European Review for Medical and Pharmacological Sciences 2014; 18: 1916-1924

Expression features of CXCR5 and its ligand, CXCL13 associated with poor prognosis of advanced colorectal cancer

X.-W. QI[1,2], S.-H. XIA[3], Y. YIN[2], L.-F. JIN[1], Y. PU[1], D. HUA[2], H.-R. WU[1]

[1]Department of General Surgery, the Second Affiliated Hospital of Soochow University, Suzhou, China
[2]Department of Pathology and Wuxi Oncology Institute, the Affiliated Hospital of Jiangnan University, Wuxi, China
[3]Department of Oncology, the First Affiliated Hospital of Soochow University, Suzhou, China

Xiaowei Qi and Suhua Xia contributed equally to this work

Abstract. – OBJECTIVE: CXCL13 plays a unique role in the trafficking and homing of B1 cells associated with its cognate receptor, CXCR5. The CXCR5-CXCL13 axis has been previously demonstrated to be a poor prognosis factor in malignancies. However, the clinical significance of the CXCR5-CXCL13 expression in colorectal cancer carcinoma (CRC) remains unclear. The aim of this study was to investigate the CXCR5-CXCL13 expression in CRC and determine its correlation with the progression and prognosis of the tumor.

PATIENTS AND METHODS: A total of 144 paraffin-embedded specimens with advanced colon cancer were assessed for CXCR5 and CXCL13 by immunohistochemistry. Patients' long-term survival was also monitored. There were significant differences in lymph node metastasis ($p = 0.0066$), neural invasion ($p = 0.0061$) and neural invasion ($p = 0.0001$) between high and low expression of CXCR5.

RESULTS: There were significant differences in distant metastasis ($p = 0.0261$), TNM stage ($p = 0.0409$), differentiation ($p < 0.0001$) and neural invasion of the CXCL13. Both CXCR5 and CXCL13 was associated with poor correlation with the overall survival (OS) and relapse-free survival (RFS).

CONCLUSIONS: Our data suggest that the CXCR5 and CXCL13 may play a crucial role in the development, metastasis and relapse of advanced colon cancer. They can be used as prognostic markers of colon cancer in clinical practice.

Key Words:
CXCR5, CXCL13, Prognosis, Colorectal cancer.

Introduction

The incidence and mortality rates of colorectal cancer (CRC) are ranked the second and third for female, the third and fourth for male in China, respectively[1]. Recently, standardized postoperative adjuvant therapy significantly improves the patients' survival[2]. However, the prognosis for most patients remains poor because of the recurrence and metastasis of the colorectal cancer. The progression of CRC is a complicated process that is associated with cumulative genomic alterations[3]. It is, therefore, necessary to explore the molecular mechanisms underlying the CRC progression and identify novel therapeutic targets needed to improve the clinical outcome of cancer patients.

Chemokines play an essential role in the recruitment of leukocytes from the circulation system to local inflammatory sites[4,5]. Chemokines exert their biological functions by binding to their cognate receptors[6]. There is overwhelming evidence that the chemokine-chemokine receptor systems regulate the tumor cell transformation, growth, neovascularization, and metastasis[7,8]. The role of the chemokines and their receptors in CRC has been studied only recently.

The chemokine receptor CXCR5 was isolated from Burkitt lymphoma and designated as the Burkitt lymphoma receptor 1 (BLR1)[9]. It is expressed mainly by mature recirculating B cells and small subsets of the T cells. We identified the ligand for CXCR5 and termed it the B-cell–attracting chemokine1 (BCA-1), and designated CXCL13. The CXCL13 is a homeostatic chemokine that is secreted by the stromal cells in the B-cell area of the secondary lymphoid tissues, where the B cells encounters the antigen and differentiate[9]. In addition to regulating lymphocyte migration and promoting inflammation, the CXCR5-CXCL13 axis has an important role in tumor development. Singh et al[9] detected that the expression of CXCR5 in prostate cancer cells contributes to tumor growth and invasion; El-Haibi et al[10] also shows that the CXCL13 can mediate prostate cancer cell proliferation through

Corresponding Author: Hao-rong Wu, MD; e-mail: haorongwusdfey@163.com

the JNK signaling, and invade through the ERK activation, in addition to CXCR5 activation. However, the clinical significance of CXCR5-CXCL13 expression in CRC remains unclear. The aim of this study was to investigate the expression of CXCR5-CXCL13 in CRC and determine its correlation with the progression of advanced colorectal cancer in patients.

