

Overexpression of GRO- β is associated with an unfavorable outcome in colorectal cancer

GUIHUA WANG¹, JIANFEI HUANG², HUIJUN ZHU², SHAOQING JU¹, HUIMIN WANG¹ and XUDONG WANG¹

¹Center of Clinical Laboratory Medicine; ²Department of Pathology, Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001, P.R. China

Received March 1, 2015; Accepted January 18, 2016

DOI: 10.3892/ol.2016.4222

Abstract. Growth-related oncogene (GRO)- β , or chemokine (C-X-C motif) ligand 2 (CXCL2), is a member of the CXC chemokine family that may mediate various functions, including attracting neutrophils to sites of inflammation, and participating in tumorigenesis and progression. However, the expression of GRO- β in colorectal cancer (CRC) and the association with the clinical outcome of the disease remains poorly understood. In the present study, CXCL2 mRNA expression in CRC was analyzed using six independent datasets from the Oncomine microarray database. The immunohistochemical analysis of tissue microarrays (TMA) was used to characterize the expression of the GRO- β protein in CRC. The association between GRO- β expression and the clinicopathological features and prognosis of patients was determined by statistical analysis. The results indicated that GRO- β was highly expressed in CRC tissues, and that high GRO- β cytoplasmic expression was associated with the tumor location, extent of the primary tumor, and lymph node metastasis. Kaplan-Meier survival and Cox regression analysis revealed that high GRO- β expression was an independent indicator of poor prognosis for CRC patients. The results indicate that high GRO- β expression in CRC may correlate with an unfavorable outcome and facilitate cancer cell invasion and metastasis.

Introduction

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in females and the third most commonly diagnosed cancer in males worldwide (1). Patients with CRC have a poor prognosis due to cancer recurrence and metastasis following surgical resection. Numerous patients are at a high risk of recurrence and may be considered candidates

for targeted therapy or chemotherapy. However, colorectal carcinogenesis is a complicated process that is associated with cumulative genomic alterations (2,3). Therefore, it is necessary to explore the molecular markers that underlie tumor progression and identify novel targets to improve therapeutic strategies and extend the survival of CRC patients.

Chemokines are a group of small proinflammatory cytokines that attract, activate and regulate leukocytes in inflamed tissues, and recent studies identified the role of chemokines in the initiation and promotion of carcinogenesis (3). Chemokines may be classified into three subfamilies, consisting of C, CC and CXC, on the basis of the number and arrangement of conserved cysteine residues. Growth-related oncogene (GRO) is a member of the CXC chemokine family, which is composed of GRO- α , GRO- β , and GRO- γ , also termed the CXCL1, CXCL2 and CXCL3 genes, respectively (4,5). The chemokines have a conserved CXC motif at the NH₂ terminus, but vary at the COOH terminus. The three GRO chemokines vary in binding affinity to CXCR2 or CXCR1 receptors, with GRO- α having the highest affinity with CXCR2 (6). GRO- α and GRO- β have been identified to be dysregulated in pre-malignant colonic adenomas (7). However, the impact of GRO- β overexpression on the clinical outcome in patients with CRC remains unclear. The present study explored the expression of GRO- β in human CRC compared with adjacent normal tissue using cDNA microarray data and tissue microarray (TMA) sections, and assessed the potential correlation with the critical clinicopathological features of CRC and patient prognosis.

Materials and methods

cDNA microarray data. CXCL2 mRNA expression data were extracted from six independent studies and the expression levels in CRC tissues were compared with matched normal tissues using publicly available gene expression data in the Oncomine Cancer Microarray database (<http://www.oncomine.org>). All data were log-transformed and median-centered per array, and the standard deviation was normalized to one per array (8,9).

