

## Original Article

# Increased expression of Rab5A predicts metastasis and poor prognosis in colorectal cancer patients

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**Abstract:** Rab5A is reported to correlate with cancer development and progression. The purpose of this study is to explore the association between Rab5A expression and the clinical characteristics of colorectal cancer (CRC). Data containing three independent investigations from Oncomine database demonstrated that Rab5A is overexpression in CRC compared with normal tissue, similar result was also found in 32 matched CRC tissue samples by qPCR. The protein expression of Rab5A was examined in 390 CRC specimens and the results showed that high expression of Rab5A was significantly correlated with tumor size ( $P = 0.008$ ), serum CEA ( $P = 0.002$ ), liver metastasis ( $P = 0.014$ ) and clinical stage ( $P = 0.010$ ). Kaplan-Meier method suggested that overexpression of Rab5A protein expression had shorter overall survival times in CRC patients ( $P < 0.001$ ). Multivariate Cox regression analysis confirmed Rab5A expression, tumor size and clinical stage as independent prognostic factor in CRC. In conclusion, the data indicated that higher expression of Rab5A was observed in CRC tissues and Rab5A may be identified as a useful predictor of metastasis and prognosis for CRC.

**Keywords:** Rab5A, colorectal cancer, metastasis, prognosis

## Introduction

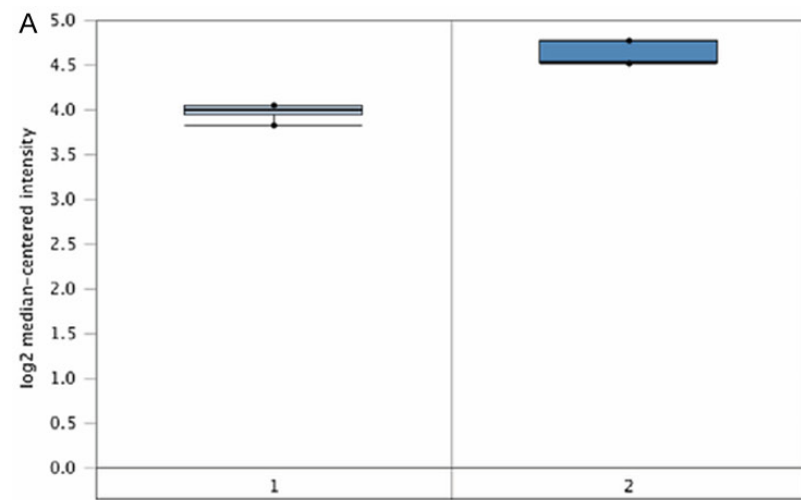
Colorectal cancer (CRC) is the third most common malignant tumor worldwide and second leading cause of cancer-related death in Europe and America [1]. Patients with distantly metastatic colorectal cancer have poor prognoses [2], and peritoneal dissemination is the most common mode of distant metastasis [3, 4]. Although there are many established therapeutic strategies including surgery, chemotherapy and radiotherapy, its prognosis remains unsatisfactory due to late diagnosis [5]. Thus, novel therapeutic strategies are urgently needed for this malignancy.

The Rab family is a type of Ras-like small GTPases which are key regulators of membrane trafficking in both exocytic and endocytic pathways [6, 7]. As soon as Rabs are activated, the vesicles are linked to effectors that are required for vesicle movement, docking, and fusion [8]. Rab5, which is a GTPase pivotal in endocytosis, consists of three isoforms, Rab5A, Rab5B, and Rab5C [9]. Rab5A is expressed in a variety of

secretory cells and is believed to play an important role in regulating secretory pathways by regulating common Rab5 effectors [10]. Recently, it is reported that aberrant expression of Rab5A contributes to cancer progression and increased distant metastasis [11]. Studies have showed that Rab5A can regulate the invasive tumor growth of breast cancer and the overexpression of Rab5A is associated with the metastatic potential of lung and gastric cancer [12-14]. Based on the above studies, Rab5A manifests oncogenic function and plays a significant role in cancer development. However, the expression of the Rab5A protein in CRC and its significance has not been examined in detail yet.

