European Review for Medical and Pharmacological Sciences 2018; 22: 4509-4517

Aberrant expression of Rab1A and its prognostic significance in human colorectal cancer

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Abstract. – OBJECTIVE: Colorectal cancer (CRC) is one of the most common types of cancer worldwide. Emerging evidence has verified that Rab1A plays an oncogenic role in several human malignancies including breast cancer, lung cancer, and hepatocellular carcinomas. However, the clinical significance and prognostic impact of Rab1A in CRC is still unclear.

PATIENTS AND METHODS: We initiated our investigation by immunohistochemistry and Western blot analysis to confirm Rab1A expression in CRC tissues. Meanwhile, the correlation of Rab1A expression and clinicopathologic features, as well as outcome in CRC patients, were retrospectively analyzed.

RESULTS: In the issue, Rab1A is overexpressed in CRC tissues compared with matched noncancerous tissues. Meanwhile, high Rab1A expression was significantly associated with the TNM stage, lymph node metastasis, and peritoneal metastasis. In addition, multivariate analyses identified Rab1A expression and TNM stage as independent predictors for CRC patients. Moreover, Kaplan-Meier survival analysis showed that patients with high Rab1A expression had a significantly worse survival time than those with low Rab1A expression, which especially affected the survival in CRC patients with advanced stage. Spearman analysis suggested that there was a positive relationship between Rab1A expression and preoperative serum carcinoembryonic antigen (CEA) for CRC patients.

CONCLUSIONS: These results suggested that Rab1A is an important diagnostic marker for CRC, and Rab1A can be used as a valuable biomarker for prognosis as well as peritoneal metastasis in CRC patients. Rab1A may prove to be clinically useful for developing a new therapeutic target of CRC treatment.

Key Words:
Colorectal cancer, Rab1A, Aberrant expression, Immunohistochemistry, Prognosis, Peritoneal metastasis.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related death worldwide, and there are projected to be 135,430 individuals newly diagnosed with CRC and 50,260 deaths from the disease in US[1]. With the improvement in curative resection and adjuvant chemotherapy, especially the emergence of molecular targeted drugs, treatment of CRC has also made substantial advances. However, there has been no significant improvement of survival for CRC with advanced or metastatic stages[2,3]. Therefore, it is urgent to identify novel therapeutic targets for prevention of CRC and establish new biomarkers useful for its early detection of high-risk populations.

Rab1A, a small guanosine triphosphatase (GTPase), is anchored on the membranes of the endoplasmic reticulum (ER) and Golgi to regulate the vesicle trafficking from ER to Golgi apparatus[4]. Previous studies discovered that Rab1A protein plays an important role in mediating signal transduction[5], cell migration[6], and regulation of autophagy[7]. Meanwhile, the aberrant expression of Rab1A also induces many diseases, such as Parkinson's disease[8], aspirin-exacerbated respiratory disease[9], and cardiomyopathy[10]. More importantly, mounting studies[11-14] conducted recently found

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Rab1A acts as an oncogene in several human malignancies. Also, Thomas et al[15] suggested that Rab1A overexpression promotes oncogenic transformation and malignant growth in CRC. Despite the importance of Rab1A in human malignancies, to date, the clinical significance of Rab1A expression in CRC is still unclear. We investigated the Rab1A expressions in CRC tissues by Western blot analysis and immunohistochemical (IHC) staining and assessed the association of Rab1A expression and clinicopathologic features in CRC patients. Furthermore, we explored the prognosis ability of Rab1A in CRC patients. It is hoped that these results can have a positive influence on the treatment and prognosis of colorectal cancer.

