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Original Article

Gab2 is a novel prognostic factor for colorectal cancer patients

Chenbo Ding[1], Junmin Luo[1], Weina Yu[1], Shaoying Gao[1], Liwen Yang[1], Chao Chen[1], Jihong Feng[2]

[1]*Department of Immunology, Zunyi Medical College, Immunology Innovation Base of Postgraduate Education in Guizhou Province, Zunyi, China;* [2]*Department of Oncology, Affiliated Hospital of Zunyi Medical College, Zunyi, China*

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Abstract: Gab2 (Grb2-associated binder 2), a member of the DOS/Gab family of scaffolding adapters, serves as a critical signal amplifier downstream of various growth factor receptors. Recent studies have identified that Gab2 is overexpressed in several cancer types and that increased Gab2 expression promotes cell proliferation, cell transformation, and tumor progression. Here, we show for the first time that Gab2 protein is overexpressed in clinical colorectal cancer (CRC) specimens. Elevated mRNA ($P = 0.014$) expression and protein ($P = 0.003$) expression of Gab2 were found in most CRC tissues compared with the matched adjacent non-tumor tissues using real-time quantitative reverse transcription PCR (qRT-PCR) and western blotting, respectively. Immunohistochemical analyses showed that Gab2 protein was upregulated in CRC tissues relative to adjacent normal tissues ($P < 0.001$), and this overexpression was significantly correlated with lymph node metastasis ($P = 0.007$), distant metastasis ($P < 0.001$) and TNM stage ($P = 0.002$). According to Kaplan-Meier model, CRC patients with Gab2-positive had a significantly poorer prognosis compared to those with Gab2-negative ($P = 0.007$). Multivariate analysis suggested that the positive expression of Gab2 protein was an independent prognostic factor for CRC patients. In conclusion, our data demonstrated that Gab2 expression may play an important role in the progression of CRC, and underscored that Gab2 has the potential value as a prognostic predictor for CRC patients.

Keywords: Gab2, colorectal cancer, qRT-PCR, western blotting, immunohistochemistry, prognosis

Introduction

It has recently been reported that global colorectal cancer (CRC) incidence and mortality are accounted for about 9% of all cancers [1]. In 2014, an estimated 71,830 men and 65,000 women will be diagnosed with CRC and 26,270 men and 24,040 women will die of the disease [2]. The survival rate for CRC is higher at early stages resulting from improved treatment and increased awareness and early detection; however, the long-term survival rate and prognosis for patients with CRC remain very poor, mainly due to local recurrence and distant metastases formation [3, 4]. Currently available cancer markers play a major role in determining the management of CRC, but are usually not suitable for current clinical practice and require further investigation. Therefore, it is hoped that useful biomarkers, which have the potential value of serving as prognostic predictors and therapeutic targets for CRC patients.

Gab2 (Grb2-associated binder 2) is a key member of Gab family proteins, which also includes mammalian Gab1, Gab3 and Gab4, Drosophila daughter of sevenless (Dos), and Caenorhabditis elegans suppressor of Clr (Soc)-1 [5]. As a scaffolding adaptor, Gab2 is involved in the amplification and integration of signal transduction, playing a pivotal role in cell growth, differentiation, migration and apoptosis. Recent it has been shown that Gab2 participates in human tumorigenesis. Gab2 has been reported to be upregulated in human breast, ovarian and lung tumors [6-8], and leukemia and melanoma [9, 10]. However, the expression of Gab2 in human CRC remains unclear. Hereby, in this study, we showed the expression of Gab2 in CRC and the relationship between the pathological characteristics and prognosis; should provide new

Table 1. Clinical characteristics of 109 CRC patients

Parameters	CRC patients (N = 109)%
Age (years)	
Mean	61
Range	33-82
Gender	
Male	64 (58.7%)
Female	45 (41.3%)
Tumor size (cm)	
Mean	5.5
Range	1.0~15.0
Histology	
Tubular	92 (84.4%)
Mucinous	15 (13.8%)
Papillary	2 (1.8%)
Tumor location	
Right colon	31 (28.4%)
Left colon	12 (11.0%)
Rectum	66 (60.6%)
Differentiation status	
Well	53 (48.6%)
Moderate	27 (24.8%)
Poor	29 (26.6%)
Lymph node metastasis	
Absent	67 (61.5%)
Present	42 (38.5%)
Distant metastasis	
Negative	71 (65.1%)
Positive	38 (34.9%)
TNM stage	
I-II	76 (69.7%)
III-IV	33 (30.3%)

insight to design effective therapies to treat CRC.

