodium citrate buffer (pH 6.0) and incubated in 2 % normal horse serum to minimize non-specific binding. The primary antibody against RKIP (1:100 dilution, rabbit anti-human polyclonal antibody, sc-28837, Santa Cruz Biotechnology) or phospho-MEK (1:200 dilution, rabbit anti-human polyclonal antibody, sc-293106, Santa Cruz Biotechnology) or phospho-ERK (1:200 dilution, goat anti-human polyclonal antibody, sc-16982, Santa Cruz Biotechnology) was then applied to sections, which were then incubated with biotinylated secondary antibody and ABC reagent. 0.5 % 3,3′-diaminobenzidine (Sigma, St Louis, MO) was used as the chromagen. For a negative control, the primary antibody was replaced with mouse IgG. Slides with normal gastric mucosa were used as a positive control for RKIP. Slides with positive staining of p-MEK and p-ERK were used as a positive control for p-MEK and p-ERK.

Immunohistochemical staining was evaluated according to a scoring method reported previously [27]. Scoring accounted for both representation of the areas and intensities of the stains. Briefly, the score is the sum of the percentage of positive cells (0, <25 % positive cells; 1, 26–50 % positive cells; 2, 51–75 % positive cells, and 3, more than 75 % positive cells) and the staining intensity (0, negative; 1, weak; 2, moderate; 3, strong). Sums between 0 and 2 were scored as negative, sums of 3 and 6 were scored as positive. All slides were examined and scored by three independent observers, who were blinded to the clinical data.

Statistical analysis

Statistical analysis was performed using SPSS10.0 software package (SPSS Company, Chicago, IL, USA). Fisher's exact test and Chi-square test were used to assess statistical significance of differences and compare categorical associations. Kaplan–Meier survival curves were constructed and the Log-rank or the Breslow tests were used as needed for the univariate comparison of RKIP methylation and expression categories. Cox's multivariate test applied in a stepwise forward method was used to adjust for potentially confounding variables (e.g., stage and UGIC family history) and to evaluate the role of RKIP as independent predictors of patient prognosis. Two-tailed P values of 0.05 or less were regarded as statistically significant for all statistic tests.

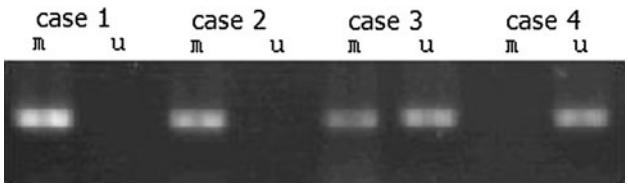

Fig. 1 Methylation analysis of RKIP in GCA tumor tissues. *Case 1* and *case 2*: RKIP is fully methylated; *case 3*: RKIP is semi-methylated; *case 4*: RKIP is unmethylated; *m* methylated; *u* unmethylated

Results

Clinicopathologic features

The GCA cases included 115 male and 30 female, age ranged from 39 to 76, mean age 58.9. Based on TNM stage and histological differentiation, 7 were stage I (4.8 %), 59 were stage II (40.7 %), 63 were stage III (43.4 %), and 16 were stage IV (11.1 %); 60 (41.4 %) of them were well differentiation, 53 (36.6 %) were moderate differentiation

and 32 (22.0 %) were poor differentiation. According to the UGIC family history, 64 of 145 GCA patients were with positive UGIC family history. Other clinicopathologic characteristics of GCA cases such as depth of invasion, LN metastasis, and distant metastasis or recurrence were listed in Table 1. The patients were followed up for a minimum period of 1.5 years (range, 1.5–7 years), with a median follow-up of 5.5 years for survivors. They were clinically assessed for signs of metastatic recurrence. Eighteen patients were lost to follow up (Table 1).