Patients and Methods

Patients and Tissues

All patients included in the study underwent surgical resection procedures at the Affiliated Hospital of Jiang Nan University between 2000 and 2003. The study was approved by the medical Ethics Committee of the Affiliated Hospital of Jiang Nan University. The patients consisted of 93 men and 51 women with a median age of 55 years and a range of 27-83 years. None of the patients received radiotherapy, chemotherapy, or other medical interventions prior to surgery. The clinicopathological findings were determined according to the classification of malignant tumors by the World Health Organization and International Union against Cancer Tumor-Node-Metastasis (TNM) staging system[10]. Formalin-fixed paraffin-embedded specimens were collected for immunohistochemistry (IHC). The median duration for follow-up for the entire sample was 56 months (ranged 15-60 months). The patients' medical records were reviewed for demographic, pathological, and survival data. For fresh tissue collection, once the surgical specimens were obtained, individual-matched normal mucosa adjacent to the proximal excision margin was harvested. All specimens were snap-frozen immediately in liquid nitrogen and stored in a freezer at −80°C until further analysis.

Immunohistochemistry

All samples were routinely fixed in 40 g/L formaldehyde solution and embedded in paraffin. Sequentially sectioned 4 μm thick slides were used for performing immunohistochemical staining in each analyzed case. Immunohistochemical staining was performed by an automate immunostainer using LSAB HRP and HRP+ kits according to the manufacturer's instructions. The rabbit polyclonal anti-CXCR5 antibody (ab46218 from Abcam) was used at 1:500 dilution. The rabbit polyclonal anti-CXCL13 antibody (ab112521 from Abcam) was used at 1:250 dilution. The negative controls were processed in a similar manner with phosphate buffered saline (PBS) instead of primary antibody. Subsequently, immunostained slides were analyzed for each antibody.

Immunohistochemistry Analysis

All slides were independently evaluated by three pathologists without the knowledge of the patients' clinical information. The immunoreactive intensity of CXCR5 and CXCL13 was observed only in the cytomembrane and/or cytoplasm of a cancer cell. The staining intensity was visually scored and stratified as follows: none stained 0(-), weakly stained 1(+), moderately stained 2(++) and strongly stained 3(+++). The quantification of the staining was expressed as an H score. The H score was determined by the formula 3 × the percentage of strongly staining cells + 2 × the percentage of moderately staining cells + the percentage of weakly staining cells, giving a range of 0 to 300 for the H scores[11,12].

Statistical Analysis

The statistical software of SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, USA) was used to conduct the statistical analyses. The *t* test was used to analyze the differences of CXCR5 and CXCL13 protein expression between cancerous tissues and paired with normal mucosa. Then, the χ^2 test was used to analyze the relationship between the CXCR5/CXCL13 expression and their clinicopathological parameters based on immunohistochemistry. The relationship between the two proteins were analyzed by Spearman's rank correlation analysis. The survival rate was calculated with the Kaplan-Meier method and Cox proportional hazards regression model, and the differences were examined by the log-rank test. Differences were considered statistically significant when p was less than 0.05.

Results

Clinicopathological Features of Patients with CRC

The expressions of CXCR5/CXCL13 were examined in 144 colorectal cancers and individual-matched normal mucosa samples by immunohistochemistry. The clinicopathological characteristics of the patients are reported in Table I. Their median age at diagnosis was 55 years (ranged 27-83 years), and the 5-year survival rate was 57.64%.

Table I. Clinicopathological data and tumor marker expressions in 144 colorectal cancers.

