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Original Article

The role of Robo1 expression in patients with colorectum cancer

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Abstract: To investigate the expression characteristics of Robo1 protein in patients with colorectum cancer. Immuohistochemistry was performed to determine the expression of Robo1 in 20 patients with colon adenoma and 42 patients with colorectum cancer, and their matched non-tumorous colonic tissues. The correlation of the expression of Robo1 and the pathologic features, as well as the prognosis in colorectum cancer was then statistically analyzed. In colon adenoma, colorectum cancer and the matched non-tumorous mucosa group, we found that the percentage of Robo1 positive expression was 45%, 59.5% and 29%, respectively, suggesting that the expression level of Robo1 was significantly different between colorectum cancer and their matched non-tumorous colonic tissues (P=0.022). In addition, our results also demonstrated that the expression level of Robo1 was associated with tumor invasion (P=0.021), lymph node metastasis (P=0.032) and distant metastasis (P=0.037). While, the expression of Robo1 has no significant correlation with age, sex, tumor size, tumor location, and tumor grade. Univariate factor analysis found that patients with higher Robo1 expression have lower survival rate. However, cox multivariate model showed that Robo1 expression was not an independent prognostic marker for patients with colorectum cancer. Robo1 might play an important role in the progression of colorectum cancer, and it may be a potential therapeutic target for colorectum cancer.

Keywords: Colorectum cancer, Robo1, prognosis

Introduction

Colorectum cancer is the second most commonly diagnosed cancer in female and the third in male worldwide [1]. In China, colorectum cancer is one of the most common causes of death for the patients with malignant. In recent years, several studies indicated that the development of colorectum cancer is a progressive process and involve multiple stages.

Roundabout (Robo) was first identified as a transmembrane receptor for the chemorepulsive ligand Slit in Drosophila. The Robo family is comprised of four members including Robo1, Robo2, Robo3 and Robo4 [2-4]. Robo1, 2, and 3 receptors share a common domain which contains five repeats of Ig functional areas, three repeats of fibronectin III (FN3), a transmembrane segment and an intracellular tail, whereas the molecular weight of Robo4 is much smaller than other three Robo receptors. Robo4 only contains two Ig and two FN3 extracellular elements [5]. Further, their expression patterning is also different, which Robol and Robo2 are expressed in mostly tissues and organs of mature individuals, Robo3 is expressed in the developing central nervous system, and Robo4 is expressed in the vascular endothelium, suggesting they might play different roles in various organs [6-8].

Many studies indicated that Robo1 expression was associated with the development of various tumors, including liver cancer, lung cancer, prostate cancer, cervical cancer, breast cancer, and renal cancer [6, 9-11]. However, the function of Robo1 in the development of colorectum cancer is still unclear. Zhang *et al.* found that the expression level of Robo1 was significantly correlated with lymph node metastasis and TNM stage in colon cancer [12]. Moreover, a different study from Zhou's group indicated that the Robo1 antigen inversely correlated with

overall survival in patients with colorectum cancer [13]. However, it is still unknown whether the expression of Robo1 was related with survival and whether Robo1 could be used as an independent factor to evaluate the prognosis of colorectum cancer. Furthermore, most of colorectum cancer commonly develops from colon adenoma [14-17], the functions of Robo1 on the development of adenoma-carcinoma transformation of colorectum cancer has not been described previously.

Here, we investigated the characteristics of the expression level of Robo1 in colorectum cancer tissues, their matched non-tumorous colonic tissues and colon adenoma. We then clarified the relationship between the expression level of Robo1 and the pathologic features in these three tissues. Our findings demonstrated that Robo1 could not be used as an independent factor to evaluate the prognosis of colorectum cancer.

Material and methods

Subjects

All of the tissues from the patients with colorectum cancer were divided in three groups: (1) 20 patients with colon adenoma (10 men and 10 women, aged from 47 to 78 years old, mean age 52 ± 11 years); (2) 42 patients with colorectum cancer (27 men and 15 women, aged from 47 to 86 years old, mean age of 63 ± 10 years) who undergone surgical resection at Tongji Hospital of Shanghai between 2008 and 2010 were enrolled in this study. No patients with colorectum cancer underwent radiation or chemotherapy before surgery; (3) Tissues from surgical resection including colonic tumor tissues and their matched non-tumorous colonic tissues were obtained. Pathological classification of the tumor stage was based on the guidelines of the Union for International Cancer Control (UICC). The study protocol was approved by the Ethics Committee of the Tongji Hospital of Shanghai.