Methylation analysis of RKIP in GCA

The methylation analysis of RKIP was successfully performed in all specimens. The frequency of RKIP methylation in GCA tumor tissues (62.1 %, 90/145) was significantly higher than that in corresponding normal tissues (4.1 %, 6/145) ($P < 0.05$) (Table 2). RKIP methylation status in GCA tumor tissues was not associated with age and gender ($P > 0.05$). When stratified for clinicopathologic characteristics, methylation frequency of RKIP was associated with TNM stage, histological differentiation, depth of invasion, LN metastasis, and distant metastasis or recurrence ($P < 0.05$). Forty-seven (73.4 %) GCA cases with positive UGIC family history were detected hypermethylation of RKIP gene, while only 43 of 81 (53.1 %) GCA cases with negative UGIC family history were detected positive methylation of RKIP. The methylation frequency of RKIP in GCA cases with positive UGIC family history was significantly higher than that in GCA cases with negative UGIC family history ($\chi^2 = 6.29$, $P = 0.01$) (Table 3).

Immunostaining of RKIP in GCA

RKIP expression was mainly observed in the cytoplasm of tumor or normal cells (Fig. 2). As shown in Table 2, positive staining of RKIP in GCA tumor tissues (34.5 %, 50/145) was significantly decreased than that in corresponding adjacent normal tissues (84.1 %, 122/145) ($P < 0.01$). RKIP protein expression in GCA tumor tissues was not associated with age and gender ($P > 0.05$). When stratified for clinicopathologic characteristics, positive protein expression of RKIP was associated with TNM stage, histological differentiation, depth of invasion, LN metastasis, and distant metastasis or recurrence ($P < 0.05$). When stratified for UGIC family history, frequency of RKIP protein expression in GCA cases with positive UGIC family history was significantly lower than that in GCA cases with negative UGIC family history ($\chi^2 = 6.19$, $P = 0.013$) (Table 3). Of 90 GCA tumor tissues which showed hypermethylation of RKIP, 71 GCA tumor tissues showed negative protein expression of RKIP, and the other 19 GCA tumor tissues which showed positive protein expression of RKIP all demonstrated incomplete methylation of RKIP. A close correlation was noted between RKIP methylation and the loss of protein expression of the gene in GCA ($R = -0.36$, $P < 0.01$) (Table 4).

Immunostaining of p-MEK and p-ERK in GCA

Expressions of p-MEK and p-ERK were found in both the cytoplasm and nucleus, and expression of p-ERK was found mainly in the nuclei of tumor cells (Fig. 2). As shown in Table 2, positive staining of p-MEK and p-ERK in GCA tumor tissues (72.4 %, 105/145 and 81.4 %, 118/145, respectively) was significantly higher than that in corresponding adjacent normal tissues (15.2 %, 22/145 and 21.4 %, 31/145, respectively) ($P < 0.01$). Protein expression of p-MEK in GCA tumor tissues was not associated with age, gender, and any other clinicopathological factor ($P > 0.05$) (Table 3). Protein expression of p-ERK in GCA tumor tissues was associated with TNM stage and distant metastasis or recurrence ($P < 0.05$), but was not related to age, gender, histological differentiation, depth of invasion, and LN metastasis ($P > 0.05$). When stratified for UGIC family history, frequency of p-ERK protein expression in GCA cases with positive UGIC family history was significantly higher than that in GCA cases with negative UGIC family history ($\chi^2 = 4.46$, $P = 0.03$) (Table 3). As shown in Table 4, protein expression of RKIP was inversely correlated with protein expression of p-MEK and p-ERK ($R = -0.20$ and -0.55, respectively, $P < 0.05$).

Survival analysis of RKIP methylation and expression in GCA

RKIP methylation was inversely correlated with GCA patients survival (Fig. 3a). In the RKIP-methylation GCA tumors, the 5-year overall survival rates (OS) were 23 % (median survival time, 34 months; $P < 0.05$; Log-rank test) as opposed to the RKIP-unmethylation GCA tumors displaying 5-year survival rates of 53 % (median survival time not reached). RKIP expression was positively correlated with GCA patients survival (Fig. 3b). In the RKIP-expression GCA tumors, the 5-year survival rates were 49 % (median survival time not reached) as opposed to the RKIP negative GCA tumors displaying 5-year survival rates of 27 % (median survival time, 35 months; $P < 0.05$; Log-rank test) (Table 5). As shown in Fig. 3c, d, GCA patients in stage III and IV, positive UGIC family history, and hypermethylation of RKIP were most likely to develop metastatic disease and also showed the worse survival.

