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ORIGINAL ARTICLE

Inverse association between Bmi-1 and RKIP affecting clinical outcome of gastric cancer and revealing the potential molecular mechanisms underlying tumor metastasis and chemotherapy resistance

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Abstract

Background B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1) and Raf kinase inhibitory protein (RKIP) are involved in cancer metastasis and chemotherapeutic resistance, respectively. In this study, we evaluated the association between Bmi-1 and RKIP and outcome of gastric cancer through clinical data analysis and in vitro experiments.

Methods Bmi-1 expression and RKIP expression were observed in 107 cases of gastric cancer through use of tissue microarray technology to identify their correlations with clinicopathological parameters, patient survival, and susceptibility to chemotherapy. The correlation was confirmed in gastric cancer cell lines, analyzed further by gene overexpression and silencing analysis, a cell invasion assay, and a chemosensitivity test.

Results Positive expression of Bmi-1 was highly correlated with T classification and clinical stage. Diminished or lost expression of RKIP was significantly associated with T classification, lymph node metastasis, distant metastasis, and clinical stage. Bmi-1 is negatively and RKIP is positively related to patient survival. Positive expression of Bmi-1 and negative expression of RKIP are associated with poor patient survival and modest efficacy of postoperative chemotherapy. A meaningfully inverse association between Bmi-1 and RKIP was found in tissue microarray studies, and was verified further in gastric cancer cell lines. Moreover, gene overexpression and silencing analysis indicated that RKIP might be regulated by Bmi-1. Furthermore, the impacts of Bmi-1 on cell invasion and chemotherapy resistance were rescued by knockdown of RKIP.

Conclusions Our study implies that detection of Bmi-1 and RKIP is valuable in predicting patient survival and therapeutic response in gastric cancer, and the inverse association between Bmi-1 and RKIP reveals the potential molecular mechanisms underlying tumor metastasis and chemotherapy resistance.

Y. Chen, G. Lian, and G. Ou are contributed equally to this work.

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Keywords B-cell-specific Moloney murine leukemia virus integration site 1 · Raf kinase inhibitory protein · Gastric cancer · Tissue microarray · Clinical outcome

Introduction

Gastric cancer (GC) is one of the most frequently diagnosed and aggressive carcinomas of the gastrointestinal tract [1]. It is estimated that the number of new cases of GC will reach 930,000 a year worldwide, of which China accounts for 42 % [2]. Although GC is usually managed by surgery or chemotherapy, the prognosis is still poor, mainly because of tumor metastasis and chemotherapy resistance, and the overall 5-year survival rate is less than 15 % [3]. Numerous studies have reported that the causes of GC include genetic factors, such as p53, E-cadherin, c-Met, and trefoil factor 1 [4, 5]. However, the molecular mechanisms underlying tumor metastasis and chemotherapy resistance, and affecting clinical outcome in GC remain unknown.

The polycomb group (PcG) genes, which are pivotal in gene expression through chromatin modifications, constitute a global system with important roles in multicellular development, stem cell biology, and cancer [6]. B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1), a transcriptional repressor member of the PcG family, plays a pivotal role in tumor progression and metastasis. Its effects on tumorigenesis involve repression of the Ink4α-Arf locus, which is an essential cell cycle regulator and encodes p16 and p19arf [7]. Aberrant Bmi-1 expression has been reported in a variety of human cancers [8–13]. In our previous study, Bmi-1 expression was correlated with tumor size, clinical stage, and prognosis for patients with gastric carcinoma [14]. Patients with Bmi-1 expression had a shorter overall survival time than those without Bmi-1 expression. This indicated that Bmi-1 expression was related to an invasive phenotype in patients with gastric carcinoma.

On the other hand, Raf kinase inhibitory protein (RKIP), a member of the phosphatidylethanolamine-binding protein family, has proved to be a significant molecule in suppressing cancer metastasis [15]. It is an evolutionarily conserved small protein, and was originally identified as a physiological inhibitor of the Raf–mitogen-activated protein kinase kinase (MEK)–extracellular-signal-regulated kinase (ERK) pathway [16]. RKIP regulates the activity of and mediates the cross talk between several important cellular signaling pathways, including the Raf–mitogen-activated protein kinase–ERK pathway [17], the nuclear factor κB (NF-κB) pathway [18], and the G protein pathway [19]. A variety of evidence suggests that reduced RKIP function may influence metastasis, angiogenesis, resistance to apoptosis, and genome integrity. Recent studies have shown that the expression levels of RKIP are frequently downregulated in various cancer types, and correlate with an invasive or metastatic phenotype [20–23]. Moreover, recent data implicated RKIP depletion in chemotherapeutic resistance both in vitro and in vivo [24].

Until now, the exact tumor markers that are associated with metastasis, chemotherapy resistance, and prognosis of GC have not been discovered. Our previous study indicated that Bmi-1 expression was related to an invasive phenotype in patients with gastric carcinoma. Further, we stably transfected the human gastric epithelial immortalized cell line GES-1 with Bmi-1, and demonstrated that overexpression of Bmi-1 enhanced the migration and invasion abilities in vitro. Besides, overexpression of Bmi-1 upregulated an epithelial–mesenchymal transition (EMT) marker [25]. Yet, the precise molecular mechanism of Bmi-1 in the motility and invasiveness of GC remains to be further elucidated. RKIP is well known for its important role in EMT and in metastasis suppression in various cancer types. As already stated, RKIP regulates the activity of and mediates the cross talk between several important cellular signaling pathways, including the NF-κB pathway. We reviewed the literature, and found that Bmi-1 promotes the aggressiveness of glioma via activation of the NF-κB pathway [26]. Moreover, Bmi-1 can directly promote metastasis in head and neck squamous cancer and nasopharyngeal carcinoma by regulating Snail [27]. Further, recent elegant experiments have revealed the NF-κB–Snail–RKIP circuitry as a possible mechanism for chemotherapeutic resistance in cancer cells [24]. Although no studies have been done on the regulatory relationships between Bmi-1 and RKIP, on the basis of the adverse effects of these two markers on cancer metastasis, and accounting for the potential correlation between Bmi-1 and RKIP by way of NF-κB and/or Snail, we hypothesized that there may be a regulation mechanism between Bmi-1 and RKIP in GC, and conducted this study. In this study, 107 cases of GC were analyzed for Bmi-1 and RKIP expression through immunohistochemistry by use of tissue microarray technology to identify their correlation with clinicopathological parameters, patient survival, susceptibility to chemotherapy, and outcome in GC. Then, the inverse relationship between Bmi-1 and RKIP was validated in GC cell lines, further analyzed by gene overexpression and silencing methods, a cell invasion assay, and a chemosensitivity test.