Materials and methods

Patients and clinical tissue samples

In this study, all the specimens were collected form CRC patients undergoing colorectal surgery at Department of gastrointestinal Surgery, The First Affiliated Hospital of Chongqing Medical University from January 2009 to December 2013. The samples included 27 paired cancerous and matched adjacent normal frozen tissue samples, and 82 paraffin-embedded tissues. After surgical resection, fresh tissues were immediately put into liquid nitrogen and frozen at -80°C until processed for RNA and protein extraction. None of these patients had received radiotherapy or chemotherapy prior to surgery. Informed consent was obtained from all participants and the study was performed in accordance with the Declaration of Helsinki. The clinicopathological features of the patients with CRC were summarized in **Table 1**.

Extraction of total RNA and qRT-PCR

Total RNA was isolated from the frozen tissues by using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The obtained RNA was used to synthesize cDNA by Superscript first strand synthesis kit as per instructions of the manufacturer (TakaRa, Japan). Real-time quantitative PCR reaction mixes were prepared using SYBR Green (TaKaRa, Japan) and run in triplicate in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems) with 25 μl PCR mix containing 12.5 μl SYBR Green master mix, 2 μl primer mix, 2 μl cDNA, and 8.5 μl deionized water. The mix was preheated at 95°C (5 min), and then amplified at 95°C (20 sec) and 60°C (1 min) for 45 cycles. The primer sequences used in the study were as follows: Gab2 forward: 5'-GTGGGGGATCTGAATGTTTTT-ATG-3'; reverse: 5'-GCCCCAGGGTAGAATGAAA-CG-3'; GAPDH forward: 5'-GAAGGTGAAGGTCG-GAGTC-3' and reverse: 5'-GAAGATGGTGATGGG-ATTTC-3'. Gene expression levels were quantified using the ABI Prism 7900HT sequence detection system (Applied Biosystems), and the results were expressed at a relative mRNA level, analyzed using the comparative threshold cycle ($2^{-\Delta CT}$) method with GAPDH as the reference gene.

Western blotting analysis

Tissues were lysed in lysis buffer and protein concentrations were measured using a BCA protein assay kit. Total protein was separated by electrophoresis on a 10% SDS-PAGE and electroblotted onto a PVDF membrane. After blocking the non-specific binding sites for 1 hour with 5% non-fat milk, the membranes were then incubated overnight at 4°C with a rabbit monoclonal antibody against Gab2 (OriGene Technologies, USA, at a 1:1000 dilution). After three washes with TBST (tris-buffered saline with tween-20) for 10 minutes, the membranes were incubated with horseradish peroxidase (HRP)-labeled Goat Anti-Rabbit IgG (Immunology Consultants Laboratory, USA, at a 1:2000 dilution) at room temperature for 1

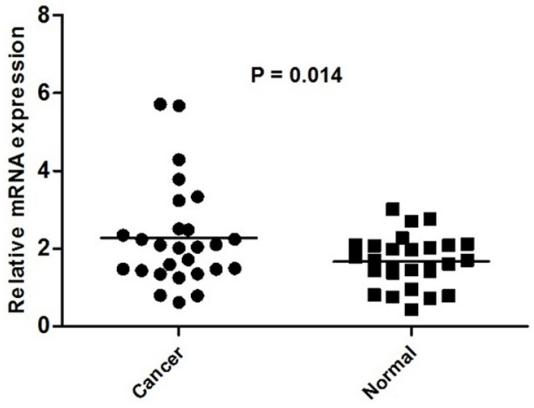

Figure 1. Gab2 mRNA was overexpressed in CRC tissues. The mRNA expression of Gab2 in CRC tissues was assessed by qRT-PCR (Gab2/GAPDH, n = 27, *P* = 0.014). Horizontal lines represent the mean.

hour. After three washes with TBST, the membranes were developed with X-ray film (Kodak, Rochester, NY). The band intensity was quantified using the Image J 1.43 software. The Gab2 protein level was normalized to the level of GAPDH detected by a mouse monoclonal antibody against GAPDH (Beyotime Institute of Biotechnology, China, at a 1:2000 dilution).