Cox multivariate analysis was done using RKIP methylation, expression, tumor stage, as well as other confounding variables such as UGIC family history, age, and patient gender. RKIP methylation status, stage and UGIC

Table 1 Clinicopathologic characteristics of gastric cardia adenocarcinoma cases

Groups	N (%)
Age	
<50	27 (18.6)
≥50	118 (81.4)
Gender	
Male	115 (79.3)
Female	30 (20.7)
TNM stage	
I	7 (4.8)
II	59 (40.7)
III	63 (43.4)
IV	16 (11.1)
Pathological differentiation of tumor	
Well	60 (41.4)
Moderate	53 (36.6)
Poor	32 (22.0)
Depth of invasion	
T1/2	46 (31.7)
T3/4	99 (68.3)
LN metastasis	
Negative (N0)	23 (15.9)
Positive (N1/2/3)	122 (84.1)
Distant metastasis or recurrence	
Negative	74 (51.0)
Positive	71 (49.0)
Family history of UGIC	
Negative	81 (55.9)
Positive	64 (44.1)
Vital statistics	
Alive	44 (30.4)
Dead GCA	76 (52.4)
Dead unrelated	7 (4.8)
Information unavailable	18 (12.4)

family history were independently associated with GCA patients' survival (Table 6).

Discussion

RKIP was originally identified as a negative regulator of the Raf-1/MEK/ERK signaling pathways and was a highly species-conserved ubiquitously expressed protein involved in growth and differentiation signaling regulation. Several studies also have identified RKIP as a suppressor of metastasis [9–13]. Although several studies have been done on RKIP expression and clinical outcome, our study is the first to evaluate the role of RKIP in GCA and with clinical outcome. Similar to the studies of RKIP in prostate cancer [9], malignant melanomas [10], breast cancer [11], insulinomas [12], colorectal cancer [13, 28], hepatocellular carcinoma [17], gastrointestinal stromal tumors [29] and gastric cancer [30, 31], decreased expression of RKIP was also found in the present study, indicating the tumor suppressor gene role of RKIP in different tumors. In the present study, we also found aberrant hypermethylation of RKIP may be one of the mechanisms that lead to loss or down expression of the gene in GCA. The methylation status of RKIP has been investigated in colorectal cancer [13, 18, 19, 28] and gastrointestinal stromal tumors [29], however, the outcome was inconsistent. Al-Mulla et al. [18, 19] found methylation of the RKIP promoter is a major mechanism by which RKIP expression is silenced in colorectal cancer, however, Martinho et al. [29] found that loss of RKIP expression was not due to the promoter methylation, Minoo et al. [13] also did not found any methylation of RKIP in colorectal cancer. The difference of sample size may be an important reason, the samples in Minoo P's study were smaller than our and Al-Mulla F's study (82 colorectal cancer patients), only included 28 colorectal cancer, the amount of samples in each subgroup was even smaller to decrease the test efficacy. Different tumor type may be the other reason, furthermore, different genetic background among different race of people may have great disparity and may be have an effect on the RKIP promoter methylation. The relatively larger samples and the reproductive methylation frequency suggested that the results in the present study may not be a chance finding.

Signaling through the RAS-MAPK pathway has emerged as a critical regulator of diverse cellular biologic functions, including growth and differentiation. Lugli et al. [32] has shown that the activated Raf-1/MEK/ERK signaling pathway is involved in tumor progression and has prognostic significance in colorectal cancer. The MEK/ERK signaling pathway is under tight regulatory control by several inhibitory proteins, including Sprouty, SPRED, and RKIP. Of all the Raf-binding proteins, RKIP is the only known inhibitor of this MAPK pathway. Yeung et al. [5] demonstrated that RKIP could bind Raf-1, and additional data from this group indicated that RKIP-1 also binds MEK and ERK. Furthermore, it appears that RKIP cannot bind Raf-1 and MEK simultaneously since the binding sites for MEK and Raf-1 overlap on RKIP-1. Protein expression of p-MEK and p-ERK was inversely correlated with protein expression of RKIP has been found in the present study,

