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Expression of SLP-2 gene and CCBE1 are associated with prognosis of rectal cancer

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Abstract. – OBJECTIVE: This study aims to investigate the clinical significance of SLP-2 gene for patients with rectal cancer. To analyze the effect of CCBE1 (Collagen and calcium-binding EGF domain-containing protein 1) on rectal cancer tissue and lymph vessels of para-carcinoma tissue.

MATERIALS AND METHODS: A total of 50 samples of rectal cancer tissues were enrolled in the experimental group, confirmed by pathological examination. 50 samples of para-carcinoma normal tissues were collected as control group. Protein expression of SLP-2 and CCBE1 was examined with immunohistochemical staining. mRNA expression of SLP-2 was examined with RT-PCR. Lymphatic vessel density (LVD) was evaluated with LYVE-1 immunohistochemical staining. Correlation analysis was performed to assess the relationship between patient survival data and clinical pathological features of rectal cancer.

RESULTS: Immunohistochemical staining showed that, compared with the control group, a positive expression rate of SLP-2 in the experimental group was significantly higher (68.0% *vs.* 24.0%, $p<0.05$), and mRNA of SLP-2 was also significantly increased ($p<0.05$). Compared with the control group, protein expression of CCBE1 in the experimental group was significantly higher ($p<0.05$). Moreover, the expression level of SLP-2 was remarkably associated with TNM classification and lymphatic metastasis. Further analysis demonstrated that a positive expression of CCBE1 was associated with lymphatic metastasis, LVD and Ducks classification, and had a negative correlation with survival rate.

CONCLUSIONS: Increased expression of SLP-2 promoted the formation of lymph vessels and exacerbated lymphatic metastasis of rectal cancer via up-regulating CCBE1. As a risk factor related to lymphatic metastasis, CCBE1 could be a novel biomarker for diagnosis and prognosis of rectal cancer.

Key Words
Rectal cancer, Lymphatic vessels, SLP-2, Ccbe1, Targeted therapy.

Introduction

Rectal cancer is one of the most common malignant tumors in clinical scenario, of which colon cancer accounts for the very great proportion. Recent reports showed that morbidity and mortality of rectal cancer has gradually increased in China. Lymphatic metastasis is the main manner for the spread of rectal cancer, which significantly influence prognosis of patients[1]. Thus, studies on lymphatic formation in the lesion of rectal cancer were of great significance for clinical treatment. Previous showed multiple factors were involved in formation and maturation of lymphatic vessels, such as collagen[2], caldesmon[3] and EGF-like domain[4,5]. Recent researches[4] indicated that CCBE1 not only regulated extracellular matrix, but also played an important role in development of lymphatic vessels, verified in human trials and zebra fish models. Moreover, up-regulation of CCBE1 promoted metastasis and increased survival of cancer cells. However, no specific regulator mechanism was elucidated for effect of CCBE1 on rectal cancer. As a member of stomatin gene family, SLP-2 was discovered as a novel gene related to growth of tumor[5]. Over-expression of SLP-2 was observed in multiple cancers, including lung cancer[4], breast cancer[5] and osteocarcinoma[6], suggesting SLP-2 was a target for cancer treatment[7,8]. Accordingly, we suggested that SLP-2 might be associated with expression of CCBE1, and factors of lymphatic vessels were influence by SLP-2 or CCBE1 in rectal cancer.

Materials and Methods

Clinical Material

A total of 50 samples of rectal cancer were enrolled in Dezhou People's Hospital from September 2014 to September 2015, and all tissues were har-

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Table I. Primers and PCR amplified condition.