Correspondence to: Dr Xudong Wang, Center of Clinical Laboratory Medicine, Affiliated Hospital of Nantong University, 20 Xisi Road, Nantong, Jiangsu 226001, P.R. China
E-mail: wangxudong88@hotmail.com

Key words: growth-related oncogene- β , colorectal cancer, tissue microarray, immunohistochemistry, prognosis

Patients and samples. Formalin-fixed, paraffin-embedded tumor tissues and corresponding tumor-adjacent specimens were obtained during surgery from 198 patients with CRC treated at the Affiliated Hospital of Nantong University

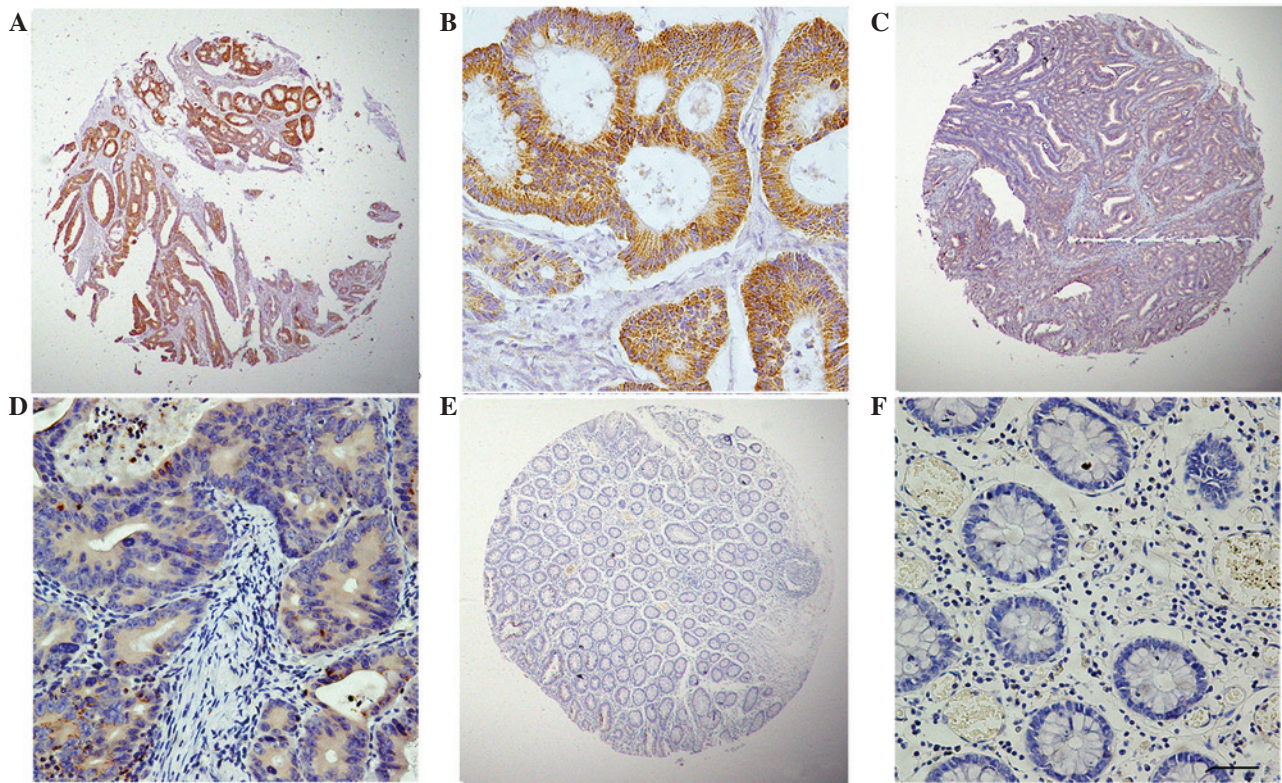


Figure 1. Various immunohistochemical staining patterns for GRO- β in CRC and peritumoral tissues. The GRO- β protein was highly expressed in the CRC tissue of a stage IIIB carcinoma; original magnification, (A) x40 and (B) x400. Low GRO- β protein expression was observed in the CRC tissue of a stage IIA carcinoma; original magnification, (C) x40 and (D) x400. The expression of GRO- β protein was negative in the adjacent normal peritumoral tissue; original magnification, (E) x40 and (F) x400. Scale bar, 50 μ m. GRO- β , growth-related oncogene- β ; CRC, colorectal cancer.