Table 2 The methylation status of RKIP and protein expression of RKIP, p-MEK and p-ERK in GCA tumor tissues and corresponding normal tissues

Groups	Methylation frequency of RKIP		Protein expression of RKIP		Protein expression of p-MEK		Protein expression of p-ERK	
	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*
Normal tissues	6 (4.1)	0.000	122 (84.1)	0.000	22 (15.2)	0.000	31 (21.4)	0.000
GCA tumor tissues	90 (62.1)		50 (34.5)		105 (72.4)		118 (81.4)	

P comparison of GCA tumor tissues versus normal tissues

Table 3 Methylation status of RKIP and immunohistochemical staining characteristics of RKIP, p-MEK and p-ERK in GCA tissues

Groups	Methylation frequency of RKIP n (%)	*P*	Protein expression of RKIP n (%)	*P*	Protein expression of p-MEK n (%)	*P*	Protein expression of p-ERK n (%)	*P*
Age								
<50	14 (51.9)	0.225	11 (40.7)	0.448	19 (70.4)	0.792	21 (77.8)	0.594
≥50	76 (64.4)		39 (33.1)		86 (72.9)		97 (82.2)	
Gender								
Male	74 (64.4)	0.268	36 (31.3)	0.115	82 (71.3)	0.558	95 (82.6)	0.457
Female	16 (53.3)		14 (46.7)		23 (76.7)		23 (76.7)	
TNM stage								
I + II	32 (48.5)	0.002	31 (47.0)	0.004	47 (71.2)	0.767	49 (74.2)	0.044
III + IV	58 (73.4)		19 (24.1)		58 (73.4)		69 (87.3)	
Pathological differentiation of tumor								
Well/ moderate	65 (57.5)	0.034	45 (39.8)	0.011	82 (72.6)	0.938	90 (79.6)	0.314
Poor	25 (78.1)		5 (15.6)		23 (71.9)		28 (87.5)	
Depth of invasion								
T1/2	22 (47.8)	0.016	22 (47.8)	0.021	35 (76.1)	0.500	34 (73.9)	0.115
T3/4	68 (68.7)		28 (28.3)		70 (70.7)		84 (84.8)	
LN metastasis								
Negative (N0)	9 (39.1)	0.013	13 (56.5)	0.015	16 (69.6)	0.739	16 (69.6)	0.195
Positive (N1/2/3)	81 (66.4)		37 (30.3)		89 (72.9)		102 (83.6)	
Distant metastasis or recurrence								
Negative	38 (51.4)	0.007	35 (47.3)	0.001	51 (68.9)	0.336	55 (74.3)	0.026
Positive	52 (73.2)		15 (21.1)		54 (76.1)		63 (88.7)	
Family history of UGIC								
Negative	43 (53.1)	0.012	35 (43.2)	0.013	56 (69.1)	0.320	61 (75.3)	0.035
Positive	47 (73.4)		15 (23.4)		49 (76.6)		57 (89.1)	

indicating the regulatory effect of RKIP on Raf-1/MEK/ERK signaling pathway. Hagan et al. [11] found that RKIP expression is reduced in lymph node metastases of 103 breast cancer patients, Martinho et al. [29] found that loss of RKIP expression was associated with poor disease-specific survival of gastrointestinal stromal tumor patients, Fujimori et al. [31] recently found that negative RKIP expression combined with positive p-ERK expression was an independent predictor of poor outcomes in patients with gastric cancer, similar results have been obtained in the studies of colorectal cancer [13, 18, 19]. In the present study, we showed that hypermethylation and expression of RKIP was significantly and directly correlated with GCA patients survival. In multivariate analysis, the combination of tumor stage, UGIC family history, and RKIP methylation and expression provided independent predictive information on GCA metastasis and poor survival. Patients with hypermethylation and down expression of RKIP, in stage III and IV, with positive UGIC family history had the worst 5-year overall survival time. Thus, loss of RKIP expression may be considered to be a marker of tumor progression and poor prognosis.