Patients and Methods

Patients and Specimens

A total of 100 human CRC tissues and 29 matched noncancerous tissues were obtained from the Department of General Surgery, the Second Affiliated Hospital of Soochow University between January 2010 and January 2014. All colorectal carcinoma cases were confirmed by pathological examination. Patients who received preoperative radiotherapy or chemotherapy were excluded. Basic clinical information was shown in Table II. Overall survival time was measured from the date of primary surgery until death or April 2017, median follow-up time for survivors was 50.1 months (range 39 to 66 months). All experiments involving human subjects were performed in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) and the relevant guidelines and regulations of Soochow University. All experimental protocols were approved by the Research Ethics Committee of the Second Affiliated Hospital of Soochow University.

Immunohistochemistry for Rab1A Expression

Immunohistochemistry (IHC) was performed to determine the protein expression level of Rab1A in CRC tissue and matched noncancerous tissues. Briefly, tissue sections were mounted on glass slides, deparaffinized, and rehydrated. Paraffin-embedded tissues were then immersed in boiling citrate buffer (Gene Tech, Shanghai, China, GT100202) for antigen retrieval. Next, tissue sections were treated with 3% hydrogen peroxide (Yonghua Chemical Technology Co. Ltd, Changshu, China) for 15 min, and nonspecific immunoglobulin binding was blocked by incubation with 5% fetal bovine serum (FBS) (Beyotime Inc, Nantong, China) for 15 min. After that, the primary antibody was added against Rab1A (1:75; Abcam, Cambridge, MA, USA, Ab97956) into each slice and incubated at 37°C for 90 min. Next, the sections were incubated with secondary antibody (Gene Tech, Shanghai, China, GK500705) for 30 min at 37°C followed by staining with fresh 0.05% 3, 3′-diaminobenzidine (DAB) (Gene Tech, Shanghai, China, GK500705). Finally, they were counterstained with hematoxylin, dehydrated, transparent, and fixed. The results of IHC were evaluated by two pathologists who were blinded to any clinical patient data for each case according to the method suggested in our previous work[16]. The staining intensity was divided as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. Then, we randomly choose five high-power fields for each slice and scored the percentage of positively stained cells as follows: 1, < 25%; 2, 25-50%; 3, 51-75%; 4, > 75%. The final score was calculated by multiplying the staining intensity score by the staining percentage score. We classified all of the tissues into the low expression group (- or +) and high expression group (++ or +++) according to the final score of Rab1A (0 = -; 1-4 = +; 5-8 =++; 9-12 = +++).

Protein Extraction and Western Blot Analysis

Whole protein extracts were lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Inc, Nantong, China) according to manufacturer's protocol. 10 μg of the protein was separated from each sample preparation using the sodium dodecyl sulphate-polyacryl amide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Biosharp, Hefei, China). Afterwards, the membranes were blocked and incubated respectively with rabbit anti-human Rab1A antibody (1:2000; Abcam, Cambridge, MA, USA, Ab97956) and mouse anti-human GAPDH antibody (1:1000, Beyotime Inc) at 4°C overnight. Then, the membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Proteintech, Wuhan, China). Following this, the membranes were washed three times with Tris-buffered saline with Tween 20 (TBST). Bands were finally visualized by us-