Materials and methods

Patients

Data from patients with GC who underwent a primary resection at Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University (Guangzhou, China) between November 2007

and January 2011 were collected. Proximal (cardia) cancers were excluded so as to reduce confounding variables. None of the patients had received chemotherapy or radiotherapy prior to surgery. All the cases were histologically diagnosed and classified using the Lauren system as intestinal-type or diffuse-type gastric adenocarcinoma. Tumors exhibiting a mixed intestinal and diffuse pattern were classified as diffuse [28, 29]. Stages were classified according to the 2010 GC staging system of the American Joint Committee on Cancer. This study was approved by the Research Ethics Committee of Sun Yat-Sen Memorial Hospital.

Tissue microarray and immunohistochemistry

Paraffin blocks containing areas consisting of unalloyed gastric carcinoma were reviewed for confirmation of diagnosis in corresponding hematoxylin–eosin-stained sections. Two different and representative areas of the tumor were determined and marked on the source block. The source block was cored and punched with 1-mm diameter, and re-embedded in a recipient paraffin block in a defined position, using a tissue arraying instrument (Beecher Instruments, Alphelys, Plaisir, France). The tissue microarray blocks were cut into 5-μm sections for immunohistochemical staining. Immunoblot analysis was performed as previously described [14] using anti-Bmi-1 antibody (1:50 dilution; Abcam) and anti-RKIP antibody (1:50 dilution; Abcam). A negative control was achieved by replacing the primary antibody with a normal IgG.

Evaluation of immunostaining

The degree of immunostaining was evaluated and scored by two independent pathologists, Jianning Chen and Haigang Li, who were blinded to both clinical and pathology data. Staining intensity was scored as four grades: 0 for a complete absence of staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The extent of Bmi-1 staining was scored as follows: 0 for completely negative staining, 1 for tumors with less than 10 % of cells staining positive, and 2 for tumors with at least 10 % of cells staining positive [14]. The extent of RKIP staining was scored as four grades: 0 for completely negative staining, 1 for tumors with less than 10 % of cells staining positive, 2 for tumors with 10–50 % of cells staining positive, and 3 for tumors with more than 50 % of cells staining positive. The final scores were derived from multiplication of the extent of staining by the intensity. For the statistical analysis, scores were further grouped into two categories as follows: negative (final scores below 4) and positive (final scores of 4 or more) [28]. The correlations of the Bmi-1 and RKIP scores between the two pathologists were good. For Bmi-1, discordant results were obtained in eight cases: the Spearman correlation coefficient was 0.836 ($p < 0.01$), and the kappa coefficient for a score below 4 versus a score of 4 or more was 0.833 ($p < 0.01$). For RKIP, discordant results were obtained in six cases: the Spearman correlation coefficient was 0.874 ($p < 0.01$), and the Kappa coefficient for a score below 4 versus a score of 4 or more was 0.871 ($p < 0.01$). In these cases, the slides were reevaluated together, and a consensus was reached.

Cell lines

Six GC cell lines—BGC-823, HGC-27, AGS, MGC80-3, NCI-N87, and SGC7901—were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The human gastric epithelial immortalized cell line GES-1 was purchased from Beijing Institute for Cancer Research Collection. All cell lines were maintained according to the respective protocols.

Plasmids, virus production, and infection of target cells

We produced pLNCX2-Bmi-1 constructs and performed retroviral transfection as previously described [25]. Stable cell lines nominated as SGC7901-Bmi-1, GES-1-Bmi-1#1, and GES-1-Bmi-1#2, respectively, were generated. Human RKIP was amplified by PCR from complementary DNA from fresh human GC and was cloned into the pcDNA 3.1+ vector. Stable cell lines expressing RKIP were generated by G418 selection (500 μg/ml) for 14 days. Small interfering RNA (siRNA) oligos targeting Bmi-1 (siBmi-1#1, 5′-UGUCUACAUUCCUUCUGUATT-3′; siBmi-1#2, 5′-GCCACAACCAUAAUAGAAUTT-3′), RKIP (siRKIP#1, 5′-CACCCAGGUUAAGAAUAGATT-3′; siRKIP#2, 5′-CCAGUUCAGUGUUGCAUGUTT-3′), and negative control siRNAs were purchased from GenePharma (Shanghai). The siRNA transfections were done with 50 nM siRNA using Lipofectamine 2000 (Invitrogen Life Technologies) following the manufacturer's instructions.

Western blotting analysis

Western blotting analysis was performed as previously described [14] using anti-Bmi-1 antibody (1:1,000 dilution; Cell Signaling Technology) and anti-RKIP antibody (1:2,000 dilution; Abcam).

In vitro cell invasion assay

Equal numbers of cells (5×10^4 cells per well) were plated on the top side of a polycarbonate Transwell filter (with Matrigel) in the upper chamber of BioCoat invasion chambers (BD), and were incubated for 40 h, followed by removal of cells from the upper chamber with cotton

swabs. Cells on the lower membrane surface were fixed in 4 % paraformaldehyde, stained with 0.1 % crystal violet, and counted under a microscope (five random fields per well, ×100 magnification). Cell counts were expressed as the mean number of cells per field. Each experiment was done independently three times.

Chemosensitivity test

Oxaliplatin (Sigma, USA) and 5-fluorouracil (5-FU; Sigma, USA) were dissolved in saline. Cells were seeded at a density of 1×10^4 cells per well in 96-well plates 24 h prior to exposure to 5-FU (62.5, 125, 250, 500, or 1,000 μM) or oxaliplatin (15.6, 31.2, 62.5, 125, or 250 μM). For siRNA-mediated-knockdown cells, cells transfected with appropriate siRNAs for 24 h were subsequently exposed to 5-FU or oxaliplatin. Phosphate-buffered saline (pH7.4) was used as the control. After incubation of cells for 72 h, the medium was removed and 10 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium solution (Promega) with 90 μl cell culture medium was added to each well. After incubation at 37 °C for 2 h, the absorbance at 492 nm was determined using an ELISA reader (Labsystem Dragon, Oy, Finland). Each experiment was done in triplicate, and was done independently three times.