Immunohistochmistry (IHC) staining

Colon adenoma, colorectum cancer, and their matched non-tumorous colonic tissues were fixed with formalin, processed and embedded in paraffin wax, and then cut to thickness of 5 mm by microtome. All of the samples were stained with hematoxylin & eosin (H&E). For immunohistochemistry, the sections were dewaxed for 10 min, then washed in Phosphoric acid buffer solution (PBS) buffer (pH 7.4) twice, for 3 min each. The sections were heated at 98°C in an Ethylene Diamine Tetraacetic Acid buffer (pH 9.0) for 15 min and then cooled naturally to room temperature. Then the sections were washed in PBS buffer (pH 7.4) twice, for 3 min each, and then incubated with 3% H_2O_2 for 15 min to block endogenous peroxidase activity. The primary anti-Robo1 antibody (1:50; Abcam, England) were incubated at 4°C overnight. After the sections were rinsed, the horseradish peroxidase-labeled secondary antibody was incubated at room temperature for 30 min. Antibody complex-bound sections were stained for 5 min with diaminobenzidine and then counterstained with hematoxylin for 30 sec, and then dehydrated, cleared and mounted.

The intensity of the IHC was evaluated blindly by two pathologists. The staining intensity of Robo1 was graded from 0 (no labeling) to 3 (strongest intensity) as follows: 0, negative; 1, weak yellow staining, slightly higher than the background; 2, moderate yellow staining, significantly higher than the background; and 3, strong brown staining. The extent was also classified to four grades according to the percentage of positive cells as follows: 0, 0-5%; 1, 6-19%; 2, 20-49%; 3, >50%. Then the staining intensity and the percentage of positive cells were summed to generate the IHC score (IS). We categorized the IS 0-3 as negative, >3 as positive.

Statistical analysis

Statistical analyses were performed by using the SPSS statistical software (SPSS 17.0), and values of $P<0.05$ were considered to be statistically significant. Quantitative values were expressed as median or range. The difference between the pathological features and Robo1 expression were assessed by using Wilcoxon signed-rank test and Mann-Whitney U test. The overall survival time was calculated from the date of surgery to the last follow-up or the date of death from colon carcinoma. Kaplan-Meier method was used to construct survival curves. In univariate survival analysis, differences in survival with respect to various medical records and survival data were performed with the log-rank test. The Cox proportional hazards regres-

