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Identification of a novel biomarker-CCL5 using antibody microarray for colorectal cancer

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ABSTRACT

Purpose: To screen novel candidate biomarkers in primary colorectal cancer (CRC), and indentify their clinical valuation in progress of colorectal cancer.
Methods: By using antibody microarray, 274 target proteins in tissue samples from primary colorectal cancer patients were detected. Among differently expressed proteins in CRC tissues, As promising candidate biomarker, RANTES/CCL5 was validated by enzyme-linked immunosorbentassay and immunohistochemistry (IHC), and the clinical significance of CCL5 was analyzed.
Results: Totally, 25 differentially expressed proteins were indentified between colorectal cancers and matched normal mucosa. CCL5 expression was significantly associated with adverse pathological progress, apt to lymph node metastasis and higher T stage.
Conclusions: CCL5 may contribute to promoting tumor growth, and CCL5 is a promising target that may help in understanding the pathogenesis of CRC.

1. Introduction

Colorectal cancer is the most common gastrointestinal cancer and the fourth leading cause of cancer death worldwide [1]. In China, the increasing colorectal cancer incidence especially among young adults, reflect an increased prevalence of risk factors such as smoking, drinking, unhealthy diet, etc. To the present, the efficacy of cytotoxic drugs and targeted therapies have been proved in the treatment of advanced colorectal cancer. However, the targeted agents, including anti-VEGF and anti-EGFR antibodies, benefit only certain patients who harbor relevant variation. Discovering new therapeutic targets for personalized treatment is still an urgent need in colorectal cancer research.

Currently, more and more studies indicate that chemokine plays a important role in tumor growth and metastasis. Different members of chemokine family eitherpromote or inhibit tumor growth by promoting or inhibiting the tumor angiogenesis. Besides leukocytes and vascular endothelial cells, the epithelial cells and colorectal cancer cells could also secrete chemokines to recruit effector cells or regulating cells with concentration dependent manners to the source of chemokines. Researches provide evidence that chemokines produced by the tumor microenvironment can induce tumor proliferation, infiltration, promote angiogenesis, and stimulate tumor cell metastasis CC chemokine motif ligand 5 (CCL5), also known as RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted), belongs to CC chemokine family whose members include monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-3, I-309, macrophage inhibitory protein-1a and macrophage inhibitory protein-1β [2]. The combination of CCL5 and its receptor C-C chemokine receptor type 5 (CCR5) is involved in malignant transformation, invasion and metastasis of tumors [3]. Expressions of CCL5 have been reported in breast cancer and cervical carcinoma specimens, and high plasma CCL5 level correlated with advanced progression [4–6]. The plasma CCL5 levels in patients with advanced ovarian cancer or advanced gastric cancer were apparently higher than those with early ovarian cancers or gastric cancers [7]. Using real time RT-PCR, Cambien et al. [8] found that the expression of CCL5 in colorectal cancer cells was higher than that in normal mucosa, systemic treatment with anti – CCL5 antibodies could delaying the progression of colon cancer. However, Szczepanik et al. [9] detected the level of CCL5 in the serum of colorectal cancer patients by Cytometric Bead Array and observed no correlation between CCL5 levels and the prognosis of colorectal cancer. Although many studies have focused on the roles CCL5 played in CRC growth and metastasis, its mechanism remains uncertain.

In this study, we detect the expression of cytokines and chenokinesin colorectal cancer using antibody microarray. Our result

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Table 1
Clinical characteristics of the colorectal cancer patients.

Age	Gender	Region	Histology	TNM
(year)				stage
46	female	Rectum	moderately differentiated	III
59	male	ascending	Moderately Differentiated	III
77	male	sigmoid	Poorly Differentiated	II
65	male	Rectum	Moderately Differentiated	III

demonstrated that RANTES/CCL5 was higher in CRC tissue than matched normal colorectal mucosa. To validate the result of the antibody microarray expression analysis, we examined CCL5 levels in flesh colorectal cancer specimens by enzyme-linked immunosorbentassay (ELISA), and measured CCL5 expression in colorectal cancer paraffin specimens by IHC. Then the associations of CCL5 expression with clinical pathologic parameters were analyzed and the influence of CCL5 expression on colorectal cancer progression was evaluated.

2. Materials and methods

Paired tissue samples of CRC and paired normal colorectal mucosa were taken from 4 patients with primary CRC. The clinicopathological data for CRC patients are documented in Table 1. The tissue samples obtained intraoperatively were immediately frozen in liquid nitrogen and stored at −70 °C until protein extraction. The study was approned by the ethical committees of Qian-Fo-Shan Hospital Affiliated Shandong University.