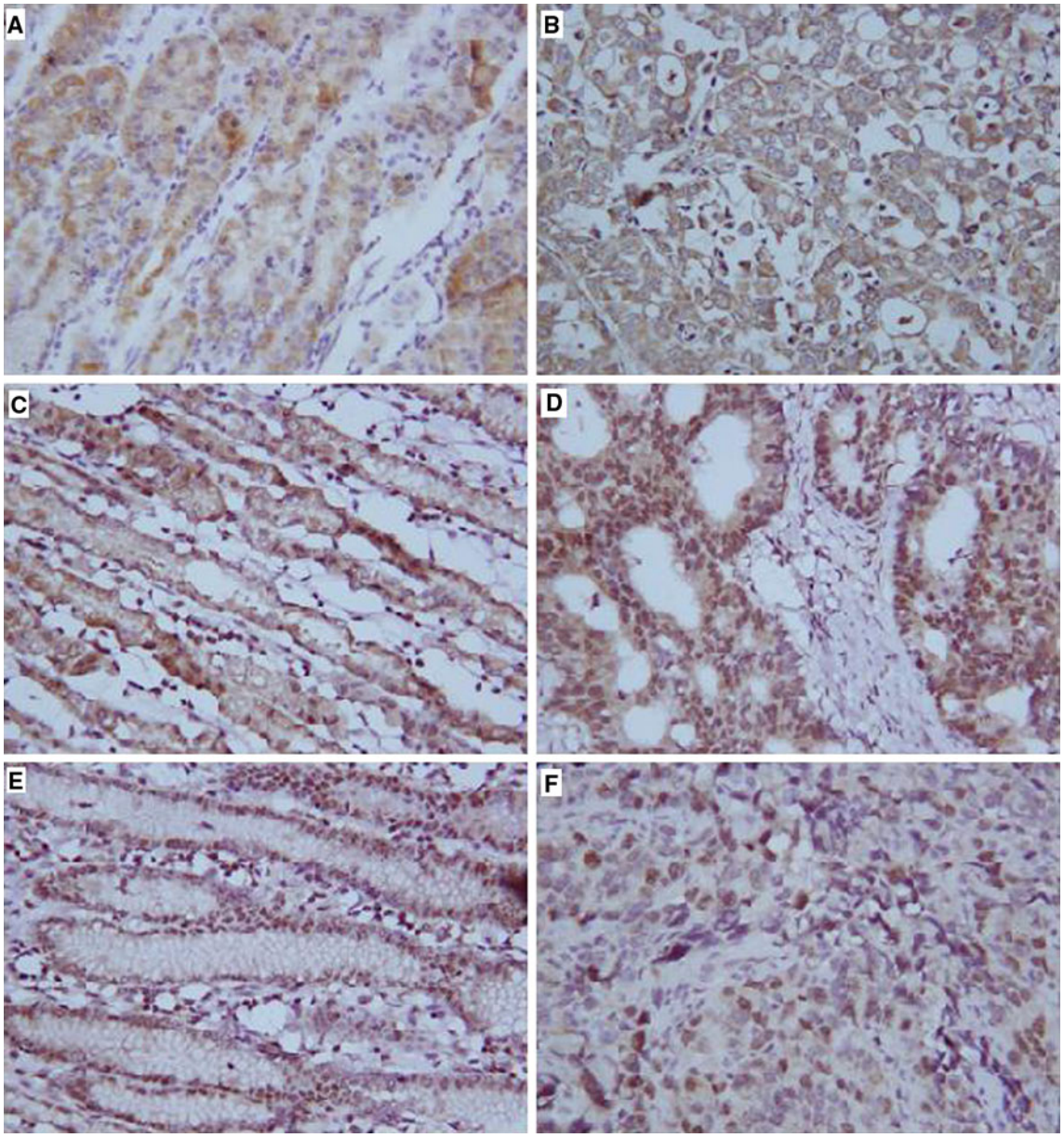

Fig. 2 Immunohistochemical staining of RKIP, p-MEK, and p-ERK in gastric normal tissues and GCA tumor tissues (SP ×400). **a** Positive staining of RKIP in normal tissue; **b** positive staining of RKIP in GCA tumor tissue; **c** positive staining of p-MEK in normal tissue; **d** positive staining of p-MEK in GCA tumor tissue; **e** positive staining of p-ERK in normal tissue; **f** positive staining of p-ERK in GCA tumor tissue

