

GATA binding protein 2 overexpression is associated with poor prognosis in KRAS mutant colorectal cancer

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Abstract. Colorectal cancer (CRC) is one of the most lethal cancers worldwide. Mutations in KRAS occur with the frequency of 30-50% in CRC leading to decreased therapeutic response to anti-epidermal growth factor receptor (EGFR) agents. Recently GATA2 was proven to be essential in the survival of KRAS mutant non-small cell lung cancer (NSCLC) cells. However, the association between KRAS mutation and GATA2 expression in CRC remains largely unknown. In the present study, dideoxy sequencing and immunohistochemistry were used to determine KRAS mutation and GATA2 expression, respectively, in a cohort of 236 patients. Cox proportional hazard regression and Kaplan-Meier survival analysis were performed to study the association between KRAS mutation or GATA2 expression and clinical outcomes. Kaplan-Meier

analysis revealed that KRAS mutant patients with high expression of GATA2 had significantly worse long-term clinical outcomes than those with low expression of GATA2 ($P < 0.001$). Further analysis showed that patients with both KRAS mutation and high GATA2 expression experienced significantly more unfavorable 5-year outcomes than patients with wild-type KRAS and low GATA2 expression ($P = 0.001$). Univariate and multivariate Cox proportional hazard regression demonstrated the GATA2 expression level was an independent risk factor for overall survival of CRC patients (HR 1.645; 95% CI 1.004-2.696; $P = 0.048$). In conclusion, the results of this study demonstrated that high expression of GATA2 is correlated with worse survival outcomes in KRAS mutant CRC patients, suggesting that GATA2 may serve as a novel biomarker for the survival of CRC patients harboring KRAS mutation.

Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide and the third ranked fatal malignancy in the United States (1,2). Although there has been progress in diagnosis, surgery, combined chemotherapy and targeted therapy (3,4), CRC remains a significant adverse influence on human health with the 5-year survival of only 65% (5). KRAS has long been known as the most frequently mutated gene in nearly all types of cancers. Particularly, approximately 40% of the CRCs harbor a mutation in KRAS (6). Mutations in codons 12 and 13 in exon 2 accounts for 90% of all KRAS mutations, and KRAS gene mutation test has been used as a predictor clinically to determine the lack of efficacy on targeting agents such as cetuximab and panitumumab (7-10). Although still controversial, several studies have demonstrated that KRAS mutation in colorectal carcinoma is correlated to unfavorable survival and enhanced tumor aggressiveness (11,12). However, recent therapeutic targeting of RAS in CRC has proven intractable, due to frequent mutation in KRAS (13). Therefore, searching for new therapeutic targets within the RAS signaling network is necessary and strongly warranted.

GATA binding protein 2 (GATA2), a key member of zinc finger transcription factors family, is identified as a critical regulator of growth, differentiation and survival of

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Abbreviations: CRC, colorectal cancer; GATA2, GATA2-binding protein 2; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; DFS, disease-free survival; PTEN, phosphatase and tensin homologue; FFPE, formalin-fixed, paraffin-embedded; PCR, polymerase chain reaction

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hematopoietic stem cells (14-16). Increasing evidence has shown that GATA2 expression is correlated with hematologic pathophysiologies and the proliferation and progression of solid tumors (17-23). It was reported that overexpression of GATA2 would contribute to the development of breast cancer through negatively regulating the transcription of phosphatase and tensin homologue (PTEN) (20). As for prostate cancer, upregulated GATA2 expression has been reported to be correlated with tumor progression and GATA2 has been suggested to be a pioneer factor in the regulation of androgen receptor related genes (21,22).

Kumar *et al* demonstrated that RAS-pathway mutant non-small cell lung cancer (NSCLC) cells depended on GATA2 for viability and regulation of the GATA2-related signaling pathways remarkably provoking regression of NSCLC (24). Further studies proved that delivery of GATA2 siRNA with selected carrier downregulated GATA2 expression and caused pronounced synthetic lethal effect of NSCLC *in vivo* (25). However, the association between KRAS mutation and GATA2 expression and its prognostic value in CRC remains unexplored.

In this study, we performed immunohistochemistry to examine GATA2 expression and dideoxy sequencing to detect KRAS mutation in the same patients in a CRC cohort. We demonstrated that GATA2 was a prospective indicator for poor prognosis in KRAS mutant CRC patients and GATA2-related pathways may be potential new therapeutic targets for KRAS mutant CRC patients.