Gene	Sense primer (5′-3′)	Anti-sense primer (5′-3′)	Product length	Amplified condition
SLP-2	CTGGAGCCTGGTTTGAACAT	GGATCTGGGCCTGTTTCTT	550 bp	94°C for 5 min, 94°C for 30 s, 55°C for 30 s, 72°C for 50 s, 30 cycles. 72°C for 7 min.
β-actin	TGAAGGTCGGTGTCAACGGA	GATGGCATGGACTGTGGTCA	262 bp	As above
CCBE1	CTTGGGAAGGCAAGACTCAC	AGCCCCTGAT-GAAGGAGTTT	500 bp	As above

vested form surgical excision. A total of 50 samples of para-carcinoma normal tissues (distance > 6 cm to edge of lesion) were collected as control group. The sex ratio was 28:22 (men: women), average age was 60 years (40 to 71). All samples were confirmed as rectal cancer by pathological examination. No patient received radiation therapy or chemotherapy. Ducks classification was as follows: 6 samples with A classification, 8 samples with B classification, 24 samples with C classification, 12 samples with D classification. 39 samples were with a diameter of tumor > 5 cm. According to WHO criteria of pathological type, 28 samples were confirmed as high differentiation cancer, 16 samples for moderate differentiation cancer and 5 samples for low differentiation cancer. Clinical staging was performed for samples according to TNM (AJCC 2010) staging criteria. There are 20 samples of rectal cancer with lymphatic metastasis. This study has been pre-approved by the Ethical Committee of Dezhou People's Hospital. All subjects have signed the consent forms before recruitment in this study.

Reagents

Mouse-anti-human SLP-2 monoclonal antibody was purchased from Biotechnology Co. Ltd. (Beijing, China). CCBE1 antibody and LYVE-1 antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kit for immunohistochemical experiment was purchased from Nanjing Haeckel Biotechnology Co. Ltd. (Nanjing, China). Kit for DAB staining was purchased from Beijing Nobleryder Biotechnology Co. Ltd. (Beijing, China). Trizol reagents, Superscript II reverse transcriptase and dNTPs were purchased from TransGen Biotech Co. Ltd. (Beijing, China). Taq DNA polymerase was purchased from Dongsheng Biotechnology Co. Ltd. (Beijing, China). DL2000 DNA markers were purchased from Beijing Bio-Lab Biotechnology Co. Ltd. (Beijing, China). Primers of CCBE1 and β-actin were purchased from Beijing Midwest Co. Ltd. (Beijing, China).

Immunohistochemical staining

Confirmed positive staining was used as positive control, and phosphate buffer solution (PBS) was added to replace primary antibodies in negative control. Protocols were described in previous reports[9,10]. Percentage of cells with positive staining was analyzed. LVD quantitative analysis was performed and repeated 3 times for average evaluation.

Real-time fluorescent quantitative PCR

Protocols of RNA extraction and RT-PCR were described previously[11,12]. All primers and PCR amplified condition were showed in Table I.

Statistical Analysis

SPSS16.0 software (SPSS Inc. Chicago, IL, USA) was used for data processing. Measurement data are normal distribution to $X \pm S$. Kaplan-Meier test was performed for survival analysis. p-value < 0.05 was considered to be statistically significant.

Results

Transcriptional level of SLP-2 was increased in rectal cancer

Compared with control group, experimental group had a significantly higher level of SLP-2 mRNA (1.45 ± 0.25 *vs.* 0.58 ± 0.13, p=0.492, Figure 1), suggesting transcriptional level of SLP-2 increased in rectal cancer.

Protein expression of SLP-2 increased in rectal cancer

Compared with control group, experimental group had a significantly higher protein ex-

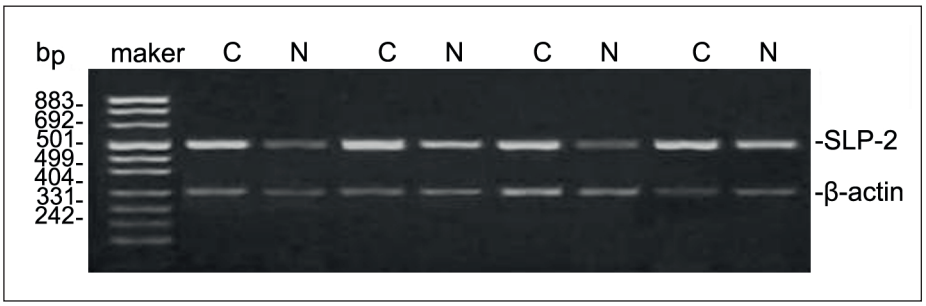

Figure 1. Level of SLP-2 mRNA. C for rectal cancer. N for normal tissues.