between 2002 and 2008. The clinical data, including gender, age, differentiation, location, extent of primary tumor, tumor-node-metastasis (TNM) classification, lymph node metastasis status, carcinoembryonic antigen (CEA) level and follow-up, including the 5-year survival rate, were obtained from the medical records of individual patients. The overall survival time was calculated as the time between the date of surgery and the date of mortality or last follow-up. The tumor stage was in accordance with the Union for International Cancer Control TNM system, but differentiation was determined following the World Health Organization standards (10). Representative 2.0-mm tissue cores from each patient were used to conduct a tissue microarray (TMA) analysis using the Manual Tissue Microarrayer (Quick-Ray WI-UT06; Unitma Co., Ltd., Seoul, Korea).

Ethics statement. The present study was performed according to the principles expressed in the Declaration of Helsinki (11). Tissue specimens were collected with full written informed consent of the patients or the patients' families, in compliance with the institutional guidelines set by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University. Ethical approval for the present study was granted by the Ethics Committee of the Affiliated Hospital of Nantong University (approval no., 2013-009).

Immunohistochemistry. For the immunohistochemical (IHC) analysis, TMA sections were deparaffinized in 100% xylene (Beyotime Institute of Biotechnology, Shanghai, China) and

rehydrated in graded ethanol solutions (80, 95 and 100%; Beyotime Institute of Biotechnology). Antigen retrieval was performed by boiling the sections under pressure (90 kPa) in citrate buffer (pH 6.0; Beyotime Institute of Biotechnology) for 5 min. The non-specific binding site was blocked by incubating the sections in 5% goat serum and phosphate buffered saline (PBS; Beyotime Institute of Biotechnology) for 15 min. The TMA sections were incubated with a primary polyclonal rabbit anti-human GRO- β antibody (dilution, 1:400; catalog no., 500-P104; PeproTech, Inc., Rocky Hill, NJ, USA) and subsequently with goat anti-rabbit horseradish peroxidase-conjugated antibody (dilution, 1:1,000; catalog no., sc-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). GRO- β immunostaining underwent two independent evaluations under blind experimental conditions. The percentage of GRO- β -positive cells was recorded as 0-100%, and the staining intensity was graded as follows: 0, no staining; 1, mild intensity; 2, moderate intensity; and 3, strong intensity. The final GRO- β staining score was a product of the intensity grading and percentage of positive cells.

The threshold for the statistically significant GRO- β expression scores in terms of overall survival (OS) time was set using the X-tile software program (<http://www.tissuearray.org/rimmlab>; Rimm Lab, Yale University, New Haven, CT, USA), as previously described (12). The degree of staining was quantified using a two-level grading system and the final GRO- β staining score was defined as follows: 0-150, low expression; 150-300, high expression. The typical expression patterns of GRO- β are illustrated in Fig. 1.