Immunohistochemistry and staining evaluation

Before staining, paraffin-embedded tissue specimens were cut into 4 μm- thick sections. The sections were deparaffinized with dimethylbenzene and rehydrated with 100%, 95%, 90%, 80% and 70% ethanol. Antigen retrieval was performed with microwave retrieval method, followed by 10 minutes incubation with 3% hydrogen peroxidase (H_2O_2) to block endogenous peroxidase activity. Nonspecific binding was blocked by incubating sections in goat serum at room temperature for 30 minutes. Subsequently, the sections were incubated with rabbit monoclonal antibody against Gab2 (OriGene Technologies, USA, at a 1:150 dilution) at 4°C overnight and then were incubated with HRP- labeled secondary goat anti-rabbit antibody (Immunology Consultants Laboratory, USA, at a 1:200 dilution) at room temperature for 30 minutes. After washing in PBS (phosphate-buffered saline), all the sections were incubated with 3, 3'-diaminobenzidine (DAB) kit (Zhongshan Goldenbridge Biotech, Beijing, China) for 5 minutes and counterstained with hematoxylin, mounted, and examined by light microscopy. As negative controls, tissue sections were processed under the same experimental conditions described above, except that they were incubated with PBS instead of primary antibody.

Immunostaining reactions were evaluated by staining intensity and the percentage of positively stained tumor cells. The immunostaining intensity was scored as: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The percentage of stained cells on each section was scored as: 0 (less than 5%), 1 (5%-25%), 2 (26%-50%), and 3 (> 51%) accordingly. Then, the total immunostaining score was calculated by multiplying stained intensity score with staining cells score and thus ranged from 0 to 9. Five random fields in each section were selected for the evaluation. For statistical reasons, a final staining scoring at least 3 points in our study were considered to be positive.

Statistical analysis

All statistical analyses were performed using SPSS software system (version 19.0; SPSS, Chicago, IL, USA). Differences in mRNA and protein expression between the CRC samples and the matched adjacent noncancerous tissue samples were evaluated with paired samples t-test. The correlation between Gab2 expression and clinicopathologic features of CRC patients was analyzed by χ^2-test. Survival curves were performed by the Kaplan-Meier model, and intergroup differences were determined by the log-rank test. Analyses of prognostic factors for overall survival were determined by univariate and multivariate Cox proportional hazards regression method. A value of $P < 0.05$ was considered to be statistically significant. All *p*-values were two-sided.

Results

mRNA expression of Gab2 in CRC tissues

To detect the mRNA expression of Gab2 in 27 CRC tissues and the matched normal tissues, qRT-PCR was applied. The result showed that the Gab2 mRNA expression level was significantly higher in tumor-bearing tissues, compared with the corresponding normal tissues (**Figure 1**). Data were analyzed by paired-samples t test ($t = 2.636$, $P = 0.014$).

High level of Gab2 protein expression in CRC tissues

We examined the expression of Gab2 protein in 19 CRC specimens and corresponding normal