Materials and methods

Ethics statement. All procedures performed in studies involving human participants were in accordance with the Ethical Standards of the Research Ethics Committee of Peking University Cancer Hospital and Institute (Beijing, China) (no. 2012071710) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Patients and tissue specimens. This retrospective study included a total of 236 patients who were diagnosed with CRC and then received primary tumor resection at the Department of Gastrointestinal Surgery IV, Peking University Cancer Hospital and Institute from 2005 to 2012. All the CRC tissues for immunochemistry analysis were obtained from surgically removed tumors and routinely embedded in paraffin. The clinicopathological characteristics and tumor stages were estimated in accordance with the American Joint Committee on Cancer (AJCC) classification guidelines. Postoperative follow-up results were available for all the 236 patients who have been followed up until March 2015. For the utility of these clinical samples for research, prior written informed consent of the patients and approval from the Research Ethics Committee of Peking University Cancer Hospital and Institute, (Beijing, China) were obtained.

KRAS mutation detection. Formalin-fixed, paraffin-embedded (FFPE) samples with $\geq 50\%$ tumor cells were collected from patients mentioned above. After extracting genome DNA from the FFPE samples, fragments contained codons 12 and 13 in KRAS exon 2 were amplified by PCR (KRAS forward,

5'-GGTACTGGTGGAGTATTTGATAG-3' and reverse, 5'-TGGTCCTGCACCAGTAATATG-3'). LA Taq polymerase was used to perform the PCR. The product size was 248 bp. The reaction mixtures recommended by the manufacturers were used. The initial denaturation step was 5 min at 94°C. The thermal profile was 45 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 20 sec. The final elongation was 10 min at 72°C. The PCR products were separated on 1% agarose gel electrophoresis and then sequenced using the same forward primer by Invitrogen 3730XL genetic analyzer. The sequencing results were analyzed with Chromas software under the condition of signal/noise $>98\%$ (26).

Immunohistochemistry. Paraffin-embedded tumor tissue blocks were cut 4- μ m thick and then baked overnight at 72°C. Then they were deparaffinized with xylene twice and rehydrated with graded ethanol. The slides were heated in antigen retrieval buffer containing 0.01 M sodium citrate-hydrochloric acid (pH 6.0) for 10 min in a high pressure apparatus at 150°C. After having been cooled in room temperature for 1 h, the slides were treated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity. After blocking nonspecific binding with goat serum, the sections were incubated with a rabbit polyclonal antibody against GATA2 (1:200; Santa Cruz Biotechnology) at 4°C overnight, followed by incubation with second antibody from the EnVision™ kit (Dako Cytomation) at room temperature for 30 min. The visualization signal was developed with diaminobenzidine (Sigma). Sections were counterstained with hematoxylin.

Evaluation of GATA2 immunohistochemistry. GATA2 staining scores were determined independently by two pathologists previously uninformed about the histopathological features and patient information to minimize subjectivity. Proportion of positive cells and staining intensity of immunohistochemistry were considered in the scoring process. The scores for GATA2 staining were determined according to the following standard (27): -, no staining or $<10\%$ positive cells; +, 10-20% weakly to moderately positive cells; ++, 10-20% intensively positive cells or 20-50% weakly positive cells; and +++, 20-50% positive cells with moderate to strong reactivity or $>50\%$ positive cells. All disagreements on results between the two pathologists were resolved after joint review and finally consensus was achieved for all the results.

Statistical analysis. Statistical analysis was carried out using SPSS software 17.0. The Pearson's χ^2 verified the correlation between either KRAS gene status or GATA2 expression and clinicopathological parameters. Kaplan-Meier method was used to estimate overall survival curve and differences between groups were compared by the log-rank test. Univariate and multivariate Cox proportional hazard regression models were used to evaluate the predictors for overall survival. A p-value of <0.05 was considered to be statistically significant.

Results

Types of KRAS mutation. Among the 236 enrolled cases, 64 (27.1%) were found to harbor mutations in the KRAS gene. As shown in Table I, the KRAS mutations were

Table I. Distribution of various KRAS mutant patterns.