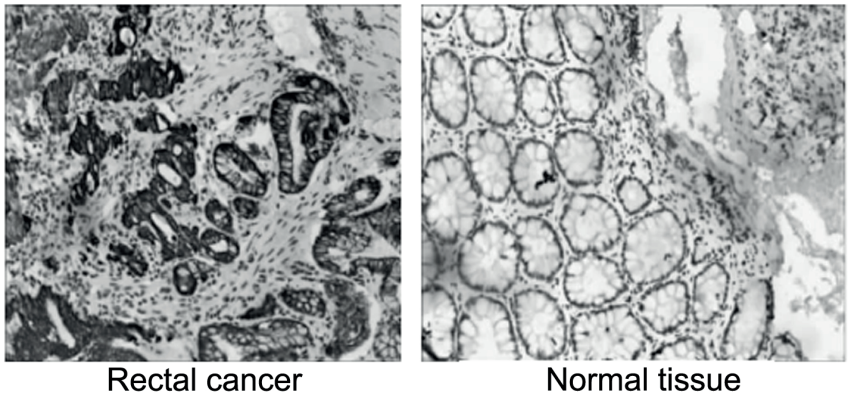

Figure 2. Protein expression of SLP-2. Immunohistochemical staining under 100×, magnification.

pression of SLP-2 (68.0% *vs.* 24.0%, χ^2=10.102, p<0.05, Figure 2), verified by immunohistochemical staining.

Protein expression of CCBE1increased in rectal cancer

Positive expression of CCBE1 presented as brown granules, distributed in cytoplasm and cytomembrane of tumor cells. A negative expression of CCBE1 was defined as no staining on both cytoplasm and cytomembrane of tumor cells. Compared with control group, experimental group had a significantly higher protein expression of CCBE1 (70.3% *vs.* 22.8%, χ^2= 9.021, p<0.05).

SLP-2 and CCBE1 were associated with clinical pathological feature

Analysis data showed that SLP-2 and CCBE1 were associated with multiple clinical factors, including Ducks classification and lymphatic metastasis (Table II).

Table II. SLP-2 and CCBE1 are associated with clinical pathological features.

Clinical pathological features		Samples	SLP-2 positive		CCBE1 positive	
			N	%	N	%
Sex	Man	28	18	64.3%	20	71.4%
	Woman	22	14	63.6%	15	68.2%
Age (year)	≤60	18	12	66.7%	14	77.8%
	>60	32	15	46.9	17	53.1%
Tumor size	≤5	39	31	79.5%	32	82.1%
(cm)	>5	11	6	54.5%	7	63.6%
Differentiated degree	Moderate to high differentiation caner	45	23	51.1%	26	57.8%
	Low differentiation	5	3	60.0%	4	80.0%
	Ducks classification					
	A+B	14	5	35.7%	8	57.1%
	C+D	36	26	72.2%	30	83.3%
Lymphatic metastasis	Y	20	15	75.0%	17	85%
	N	25	8	32.0%	10	40.0%

CCBE1 was positively associated with LVD

Compared with control group, CCBE1 positive group had a significantly higher level of LVD (16.11 ± 1.03 *vs.* 9.22 ± 0.17, T=6.049, $p<0.05$).

SLP-2 and CCBE1 enhanced lymphatic metastasis

Compared with experimental subgroup without lymphatic metastasis, cancer samples with lymphatic metastasis had higher levels of SLP-2 protein, CCBE1 mRNA ($p<0.05$), suggesting both SLP-2 and CCBE1 enhanced lymphatic metastasis.

CCBE1 was negatively associated with survival rate

As showed in Figure 3, compared with CCBE1 negative patients, survival rate of CCBE1 positive patients was remarkably decreased (60.22 ± 4.02 months *vs.* 41.15 ± 3.46 months, $\chi^2= 5.203$, $p<0.05$).

SLP-2 regulated expression of CCBE1

Analysis of Spearman showed that SLP-2 had a positive correlation with the expression of CCBE1 (r=0.425, $p<0.05$). Moreover, both SLP-2 and CCBE1 had positive correlation with LVD ($p<0.05$).