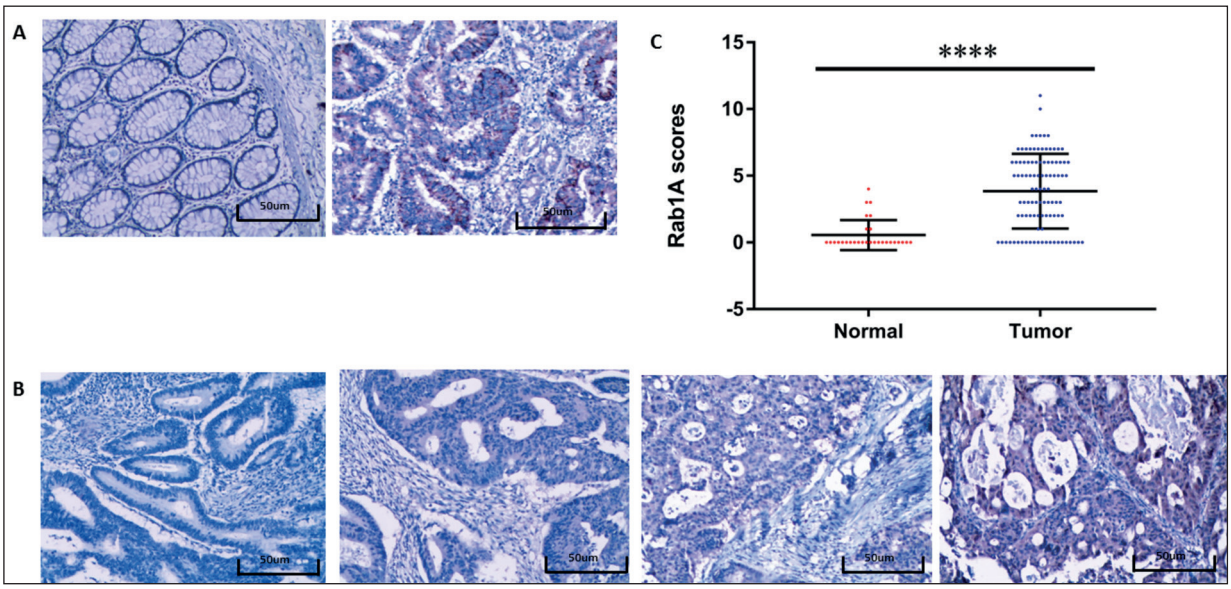

Figure 1. Immunohistochemistry (IHC) of Rab1A expression in CRC tissues and adjacent normal tissues. ***A,*** Representative IHC staining of Rab1A in CRC and adjacent normal tissues, Rab1A was predominantly expressed in the cytoplasm. (magnification, ×400). *Left:* Rab1A expression in normal tissues. *Right:* expression of Rab1A in CRC tissues. ***B,*** Representative photomicrographs showing immunohistochemical staining using an anti-Rab1A antibody. (magnification, ×400). From left to right are negative, mild positive, positive, strong positive. ***C,*** Scatter plot analysis of Rab1A expression in 100 CRC tissues and 29 adjacent normal tissues. ****$p < 0.0001$.

ing an ECL + PlusTM Western blot system kit (Amersham, GE Healthcare, Chicago, IL, USA). The expression levels of the target protein were normalized to those of GADPH.

Statistical Analysis

Statistical analyses were carried out using SPSS 22.0 (IBM Armonk, NY, USA). The relationship between IHC expression and clinic-pathological feature were analyzed using x^2-statistical test. Survival curves were performed using the Kaplan-Meier method, and the significance was established using the log-rank test. Cox proportional hazard model was used to determined univariate analysis and multivariate survival analysis. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of Rab1A is Elevated in Colorectal Cancer Tissues

To investigate the potential role of Rab1A in the development of CRC, immunohistochemical staining was performed to evaluate Rab1A expression profiles in 100 CRC tissues and 29 matched noncancerous tissues (Figure 1). In result, Rab1A showed positive staining in 78% of CRC tissues. In contrast, we only observed positive staining in 24% of matched noncancerous tissues ($p < 0.001$, Table I). Next, Western blot analysis was completed to further verify our outcome in 6 randomly selected pairs of CRC tumors and matched noncancerous tissues. These results revealed that Rab1A was low expressed

Table I. Expression of Rab1A in CRC tissues and adjacent normal tissues.

Tissues	Case	Rab1A		χ^2	*p*-value
		Negative	Positive		
Colorectal cancer tissues	100	22	78	29.019	< 0.001***
Adjacent normal tissues	29	22	7		

Abbreviations: CRC, colorectal cancer. ***$p < 0.001$.

in matched noncancerous tissues, whereas their expression levels were more elevated in CRC tissues ($p = 0.041$, Figure 2), which was consistent with the results of the IHC.