Statistical analysis

Statistical analyses were performed using SPSS 18.0. For categorical, nonordered variables, cross tabulations were analyzed using the χ^2 test, or when the χ^2 test was not valid, Fisher's exact test was used. The associations between Bmi-1 expression and RKIP expression were analyzed using the Spearman rank test. Survival curves were plotted by the Kaplan–Meier method, and were compared by the log-rank test. Multiple Cox proportional hazards regression was performed to identify the independent factors which had a significant impact on patient survival. For continuous variables, data were presented as the mean ± the standard error of the mean, and were analyzed by one-way ANOVA. A p value below 0.05 was considered significant.

Results

Bmi-1 expression in GC

Bmi-1 expression was detected by immunohistochemistry on gastric tissue microarrays. Among the 107 GC samples, 70 (65.4 %) showed moderate to strong nuclear staining of Bmi-1 in most of the tumor cells in the form of yellow–brown granules (Fig. 1).

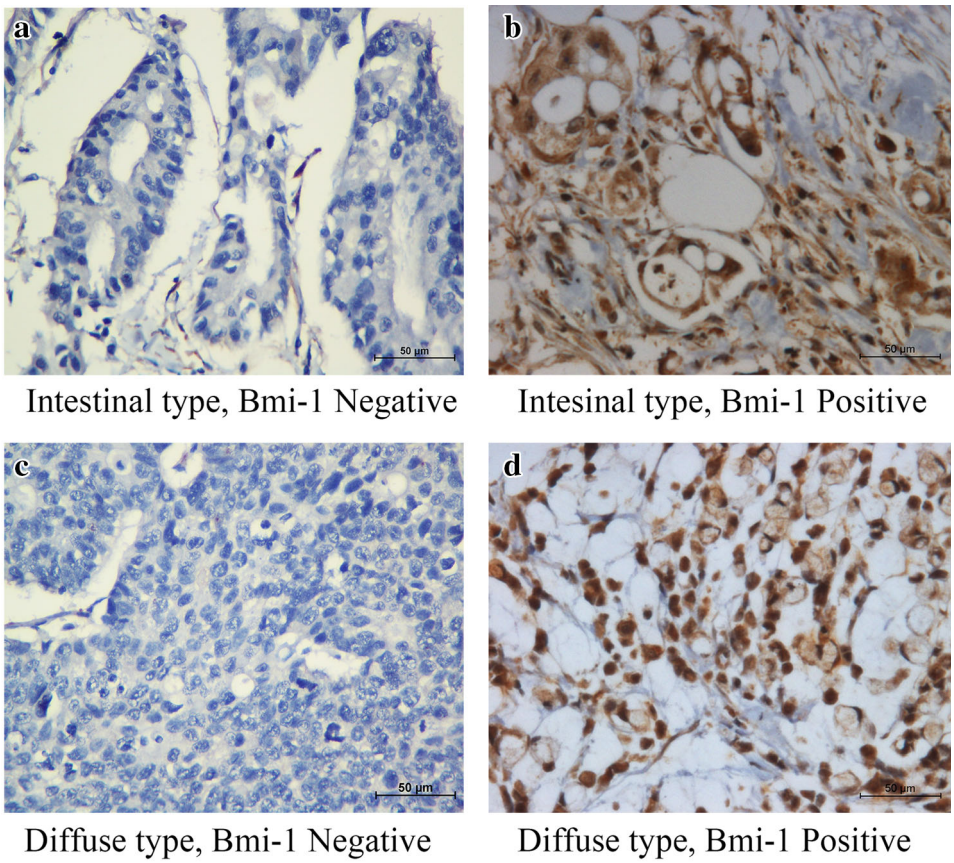

Fig. 1 Analysis of B-cell-specific Moloney murine leukemia virus integration site 1 (*Bmi-1*) protein expression by immunohistochemistry in gastric carcinomas. **a**, **b** Immunohistochemical staining for Bmi-1 in the intestinal gastric histological type. Bmi-1-negative (**a**) and Bmi-1-positive (**b**) tumors are shown (magnification ×400). **c**, **d** Immunohistochemical staining for Bmi-1 in the diffuse gastric histological type. Bmi-1-negative (**c**) and Bmi-1-positive (**d**) tumors are shown (magnification ×400). *Scale bar* 50 μm

We further analyzed the relationship between the expression of Bmi-1 and clinical characteristics of the patients (Table 1). Expression of Bmi-1 was highly correlated with T classification and clinical stage ($p < 0.05$). Correlation with T classification implies that Bmi-1 promotes the spread of the primary tumor. Although there was no statistically significant difference between Bmi-1 expression and lymph node and distant metastasis, Bmi-1 expression was positive in 69.5 % (57/82) and negative in 30.5 % (25/82) of node-positive tumors, and was positive in 81.8 % (9/11) and negative in 18.2 % (2/11) of distant metastasis tumors. There was no meaningful correlation between the expression level of Bmi-1 and gender, age, and tumor type.

RKIP expression in GC

By immunohistochemical analysis, 35 of 107 GC samples (32.7 %) showed positive RKIP staining. Representative examples of RKIP staining in GC are shown in Fig. 2.

The relationship between the expression of RKIP and clinical characteristics of the patients was further analyzed (Table 1). Diminished or lost expression of RKIP was strongly correlated with T classification, lymph node metastasis, distant metastasis, and clinical stage ($p < 0.05$). Consistent with the findings for Bmi-1, there was no statistically significant difference in RKIP expression for diffuse type compared with intestinal type ($p = 0.593$). Further, there were no noteworthy associations between the expression level of RKIP and gender, age, and tumor type.