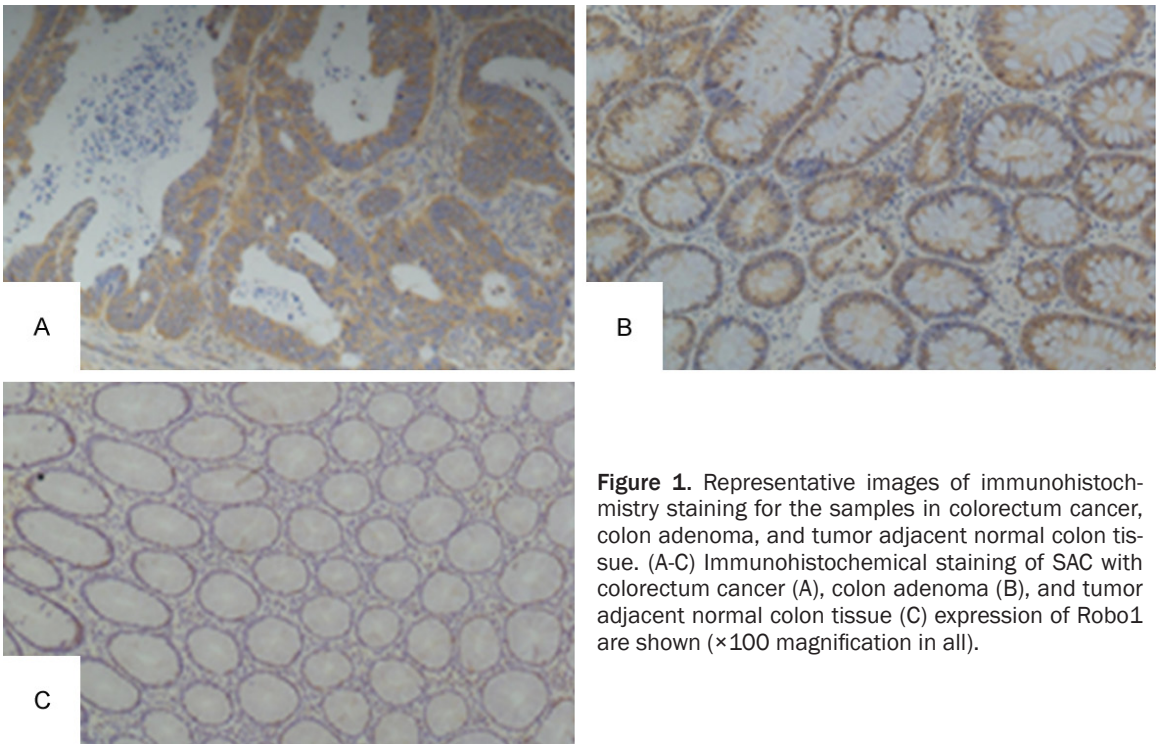

Figure 1. Representative images of immunohistochemistry staining for the samples in colorectum cancer, colon adenoma, and tumor adjacent normal colon tissue. (A-C) Immunohistochemical staining of SAC with colorectum cancer (A), colon adenoma (B), and tumor adjacent normal colon tissue (C) expression of Robo1 are shown (×100 magnification in all).

Table 1. Expression of Robo1 in colon adenoma, colorectum cancer and their matched non-tumorous colonic tissues

		Robo1 expression		
	Cases	Negative	Positive	*P* value
Colon adenoma	20	11 (55%)	9 (45%)	<0.05*
Colorectum cancer	42	17 (40.5%)	25 (59.5%)	>0.05**
Tumor adjacent normal colon tissues	42	30 (71%)	12 (29%)	<0.05***

*The matched non-tumorous colonic tissues and colon adenoma; **colorectum cancer and colon adenoma; ***the matched non-tumorous colonic tissues and colorectum cancer.

sion model was used to multivariate survival analysis to evaluate factors were independently associated with survival time after surgery.

Result

Expression of Robo1 in colon adenoma, colorectum cancer, and their matched non-tumorous colonic tissues

To investigate whether the expression level of Robo1 is correlated with the progression of colorectum cancer, we determined Robo1 expression in colon adenoma, colorectum cancer, and their matched non-tumorous colonic tissues by IHC staining (**Figure 1**). The expression levels of Robo1 were increased gradually in the matched non-tumorous colonic tissues, colon adenoma, and colorectum cancer samples. Our results demonstrated that in colon cancer tissues (colon adenoma and colorectum cancer), the expression levels of Robo1 were much higher than their matched that in non-tumorous colonic tissues (**Table 1**). In addition, the percentages of positive expression of Robo1 in the samples of colorectum cancer, colon adenoma, and their matched non-tumorous colonic tissues, were 59.5%, 45% and 29%, respectively. There was significant difference between colorectum cancer and their matched non-tumorous colonic tissues (P=0.022), whereas there was no significant difference between colorectum cancer and colon adenoma.

Depending on the area of positive immunoreactivity, a final overall score (no or low tumor Robo1 or high tumor Robo1 expression) was established as described. In the total 42 tumor samples from patients with colorectum cancer, 17 samples displayed no or low Robo1 expression, whereas other 25 samples had high Robo1 expression.

Table 2. Association between Robo1 expression level and clinical pathological features in the patients with colorectum cancer (n=42)

Variable	n	Robo1 expression		*P* value
		Low	High	
Age (years)				0.849
<55	8	3	5	
≥55	34	14	20	
Genders				0.324
Male	26	9	17	
Female	16	8	8	
Tumor diameter (cm)				0.569
<5	22	8	14	
≥5	20	9	11	
Tumor location				0.346
Distal/rectum	21	10	11	
Proximal	21	7	14	
Tumor grade				0.474
G1	17	8	9	
G2+G3	25	9	16	
Primary tumor classification (pT)				0.021
T1+T2	6	5	1	
T3+T4	36	12	24	
Lymph node metastasis (pN)				0.032
N0	20	12	8	
N1+N2	22	6	16	
Distant metastases (pM)				0.037
M0	24	13	11	
M1	18	4	14	
Dukes' stage				0.046
A+B	17	10	7	
C+D	25	7	18	