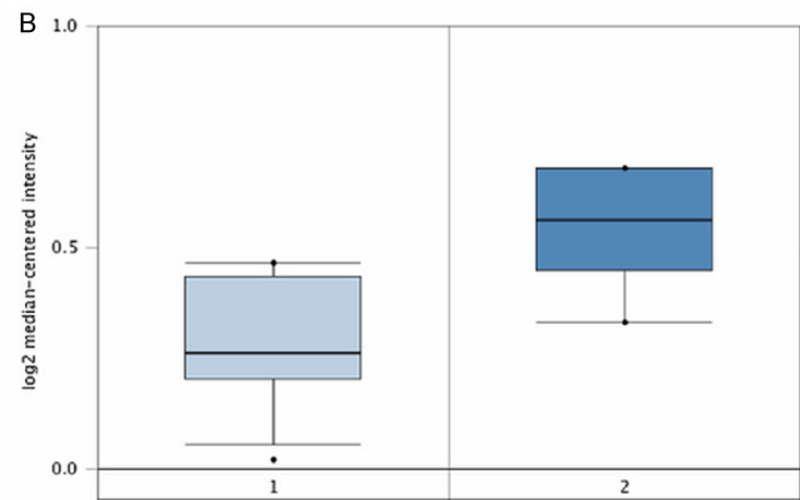
In this study, we evaluated Rab5A mRNA in both CRC tissue and adjacent normal mucosa, and also its relationship with clinicopathological parameters. Our results revealed that increased expression of Rab5A was significantly correlated with poor prognosis in colorectal cancer patients.

# Prognostic value of Rab5A in colorectal cancer



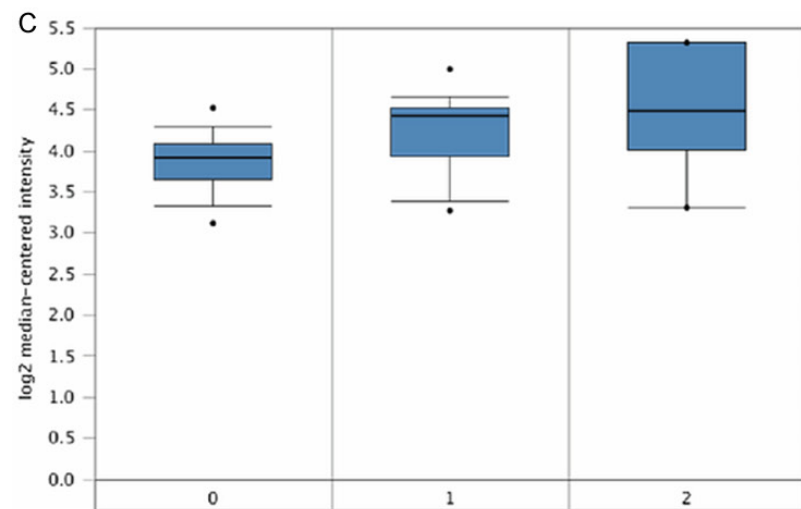
## Legend

- 1. Colon (5)
- 2. Rectal Mucinous Adenocarcinoma (4)



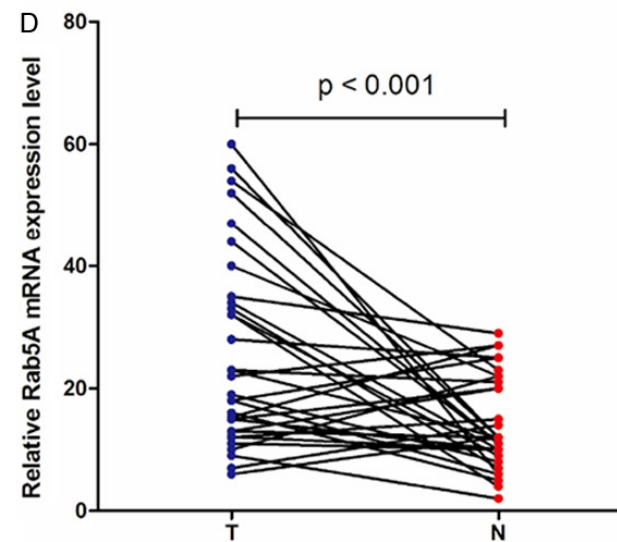
## Legend

- 1. Colon (10)
- 2. Colon Adenoma (5)