Table 4 The association of RKIP protein expression with RKIP methylation status and p-MEK, p-ERK protein expression

Protein expression of RKIP	Methylation status of RKIP		*P*	Protein expression of p-MEK		*P*	Protein expression of p-ERK		*P*
	M	U		+	−		+	−	
+	19	31	0.000	30	20	0.015	26	24	0.000
−	71	24		75	20		92	3	

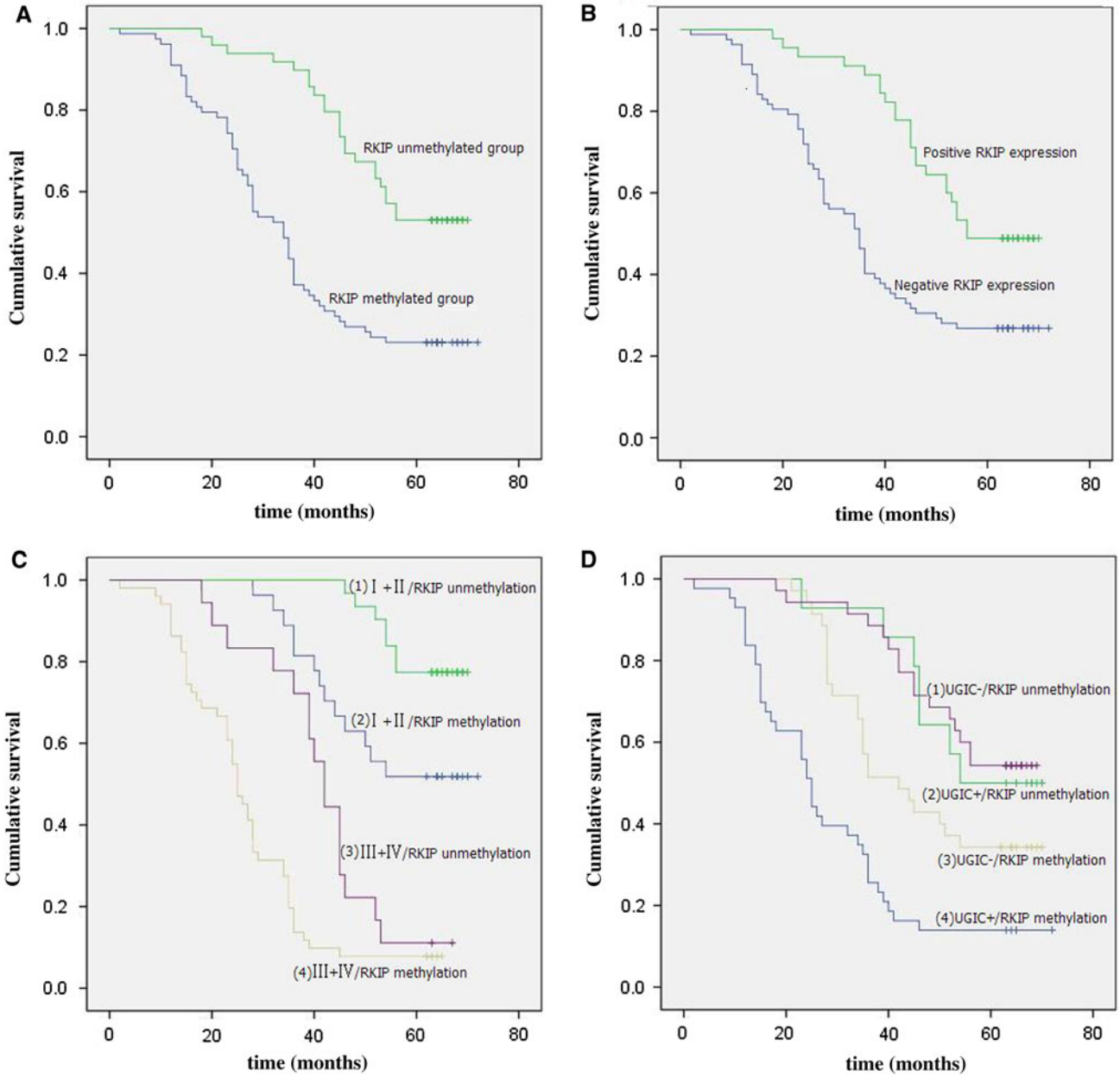

Fig. 3 Kaplan–Meier univariate survival analysis of RKIP methylation and expression in gastric cardia adenocarcinoma. **a** Kaplan–Meier curves for cumulative survival stratified by RKIP methylation status: methylation and unmethylation of RKIP in GCA showing consistently a direct correlation between RKIP methylation and poor patient survival. **b** Kaplan–Meier curves for cumulative survival stratified by RKIP expression status: positive and negative staining of RKIP in GCA showing a direct correlation between negative RKIP expression and poor patient survival. **c** Kaplan–Meier curves for cumulative survival stratified by tumor stages and RKIP methylation status: patients in stage III and IV with RKIP methylation showing poor patient survival. **d** Kaplan–Meier curves for cumulative survival stratified by UGIC family history and RKIP methylation status: patients with positive UGIC family history and RKIP methylation showing poor patient survival, $P = 0.000$

Many studies have shown that the occurrence of GCA was associated with UGIC family history, suggesting that genetic background may play important roles in the progression of GCA [26, 33]. The association of cancer-related genes methylation and family history also remains somewhat unclear. In the present study, the methylation frequency of RKIP in GCA patients with positive UGIC family history was significantly higher than that in the GCA patients with negative UGIC family history, and positive UGIC family history predicted poor prognosis.