Correlation between Robo1 expression and the colinicopathological characteristics of colorectum cancer

To clarify the correlation between Robo1 expression and the clinical pathologic features of colorectum cancer, further evaluation were performed to analysis the relationship between the expression level of Robo1 and age, sex, tumor size, tumor location, tumor grade, lymph node. As showed in **Table 2**, the expression level of Robo1 in colorectum cancer tissues were significantly correlated with depth of invasion (P=0.021), lymph node metastasis (P=0.032) and distant metastasis (P=0.037). However, there was no correlation between Robo1 and age, sex, tumor location, and tumor grade (P>0.05), suggesting that Robo1 might be a potential biomarker for evaluating the clinical pathologic feature of colorectum cancer.

The expression level of Robo1 is associated with the survival time of the patients with colorectum cancer

To examine the relationship between the expression level of Robo1 and the survival time of patients with colorectum cancer, we then analyzed the survival times of the determined tumor tissues from the patients with colorectum cancer. As shown in **Figure 2**, our results demonstrated that Robo1-negative tumor from the patients with colorectum cancer exhibit much longer survival times (5-year survival rate; P=0.039). Furthermore, Multivariate analysis using the Cox regression model showed that Robo1 immunolabeling could not be considered as an independent prognostic factor (P=0.873; **Table 3**). All these results suggested that the lower expression level of Robo1 is essential for the survival times of the patients with colorectum cancer, indicating that Robo1 might be a potential target for the clinical therapy for the patients with colorectum cancer.

Discussion

The family of Robo was originally identified in the nervous system. It was found to play important roles for the development of axonal guidance in neurogenesis, promoting axon branching, and controlling neuronal migration. Recent studies indicated that Robo proteins, especially Robo1, were associated with the progression of tumorigenesis. The functions of Robo1 were involved in several processes of tumor progression, including the promotion and suppression of tumor cell survival, proliferation and migration. Besides, Robo1 also acts as both oncogene and cancer suppressor [4], and its expression levels are different in various types of cancer. In hepatic cell carcinoma, the expression of Robo1 is high-expressed. And over 80% of patients with hepatic cell carcinoma display Robo1-positive, suggesting that Robo1 might be a potential sensitive marker to diagnose hepatic cell carcinoma [18]. Further, the high-level expression of Robo1 was also

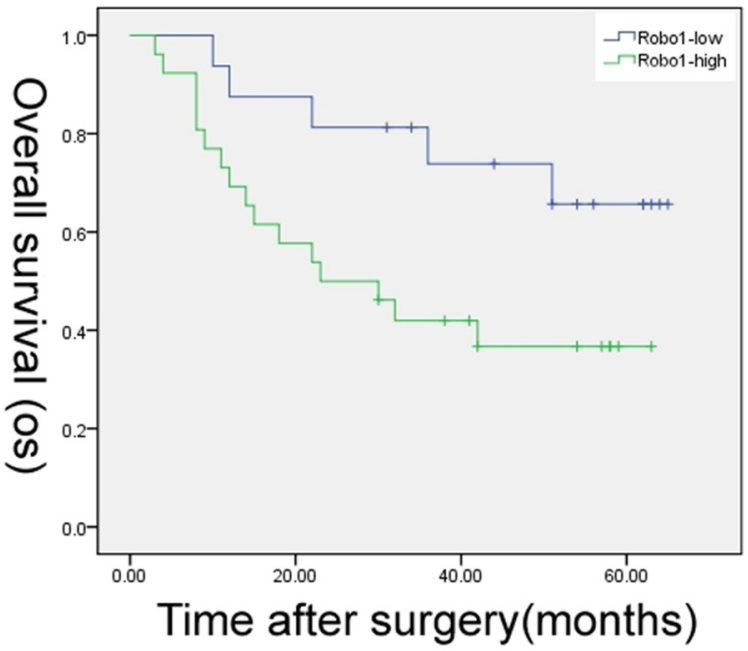

Figure 2. Expression level of Robo1 is correlated with the survival times of the patients with colorectum cancer.