KRAS status	No. of cases (%)
Wild	172 (72.9)
C12GAT	31 (13.1)
C13GAC	12 (5.1)
C12GTT	12 (5.1)
C12TGT	5 (2.1)
C12AGT	2 (0.9)
C12GCT	1 (0.4)
C13TGC	1 (0.4)

distributed between codon 12 [51/64 (79.7%)] and codon 13 [13/64 (20.3%)]. A total of seven types of mutation in exon 2 were detected. The G>A transitions at nucleotides, namely C12GAT and C12AGT in codon 12 as well as C13GAC in codon 13, were the most frequent mutations in this cohort (70.3%).

Association between KRAS mutation or GATA2 expression and clinicopathological features. All 236 patients with CRC were included in this analysis. GATA2 expression was examined in all of the 236 CRC patients (Fig. 1). Table II shows the correlation between either KRAS mutation or GATA2 expression and various clinicopathological features. Statistical analysis indicated that KRAS mutation was significantly correlated with gender ($P=0.011$), tumor location ($P=0.024$) and overall survival ($P=0.012$). Nevertheless, no associations were found between KRAS mutation and age, tumor size, depth of invasion, lymph node metastasis, TNM stage, histological type, and tumor differentiation ($P>0.05$). On the other hand, GATA2 expression was significantly linked with age ($P=0.012$), depth of invasion ($P=0.002$), lymph node metastasis ($P=0.018$), TNM stage ($P=0.001$) and overall survival ($P=0.001$). No correlation was observed between the level of GATA2 expression and gender, tumor location, tumor size, histological type, and tumor differentiation ($P>0.05$). However, KRAS mutation was not significantly associated with GATA2 expression ($P=0.766$).

Correlation between KRAS mutation or GATA2 expression and overall survival. Kaplan-Meier survival curves with log-rank test were performed to evaluate the association between KRAS mutation or GATA2 expression level and prognosis. The results revealed that KRAS mutation in CRC was significantly associated with worse overall survival ($P=0.011$, Fig. 2A). Additionally, GATA2 high expression was significantly correlated with shorter overall survival compared with GATA2 low expression group ($P=0.012$, Fig. 2B).

For further analysis, the CRC patients enrolled were separated into different groups according to KRAS status (wild-type and mutant) and GATA2 expression (low and high). The Kaplan-Meier curves in Fig. 2C demonstrated that KRAS mutant/GATA2 high cancers had poor ($P=0.001$, Fig. 2C) long-term clinical outcomes in comparison with KRAS wild/GATA2 low cancers.

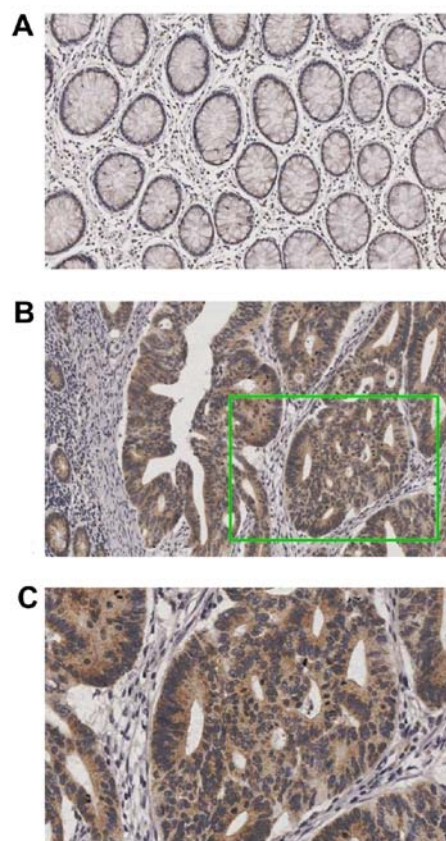


Figure 1. Immunohistochemical results of GATA2 protein expression in normal tissue and colorectal tissue. (A) Representative images revealed negative GATA2 staining in normal tissue, (B) strong GATA2 staining in CRC tissue. Magnification x100. (C) CRC tissue with at magnification x200.

Stratified analysis by KRAS mutation (KRAS wild-type and KRAS mutant type) showed that in the wild-type KRAS subgroup, no statistical significance was observed between the levels of GATA2 expression and overall survival of CRC patients ($P=0.748$, Fig. 3A). However, in the mutant KRAS subgroup, patients with high expression of GATA2 experienced significantly shorter overall survival, compared with those with low expression of GATA2 ($P<0.001$, Fig. 3B).