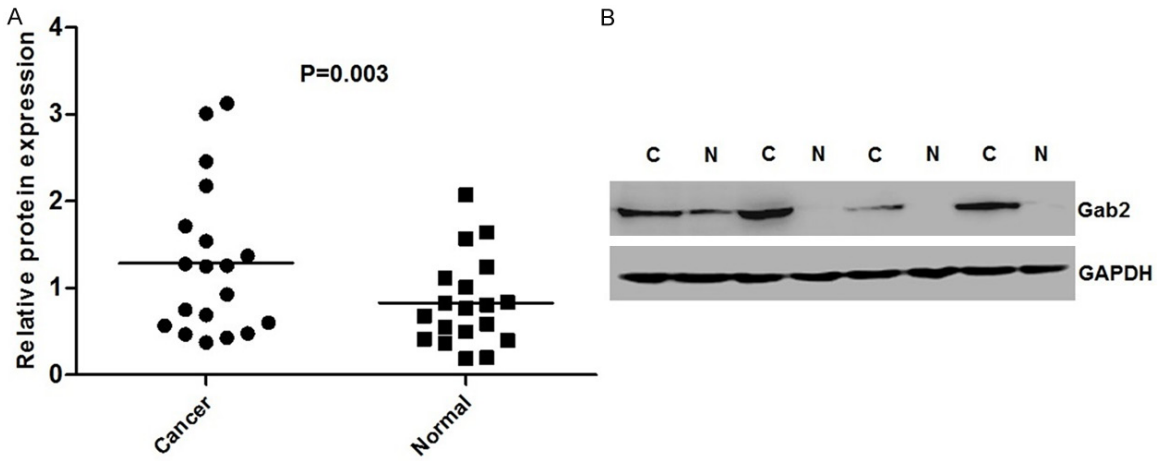

Figure 2. Gab2 protein was upregulated in CRC tissues. The protein expression of Gab2 in CRC tissues was detected by western blotting analysis. A. Relative Gab2 protein expression levels in CRC tissues and noncancerous tissues (Gab2/GAPDH, n = 19, $P = 0.003$). Horizontal lines represent the mean. B. Representative result of Gab2 protein expression in 4 paired colorectal cancer and the matched adjacent nontumorous tissues (C, CRC tissues; N, matched noncancerous tissues).

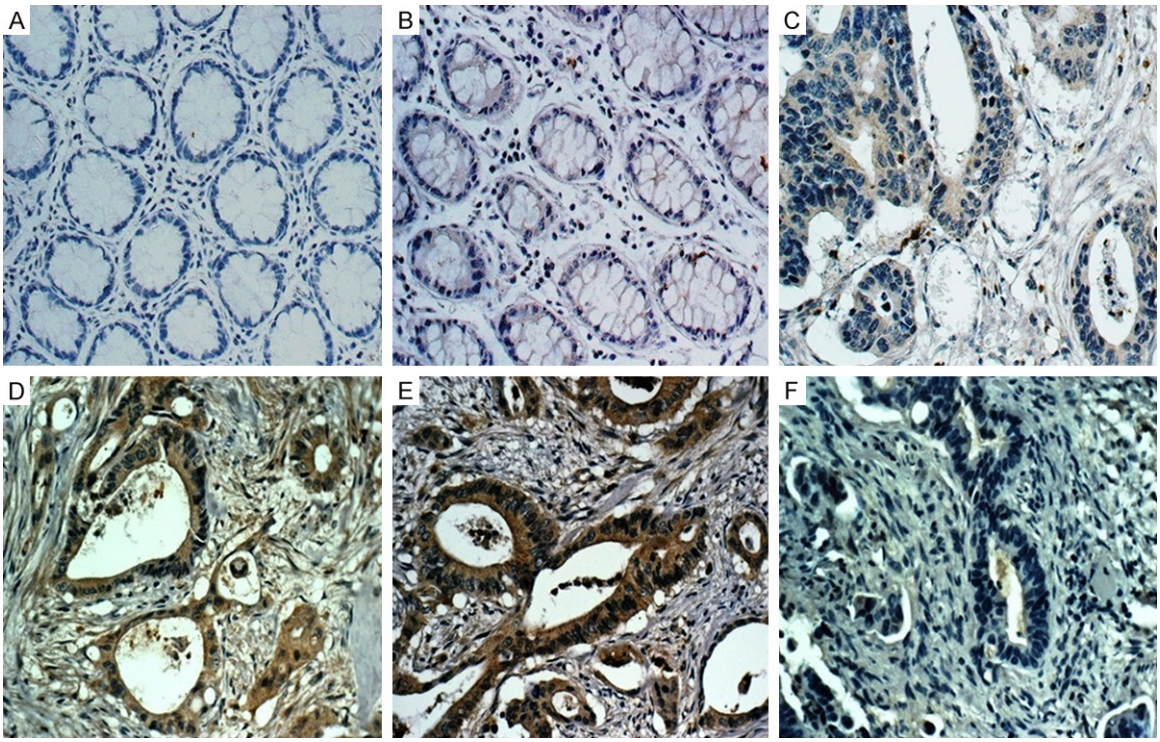

Figure 3. Immunohistochemical staining for Gab2 in CRC tissue and adjacent normal tissue. A. Adjacent normal tissue (no stain). B. Adjacent normal tissue (weak). C. CRC tissue (weak). D. CRC tissue (moderate). E. CRC tissue (strong). F. Negative control in CRC tissue (SP × 400).

tissues from 27 paired samples by western blotting. Consistent with the qRT-PCR results, high level of Gab2 protein expressed in 10 (52.6%) of CRC tissues, compared with the matched adjacent normal tissues ($P = 0.003$, **Figure 2**).