Table 3. Univariate and multivariate Cox-regression analysis showing Robo1 expression on the cancer-specific death of the patients with colorectal cancers (n=42)

Variable	*P*-value, Univariate Cox-regression	*P*-value, Multivariate Cox-regression
Robo1	0.039	0.873

P-value: low expression VS high expression.

found in glioma, head and neck cancer, and small-cell lung cancer [19-21].

Interestingly, the conflictive result from Parray et al. showed that Robo1 expression was progressively undetectable during progression of prostate cancer [22], suggesting that Robo1 could be a tumor suppressor in human prostate cancer. Moreover, several studies demonstrated that the expression of Robo1 was down-regulated in several cancers, including non-small cell lung cancer, breast cancer, cervical cancer, renal carcinoma and acute lymphocytic leukemia [9, 11, 23-25]. During the progression of tumorigenesis, the down-regulation of Robo1 expression can result in the escapement of tumor cells from the safeguarding mechanisms [4]. Therefore, we expected that these conflictive functions of Robo1 might be due to its different roles for different processes in various cancers.

Although advances in surgical techniques, radiotherapy and chemotherapy have improved overall life quality and morbidity in patients with colon cancer, metastasis is the main cause of treatment failure and death. Our results demonstrated that the expression level of Robo1 was associated with depth of invasion, lymph node metastasis and distant metastasis (**Table 2**), suggesting that Robo1 might be a potential biomarker to evaluate the possibility of the metastatic tumor for the patients with colorectum cancer.

The studies for the functions of Robo1 on the development of adenoma-carcinoma transformation of colorectum cancer are still very limited. Comparing with the normal mucosal cells, the higher expression level of Robo1 was determined in colorectum cancer [5]. There are many molecular events for the progression of the transition from normal epithelium to adenoma, and malignant transformation in the human colon [26]. Our results indicated that the expression level of Robo1 increased gradually in the normal colorectal tissues, colon adenoma tissues and colorectum cancer tissues. The expression level of Robo1 was significant higher in the colorectum cancer tissues and adenoma tissues than that in the normal tissues. We found that Robo1 was associated with Dukes stage, the expression level of Robo1 in the colorectum cancer of Dukes C and D stage were higher than that in Dukes A and B (**Table 1**), suggesting that Robo1 may promote the occurrence and development processes of colorectum cancer. All these results suggested that the increased expression of Robo1 is likely affecting colon physiology. The higher expression level of Robo1 in colon tissue, the higher malignant transformation was occurred in colorectum cancer. Therefore, we expected that the expression level of Robo1 was involved in colon tumorigenesis, and the high-expression of Robo1 probably promote the progression of tumorigenesis in colorectum cancer.

Previous studies indicated that although the patients with colorectum cancer are at the

same clinical stage and pathological type, their response to treatment and prognosis are very different, suggesting that many prognostic factors such as age, lymph node metastasis, pathologic grade, stage, were associated with the survival time for the patients with colorectum cancer. Therefore, it is important to find a reliable biomarker which can classify and predict the prognosis for the patients with colorectum cancer [27, 28]. Zhou et al. demonstrated that Robo1 expression shortened survival in patients with colorectum cancer by univariate factor analysis [13]. But whether Robo1 could be used as an independent factor to evaluate the prognosis of colorectum cancer is still unknown. Cox multivariate analysis was performed to investigate whether Robo1 could be used as a clinical biomarker to evaluate the prognosis of colorectum cancer independently. **Table 3** showed that Robo1 could not be considered as an independent prognostic factor (P=0.873). We expected that there may be two reasons to account for this result. The numbers of tissues from the patients with colorectum cancer were not enough for the evaluation of the functions of Robo1 in colorectum cancer. Besides, previous study demonstrated the upregulation of Robo4 in colorectum cancer tissue [5]. Slit2 is considered as the corresponding candidate ligand for the Robo1 and Robo4 receptors. And Robo4 may participate in the functions of Robo1 through Slit-Robo signaling pathway which plays an important role in the colorectum cancer. Therefore, to confirm whether Robo1 is an independent factor to evaluate the prognosis of colorectum cancer, further studies with more samples are needed in the future.

Disclosure of conflict of interest

None.

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