Bmi-1 expression and RKIP expression inversely correlate in GC samples

We further assessed the relationship between the expression of Bmi-1 and that of RKIP. We found that 50.5 % of tumors (54 of 107) that exhibited positive Bmi-1 expression were negative for RKIP, whereas 17.8 % of tumors (19 of 107) with negative Bmi-1 expression were positive

Table 1 Correlation between patients' clinicopathological features and the expression of B-cell-specific Moloney murine leukemia virus integration site 1 (*Bmi-1*) or Raf kinase inhibitory protein (*RKIP*)

Characteristics	*n*	Bmi-1			RKIP		
		Negative	Positive	*p*	Negative	Positive	*p*
Gender							
Male	66	26 (39.4 %)	40 (60.6 %)	0.184	41 (62.1 %)	25 (37.9 %)	0.148
Female	41	11 (26.8 %)	30 (73.2 %)		31 (75.6 %)	10 (24.4 %)	
Age (years)							
<60	64	19 (29.7 %)	45 (70.3 %)	0.194	44 (68.8 %)	20 (31.3 %)	0.694
≥60	43	18 (41.9 %)	25 (58.1 %)		28 (65.1 %)	15 (34.9 %)	
Tumor type							
Intestinal	45	20 (44.4 %)	25 (55.6 %)	0.068	29 (64.4 %)	16 (35.6 %)	0.593
Diffuse/mixed	62	17 (27.4 %)	45 (72.6 %)		43 (69.4 %)	19 (30.6 %)	
T classification							
T1/2	27	15 (55.6 %)	12 (44.4 %)	0.008	10 (37.0 %)	17 (63.0 %)	0.000
T3/4	80	22 (27.5 %)	58(72.5 %)		62 (77.5 %)	18 (22.5 %)	
Lymph node metastasis							
Positive	82	25 (30.5 %)	57 (69.5 %)	0.107	60 (73.2 %)	22 (26.8 %)	0.019
Negative	25	12 (48.0 %)	13 (52.0 %)		12 (48.0 %)	13 (52.0 %)	
Distant metastasis							
Positive	11	2 (18.2 %)	9 (81.8 %)	0.227	11 (100.0 %)	0 (0.0 %)	0.015
Negative	96	35 (36.5 %)	61 (63.5 %)		61 (63.5 %)	35 (36.5 %)	
Clinical stage							
I/II	59	26 (44.1 %)	33 (55.9 %)	0.022	30 (50.8 %)	29 (49.2 %)	0.000
III/IV	48	11 (22.9 %)	37 (77.1 %)		42 (87.5 %)	6 (12.5 %)	

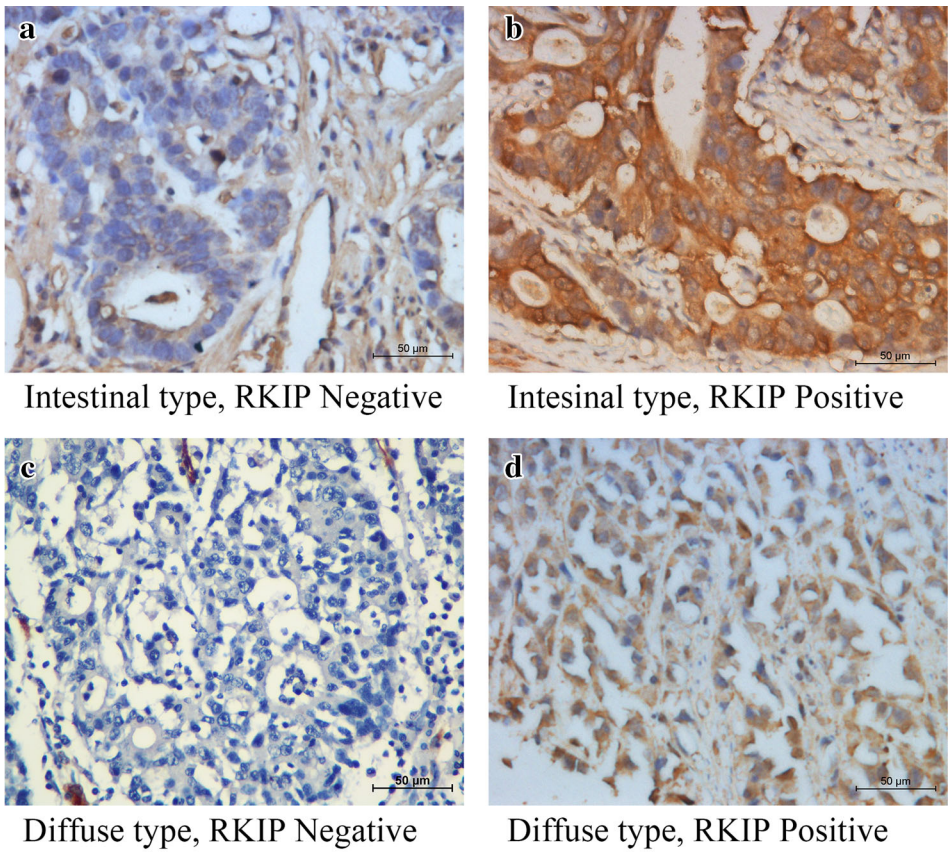

Fig. 2 Analysis of Raf kinase inhibitory protein (*RKIP*) protein expression by immunohistochemistry in gastric carcinomas. **a**, **b** Immunohistochemical staining for RKIP in the intestinal gastric histological type. RKIP-negative (**a**) and RKIP-positive (**b**) tumors are shown (magnification ×400). **c**, **d** Immunohistochemical staining for RKIP in the diffuse gastric histological type. RKIP-negative (**c**) and RKIP-positive (**d**) tumors are shown (magnification ×400). *Scale bar* 50 μm

for RKIP. Sixteen of 107 cases (14.9 %) exhibited a positive staining pattern for both Bmi-1 and RKIP. In 16.8 % of cases (18 of 107), staining for both Bmi-1 and RKIP was negative. Two discordant examples are shown in Fig. 3a. Statistically, these findings clearly indicated the existence of a remarkably inverse association between Bmi-1 expression and RKIP expression ($p = 0.003$; $r = -0.289$) (Fig. 3b).

Survival analysis of RKIP and Bmi-1 expression in GC

For 20 patients, the cause of death or the status at the last follow-up was unknown, and these patients were excluded from the survival analysis. The remaining 87 patients were followed up for 1–64 months (median 29 months) to obtain survival data.