Characteristics, n (%)	Total
Age at diagnosis, median (range)	55 (27, 83)
Gender	
Male	93
Female	51
Tumor site	
Colon	88
Rectum	56
Tumor stage	
T2	21
T3	96
T4	27
Lymph node stage	
N0	28
N1	59
N2	57
Distant metastasis	
M0	103
M1	41
TNM stage	
II	28
III	75
IV	41
Differentiation	
Well	22
Moderately	67
Poor	55
Lymph vascular invasion	
No	57
Yes	87
Neural invasion	
No	65
Yes	79
Ki-67	
No	54
Yes	90
CXCR5 staining	
Positive	89
Negative	55
CXCL13 staining	
Positive	94
Negtative	50
Relapse	
Yes	109
No	35
Death	
Yes	61
No	83

Expression and Correlation of CXCR5 and CXCL13 Protein in CRC Specimens

The immunohistochemical analysis revealed that CXCR5 staining was mainly localized in the epithelial cells. The number of cases of CXCR5 positive-expression staining were 89 (61.81%) in tumor tissue and was 13 (9.03%) in normal mucosa. CXCL13 was mainly localized in mesenchymal cells and the number of CXCL13 positive-expression staining were 94 (65.28%) in tumor tissue and 9 (6.25%) in normal mucosa (Table I). Positive expression rates were significantly higher than that of normal tissue, the difference was statistically significant (Figure 1, A and B) ($p < 0.05$). The expression levels of CXCR5 and CXCL13 were associated with tumor progression (Figure 2). The expression levels of CXCR5 and CXCL13 protein in CRC tissues with high stage (III-IV) were significantly stronger than those with low stage (II) (Figure 2, A-C and Figure D-F). Moreover, it showed a statistically significant correlation between the concentrations of CXCR5 and CXCL13 in CRC (Figure 1C).

Association of CXCR5 and CXCL13 Expression with the Clinicopathological Features of CRC

The correlations between immunohistochemical expression of the CXCR5 and CXCL13 with clinical-pathological variables are examined in Table II. As a result, the expression of CXCR5 differed significantly according to lymph node metastasis ($p = 0.0066$), neural invasion ($p = 0.0061$) and neural invasion ($p = 0.0001$), while the expression of CXCL13 differed significantly according to distant metastasis ($p = 0.0261$), TNM stage ($p = 0.0409$), differentiation ($p < 0.0001$) and neural invasion ($p < 0.0001$).

Prognostic Values of CXCR5 and CXCL13 Expression in CRC

Sixty-one patients died during the postoperative follow-up period. We investigated the prognostic value of various factors with Kaplan-Meier analysis, and observed lymph node metastasis, distant metastasis, TNM stage, neural invasion, Ki-67 as well as CXCR5 and CXCL13 were correlated with the survival and relapse-free survival ($p < 0.05$) (Tables III and V). It showed that patients with positive CXCR5 and CXCL13 expression were significantly poorer than that in the group negative CXCR5 and CXCL13 expression, both in terms of 5-year overall survival (Figure 3) and 5-year relapse-free survival (Figure 4). In the further Cox multivariate regression analysis, after adjusting for patients' gender, age, location, maximum tumor size, depth of invasion, differentiation, and TNM stage, CXCR5 expression was still an independent poor prognostic factor, for both 5-year overall survival (95% con-