Furthermore, the prognostic value of KRAS gene status in different GATA2 protein expression levels were also evaluated. In patients with low GATA2 expression, only a borderline significance was observed in overall survival between KRAS wild-type and KRAS mutant groups ($P=0.067$, Fig. 4A). Whereas in patients with high GATA2 expression, mutant KRAS was significantly correlated with poor overall survival, compared with wild-type KRAS ($P=0.020$, Fig. 4B).

Cox proportional hazard regression analysis demonstrated GATA2 as an independent prognostic factor. In the univariate analysis, patients with KRAS mutation or GATA-high carcinoma tended to have a shorter overall survival (HR 1.704; 95% CI 1.123-2.583; $P=0.012$, HR 1.818; 95% CI 1.129-2.927; $P=0.014$, respectively, Table III) than those with KRAS wild-gene type or GATA2-low tumors. Gender, tumor location, tumor size, depth of tumor invasion and TNM stage also showed significant associations with overall survival in univariate

Table II. Correlations between KRAS mutation or GATA2 expression and clinicopathological features in colorectal cancer patients.

Variables	Cases	KRAS mutation		P-value	GATA2 expression		P-value
		Wild-type	Mutant		Low	High	
Age (years)							
<60	140	106	34	0.237	57	83	0.012
≥60	96	66	30		24	72	
Gender							
Female	94	60	34	0.011	35	59	0.443
Male	142	112	30		46	96	
Tumor location							
Colon	134	90	44	0.024	44	90	0.582
Rectum	102	82	20		37	65	
Tumor size (cm)							
≤4	142	108	34	0.178	49	93	0.941
>4	94	64	30		32	62	
Depth of invasion							
T1/T2	35	30	5	0.064	20	15	0.002
T3/T4	201	142	59		61	140	
Lymph node metastasis							
Negative	73	53	20	0.949	33	40	0.018
Positive	163	119	44		48	115	
TNM stage							
I/II	54	39	15	0.901	29	25	0.001
III/IV	182	133	49		52	130	
Histological type							
Adenocarcinoma	213	151	62	0.110	74	139	0.893
Mucinous	14	13	1		4	10	
Others	9	8	1		3	6	
Tumor differentiation							
Well	2	1	1	0.467	0	2	0.259
Moderate	160	116	44		50	110	
Poor	53	37	16		21	32	
Unknown	21	18	3		10	11	
Survival							
Alive	138	109	29	0.012	59	79	0.001
Dead	98	63	35		22	76	

analysis (HR 1.639; 95% CI 1.102-2.437; $P=0.015$, HR 1.663; 95% CI 1.097-2.521; $P=0.017$, HR 1.586; 95% CI 1.066-2.359; $P=0.023$, HR 2.575; 95% CI 1.193-5.559; $P=0.016$, HR 3.447; 95% CI 1.832-6.488; $P<0.001$, respectively, Table III). In the multivariate analysis, the potential risk factors mentioned above were included into the Cox proportional hazard regression model in order to avoid covariation. The results showed that GATA2 high expression, advanced TNM stage (III/IV) and female patients were retained as independent prognostic factors for poor prognosis (HR 1.645; 95% CI 1.004-2.696; $P=0.048$, HR 3.058; 95% CI 1.594-5.865; $P=0.001$, HR 1.519; 95% CI 1.010-2.283; $P=0.045$, Table III).

Discussion

In this study, we analyzed the influence of KRAS mutation and GATA2 expression on the overall survival based on a cohort of CRC patients. Our data demonstrated that although there was no significant correlation between KRAS mutation and expression of GATA2, high level of GATA2 expression and KRAS mutation were significantly associated with unfavorable prognosis in CRC patients, compared to those bearing GATA2-low and wild-type KRAS tumors, respectively. Moreover, we determined that combined KRAS mutation and overexpression of GATA2 led to more adverse overall survival