The survival analysis showed that Bmi-1 negatively correlated with survival (Fig. 4a). The 5-year survival rate was only 29.2 % in the Bmi-1-positive group (median 31 months), whereas it was 63.5 % in the Bmi-1-negative group (median 48 months; $p = 0.002$). In contrast, the survival analysis showed that RKIP positively correlated with survival (Fig. 4b). For the RKIP-positive tumors, the 5-year survival rate was 59.8 % (median 47 months), whereas for the RKIP-negative tumors, the 5-year survival rate was 29.1 % (median 29 months; $p = 0.006$). There was also a statistically significant difference in the 5-year survival rate between Bmi-1-positive combined with RKIP-negative tumors and Bmi-1-negative combined with RKIP-positive tumors. For Bmi-1-positive combined with RKIP-negative tumors, the 5-year survival rate was not observed (median 26.5 months), as opposed to Bmi-1-negative combined with RKIP-positive tumors, for which the 5-year survival rate was 76.6 % (median 51.2 months; $p = 0.001$) (Fig. 4c).

The susceptibility of tumors that were positive or negative for Bmi-1 and RKIP to postoperative chemotherapy was also estimated in GC. The survival analysis of patients receiving chemotherapy showed that the 5-year survival rate in the Bmi-1-positive group was 34.6 % (median 35.3 months), as opposed to 68.9 % (median 52.6 months; $p = 0.022$) in the Bmi-1-negative group (Fig. 4d). Differently, RKIP negativity resulted in worse survival in patients receiving chemotherapy. The 5-year survival rate was not observed in the RKIP-negative group, and the median 5-year survival time was 31.1 months, whereas in the RKIP-positive group, the 5-year survival rate was 78.8 %, and the median 5-year survival time was 56.6 months ($p = 0.002$) (Fig. 4e). When the Bmi-1-positive combined with RKIP-negative group was compared

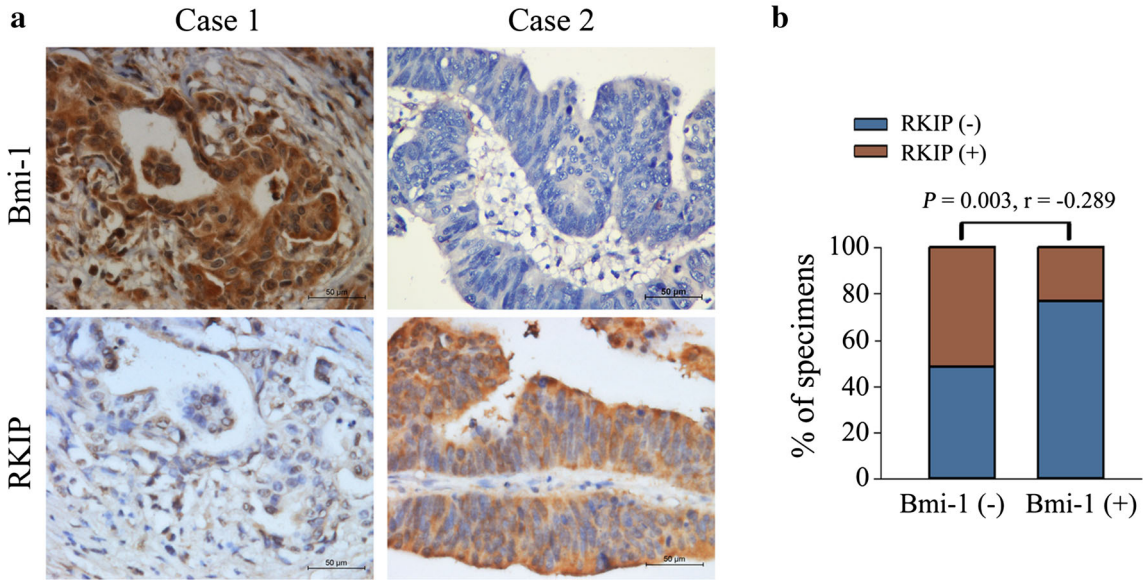

Fig. 3 Inverse expression pattern of B-cell-specific Moloney murine leukemia virus integration site 1 (*Bmi-1*) and Raf kinase inhibitory protein (*RKIP*). Visualization of two representative cases of 107 clinical gastric carcinoma specimens (**a**; magnification ×400; *scale bar* 50 μm) and percentage of samples showing negative or positive Bmi-1 expression relative to RKIP levels (**b**)

with the Bmi-1-negative combined with RKIP-positive group, the 5-year survival rate was not observed in the former (median 29.1 months), whereas it was 88.9 % in the latter (median, 58.1 months; $p = 0.005$) (Fig. 4f).

Endogenous inverse expression patterns of Bmi-1 and RKIP in GC cell lines

On the basis of the inverse association of Bmi-1 and RKIP in GC tissue, the coexpression patterns of Bmi-1 and RKIP in six GC cell lines—BGC-823, HGC-27, AGS, MGC80-3, NCI-N87, and SGC7901—were checked by Western blotting analysis. Similarly, negative correlation of Bmi-1 and RKIP was found in GC cell lines. As indicated in Fig. 5a, a relatively high level of Bmi-1 was detected in the HGC-27 and MGC80-3 cell lines, whereas relatively low levels of RKIP were found in these cell lines. Further, a low level of Bmi-1 and a high level of RKIP were exhibited in the BGC-823 and NCI-N87 cell lines. Thus, a certain regulation relationship existed between Bmi-1 and RKIP.

Gene overexpression and silencing analysis of Bmi-1 and RKIP

To investigate the role of Bmi-1 in RKIP expression, we expressed Bmi-1 or siRNA for Bmi-1 in GES-1 cells and the GC cell line SGC7901. As shown in Fig. 5b, ectopic expression of Bmi-1 in GES-1 and SGC7901 cells compared with vector control cells resulted in a significant decrease of RKIP expression. Conversely, silencing of Bmi-1 resulted in upregulation of RKIP in SGC7901 and BGC-823 cells (Fig. 5c). To determine whether Bmi-1 expression might be regulated by RKIP, RKIP was overexpressed or knocked down in SGC7901 and BGC-823 cells, but Bmi-1 expression did not change correspondingly (Fig. 5d).

RKIP knockdown rescues the effects of Bmi-1 silencing in GC cells

It is well known that Bmi-1 can enhance the invasiveness and chemotherapy resistance of tumor cells. Therefore, we asked whether knockdown of RKIP in Bmi-1-silenced cancer cells could rescue the invasive phenotype and restore chemosensitivity. As expected, knockdown of RKIP partially restored the invasiveness of Bmi-1-silenced cells (Fig. 6a). In addition, ablation of RKIP expression restored the sensitivity of Bmi-1-silenced GC cells to various concentrations of 5-FU and oxaliplatin (Fig. 6b).