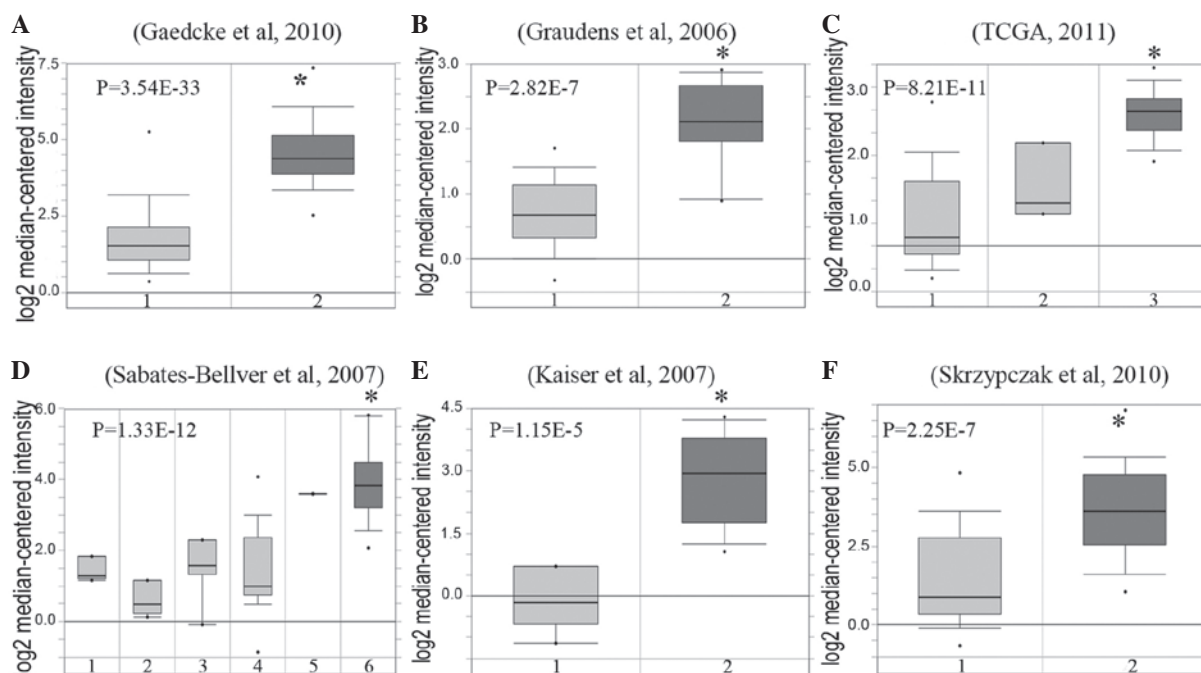


Figure 2. GRO- β mRNA is overexpressed in colorectal cancer. GRO- β expression in colorectal cancer tissues and normal tissues. Data sets in a single panel were from the same study. GEP data are log transformed and normalized. (A) Gaedcke *et al* (16): 1, 65 rectum samples; 2, 65 rectal adenocarcinoma samples. (B) Graudens *et al* (13): 1, 12 colon samples; 2, 18 colorectal carcinoma samples. (C) TCGA (18): 1, 19 colon samples; 2, 3 rectum samples; 3 22 colon mucinous adenocarcinoma samples. (D) Sabates-Bellver *et al* (15): 1, 4 ascending colon samples; 2, 5 descending colon samples; 3, 7 rectum samples; 4, 15 sigmoid colon samples; 5, 1 transverse colon sample; 6, 25 colon adenoma samples. (E) Kaiser *et al* (14): 1, 5 colon samples; 2, 17 cecum adenocarcinoma samples. (F) Skrzypczak *et al* (17): 1, 24 colorectal tissues samples; 2, 45 colorectal adenocarcinoma samples. GRO- β , growth-related oncogene- β ; GEP, gene expression profiling. * $P < 0.05$.

Statistical analysis. SPSS version 20.0 software (IBM Corporation, Armonk, NY, USA) was used to conduct the statistical analyses. The comparison between the CXCL2 mRNA levels in the microarray was performed using the Mann-Whitney U or Student's *t*-tests. A χ^2 test was used to analyze the association between GRO- β expression and clinicopathological parameters, based on the IHC analysis. For the TMA slides, the gender, age, differentiation, location, extent of primary tumor, TNM classification, lymph node metastasis status and CEA level were assessed. The survival rate was calculated using the Kaplan-Meier method and Cox proportional hazards regression model, and the statistical differences were examined using the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical tests were two-sided.

Results

CXCL2 mRNA is overexpressed in CRC. Using the Oncomine microarray database, the expression levels of CXCL2 mRNA in CRC tissues were increased compared with normal tissues from six independent studies (13-18). There was a significant difference between tumor tissue and normal tissue according to the mean expression value ($P < 0.0001$ in all six datasets; Fig. 2).

Expression of GRO- β in CRC and peritumoral tissues, determined by IHC analysis. In order to investigate the expression of GRO- β protein in colorectal carcinoma and the corresponding adjacent tissues, an IHC analysis on the primary colorectal tumors and normal colorectal mucosa was performed. As

shown in Fig. 1, GRO- β staining was detected at various levels, primarily in the cytoplasm of CRC cells. The staining index of cytoplasmic expression of GRO- β in all 86 normal colorectal mucosa tissues was < 150 . High GRO- β expression was detected in 31.31% (62/198) of CRC samples. Therefore, in accordance with the data of the CXCL2 mRNA analysis, the GRO- β protein was confirmed to be highly expressed in CRC tissues.