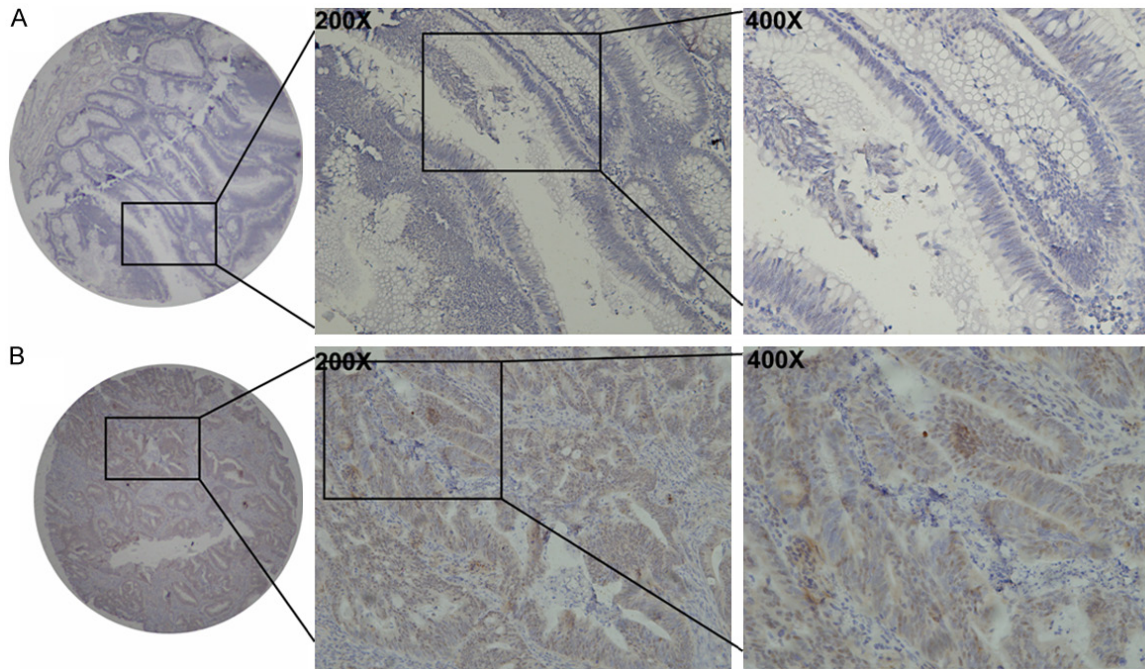


## Legend

- 0. No value (32)
- 1. Colon Adenoma (25)
- 2. Rectal Adenoma (7)



**Figure 1.** Rab5A expression in CRC at mRNA level. Rab5A expression in Kaiser colon (A), Skrzypczak Colorectal 2 (B) and Sabates-Bellver Colon (C). (D) Increased Rab5A mRNA expression in 32 matched tumor (T) and non-tumor tissue (N) was detected by Real-time quantitative PCR. *P*-values were calculated by Paired t-test.



**Figure 2.** Rab5A expression in CRC was determined by immunochemistry. A: Positive expression of Rab5A; B: Negative expression of Rab5A. Representative images are shown at 200 × and 400 × magnification, respectively.

## Materials and methods

### *Patients and tissue samples*

A total of 390 formalin-fixed, paraffin-embedded CRC tissues to perform immunohistochemical staining, which were collected from January 2004 to November 2013 at Renji Hospital, Shanghai Jiao Tong University School of Medicine. Important clinical data, such as tumor location, serum CEA level, TNM stage, were collected from each patient's medical records. Clinical staging was performed according to the latest revision of American Joint Committee on Cancer [15]. The follow-up time was calculated from the date of surgery to the date of death, or the last known follow-up. Before surgical therapy, none of the patients had received neoadjuvant chemotherapy, radiation therapy, or other related anti-tumor therapies. Moreover, an additional 32 paired freshly frozen CRC tissues and corresponding adjacent noncancerous tissues to perform qPCR, which obtained from Renji Hospital, were enrolled in this study simultaneously. All CRC tissue samples in this study were obtained with patients'

written informed consent and all experiments have been approved by the ethics committee at local Hospital.