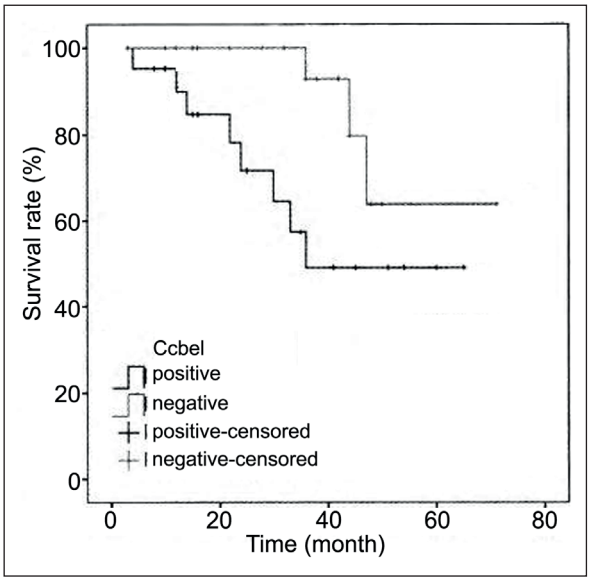

Figure 3. Survival analysis for patients with different CCBE1 expression.

Discussion

As a hotspot of clinical study in recent years, SLP-2 was firstly discovered by Wang et al[8], which was cloned from human erythrocytes and confirmed with function of encoding cell membrane associated proteins[13,14]. SLP-2 was found with over expression in human malignant tumors, suggesting SLP-2 was related to oncogenesis. On the other hand, studies proved that SLP-2 was associated with signal transduction pathways, and regulated mitochondria energy metabolism[15,16]. Moreover, multiple signal transduction molecules existed in mitochondria, and various cellular events of cancer depended on mitochondria energy metabolism, including proliferation, apoptosis, invasion and migration. Thus, SLP-2 has been indicated to regulate oncogenesis via mitochondria metabolism[17,18]. Furthermore, Christie et al[15] found that, compared with normal esophageal tissue, expression of SLP-2 increased by 6 times in esophagus cancer, verified by chip inspection. SLP-2 was proved with increased expression in other cancers, such as breast cancer[19] and laryngocarcinoma[18]. Cao et al[20] further demonstrated that SLP-2 in esophageal squamous cancer significantly increased in clinical scenario. Intriguing, previous studies[19,20] indicated that SLP-2 was associated with clinical staging and lymphatic metastasis. Also, Hogan et al[2] found SLP-2 influenced metastasis of lung cancer and up-regulation of SLP-2 was associated with poor prognosis. Our study demonstrated both transcription level and translation level of SLP-2 increased in rectal cancer, and analysis of follow-up data showed that the level of SLP-2 was further elevated in rectal cancer patients with lymphatic metastasis, suggesting that SLP-2 was associated with clinical progress of rectal cancer. CCBE1 was a lately identified protein with 44 kD molecular weight and multiple structural units recognizing cellular elements; for example, CCBE1 had EGF-like domain, which effectively bound with aspartic acid to stimulate reproduction[4,21]. Recent reports[1,22] found that, although the mechanism was unclear, CCBE1 influenced remodeling of extracellular matrix and cell migration, suggesting that CCBE1 promoted the survival of tumor cells. Our work showed that the expression of CCBE1 had a positive association with SLP-2, and CCBE1 enhanced lymphatic metastasis, verified by the correlation between CCBE1 and LVD. Further clinical data indicated that CCBE1 promoted lymphatic proliferation in tumor lesion, and exacerbated infiltration, consistent with previous reports. For example, Hogan et al[22] proved that CCBE1 played a key role in affecting lymphatic formation and cancer migration via veins, and inhibition of CCBE1 decreased lymphatic formation and attenuated tumor lesion.

Conclusions

SLP-2 was over-expressed in rectal cancer and promoted expression of CCBE1. SLP-2 enhanced lymphatic metastasis via up-regulating CCBE1. Both SLP-2 and CCBE1 were risk factors of rectal cancer, and for rectal cancer patients SLP-2 could be a potential target for molecular target therapy.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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