Association of Rab1A Expression and Clinicopathologic Parameters in Colorectal Cancer Patients

To further estimate the association between Rab1A expression and the clinicopathologic features in CRC patients, clinical features of CRC patients were summarized in Table II. Among the 100 patients, high expression of Rab1A was significantly associated with TNM stage ($p = 0.010$) and lymph node metastasis ($p = 0.037$). Meanwhile, the proportion of peritoneal metastasis was significantly elevated in the high Rab1A group than in the low Rab1A group ($p = 0.032$). But no significant differences were observed between Rab1A expression and other clinicopathologic variables such as age, gender, tumor size, differentiation, vascular metastasis, neural invasion, and depth of tumor invasion ($p > 0.05$).

Association Between Rab1A Expression and Preoperative Serum CEA

Carcinoembryonic antigen (CEA) is currently employed as a routine marker for CRC prognosis and disease-free survival. In our paper, preoperative increased serum CEA (> 6.5 ng/ml) was observed in half of CRC patients. Spearman analysis was used to clarify the relationship between Rab1A expression and preoperative serum CEA levels, which indicated that there was a positive association between Rab1A expression and CEA levels in CRC patients ($p = 0.028$) (Table III).

Prognostic Significance of Rab1A High Expression

As shown in Table IV, univariate analysis revealed that size of cancer, depth of tumor invasion, lymph node metastasis, neural invasion, degree of differentiation, Rab1A expression, TNM staging were related with poor prognosis ($p < 0.05$). However, the result of multivariate survival by the Cox proportional hazard model indicated that only Rab1A expression and TNM staging were independent prognostic factors in CRC patients ($p < 0.05$). The result indicated that Rab1A high expression is correlated with poor prognosis of CRC patients. To further evaluate the value of Rab1A in prognosis of patients with CRC, we accomplished the survival curves in CRC patient according to Rab1A expression level, which suggested that survival time of patients with high Rab1A expression were significantly lower than those with mild expression ($p = 0.001$, Figure 4A). More precisely, median survival time for the Rab1A high expression was 38 months compared with 56 months in Rab1A mild expression group. Furthermore, Kaplan-Meier survival curves according to the combination status with Rab1A and CEA indicated that Rab1A+/CEA+ patients have a significantly worsened survival than Rab1A-/CEA-patients ($p < 0.001$, Figure 3). Next, we performed a stage-stratified analysis to confirm the influence of different Rab1A expres-