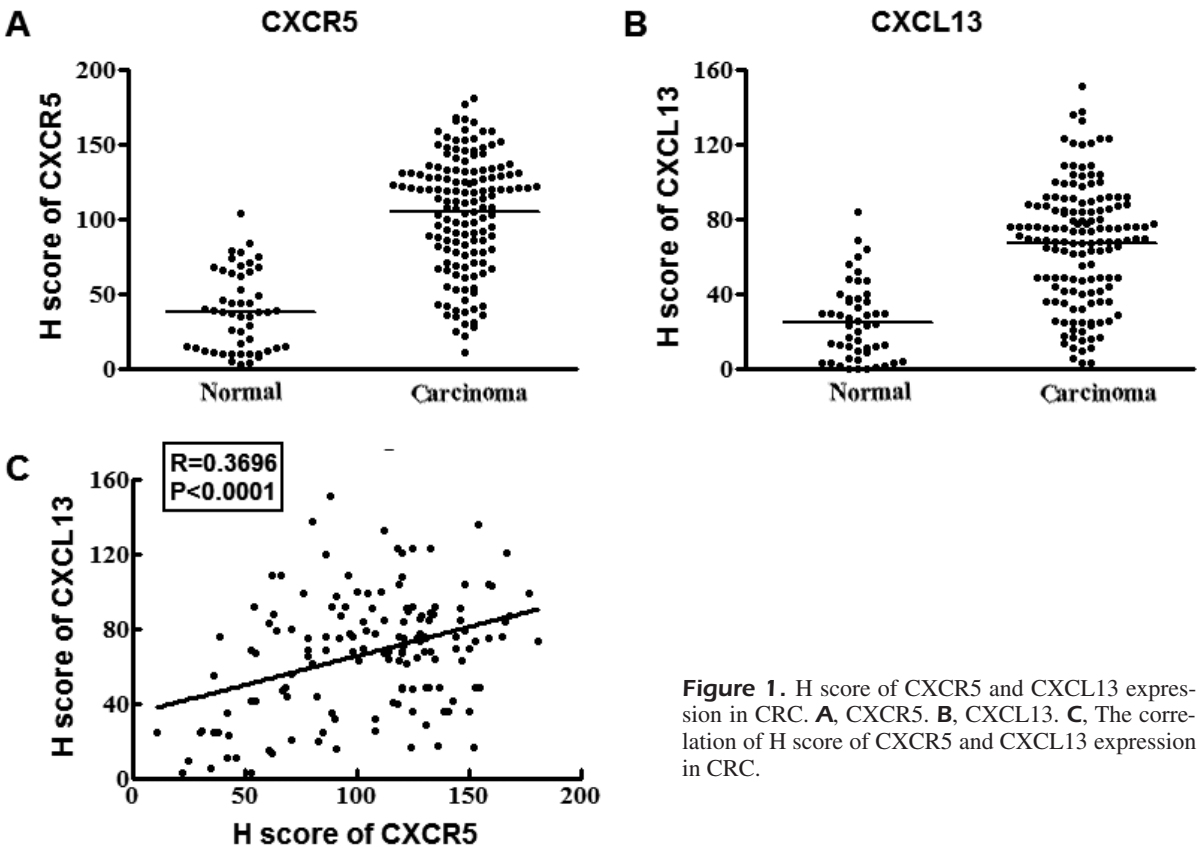

Figure 1. H score of CXCR5 and CXCL13 expression in CRC. **A**, CXCR5. **B**, CXCL13. **C**, The correlation of H score of CXCR5 and CXCL13 expression in CRC.