To further investigate the expression of Gab2 protein in CRC tissues and adjacent non-tumor tissues, immunohistochemistry staining was performed. We found that positive signals of Gab2 were mainly localized in the cytoplasm of CRC cells. According to the established evalua-

Table 2. Correlation between Gab2 protein expression and clinic pathological characteristics of patients with CRC

Characteristics	Total	Gab2 expression		χ^2 value	*P* value
		Positive	Negative		
Gender				0.328	0.567
Male	51	28	23		
Female	31	15	16		
Age (years)				2.221	0.136
≤ 60	33	14	19		
> 60	49	29	20		
Tumor size				1.400	0.237
≤ 5 cm	47	22	25		
> 5 cm	35	21	14		
Histology				0.027	0.869
Tubular	72	38	34		
Mucinous, Papillary	10	5	5		
Tumor location				1.565	0.211
Colon	28	12	16		
Rectal	54	31	23		
Differentiation status				3.759	0.053
Well-moderate	59	27	32		
Poor	23	16	7		
Lymph node metastasis				7.178	0.007*
Absent	53	22	31		
Present	29	21	8		
Distant metastasis				13.874	< 0.001*
Negative	60	24	36		
Positive	22	19	3		
TNM stage				9.151	0.002*
I-II	56	23	33		
III-IV	26	20	6		

*$P < 0.05$

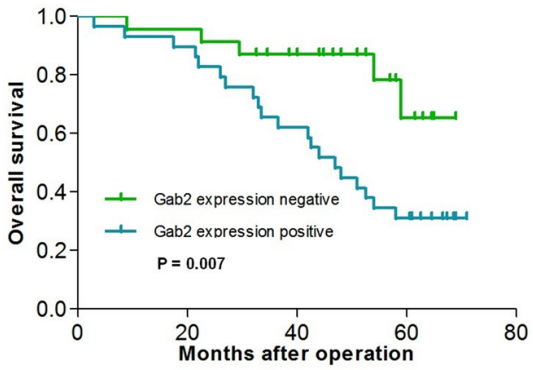

Figure 4. Kaplan-Meier graph showing the overall survival for the patients with CRC based on the immunohistochemical staining results of Gab2. Patients with Gab2-positive had a significantly poorer prognosis than those with Gab2-negative ($P = 0.007$).

tion principle for immunostaining, Gab2 protein positive expression rate was 52.4% (43/82) in CRC tissues; whereas was negative expression or weak in the adjacent normal tissues, exhibiting a significant difference of comparison within this result ($P < 0.001$, **Figure 3**).

Correlation between Gab2 expression and clinicopathological characteristics of CRC

As shown in **Table 2**, Gab2 expression was indicated to have a marked association with lymph node metastasis ($P = 0.007$), distant metastasis ($P < 0.001$) and TNM stage ($P = 0.002$). However, our findings revealed no significant correlation between Gab2 expression and other clinicopathological parameters, including gender, age, histology, tumor size, location and differentiation. Data were analyzed by χ^2-test.

Prognostic significance of Gab2 expression in CRC

Kaplan-Meier survival analysis revealed that the patients with Gab2-negative had significantly longer survival than those with Gab2-positive ($P = 0.007$, **Figure 4**). To determine whether Gab2 could be used as an independent risk factor for poor prognosis of CRC patients, conventional clinicopathological factors and Gab2 protein expression were analyzed by Cox's univariate and multivariate hazard regression model. As shown in **Table 3**, univariate analysis and multivariate analysis indicate that Gab2 protein expression, together with lymph node metastasis, distant metastasis and advanced clinical stage, were to be significant independent prognostic factors of CRC.