The antibody microarray was used according to manufacturer's protocol with AAH-CYT- G4000 kit (RayBiotech, USA). After antibody microarray slides (RayBio® Human Cytokine Antibody Microarray slides, USA) with 274 antibodies were dried at room temperature for 2 h, they were treated with 100 u L of blocking solution and incubated on shaker for 30 min at room temperature. After blocking, the array slides were incubated with 100 u L diluted samples on shaker overnight at room temperature. After washing step (Thermo Scientific Wellwash Versa, USA), the arrays were incubated with Biotin-conjugated antibody. Then the array members were incubated with Cy3 equivalent dye labeled-streptavidin in dark on shaker for 2 h at room temperature. The slide scanning was performed using GenePix 4000B Microarray Scanner (Molecular Devices, LLC ; 1311 Orleans Drive Sunnyvale, CA 94089-1136, United States) and ArraySifter Express 1.3 (Vidar Systems). The slide was scanned at 10 μm resolution (PMT: 650, Wavelengh : 532 nm). The numeric data were analyzed using analytic software (AAH-CYT-G6-10, RayBiotech, USA).

3. Elisa

Paired samples of colorectal cancer and mucosa were obtained from 38 patients during surgery in Qian-Fo-Shan Hospital Affiliated Shandong University. Tumor samples were taken from vital areas of histopathologically confirmed adenocarcinomas. Mucosa samples were taken from unaffected mucosa, 5 cm distal to the tumor margin. The tissue was harvested immediately after resection of the colon and rectal, washed in ice cold phosphate-buffered saline (PBS). Tissue samples were minced and homogenized on ice in PBS. Homogenates were centrifuged at 5000 *g*, 4 °C for 10 min, and supernatant liquid was collected. Protein concentration was assayed by the method by Bradford (Bio-Rad

Protein Assay, Bio-Rad, München, Germany). RANTES/CCL5 was determined by ELISA kit (RayBio® Human I-TAC, USA), according to the instructions of the producer. CCL5 levels were indicated as CCL5 [mg]/sample volume [ml].

4. Immunohistochemistry

Immunohistochemical studies were performed on tissue specimens from 60 patients who had been clinically diagnosed with primary CRC between 2014 and 2015 at Yantaishan Hospital. Tissues (IHC-P - paraformaldehyde-fixed, paraffin-embedded sections) underwent heat mediated antigen retrieval in sodium citrate buffer (pH 6.0). Sections were reacted with Polyclonal mouse Anti-RANTES antibody (dilution 1:150; abcam) and incubated with sample at 4 °C overnight. A HRP-labeled polymer detection system was used with an FAST-RED chromogen. The optimal primary antibody dilutions were predetermined using appropriate positive controls (T cells in CRC tissues) and negative controls (omission of primary antibody).

The extent and location of immunohistochemical staining for CCL5 was assessed according to the immunoreactive score (IRS) that evaluated the proportion and intensity of positive staining cells. Scores of the two parameters were independently assessed by two pathologists. Briefly, intensity of immunostaining in cancer cells was graded as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong); staining extent was graded as 0 (0%–4%), 1 (5%–24%), 2 (25%–49%), 3 (50%–74%) and 4 (> 75%). The two scores were multiplied and the IRS was determined as low (scores, 0–4), medium (scores, 6–8) and high (scores, 9–12). Disagreements among the researchers were resolved by consensus.

5. Statistical analysis

All statistical analyses were performed using the IBM SPSS Statistics 20.The differences of target proteins levels detected by antibody microarray between groups were assessed by paired t-tests. CCL5 protein concentration determined by an ELISA assay were shown as MEAN ± SEM and analyzed by Wilcoxon matched-pair signed-rank (two samples). Spearman Rank Correlation Test was used to evaluate the association between CCL5 expression and clinicopathological characteristics.

6. Results

Among 274 human proteins detected in 4 CRC tissues, 25 differently expressed proteins were found in tissues of colorectal cancer compared to their matched normal colorectal mucosa, including 14 up-regulated and 11 down –regulated proteins (Fig. 1), based on the significant difference ($P < 0.05$) of light signal intensities between two samples. The up-regulated proteins were shown in red and down –regulated proteins in green (Fig. 1a). Bar graph shows the relative expression levels of target proteins which were statistically significantly upregulated and downregulated in CRC compared with normal colon tissues ($P < 0.05$) (Fig. 1b).