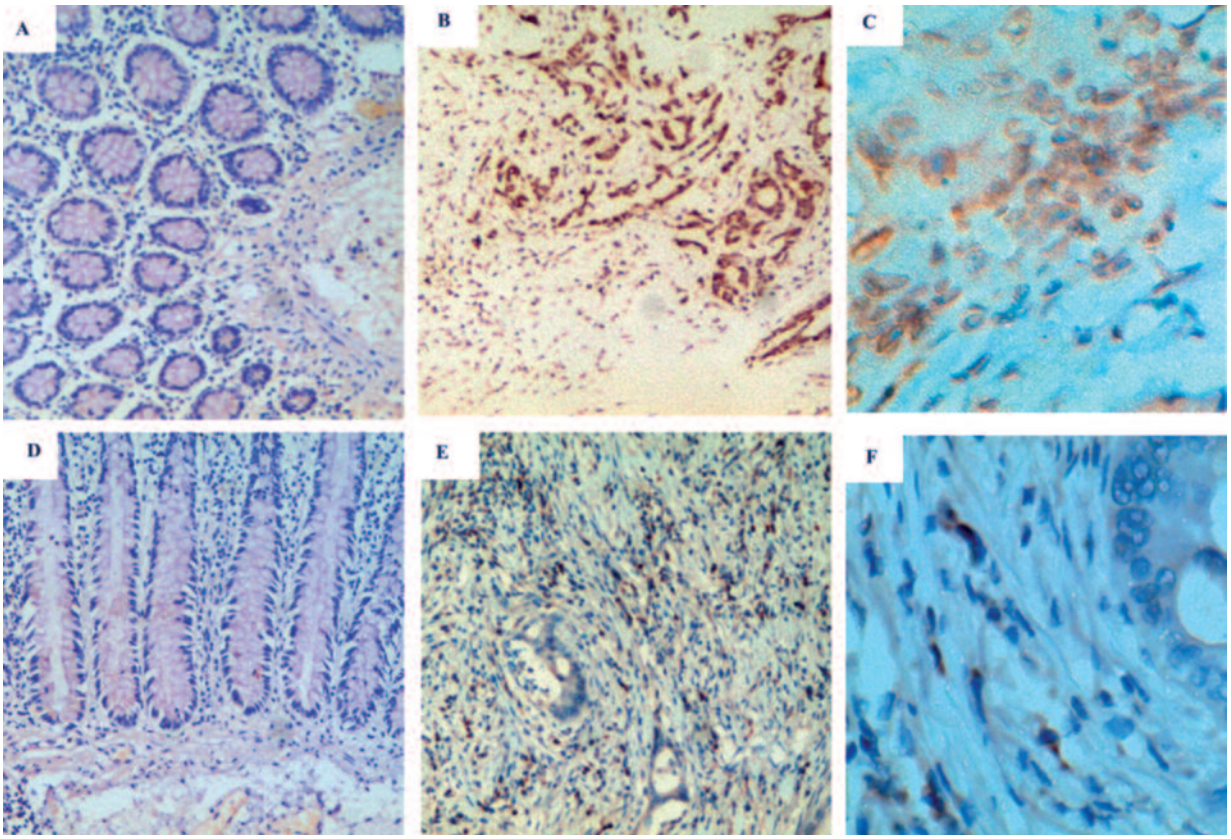

Table II. Association of CXCR5 and CXCL13 expression with the clinicopathological features of CRC.

Clinnicopathological characteristics	CXCR5		p	CXCL13		p
	+ (89)	- (55)		+(94)	- (50)	
Gender						
Male 93	55	38	0.4778	57	36	0.2403
Female 51	34	17		37	14	
Age (years)						
≥55 67	37	30	0.0588	43	24	0.9340
<55 87	62	25		51	26	
Tumor site						
Colon 88	56	32	0.6959	69	29	0.0892
Rectum 56	33	23		35	21	
Tumor stage						
T2 21	14	7		12	9	
T3 96	63	33	0.1194	67	29	0.2723
T4 27	12	15		15	12	
Lymph node stage Metastasis						
N0 28	10	18		15	13	
N1 59	40	19	0.0066	36	23	0.0925
N2 57	39	18		43	14	
Distant Metastasis						
M0 103	59	44	0.1139	61	42	0.0261
M1 41	30	11		33	8	
TNM stage						
II 28	10	18		15	13	
III 75	50	25	0.0061	46	29	0.0409
IV 41	29	12		33	8	
Differentiation						
Well 22	14	8		0	22	
Moderately 67	32	25	0.8144	44	23	<0.0001
Poor 55	33	22		50	5	
Lymphvascular invasion						
No 57	33	24	0.5442	37	20	0.9169
Yes 87	56	31		57	30	
Neural invasion						
No 65	28	37	0.0001	25	40	<0.0001
Yes 79	61	18		69	10	
Ki-67						
No 54	30	24	0.3084	33	21	0.5269
Yes 90	59	31		61	29	

fidence interval, CI, 1.318-5.569) and 5-year relapse-free survival (95% confidence interval, CI, 1.528-3.788). And CXCL13 expression was still an independent poor prognostic factor, for both 5-year overall survival (95% confidence interval, CI, 1.808-12.695) and 5-year relapse-free survival (95% confidence interval, CI, 2.821-9.461) (Tables IV and VI). Taken together, our results indicate that CXCR5 and CXCL13 could serve as prognostic markers for CRC.