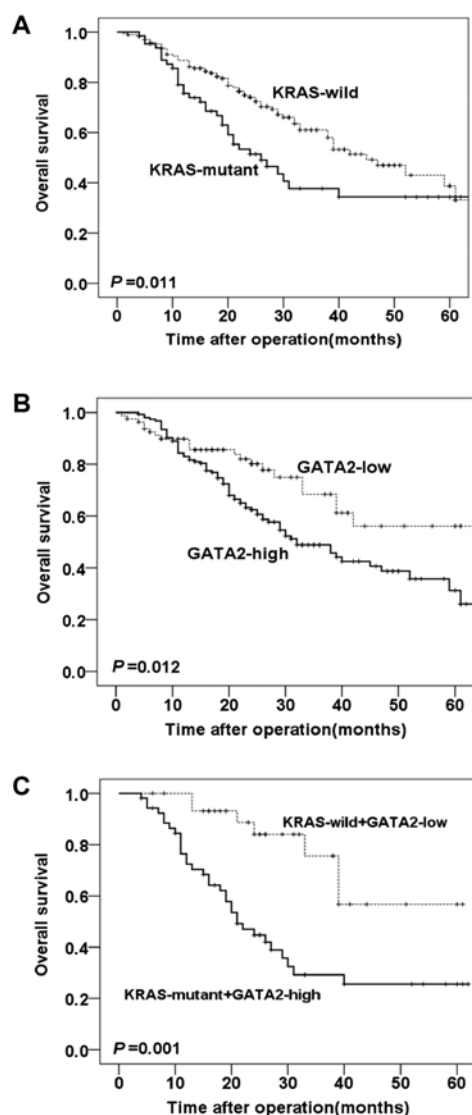


Figure 2. Kaplan-Meier analysis of overall survival in CRC patients. (A) According to KRAS status, (B) according to the levels of GATA2, (C) according to their combination. The log-rank test was used to calculate the p-values.

over the combination of KRAS wild-type and GATA2 low expression. Univariate and multivariate Cox regression analysis demonstrated that the GATA2 expression level was a possible independent predictor for poor overall survival in CRC patients. The results in all showed that high levels of GATA2 expression predicted significant adverse clinical outcomes when KRAS mutation occurred in the same patient, suggesting GATA2 protein may be prone to tumorigenesis of KRAS mutant CRC.

The role of GATA2 in hematopoietic malignant disorders has been well elucidated (15,19). In contrast, only a few studies in gastrointestinal malignancies involving GATA2 transcription factor have been done (28). Previous studies suggested that GATA2 expression had influence on the clinical outcomes in patients with several solid carcinomas, such as breast, prostate, colorectal, renal and hepatocellular carcinoma (20-23,29,30). High levels of GATA2 expression predict tumor recurrence, metastasis, or poor survival except for renal and hepatic cancer (29,30). Moreover, the role of GATA2 in lung cancer

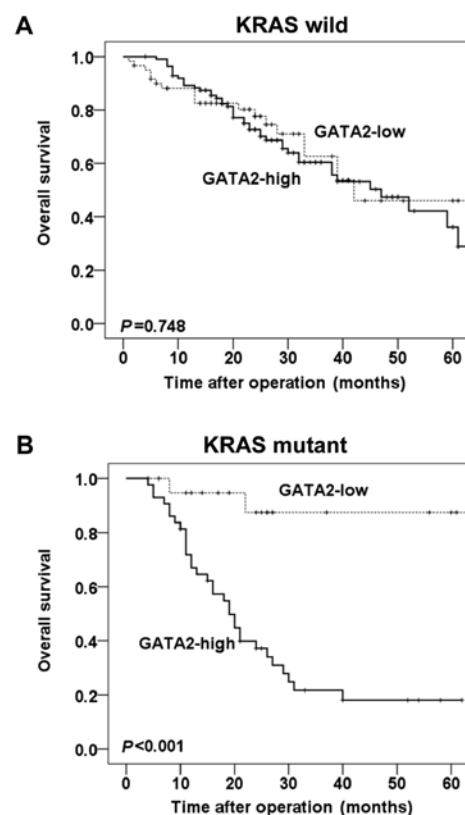


Figure 3. Kaplan-Meier curves for overall survival stratified by KRAS status. (A) KRAS wild-type subgroup according to the expression level of GATA2. (B) KRAS mutant subgroup according to the expression level of GATA2. The log-rank test was used to calculate the p-values.