Association between GRO- β expression and clinicopathological features. The association between GRO- β cytoplasmic expression and clinicopathological features of 198 cases of CRC was studied (Table I). The results revealed that high GRO- β cytoplasmic expression in the primary CRC was significantly associated with the tumor location ($P = 0.022$), extent of primary tumor ($P = 0.005$) and lymph node metastasis ($P = 0.017$). However, no significant association between GRO- β levels and other clinicopathological characteristics, including patient gender, patient age, tumor differentiation, TNM stage and CEA level, were observed (Table I).

Prognostic value of high GRO- β expression in CRC. In total, 68 patients succumbed during the postoperative follow-up period. The prognostic value of various factors was investigated using Kaplan-Meier analysis, and differentiation, TNM classification, extent of primary tumor, lymph node metastasis, CEA level and high GRO- β expression were correlated with overall survival ($P < 0.05$) (Table II). Kaplan-Meier survival curves demonstrated that patients with high GRO- β expression possessed a significantly shorter survival time compared with

Table I. Correlation of growth-related oncogene- β expression in tumor tissues of colorectal cancer patients by clinicopathological characteristic.

Characteristic	N	Low expression, n (%)	High expression, n (%)	Pearson χ^2	P-value
Total	198	136 (68.69)	62 (31.31)		
Gender					
Male	126	86 (68.25)	40 (31.75)	0.030	0.862
Female	72	50 (69.44)	22 (30.56)		
Age, years					
<60	65	45 (69.23)	20 (30.77)	0.013	0.908
\geq 60	133	91 (68.42)	42 (31.58)		
Location					
Colon	145	93 (64.14)	52 (35.86)	5.212	0.022 ^a
Rectum	53	43 (81.13)	10 (18.87)		
Differentiation					
Well - middle	166	113 (68.07)	53 (31.93)	0.180	0.671
Poor	32	23 (71.88)	9 (28.13)		
TNM stage					
I	46	38 (82.61)	8 (17.39)	5.462	0.065
II	78	51 (65.38)	27 (34.62)		
III+IV	74	47 (63.51)	27 (36.49)		
T					
Tis+1+2	51	43 (84.31)	8 (15.69)	7.799	0.005 ^a
3+4	147	93 (63.27)	54 (36.73)		
N					
0	124	89 (71.77)	35 (28.23)	8.176	0.017 ^a
1a+1b	57	40 (70.18)	17 (29.82)		
2a+2b	17	7 (41.18)	10 (58.82)		
CEA					
No	119	81 (68.07)	38 (31.93)	1.713	0.191
Yes	24	13 (54.17)	11 (45.83)		
Unknown	55	42 (76.36)	13 (23.64)		

^aP<0.05. TNM, tumor-node-metastasis; T, size of the primary tumor; Tis, carcinoma *in situ*; N, degree of spread to lymph nodes; CEA, carcinoembryonic antigen.

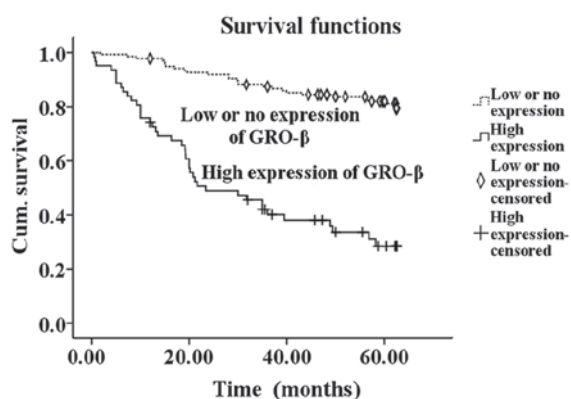


Figure 3. Kaplan-Meier survival curves of colorectal cancer patients with high and low GRO- β expression. GRO- β , growth-related oncogene- β ; Cum., cumulative.

the low GRO- β expression group (P<0.001) (Fig. 3). In the additional multivariate Cox regression analyses, subsequent to adjustment for gender, age, location, differentiation, tumor stage

and lymph node metastasis, high GRO- β expression (P<0.001), TNM classification (P=0.002) and CEA level (P=0.027) remained as independent predictive factors of a poor outcome in CRC (Table III). Overall, the results indicate that high GRO- β expression may act as a prognostic marker for CRC.