Discussion

In the present study, we detected the expression of CXCR5 and CXCL13, then explored the clinical prognostic value of CXCR5-CXCL13 axis by using complete long-term follow-up data of a cohort of CRC samples. The expression of CXCR5 and CXCL13 showed a statistically significant correlation, and their immunoreactivities were increased in a substantial proportion of

Figure 2. IHC staining shows expression of CXCR5 and CXCL13 in CRC. CXCR5 staining in adjacent normal tissue (**A**, 100×) and B, CRC (**B**, 100×; **C**, 400×); CXCL13 staining in adjacent normal tissue (**D**, 100×) and CRC (E, 100×; F, 400×).

Table III. Kaplan-Meier univariate analysis of overall survival in CRC patients ($p < 0.05$).

Variable	Univariate analysis			
	M ± se (month)	95% CI		p value
Overall survival after surgery				
Lymph node stage metastasis				
N0	53.8±7.3	39.4	68.2	
N1	27.0±1.3	24.5	29.5	0.020
N2	26.7±1.5	23.7	29.7	
Distant metastasis				
M0	35.5±0.9	33.8	37.2	<0.001
M1	25.0±1.4	22.3	27.7	
TNM stage				
II	53.8±7.3	39.4	68.2	
III	29.4±4.3	21.0	37.8	<0.001
IV	25.0±1.4	22.3	27.7	
Neural invasion				
–	35.8±2.1	31.8	39.8	<0.001
+	26.5±1.2	24.1	28.9	
Ki-67				
–	26.9±2.8	21.4	32.4	0.037
+	28.1±0.8	26.6	29.6	
CXCR5				
–	38.2±9.5	19.5	56.9	<0.001
+	26.5±1.1	24.3	28.7	
CXCL13				
–	28.3±4.3	19.8	36.6	<0.001
+	27.5±1.0	25.5	29.5	

CRC cases compared with their corresponding normal tissues. The stronger levels of CXCR5 and CXCL13 were observed in tumor tissues with higher tumor stage and grade. Furthermore, univariate and multivariate regression analyses indicated that CXCR5 and CXCL13 were correlated with the overall and relapse-free survival of patients with CRC. When combined, our data suggest that the CXCR5 and CXCL13 might represent novel indicators for CRC.

Chronic inflammation is a risk factor for several gastrointestinal malignancies, including CRC. There is an increased risk of developing CRC in ulcerative colitis (UC) and Crohn's disease patients[13]. Chronic inflammation associated with development of cancer is partly driven by the chemokine-chemokine receptor system[4,14]. Recent studies have elucidated the role of chemokine in virtually all steps of colon tumor genesis[15]. Zipin-Roitman reported that CXCL10

Table IV. Cox multivariate regression analysis of overall survival in CRC patients.

	Variables in the Equation							
	B	SE	Wald	Df	Sig. (p value)	Exp (B)	95.0% CI for Exp (B)	
							Lower	Upper
Lymph node stage metastasis	0.295	0.348	0.720	1	0.396	1.343	0.679	2.656
Distant metastasis	11.654	73.556	0.025	1	0.874	115121.325	0.000	4.704E+067
TNM stage	-0.265	0.712	0.139	1	0.709	0.767	0.190	3.094
Neural invasion	0.372	0.407	0.836	1	0.360	1.451	0.654	3.220
Ki-67	0.357	0.349	1.045	1	0.307	1.428	0.721	2.829
CXCR5	0.997	0.368	7.346	1	0.007	2.709	1.318	5.569
CXCL13	1.567	0.497	9.931	1	0.002	4.791	1.808	12.695