Discussion

GC is a common malignant disease of the gastrointestinal tract with a poor prognosis. Adjuvant chemotherapy can improve prognosis in patients with resected GC. However, tumor metastasis and chemotherapy resistance are the foremost causes of poor prognosis of GC, and are the most serious challenges to therapeutic intervention. This study was undertaken to identify independent clinicopathological factors and tumor markers leading to the identification of patients at risk of developing metastasis, and consequently, more likely to benefit from chemotherapy. Numerous gene

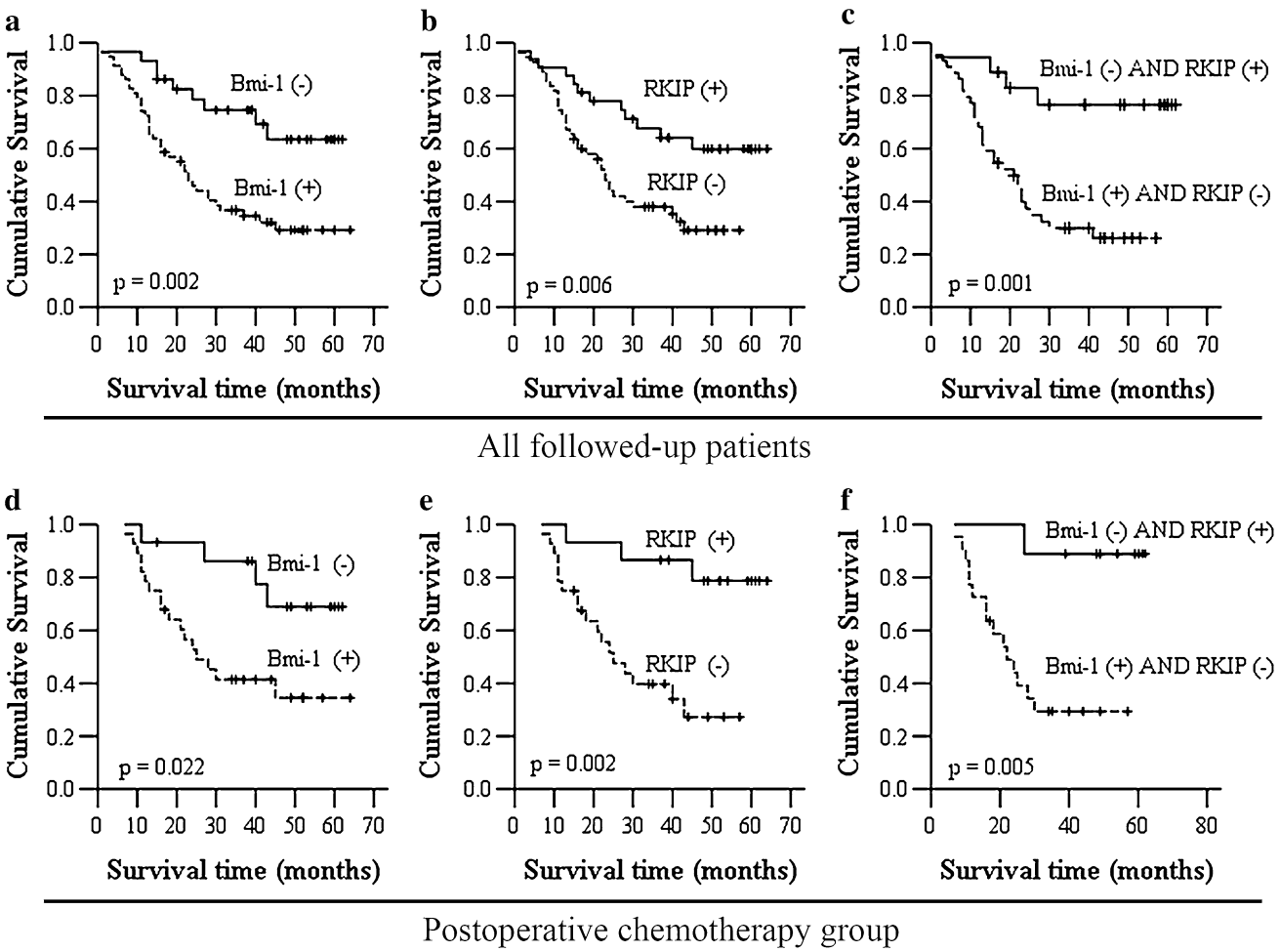

Fig. 4 Kaplan–Meier analysis of overall survival time. **a–c** Overall survival time of all gastric cancer patients followed up according to expression of B-cell-specific Moloney murine leukemia virus integration site 1 (*Bmi-1*) and Raf kinase inhibitory protein *(RKIP)*. Kaplan–Meier curves for cumulative survival stratified by Bmi-1 positive/negative (**a**; $p = 0.002$), RKIP positive/negative (**b**; $p = 0.006$), and Bmi-1 positive, RKIP negative/Bmi-1 negative, RKIP positive (**c**; $p = 0.001$). **d–f** Overall survival time of gastric cancer patients receiving postoperative chemotherapy according to expression of Bmi-1 and RKIP. Kaplan–Meier curves for cumulative survival stratified by Bmi-1 positive/negative (**d**; $p = 0.022$), RKIP positive/negative (**e**; $p = 0.002$), and Bmi-1 positive, RKIP negative/Bmi-1 negative, RKIP positive (**f**; $p = 0.005$)

profiling studies have attempted to clarify the correct definition of the molecular profile of GC, in order to establish better stratification of patients for therapeutic purposes. Bmi-1 is a member of the PcG family of transcriptional repressors, and has been identified as a proto-oncogene in tumor progression and metastasis, whereas RKIP is well known for its metastasis suppression function in various cancer types. Our study strengthens the relationship between Bmi-1 and RKIP and their clinical values to predict clinical outcome of gastric carcinoma, and hints at the molecular mechanisms underlying tumor metastasis and chemotherapy resistance.