In vitro, Cambien et al. [8] found that RANTES/CCL5 increased the growth and migratory responses of colon cancer cells from both human and mouse origins. In addition, systemic treatment of mice with CCL5-directed antibodies reduced the extent of development of subcutaneous. Analysis of levels of expression of human targets was performed by quantitative RT-PCR in surgical resection pieces of human colorectal carcinoma, increased levels of CCL5 expression were observed in colorectal tumors (6 fold, P,0.05).In our study, the mean level of CCL5 detected by Enzyme-linked immunosorbent assay (ELISA) in 38 CRC tissues were157.50 ± 89.88 mg/ml, in contrast, the levels of CCL5 in corresponding 38 normal colon mucosa was114.02 ± 75.57 mg/ml. The average expression level of CCL5 in tissue of colorectal cancer was significantly higher than that in corresponding normal colorectal mucosa ($P = 0.037$) (Fig. 2).

Immunohistochemistry revealed the positive location of CCL5 is cytoplasm of CRC cells and part of infiltrating lymphocytes in tumor. The distribution of all the CRC cases was 26.7% (16/60), 23.3%(14/60), 50%(30/60) according to expression score weak, medium and high respectively. Representative pictures of CCL5 positive expression were

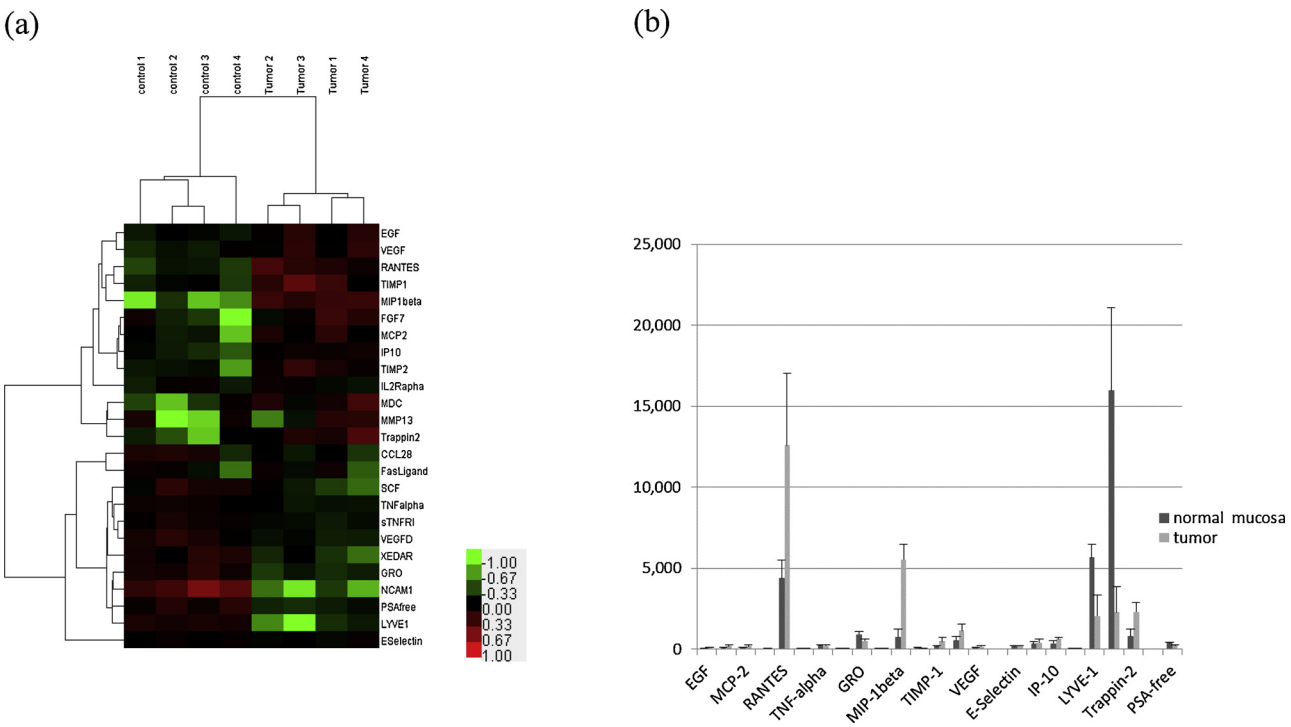

Fig. 1. (a) Protein expression was analyzed by antibody microarray. Differential protein expression between human colorectal cancer (CRC) tissues and normal mucosa identified by antibody microarray profiling. Four paired CRC tissue and normal mucosa samples were obtained from 4 patients with primary CRC following resection of the tumor. Proteins shown in the left column are those that the difference of light signal intensities beween two samples was statistically significant ($P < 0.05$). Red indicates higher expression in cancer tissues compared to normal colorectal mucosa; green indicates lower expression in cancer tissues. (b) The relative expression levels (fluorescent signal intensity) of target proteins in normal mucosa and CRC.