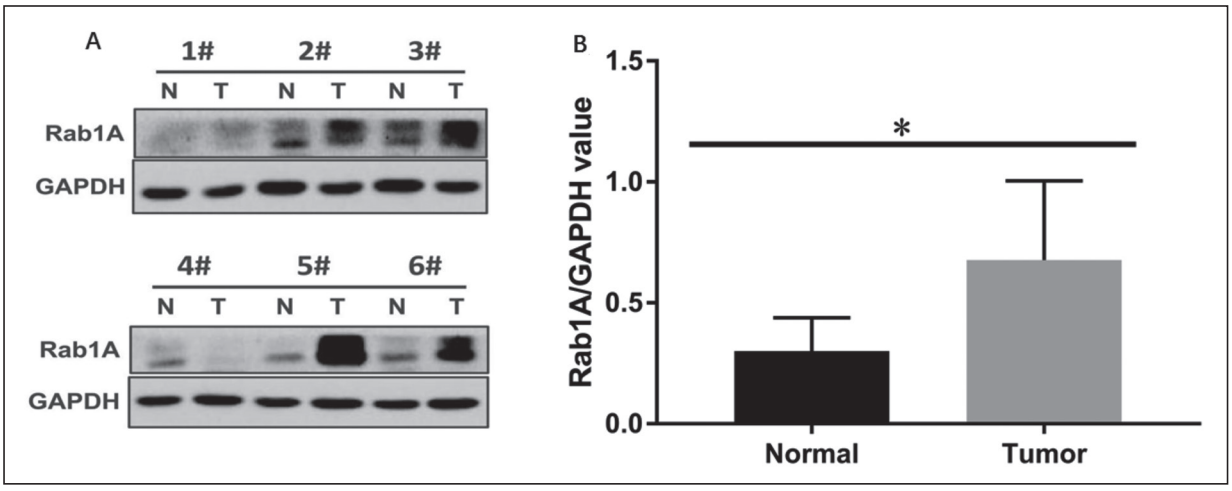

Figure 2. ***A-C,*** Western blot of Rab1A expression in CRC tissues and adjacent normal tissues. ***A,*** Six randomly selected pairs of CRC tissues (T) and adjacent normal tissues (N) are presented. ***B,*** The mean and standard deviation of the two groups, which the difference is statistically significant. *$p < 0.05$.

Table II. The relationship between Rab1A expression and clinic-pathological factors in CRC patients.

Clinic-pathological factors	Rab1A		χ^2	*p*-value
	Low (%)	High (%)		
Age (years)			3.164	0.075
< 65	25 (47.2%)	14 (29.8%)		
≥ 65	28 (52.8%)	33 (70.2%)		
Gender			0.589	0.443
Male	31 (58.5%)	31 (66.0%)		
Female	22 (41.5%)	16 (34.0%)		
Size (cm)			0.877	0.349
< 5 cm	32 (60.4%)	24 (51.1%)		
≥ 5 cm	21(39.6%)	23 (48.9%)		
Lymph node metastasis			6.577	0.037*
N0	33 (62.3%)	20 (42.6%)		
NI	11 (20.8%)	21 (44.7%)		
N2	9 (17.0%)	6 (12.8%)		
Vascular metastasis			0.916	0.339
Negative	30 (56.6%)	31 (66.0%)		
Positive	23 (43.4%)	16 (34.0%)		
Neural invasion			0.672	0.412
Negative	47 (88.7%)	39 (83.0%)		
Positive	6 (11.3%)	8 (17.0%)		
Degree of differentiation			1.451	0.228
Poor	7 (13.2%)	9 (19.1%)		
Moderate	44 (83.0%)	38 (80.9%)		
Well	2 (3.8%)	0 (0.00%)		
Depth of tumor invasion			1.790	0.181
T1-2	15 (28.3%)	8 (17.0%)		
T3-4	38 (71.7%)	39 (83.0%)		
TNM stage			6.670	0.010*
I-II	34 (64.2%)	18 (38.3%)		
III-IV	19 (35.8%)	29 (61.7%)		
Peritoneal metastasis			4.575	0.032*
Negative	48 (90.6%)	35 (74.5%)		
Positive	5 (9.4%)	12 (25.5%)		

Abbreviations: CRC, colorectal cancer; TNM, tumor-lymph node-metastasis. *$p < 0.05$.

sion on other independent prognostic factors (Table V), for patients with stage T3-T4, the survival time was worse in patients with high Rab1A expression than those with low Rab1A expression ($p = 0.042$, Table V). In Figure 4C, the results clearly showed that the expression of Rab1A significantly shortened the survival time of stage T3-T4 patients ($p = 0.036$). However, significant differences were not observed in stage T1-T2 patients ($p = 0.283$, Figure 4B). These results indicated that at the same stage, patients with high Rab1A expression could have a significantly poorer survival than others.

Discussion

Colorectal cancer is one of the most common cancers in the world. The global burden of CRC

Table III. Relationship between Rab1A and preoperative serum CEA.

Preoperative serum CEA	Case	Rab1A		*r*-value	*p*-value
		Negative	Positive		
Negative	50	32	18	0.220	0.028*
Positive	50	21	29		

Abbreviations: CEA, carcinoembryonic antigen. *$p < 0.05$.

Table IV. Cox regression analysis of Rab1A expression and clinicopathologic features.