Here, we detected the status of Bmi-1 expression through immunohistochemistry by tissue microarray technology in 107 cases of GC. Nuclear Bmi-1 staining was positive in 70 cases (65.4 %), and Bmi-1 expression was strongly correlated with T classification and clinical stage. There was no meaningful correlation between the expression level of Bmi-1 and gender, age, tumor type, lymph node metastasis, and distant metastasis. Despite the fact that the Bmi-1-positive distribution was statistically not significant, neither in node-positive tumors nor in distant metastasis cases, a higher positive rate of Bmi-1 implied that primary tumors with increased levels of Bmi-1 expression had a higher tendency to metastasize.

Staining for RKIP was negative in 72 cases (67.3 %) of the same GC samples. Our results identified that negative expression of RKIP was significantly associated with the presence of metastasis in GC. Differently from Bmi-1, diminished or lost expression of RKIP was correlated strongly with T classification, lymph node metastasis, distant metastasis, and clinical stage ($p < 0.05$).

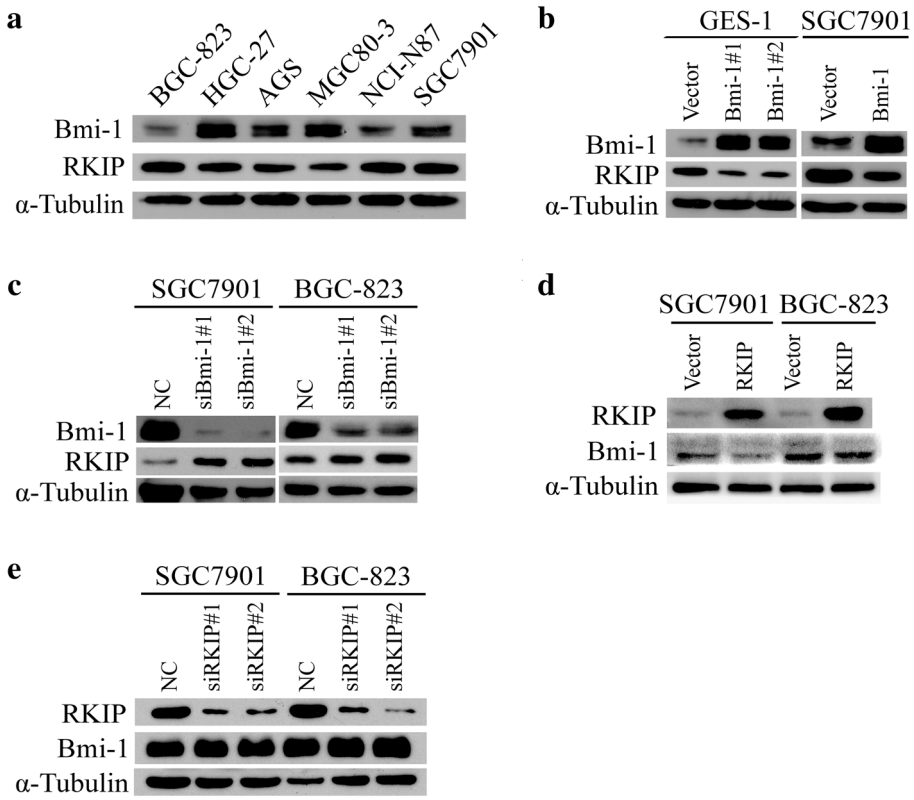

Fig. 5 Analyses of ectopic expression or silencing of B-cell-specific Moloney murine leukemia virus integration site 1 (*Bmi-1*). Endogenous expression patterns of Bmi-1 and Raf kinase inhibitory protein (*RKIP*) in six gastric cancer cell lines were detected through Western blotting, and α-tubulin was used as a loading control (**a**). Ectopic expression of Bmi-1 led to a significant downregulation of RKIP in both GES-1 and SGC7901 cells (**b**), whereas silencing of Bmi-1 increased RKIP expression in both SGC7901 and BGC-823 cells (**c**). RKIP was overexpressed (**d**) or silenced (**e**) in SGC7901 and BGC-823 cells, whereas Bmi-1 expression did not change. *NC* negative control

Moreover, we correlated the immunohistochemistry expression data for both Bmi-1 and RKIP with GC patient survival to establish their potential role in tumor evolution and progression. A negative trend of statistical significance between Bmi-1 expression and survival is shown in Fig. 4a, whereas a positive trend of statistical significance between RKIP expression and survival is shown in Fig. 4b. Kaplan–Meier analysis demonstrated that patients positively expressing Bmi-1 and negatively expressing RKIP were predicted to have worse survival prognosis of GC (Fig. 4c). The susceptibility of tumors positively and negatively expressing Bmi-1 and RKIP to postoperative chemotherapy was also estimated in GC. Our study showed that the susceptibility of patients with tumors positive or negative for Bmi-1 and RKIP to postoperative chemotherapy is different. Bmi-1-positive and RKIP-negative status showed worse survival in patients receiving chemotherapy, whereas the Bmi-1-negative and RKIP-positive group had better survival in patients receiving postoperative chemotherapy (Fig. 4f). Therefore, these patients may benefit more from chemotherapy.

In multivariate analysis of survival (Table S2), the combination of Bmi-1 expression, clinical stage, and chemotherapy provided independent predictive information on patient survival. In general, patients with advanced clinical stage, positive expression of Bmi-1, and not receiving chemotherapy were more likely to have a lower survival rate and a shorter survival time. For the tumor markers analyzed (Bmi-1 and RKIP), Bmi-1 expression is a powerful independent indicator of adverse prognosis in GC, and the role of RKIP as a correlated marker of metastasis, rather than an independent prognostic factor, is highlighted by these results.

Finally, the hypothesis that there was some definite connection between the expression of Bmi-1 and that of RKIP in GC was confirmed. As shown in Fig. 3, Bmi-1 expression is inversely related to RKIP expression in tissue microarrays. Endogenous inverse expression patterns of Bmi-1 and RKIP in GC cell lines were further detected (Fig. 5a). In light of the inverse correlation between Bmi-1 and RKIP, we predicted that there may be a regulation mechanism between Bmi-1 and RKIP in GC. Overexpression or silencing of Bmi-1 results in downregulation or upregulation of RKIP (Fig. 5b, c), whereas silencing of RKIP is not accompanied by a change in Bmi-1 expression (Fig. 5d), suggesting that RKIP is likely to be regulated by Bmi-1. Furthermore, the impacts of Bmi-1 silencing on cell invasion and chemotherapy resistance were rescued by knockdown of RKIP (Fig. 6). Collectively, we find that RKIP expression is likely to be regulated by Bmi-1 in GC cells.