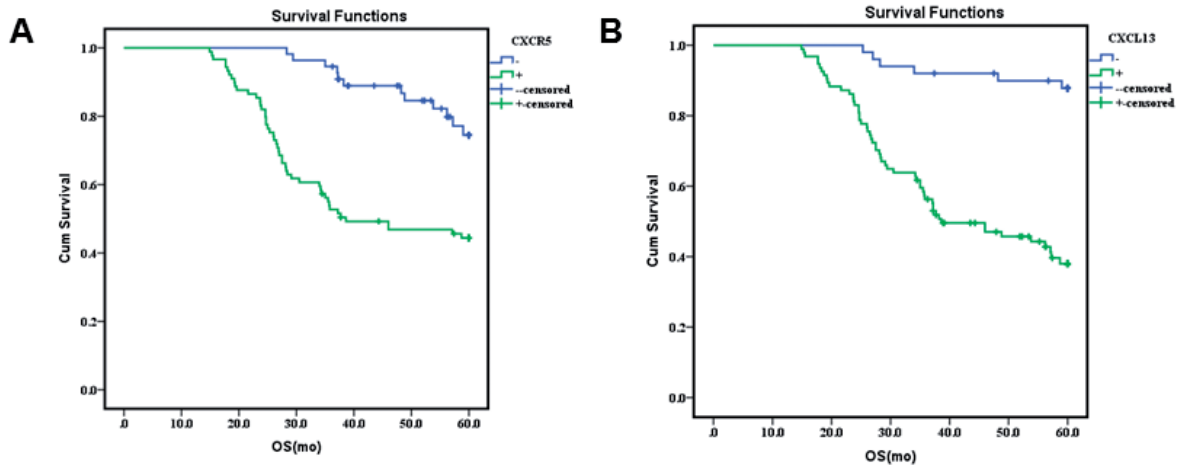

Figure 3. Association of the overall survival of patients with CRC with CXCR5 and CXCL13. (**A**) CXCR5; (**B**) CXCL13.

promotes invasion in human colorectal carcinoma cells by activating the ERK pathway[15]; and immunohistochemical staining of 96 pairs of CRC tumor tissues confirmed that, the strong expression of CXCR4 is significantly associated with lymphatic and distant dissemination in patients with CRC[16].

CXCL13 and CXCR5 are a chemokine and receptor pair whose interaction is critical for naive B-cell trafficking and activation within germinal centers[17]. Our study revealed that CXCR5 staining was localized in the epithelial cells and the CXCL13 was localized in mesenchymal cells, which was consistent with the research of Burkle et al[18]. They detected the CXCL13+ expression by CD68+ macrophages *in situ* within the CLL lymph nodes, and the CXCR5 expression by tumor cells in chronic lymphocytic leukemia (CLL) patients. It suggests that the CXCR5-CXCL13 axis may interact between the tumors associated macrophages and tumor cells. We also found that the CXCR5-CXCL13 expressions was associated with the lymph node metastasis, distant metastasis, TNM stage, neural invasion, and tumor recurrence. Recurrence and poor prognosis of the CRC was mainly due to the tumor cells resistance. The tumor associated macrophages can regulate the antitumor efficacy of cytotoxic