### *Real-time quantitative PCR*

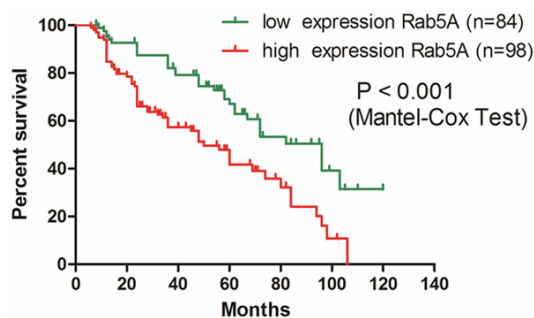
Total RNA from primary tumor and adjacent noncancerous tissue samples was extracted using Trizol reagent (Invitrogen, CA, USA), and according to the manufacturer's instructions, reverse transcription was performed by PrimeScript RT-PCR kit (Takara, Japan). Quantitative real-time PCR was performed using a 7500 Real-time PCR system (Applied Biosystems, Inc. USA). The primers for Rab5A were as follows: forward: 5'-ACGGGCCAAA-TACGGGAAAT-3'; reverse: 5'-TCAAACCTTACCC-CAATGGTACTC-3'. GAPDH mRNA was used to standardize the relative expression of Rab5A. The primers for GAPDH were as follows: forward: 5'-GCATTGCCCTCAACGACCAC-3', reverse: 5'-CCACCACCCTGTTGCTGTAG-3'.

### *Immunohistochemical staining*

Tissue microarray (TMA) was constructed by Suzhou Xinxin Biotechnology limited company

**Table 1.** Relationship between Rab5A expression and clinico-pathological features in 390 colorectal cancer patients

Variable		Rab5A (n)		P
		Low	High	
		N = 171	N = 219	
Age	≤ 65 years	90 (23.08)	117 (30.00)	0.876
	> 65 years	81 (20.77)	102 (26.15)	
Gender	Male	87 (22.31)	125 (32.05)	0.223
	Female	84 (21.54)	94 (24.10)	
Tumor size	≤ 5 cm	99 (25.38)	97 (24.87)	0.008
	> 5 cm	72 (18.46)	122 (31.28)	
Tumor location	Rectum	104 (26.67)	134 (34.36)	0.941
	Colon	67 (17.18)	85 (21.79)	
Serum CEA	≤ 5 ng/ml	107 (27.37)	102 (26.09)	0.002
	> 5 ng/ml	65 (16.62)	117 (29.92)	
Depth of tumor invasion	T1	27 (7.11)	41 (10.79)	0.969
	T2	43 (11.32)	57 (15.00)	
	T3	60 (15.79)	79 (20.79)	
	T4	31 (8.16)	42 (11.05)	
Lymph node metastasis	N0	79 (20.26)	81 (20.77)	0.066
	N1-N2	92 (23.59)	138 (35.38)	
Liver metastasis	M0	146 (37.44)	165 (42.31)	0.014
	M1	25 (6.41)	54 (13.84)	
Clinical stage	I	36 (9.23)	24 (6.15)	0.010
	II	42 (10.77)	49 (12.56)	
	III	68 (17.44)	92 (23.59)	
	IV	25 (6.41)	54 (13.85)	

**Figure 3.** Rab5A is correlated with overall survival rate in CRC patients. Kaplan-Meier survival curves show high expression level of Rab5A was significantly correlated with poor survival of CRC. P-values were calculated by log-rank test.

(xinxin Biotechnology Co, Jiangsu, China). Cores with 2 mm diameter were collected from individual paraffin-embedded CRC sections and arranged in the recipient paraffin blocks. Then, four-micrometer-thick sections placed on Superfrost charged glass microscope slides.