In conclusion, Bmi-1 is positively correlated with tumor progression, whereas RKIP is negatively associated with tumor metastasis in GC. There is a remarkable inverse

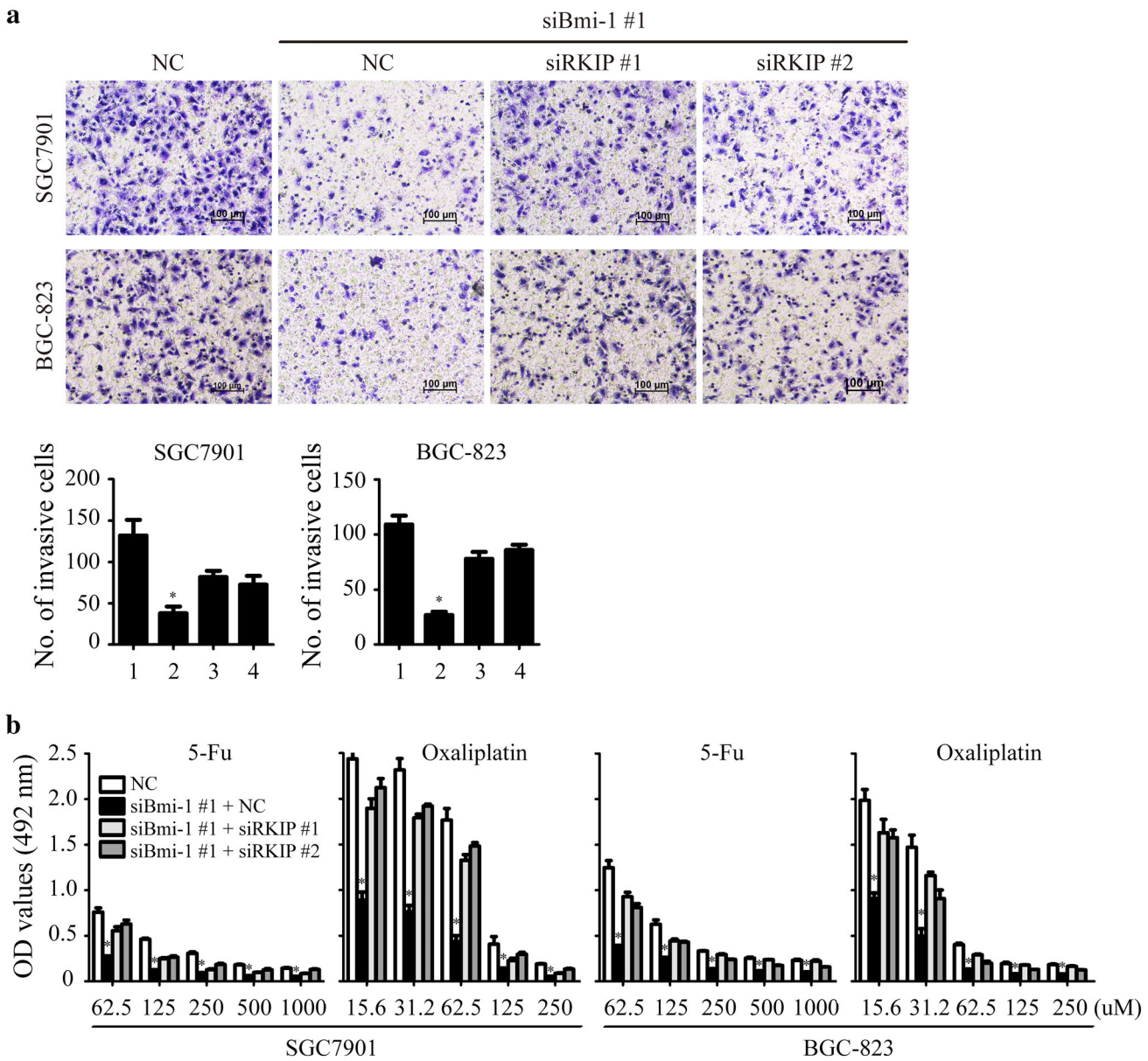

Fig. 6 Inhibition of Raf kinase inhibitory protein (*RKIP*) expression rescues invasiveness and drug sensitivity in B-cell-specific Moloney murine leukemia virus integration site 1 (*Bmi-1*)-silenced cells. **a** The invasiveness properties of RKIP small interfering RNAs (siRNAs) or negative control (*NC*) siRNA in Bmi-1-knockdown cells using a Matrigel-coated Boyden chamber. Original magnification ×100. *Error bars* represent the standard error of the mean. *1* NC siRNA, *2* Bmi-1 siRNA#1 and NC siRNA, *3* Bmi-1 siRNA#1 and RKIP siRNA#1, *4* Bmi-1 siRNA#1 and RKIP siRNA#2. **b** The 5-fluorouracil (*5-Fu*) and oxaliplatin sensitivity of RKIP siRNAs or NC siRNA in Bmi-1-knockdown cells. *Error bars* represent the standard error of the mean. *Asterisk* $p < 0.05$, compared with Bmi-1 siRNA#1 and NC. *OD* optical density

association between Bmi-1 expression and RKIP expression. Positive Bmi-1 expression combined with negative RKIP expression was predicted to have the worse survival prognosis in GC patients. Moreover, Bmi-1-negative and RKIP-positive patients benefit more from chemotherapy. Furthermore, our study demonstrated that the combined analysis of Bmi-1 expression, clinical stage, and chemotherapy is highly predictive of survival in patients with GC. We therefore recommend the evaluation of expression of Bmi-1 and RKIP as biomarkers for prospective validation in randomized multicenter trials. In addition, the strong and inverse association between Bmi-1 and RKIP is a point of special significance, and may be a piece to include in the complex puzzle that characterizes molecular profiles of GC. The regulation mechanism between Bmi-1 and RKIP in GC merits further investigation.

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Conflict of interest The authors declare that they have no conflict of interest.

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