Discussion

Previously, the potential oncogenic role of GRO- β in the promotion of several human cancers has been examined, including in esophageal squamous cell carcinoma and melanoma (19,20). Little is understood regarding the role of GRO- β in CRC. To the best of our knowledge, only two studies have investigated the potential role of GRO- β in colorectal tumors at present. These studies indicated that the expression of CXCL2 mRNA was significantly increased in CRC compared with normal colon tissue using quantitative reverse transcription-polymerase chain reaction (6) and that CXCL2 mRNA was also enhanced in premalignant adenomas in a previous small cohort study (7), which suggests that the dysregulation of GRO- β may be an early event in the tumorigenesis of CRC. Additional statistical

Table II. Kaplan-Meier univariate analysis of the overall survival time of colorectal cancer patients following surgery.

Variable	Univariate analysis		
	Survival in months \pm standard error	95% confidence interval	P-value
GRO- β expression			
Low	56.24 \pm 1.29	53.71-58.77	<0.001 ^a
High	33.02 \pm 2.99	27.16-38.88	
Gender			
Female	51.68 \pm 2.35	47.07-56.29	0.153
Male	47.67 \pm 1.92	43.91-51.42	
Age, years			
<60	48.97 \pm 2.64	43.80-54.14	0.929
\geq 60	49.21 \pm 1.84	45.61-52.81	
Location			
Rectum	50.11 \pm 1.74	46.70-53.52	0.169
Colon	46.59 \pm 2.89	40.92-52.27	
Differentiation			
Well - middle	50.53 \pm 1.57	47.45-53.61	0.044 ^a
Poor	41.70 \pm 4.18	33.51-49.89	
TNM stage			
I	59.73 \pm 1.55	56.69-62.76	<0.001 ^a
II	49.46 \pm 2.30	44.95-53.97	
III +IV	42.08 \pm 2.75	36.68-47.48	
T			
Tis+1+2	59.36 \pm 1.52	56.39-62.33	<0.001 ^a
3+4	45.52 \pm 1.86	41.88-49.17	
N			
0	53.34 \pm 1.62	50.18-56.51	<0.001 ^a
1a+1b	45.46 \pm 3.14	39.29-51.62	
2a+2b	30.85 \pm 4.91	21.22-40.47	
CEA			
No	51.59 \pm 1.73	48.20-54.98	0.001 ^a
Yes	38.13 \pm 5.12	28.10-48.15	

^aP<0.05. GRO- β , growth-related oncogene- β ; TNM, tumor-node-metastasis; T, size of the primary tumor; Tis, carcinoma *in situ*; N, degree of spread to lymph nodes; CEA, carcinoembryonic antigen.

Table III. Results of the Cox multivariate regression analysis of the overall survival of colorectal cancer patients.

Factor	β	SE(β)	Wald	P-value	e ^{β} hazard ratio	95.0% CI for e ^{β} hazard ratio
GRO- β	1.778	0.306	33.702	0.000	5.920	3.248-10.791
TNM	0.653	0.222	8.613	0.003	1.921	1.242-2.970
CEA	0.758	0.325	5.461	0.019	2.135	1.130-4.032

Degrees of freedom = 1 (for all factors). GRO- β , growth-related oncogene- β ; TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen; β , regression coefficient; SE(β), standard error of the regression coefficient; Wald, Wald test score; e ^{β} hazard ratio, relationship between treatment effect and hazard ratio; CI, confidence interval.

analyses revealed that CXCL2 mRNA was overexpressed in malignant colorectal tissues compared with normal adjacent tissues using microarray data from six independent datasets (13-18).