Immunohistochemical staining was performed as previously described [16]. Tissue sections were incubated by Anti-Rab5A antibody (1:200, Abcam, Cambridge, UK) at 4°C overnight. Immunohistochemical staining was scored by two independent pathologists according to intensity and percentage of positive cells simultaneously. Staining intensity was scored as follows: 0: negative; 1: weak staining; 2: moderate staining; 3: strong staining, and the percentage of positive cells was also scored according to 4 categories, in which 1 was given for 0-25%, 2 for 26-50%, 3 for 51-75%, and 4 for 76-100%. And the final score was designated as low or high expression group using the percent of positive cell score x staining intensity score as follows: low expression was defined as a total score < 4 and high expression with a total score ≥ 4.

#### Statistical analysis

Statistical analyses were performed by SPSS 19.0 (SPSS Inc.; Chicago, USA). The expression of Rab5A mRNA in fresh frozen CRC tissues and corresponding noncancerous tissues was analyzed with the paired t-test. The chi-square test was used to analyze the relationship between Rab5A expression and clinicopathological features. Survival rate was evaluated by Kaplan-Meier method and differences between survival curves were tested by the log-rank test. Univariate and multivariate Cox regression analyses were performed to identify clinical factors that had a significant influence on survival. For all tests, the significance level for statistical analysis was set at  $P < 0.05$ .

#### Results

##### *Rab5A is overexpressed in CRC at mRNA level*

To fully investigate Rab5A expression in CRC, we analyzed three independent microarray datasets from Oncomine database [17-19]. The results showed that the mRNA expression lev-



**Table 2.** Univariate and multivariate analysis showing the overall survival in colorectal cancer

Prognostic parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Rab5A (high vs. low)	2.189	1.420-3.373	<b>0.000</b>	1.633	1.019-2.515	<b>0.041</b>
Age (> 65 vs. ≤ 65)	1.493	0.969-2.300	0.069	-	-	-
Gender (male vs. female)	1.256	0.831-1.897	0.279	-	-	-
Tumor size (> 5 cm vs. ≤ 5 cm)	1.713	1.102-2.661	<b>0.015</b>	1.656	1.056-2.597	<b>0.028</b>
Tumor location (colon vs. rectum)	0.800	0.510-1.255	0.332	-	-	-
Serum CEA (> 5 ng/ml vs. ≤ 5 ng/ml)	1.578	1.030-2.418	<b>0.036</b>	1.365	0.887-2.101	0.157
Depth of tumor invasion (T1 vs. T2 vs. T3 vs. T4)	1.219	0.975-1.524	0.817	-	-	-
Lymph node metastasis (present vs. absent)	1.482	0.963-2.280	<b>0.074</b>	-	-	-
Liver metastasis (present vs. absent)	4.554	2.435-8.515	<b>0.000</b>	2.193	0.951-5.057	0.065
Clinical stage (I vs. II vs. III vs. IV)	2.057	1.592-2.657	<b>0.000</b>	1.593	1.154-2.199	<b>0.005</b>

HR: Hazard ratio; CI: Confidence interval. The bold number represents the P-values with significant differences.

els of Rab5A were upregulated in the most of CRC tissue compared with normal tissue (**Figure 1A-C**). In this study, 32 pairs of CRC and corresponding adjacent noncancerous tissues were collected and subjected to real-time quantitative PCR. Consistent with the data from Oncomine database, Rab5A was also overexpression in 68.75% (22/32) of CRC patients at mRNA level (**Figure 1D**).

#### *Rab5A is expressed diversely at protein level in CRC tissues*

To observe the expression of Rab5A in CRC, we tested 390 CRC tissue samples by using the method of immunohistochemical staining and found Rab5A was low expressed in the 171 (43.85%) (**Figure 2A**) of the total 390 CRC samples while the rest 219 (56.15%) samples remained a high expression level (**Figure 2B**).