Discussion

It has become evident that Gab proteins are crucial signaling elements and have been implicated in several hematological neoplasias and solid cancers. Recently, increasing studies

Table 3. Univariate and multivariate analysis for factors affecting the overall survival rate of CRC patients

Parameters	Univariate			Multivariate		
	HR	95 % CI	*P*	HR	95 % CI	*P*
Age	1.464	0.643-3.332	0.364	0.990	0.441-2.224	0.981
Gender	2.019	0.816-4.997	0.129	1.660	0.668-4.123	0.275
Tumor size	0.705	0.310-1.599	0.403	0.498	0.219-1.131	0.096
Tumor location	0.868	0.357-2.110	0.755	0.709	0.292-1.724	0.448
Tumor differentiation	2.715	0.882-5.363	0.092	1.772	0.718-4.375	0.215
Lymph node metastasis	3.214	1.403-7.362	0.006*	2.702	1.178-6.195	0.019*
Distant metastasis	4.407	1.815-10.700	0.001*	3.613	1.487-8.780	0.005*
TNM stage	3.365	1.432-7.908	0.005*	2.856	1.211-6.731	0.017*
Gab2 expression	3.626	1.344-9.785	0.011*	2.875	1.061-7.796	0.038*

*$P < 0.05$. HR, hazard ratio; CI, confidence interval.

have established that Gab proteins promote human tumorigenesis by functioning as "accomplices" of certain oncoproteins or by amplifying signaling upon the overexpression of Gab proteins. Similar to the Gab proteins, Gab2 is a key member of Gab protein family and has shown that plays an important role in human tumorigenesis. For example, overexpression of Gab2 was detected in estrogen receptor-positive cells [11], and a subset of breast cancers is driven by Gab2 overexpression coupled with ErbB2 (also known as Neu or HER2) receptor signaling [12]. In metastatic melanoma, high level of Gab2 expression and Gab2-mediated signaling has been identified as a pivotal mechanism regulating cell proliferation and migration [10, 13]. Similarly, Gab2's overexpression can regulate cell migration and transform in a subset of ovarian cancers through the activation of PI3K signaling [14, 15]. Furthermore, an interesting study in a model of chronic myelogenous leukemia (CML) has demonstrated that myeloid progenitors from $Gab2^{-/-}$ mice are resistant to transformation by Bcr-Abl, suggesting that Gab2 is required to sustain the leukemogenesis evoked by this oncogenic fusion protein [16]. Gab2 is also important in the progression of other tumorigenesis, such as acute myelocytic leukemia (AML), juvenile myelomonocytic leukemia (JMML), lung cancer, glioma and gastric cancer [17-21]. So far, no investigation of Gab2 protein has been carried out in CRC tissue samples from CRC patients.

In the present study, we performed qRT-PCR and western blotting to investigate the expression of Gab2 in CRC tissues and corresponding normal tissues. We found that the Gab2 expression of CRC tissues was significantly higher than that of corresponding normal tissues, suggesting a possible involvement of Gab2 in CRC development. To further explore the role of Gab2, immunohistochemistry assay was performed. We demonstrated that Gab2 was elevated in CRC tissues compared with the levels in the adjacent normal tissues, and the expression of Gab2 was significantly associated with clinical parameters, including lymph node metastasis, distant metastasis and TNM stage (all $P < 0.05$). These findings shown here have been supported by previous studies in breast cancer, ovarian cancer and metastatic melanomas, which have also indicated that overexpression of Gab2 is vital for the progress and metastasis of malignancies. To investigate whether Gab2 is a significant clinical predictor of CRC, we preformed Kaplan-Meier method as well as Cox's univariate and multivariate hazard regression model. We found that the CRC patients with Gab2-positive were associated with a shorter overall survival; furthermore, both univariate and multivariate Cox proportional hazards regression revealed that the expression of Gab2-positive may be a useful prognostic marker for CRC.

Several kinds of bioactive molecules, such as cytokines, microRNAs and oncogenes, have been shown to participate in both the initiation and progress of cancer [22-25]. In addition, SHP2/Ras and PI3K/Akt pathways are considered as the two major signaling transduction pathways of Gab2 protein [26]. Based on this study, we do not indicate which bioactive mole-

cules combining with which Gab2 mediated signaling transduction pathway are involved in CRC. Thus, it's worthy to further detect the value and underlying mechanisms of Gab2 in CRC.

Conclusively, the results in this study showed that Gab2 is overexpressed in CRC tissues and is associated with the progression of CRC. Moreover, we found that elevated Gab2 expression is an independent prognostic factor for the poor survival of CRC patients. The practical value of Gab2 and its underlying molecular mechanisms in regulation of CRC progression require further investigations.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jihong Feng, Department of Oncology, Affiliated Hospital of Zunyi Medical College, 149 Dalian Road, Huichuan District, Zunyi 53003, China. E-mail: m15527270-734_1@163.com

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