Clinicopathologic features	Univariate analysis			Multivariate analysis		
	OR	95.0% CI	*p*	OR	95.0% CI	*p*
Age ≥ 65/< 65 years)	1.028	0.553-1.911	0.931			
Gender (F/M)	0.746	0.392-1.418	0.371			
Size of cancer (≥ 5/< 5 cm)	2.019	1.097-3.716	0.024*	1.692	0.899-3.184	0.103
Depth of tumor invasion (T1-2/T3-4)	2.778	1.090-7.081	0.032*	1.869	0.710-4.915	0.205
Lymph node metastasis (N0/N1/N2)	1.992	1.379-2.879	< 0.001***	0.941	0.474-1.869	0.863
Vascular metastasis (negative/positive)	1.327	0.723-2.438	0.362			
Neural invasion (negative/positive)	2.615	1.277-5.354	0.009**	1.367	0.602-3.088	0.458
Degree of differentiation (poor/moderate/well)	0.489	0.242-0.984	0.045*	1.018	0.446-2.324	0.966
Rab1A expression (low/high)	2.997	1.559-5.762	0.001**	2.060	1.010-4.202	0.047*
TNM stage (I-II/III-IV)	5.415	2.634-11.133	<0.001***	4.152	1.354-12.731	0.013*
Peritoneal metastasis (negative/positive)	2.415	1.210-4.823	0.012*	1.535	0.730-3.228	0.258

Abbreviations: CRC, colorectal cancer; CEA, carcinoembryonic antigen. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

is expected to increase by 60% to more than 2.2 million new cases and 1.1 million deaths by 2030[17,18]. Advanced CRC patients still have poor prognosis all over the world, especially in Asian population[19,20]. In the last few decades, many molecular markers have been applied in CRC for diagnosis and prognosis including CEA, carbohydrate antigen 19-9 (CA19-9) and carbohydrate antigen 125 (CA125)[21-23]. Nevertheless, all of them have the restriction of sensitivity and accuracy to predict and provide information to patient prognosis. Therefore, the discovery of new biomarkers is important to predict prognosis and guide postoperative treatment for CRC patients[24]. Rab1A is a member of the Ras oncogene super family[25], which has been well established to adjust vesicular trafficking from endoplasmic reticulum (ER) to Golgi apparatus[4]. A growing body of evidence has reported that Rab1A is closely related to several human malignancies[11-15]. Shimada et al[12] study identified that Rab1A is overexpressed not only in tongue squamous cell carcinomas (TSCCs) but also in premalignant lesions, and elevated expression of Rab1A in lung cancer tissues is associated with tumor size and T stage[13]. Downregulation of Rab1A inhibited cellular growth, cell migration, cell invasion and cell epithelial-mesenchymal transition (EMT) in breast cancer[14], and this study suggested that Rab1A acts as an oncogene in triple-negative breast cancer. Moreover, Rab1A has been found to promote malignant growth and metastasis of hepatocellular carcinoma (HCC) *in vitro* and *in vivo*[11]. Strikingly, overexpression of Rab1A is significantly associated with poor prognosis in HCC. These results suggested that aber-

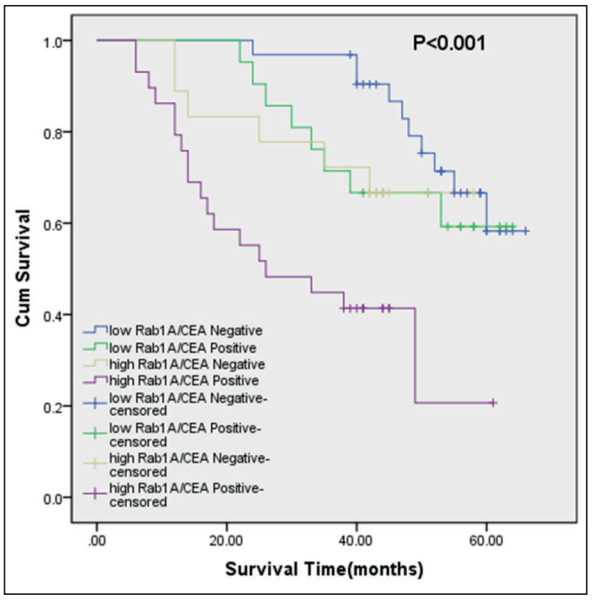

Figure 3. Kaplan-Meier survival curves for CRC patients according to the expression of Rab1A and CEA. The logrank test was used to calculate p-value. ***$p < 0.001$.