#### *Relationship between Rab5A expression and corresponding clinical parameters*

To evaluate the clinical significance of Rab5A, the Chi-square test was used to analyze correlations between Rab5A protein expression and clinicopathological parameters in CRC, including age, gender, tumor size, serum CEA level, depth of tumor invasion, lymph node metastasis, liver metastasis, and clinical stage. The results indicated that overexpression of Rab5A in CRC tissues are closely correlated with tumor size ( $P = 0.008$ ), serum CEA level ( $P = 0.002$ ), liver metastasis ( $P = 0.014$ ) and clinical stage ( $P = 0.010$ ). No significant difference was found

in age, gender, tumor location, depth of tumor invasion and lymph node metastasis (**Table 1**).

#### *Correlation between Rab5A expression and prognosis in CRC patients*

To investigate the prognostic influence of Rab5A, the overall survival (OS) rate of CRC patients was analyzed using Kaplan-Meier survival curves and the log-rank test. The result revealed that high expression of Rab5A was a significant prognostic factor for poor overall survival of CRC patients ( $P < 0.001$ ) (**Figure 3**). Furthermore, univariate and multivariate analyses were performed to confirm the possibility of Rab5A used as an independent risk factor for poor prognosis in the 182 cases of CRC. Univariate Cox regression analyses showed that Rab5A expression, tumor size, serum CEA, liver metastasis and clinical stage were significantly associated with OS (**Table 2**). Furthermore, a multivariate Cox regression analysis confirmed Rab5A expression, tumor size and clinical stage as independent predictors of the OS in CRC patients (**Table 2**).

#### **Discussion**

To date, several members of the Rab family have been indicated to act substantially in cancer development. Rab25 has been demonstrated to promote the proliferation and invasion of bladder, gastric and ovarian cancer [20-22]. Rab27A is reported to be overexpressed in pancreatic and breast cancer and function importantly in tumor proliferation and invasion [23, 24]. Similarly, Rab5A, which is involved in the

regulation of vesicular traffic, particularly in endocytosis, has emerged as a major tumor promotor in various tumor types, such as hepatocellular, lung and breast cancer [12, 13, 25]. How does Rab5A perform oncogenically and facilitate tumorigenesis? Zhao Z et al. thought Rab5A may be associated with the APPL1-related epidermal growth factor (EGF) signaling pathway to increase cell cycle and promote ovarian cancer cells proliferation [14]. Similarly, Fukui K et al. demonstrated that Rab5A enhance the epidermal growth factor (EGF) signaling and overexpression of Rab5A predict poor prognosis in hepatocellular cancer patients [26]. While Lu Y et al. explained vacuolin-1 activated Rab5A to block autophagosome-lysosome fusion in cancer cells [27]. Here, we attempted to illustrate the relationship between Rab5A expression and clinicopathological features of colorectal cancer, especially the prognosis significance.

In the current study, we firstly assessed Rab5A expression at mRNA level. Both data from Oncomine database and our results showed that Rab5A expression was higher in CRC tissues than in normal colorectal or adjacent non-tumor tissues. Subsequently, we prepared TMA and performed IHC analysis for further evaluation and the data showed high Rab5A protein expression was closely correlated with an aggressive phenotype of CRC, including serum CEA level, lymph node metastasis, distant metastasis, and TNM stage.

In addition, it is well known that metastases are severely responsible for most cancer-related mortalities. As for CRC patients, the lymph node metastasis and distant metastasis are two main reasons for the extremely low and desperate 5-year survival rate. In the study, we also found that those CRC patients with higher Rab5A expression are prone to have lymph node metastasis and distant metastasis and hence we hypothesize that Rab5A may be identified as a new predictor of metastasis status in CRC.

Furthermore, univariate analyses showed that elevated Rab5A expression was significantly associated with the overall survival duration in CRC patients; multivariate analysis demonstrated that Rab5A expression, tumor size and the clinical stage are independent risk factors for the prognosis of CRC patients. Collectively, these results suggest that Rab5A might serve as a novel prognostic marker for CRC patients.

In conclusion, our study demonstrated that Rab5A overexpression can serve as an independent predictor of poor clinical and decreased survival. Therefore, Rab5A may be an important clinical marker of therapy for CRC.

## Disclosure of conflict of interest

None.

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