The present study explored the expression of the GRO- β protein in human CRC compared with adjacent tissues, and

assessed the potential correlation with the critical clinicopathological features of CRC and the patient outcome. Several notable observations were made from the results.

First, the GRO- β protein was demonstrated to be highly expressed in a series of 198 CRCs comprising all stages of disease using an IHC approach. In addition, overexpression was

found to be associated with neoplastic epithelial cells rather than inflammatory cells or non-epithelial stroma. Notably, the division of the data into two categories according to localization indicated that increased GRO- β expression in the colon was significantly more frequent compared with the rectum ($P=0.022$). Previously, cancers of the rectum and colon have been implicated as distinct tumors, as they have a dissimilar prevalence and variations in the clinical presentation, prognosis and possibly genetic and environmental epidemiology (21,22). Therefore, GRO- β expression may affect carcinogenesis of the colorectal tissues in a site-specific manner. The findings of the present study provide additional evidence that colon and rectal cancers possessed varied etiologies. In addition, GRO- β expression was significantly associated with the extent of the primary tumor and lymph node metastasis. Increased GRO- β expression is associated with a more advanced stage of disease and the propensity to develop lymph node metastasis. These results suggest that the overexpression of cytoplasmic GRO- β in CRC may facilitate cancer cell invasion and metastasis. Previous studies have also reported that the increased expression of GRO- β was involved in the development and invasion of several types of carcinomas, including esophageal squamous cell carcinoma (19) and melanoma (20). The data in the present study clearly revealed that a high cytoplasmic expression of GRO- β was associated with significantly poorer survival time. Multivariate analyses revealed that GRO- β expression was regarded as an independent prognostic factor for CRC patients. In addition to increased GRO- β expression, TNM classification and high CEA levels are also considered to be independent factors for a poor prognosis in CRC. Overall, GRO- β expression may be an important prognostic factor for aggressive human CRC. GRO- β has potential value as a therapeutic target in patients with CRC.

In conclusion, the role of GRO- β in the pathophysiology of CRC carcinogenesis and progression is unclear. GRO- β is a classical neutrophil chemoattractant and was the first chemokine to be identified as a product of neutrophils, and to be demonstrated to mediate neutrophil recruitment, the release of granule enzymes and the expression of adhesion molecules, which multiply inflammatory effects (7). Chronic inflammation may increase the risk of cancer development (23,24). GRO- β is excessively expressed during inflammation and is chemotactic for neutrophils in combination with the CXCR2 receptor. GRO- β is crucial in the initiation and progression of colitis-associated colon cancer, and the GRO- β -CXCR2 axis may be useful in decreasing the risk of ulcerative colitis-associated colon cancer (6,7). Sufficient evidence demonstrates that chemokines are also significant in cancer, in addition to having a role in the development and inflammatory responses (25,26). GRO increases matrix metalloproteinase production in oral squamous cell carcinoma by binding to CXCR2, which may participate in cancer progression (26). Wang *et al* (27) also indicated that GRO- β /CXCR2 forms an autocrine loop by activating the ERK1/2 pathway, and contributes significantly to proliferation in primary esophageal squamous cell carcinoma.

The findings of the present study suggest that GRO- β was overexpressed in the cytoplasm of CRC cells rather than inflammatory cells. However, the association between GRO- β protein expression and the clinical characteristics of CRC

was confined to clinical observations using a tumor tissue microarray. The association between GRO- β in colorectal carcinogenesis and the autocrine or paracrine mechanisms and the signal pathways involved remains to be elucidated. Additional *in vitro* and *in vivo* studies are required in order to investigate the biological functions of GRO- β in CRC.

Acknowledgements

This study was supported by grants from the Translational Medicine Program of Affiliated Hospital of Nantong University (grant no. TDFzh2014014), the Six Talent Peaks Program of Jiangsu Province (grant no. 2012-ws-064) and the 226 Talent Training Project of Nantong City (The Fourth Batch).

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