Table V. Results of TNM stage-stratified analysis according to Rab1A expression.

Variable	OR	95% CI	*p*-value
TNM stage			
I-II	2.047	0.539-7.776	0.293
III-IV	2.198	1.028-4.698	0.042*

Abbreviations: TNM, tumor-lymph node-metastasis. *$p < 0.05$.

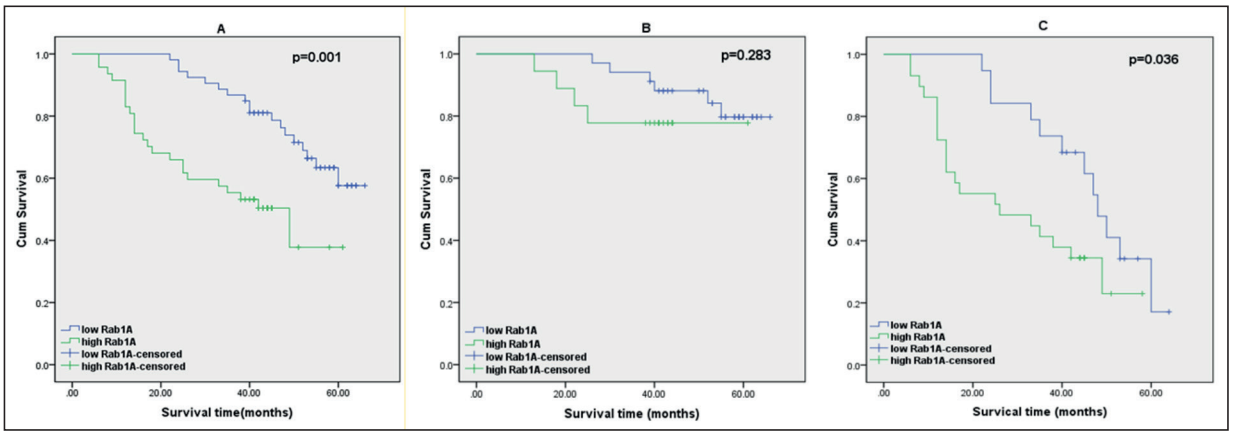

Figure 4. Kaplan-Meier survival curves for CRC patients according to the status of Rab1A expression. The log-rank test was used to calculate *p*-value. ***A,*** The survival for the 53 patients with weak Rab1A expression was significantly better than the 47 patients with stronger expression (**$p < 0.01$). ***B-C,*** Kaplan-Meier survival curves for the 100 patients with different TNM stage of CRC according to Rab1A expression. ***B,*** Survival curves for the 59 patients with stage T1-T2 according to Rab1A expression. No significant survival difference was found between the CRC with mild and high Rab1A expression ($p = 0.283$). ***C,*** Survival curves for the 41 patients with stage T3-T4 according to Rab1A expression. The survival in patients with well Rab1A expression was significantly poorer than low groups ($p = 0.036$). *$p < 0.05$.

rant Rab1A expression is a general phenomenon in human malignancies and these consistent results led us to explore Rab1A expression in CRC and its prognostic significance.