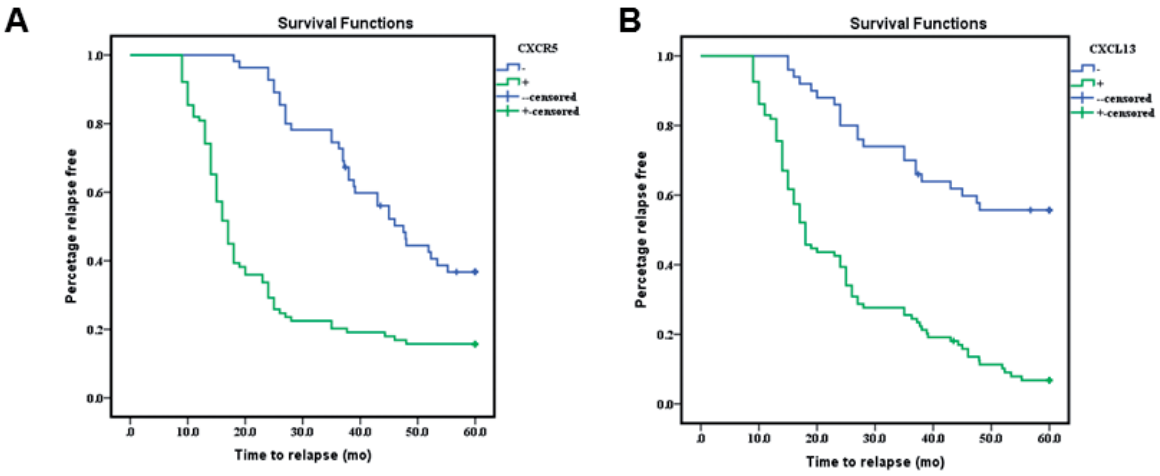

Figure 4. Association of the disease free survival of patients with CRC with CXCR5 and CXCL13. (**A**) CXCR5; (**B**) CXCL13.

Table V. Kaplan-Meier univariate analysis of disease-free survival in CRC patients ($p < 0.05$).

Variable	Univariate analysis			
	M ± se (month)	95% CI		p value
Disease-free survival after surgery				
Lymph node stage metastasis				
N0	47.5±2.4	42.7	52.3	0.003
N1	25.0±1.1	22.9	27.1	
N2	18.0±3.4	11.4	24.6	
Distant metastasis				
M0	35.0±4.7	25.7	44.3	0.002
M1	16.0±1.1	13.9	18.1	
TNM stage				
II	47.5±2.4	42.7	52.3	0.001
III	26.0±1.4	23.2	28.8	
IV	16.0±1.1	13.9	18.1	
Neural invasion				
–	47.5±4.9	38.0	57.0	<0.001
+	17±1.3	14.4	19.6	
Ki-67				
–	43±11.5	20.5	65.5	0.015
+	24±2.4	19.4	28.7	
CXCR5				
–	47.5±2.9	41.7	53.3	<0.001
+	17.0±0.9	15.3	18.7	
CXCL13				
–	27.0±1.9	23.2	30.8	<0.001
+	17.0±1.0	15.2	18.8	

chemotherapy. Selective depletion of the macrophages in tumors resulted in an associated reduction of chemotherapy resistance[19,20]. As the CXCR5-CXCL13 may play an important role between macrophages and tumor cells, which means that the interruption of the interaction may be able to reverse resistance to treatment and improve the prognosis of patients. Our data may offer new insight into CXCR5 and CXCL13 which are potentially important in the progression of CRC, as well as novel therapeutic strategies in the diseases.

Conclusions

This study demonstrated that CXCR5 and CXCL13 are upregulated in the CRC tissues com-

Table IV. Cox multivariate regression analysis of overall survival in CRC patients.

	Variables in the Equation							
	B	SE	Wald	Df	Sig.	Exp (B)	95.0% CI for Exp (B)	
							Lower	Upper
Lymph node stage metastasis	0.034	0.248	0.019	1	0.891	1.035	0.636	1.684
Distant metastasis	-0.504	0.658	0.586	1	0.444	0.604	0.166	2.195
TNM stage	0.891	0.497	3.216	1	0.073	2.438	0.920	6.458
Neural invasion	0.279	0.257	1.182	1	0.277	1.322	0.799	2.188
Ki-67	0.229	0.251	0.829	1	0.363	1.257	0.768	2.058
CXCR5	0.878	0.232	14.365	1	0.000	2.406	1.528	3.788
CXCL13	1.642	0.309	28.295	1	0.000	5.166	2.821	9.461

pared with their benign counterparts and associated with tumor progression. The positive CXCR5 and CXCL13 expressions correlate with the metastasis and predicts poor prognosis in CRC patients. Our findings indicate that CXCR5 and CXCL13 appears to be an independent predictor of survival for patients with CRC, and may provide a potential therapeutic strategy by targeting the CXCR5-CXCL13.

Founding

Foundation of Wuxi Hospital management center (Nos. CSEY1N1106), National Natural Science Foundation of China (Nos. 81301784)

Conflict of interest

The Authors declare that they have no conflict of interests.

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