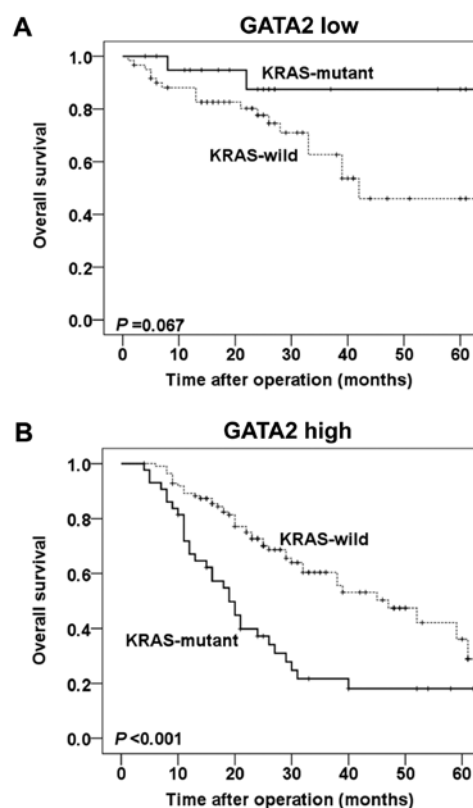


Figure 4. Kaplan-Meier analysis of overall survival stratified by GATA2 expression. (A) Low levels of GATA2 expression subgroup according to KRAS gene status. (B) High levels of GATA2 expression subgroup regarding KRAS gene status. The log-rank test was used to calculate the p-values.

Table III. Univariate and multivariate analysis of KRAS status and GATA2 expression with respect to overall survival.

Variables	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (≥ 60 vs. < 60 years)	1.386	0.933-2.060	0.106			
Gender (female vs. male)	1.639	1.102-2.437	0.015	1.519	1.010-2.283	0.045
Tumor location (colon vs. rectum)	1.663	1.097-2.521	0.017	1.508	0.973-2.335	0.066
Tumor size (> 4 vs. ≤ 4 cm)	1.586	1.066-2.359	0.023	1.368	0.902-2.075	0.140
Depth of invasion (T3/T4 vs. T1/T2)	2.575	1.193-5.559	0.016	1.165	0.505-2.691	0.720
TNM stage (III/IV vs. I/II)	3.447	1.832-6.488	< 0.001	3.058	1.594-5.865	0.001
Histological type (mucinous vs. adenocarcinoma)	0.578	0.183-1.826	0.350			
Tumor differentiation (poor vs. well/moderate)	1.467	0.930-2.315	0.099			
KRAS mutation (mutant vs. wild)	1.704	1.123-2.583	0.012	1.499	0.974-2.305	0.065
GATA2 expression (high vs. low)	1.818	1.129-2.927	0.014	1.645	1.004-2.696	0.048

remains controversial. A recent study demonstrated that GATA2 levels were indispensable for survival of KRAS mutant NSCLC cells (24). Co-inhibition of GATA2-regulated proteasome and Rho-signaling pathway significantly suppressed the proliferation of lung tumor cells with KRAS mutation. These results indicated that application of novel strategy targeting GATA2 related pathways may benefit patients with RAS pathway mutated NSCLC. On the contrary, Tessema *et al* reported that GATA2 was not requisite for the survival of lung cancer patients with KRAS mutation due to epigenetic repression (31). Therefore, it is critical to determine the association between KRAS mutation and GATA2 expression, and verify their clinical significance in CRC patients. Consistent with previous studies (23,24), our findings showed that GATA2 played a crucial role in the long-term outcomes of CRC patients with KRAS mutation, which raised the possibility that GATA2 protein may influence carcinogenesis and proliferation of KRAS mutant CRC cells. Accordingly, GATA2 could be a potential target for the treatment of CRC patients with KRAS mutation, which indicates resistance to EGFR-targeting antibodies, including cetuximab and panitumumab. However, GATA2 is traditionally considered to be undruggable (32). Thus, therapies against GATA2-regulated pathways may become an alternative strategy for the treatment of CRC patients with KRAS mutation.

In this cohort, as expected, TNM stage was identified as an independent prognostic factor to predict patient clinical outcomes (HR 3.058; 95% CI 1.594-5.865; $P=0.001$, Table III). Additionally, female gender was observed as an adverse factor in our study (HR 1.519; 95% CI 1.010-2.283; $P=0.045$, Table III), which was in accordance with previous studies (33).

However, potential limitations still exist in this study. To validate the role of GATA2 expression in CRC patients harboring KRAS mutation, replication cohorts containing detailed clinical data are required. In addition, further functional studies are needed to understand the role of GATA2 and KRAS in CRC.

In conclusion, we demonstrated that elevated GATA2 expression correlated with poor overall survival in CRC patients. Furthermore, GATA2 overexpression in combination

with KRAS mutation is significantly associated with poor prognosis. Our results suggested that GATA2 is a promising predictor to identify individuals with worse long-term clinical outcomes, especially in KRAS mutant CRC patients. Also, we consider GATA2-related pathways as potential targets for the development of novel therapies for KRAS mutant CRC.

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