In our work, IHC analysis demonstrated that Rab1A showed positive staining in 78% of CRC tissues, which provided supports to these previous researches. Thomas et al[15] suggested that Rab1A is scored higher in approximately 80% CRC patients. Western blot analysis also confirmed the overexpression of Rab1A in CRC, which in accorded with the results of IHC. Moreover, our study for the first time showed that high expression of Rab1A was significantly correlated with some important clinicopathologic variables such as TNM stage and lymph node metastasis as well as peritoneal metastasis. Peritoneal metastasis has been considered a terminal state for CRC patients and there has been no effective therapy for them[26,27]. A novel biomarker is needed to recognize and forecast peritoneal metastasis to improve the prognosis of these patients. Our study found that peritoneal metastasis was more frequently in high Rab1A group than mild Rab1A group, which suggested that Rab1A might be a possible predictive marker for CRC patients with peritoneal dissemination. Our present data also suggested that Rab1A overexpression might be associated with tumorigenesis and progression of CRC. Besides, Rab1A protein level is a strong independent prognostic factor for CRC patients according to multivariate survival analysis by the Cox proportional hazard model. Meanwhile, for patients with stage III-IV, the effect of high Rab1A expression was more significant in postoperative survival as a result of the stages-stratified analysis. However, we have not found significant connection between Rab1A and survival in patient with stage I-II, this may due to some other more significant factors for prognosis in the early stage, such as lymph node metastasis[28]. In the present investigation, we performed an additional statistical work to analysis relationship between Rab1A expression and preoperative serum CEA, which suggested that there was a positive correlation between Rab1A expression and CEA levels. Moreover, Kaplan-Meier survival curves according to the joint observation of Rab1A and CEA indicated that combination monitoring is a meaningful indicator of the prognosis for CRC patients. This research implied that the Rab1A might play an important role in diagnose and prognosis of CRC just like serum CEA. According to these findings, Rab1A should be combined with the TNM stage to predict the prognosis of CRC. These results suggested that further studies should focus on exploring the mechanism and function of Rab1A in advanced CRC.

To date, the mechanism of Rab1A in human malignant tumors is still unclear. However, many studies[11,13-15] recently found Rab1A is involved in the regulation of mammalian target of rapamycin complex 1 (mTORC1) signaling. Xu et al[11] found that overexpression of Rab1A causes a significant increase in mTORC1 signaling and Rab1A knock-

down attenuates mTORC1 signaling in HCC, which suggested that activation of mTORC1 signaling is critically important for Rab1A to promote growth and metastasis in HCC. Similarly, Rab1A siRNA transfected breast cancer cell has been found to inhibit the phosphorylation of p-P70S6K, the effector molecular of mTORC1, which revealed a close relationship between Rab1A and mTORC1 pathway in breast cancer cells[14]. Conversely, Rab1A protein levels were not correlated with mTORC1 and Rab1A knockdown had no effect on mTORC1 pathway in human lung cancer[13].

We acknowledge limitations of our present paper. First, the mRNA expression of Rab1A in CRC was not further performed by PCR, and we only completed Western blot in six randomly selected pairs of CRC tissues and adjacent normal tissues. Second, it is necessary to downregulate Rab1A in CRC cells to observe its effect on cancer cells. Moreover, the molecular mechanism of Rab1A in CRC is still not clearly explained. Therefore, more studies are required to explore the molecular mechanisms regulating Rab1A and direct downstream effect targets.

Conclusions

We demonstrated that the expression of Rab1A was significantly elevated in CRC tissues compared with the matched adjacent normal tissues. We also showed that Rab1A is an independent prognostic factor for CRC patients and increased Rab1A expression is associated with shorter survival time. These findings might give us a new insight into the pathogenesis of CRC and provide us a novel therapeutic strategy in the treatment of CRC.

Acknowledgements

The study was supported by Project of Nature Science Foundation of China (81672348), the National Science Foundation of Jiangsu Province, China (BK2016255), the Special Clinical Research Fund from Wu Jie Ping Medical Foundation (320.6750.17276), the Six Major Talent Peak Project of Jiangsu Province, China (2015-WSW-014), the Six One Project for Advanced Medical Talent of Jiangsu Province, China (LGY2016031), and Jiangsu Provincial Medical Youth Talent (QNRC2016735).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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