Table 5 Univariate analysis of survival in GCA cases (Log-rank test)

Groups	Univariate	
	5-year OS	*P*
Age		
<50	20	0.243
≥50	38	
Gender		
Male	34	0.123
Female	39	
TNM stage		
I + II	48	0.002
III + IV	24	
Pathological differentiation of tumor		
Well/moderate	38	0.254
Poor	20	
Depth of invasion		
T1/2	48	0.002
T3/4	29	
LN metastasis		
Negative (N0)	64	0.012
Positive (N1/2/3)	31	
Distant metastasis or recurrence		
Negative	44	0.001
Positive	25	
Family history of UGIC		
Negative	40	0.010
Positive	29	
RKIP methylation status		
Methylation	23	0.000
Unmethylation	53	
RKIP protein expression		
Positive	49	0.001
Negative	27	
p-MEK protein expression		
Positive	34	0.064
Negative	38	
p-ERK protein expression		
Positive	33	0.069
Negative	42	

Table 6 Multivariate analysis of survival in GCA cases (Cox's test)

Variables	B	SE	P	Odds ratio (95 % CI)
Methylation	−1.494	0.755	0.042	0.224 (0.094–0.896)
TNM stage	0.873	0.136	0.000	2.394 (1.832–3.127)
Family history of UGIC	0.745	0.238	0.002	2.107 (1.322–3.357)

One possibility is that shared environmental risk factors, acting independently or in conjunction with genetic factors, reinforce the aggregation of GCA within families. Our findings reinforced the fact that individuals with family history of UGIC should take a regular examination for cancer screening, especially in the high incidence regions.

In all, our study suggested that epigenetic silencing of the RKIP promoter via hypermethylation may be one of the

mechanisms for inactivation of RKIP in GCA, especially in GCA patients with UGIC family history of North China. Additionally, hypermethylation and down expression of RKIP, in stage III and IV, with positive UGIC family history is highly predictive of metastasis and poor prognosis in GCA. Further studies need to be done to determine if RKIP can be used as a target to improve clinical outcome of GCA.

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