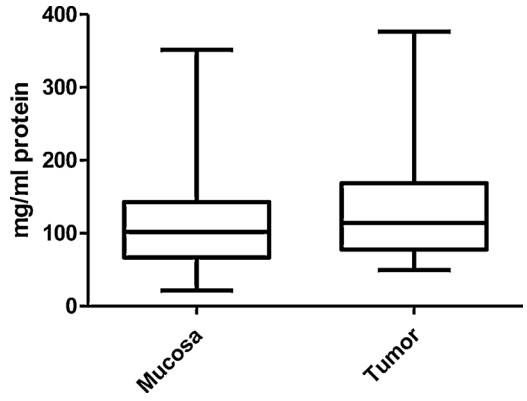

Fig. 2. Comparison of CCL5 levels in 38 colorectal carcinomas and corresponding normal mucosa.

showed as below (Fig. 3A–C). Positive CCL5 expression was positive associated with adverse pathological characteristics, including lymph node metastasis and higher T stage ($P = 0.02$ and 0.028, respectively). We have found no statistically significant correlations between high expression of CCL5 and other clinicopathological factors including gender, age, tumor size, region, histology and plasma levels of CEA (Table 2).

7. Discussion

In 2013, Japanese scholars Hisaaki Miyoshi et al. detected the expression of 506 target proteins of colorectal cancer with biotin-labeled antibody microarray and found that, in CRC tissues, IL-1α, GRO, Glut5, MIG, ICAM-5, VE-cadherin, uPA and Leptin R were increased when comparing to normal colon tissues, MPIF-1/CCL23, FGF R5, MIP2, SAA and IL-18 Rβ were strongly up-regulated in rectal cancer when comparing to the levels in non-rectal cancer [10].

In this study, we used a novel antibody microarray to analyze the expression of different proteins in CRC tissues. Using our assay, we have analyzed 207 target proteins, including chemokines, cytokines, growth factors, soluble enzymes, blood vessel formation factors and soluble receptors. Of 207 proteins screened, we got 25 differently expressed proteins in colorectal cancer compared to matched normal colorectal mucosa, including 14 up-regulated and 11 down –regulated proteins.

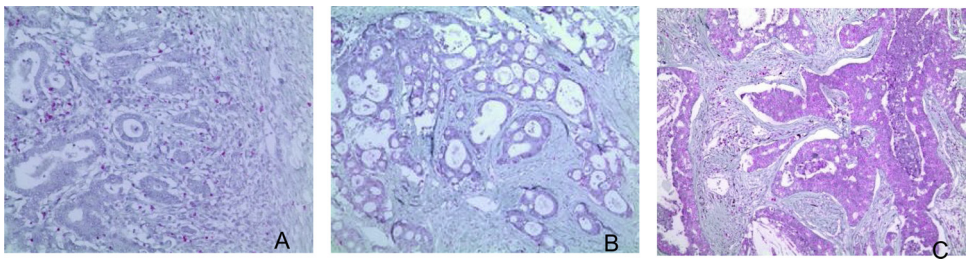

Fig. 3. Immunohistochemistry (IHC) for CCL5 in the cytoplasm of cancer colorectal cancer cells (Fast Red chromogen ×100): (A) low immunoreactivity (B) moderate immunoreactivity (C) high immunoreactivity.

Table 2
Associations between CCL5 expression and clinicopathological characteristics.

	total	CCL5 expresson			P	CC
		low	medium	high		
Gender						
Male	34	10	8	16	0.563	0.074
Female	26	6	6	14		
Age(years)						
≤50	11	3	3	5	0.512	0.086
>50	49	12	8	29		
Region						
Rectum	33	9	4	20	0.535	0.082
Colon	27	7	7	13		
Tumor size (cm)						
≤3	13	1	2	10	0.109	−0.209
>3 ≤ 5	30	7	8	15		
>5	17	7	1	9		
Histology						
poorly	20	7	4	9	0.369	0.118
Moderate	34	8	6	20		
well	6	1	2	3		
T stage						
T1, T2	15	4	8	3	0.028	0.285
T3, T4	45	7	13	25		
Lymph node metastasis						
No	35	19	9	7	0.002	0.400
Yes	25	5	6	14		
CEA(ng)						
≤5	31	11	5	15	0.156	0.187
>5	28	5	5	18		

Among the 25 proteins sifted, RANTES/CCL5, a novel protein highly expressed in CRC tissues, was selected as the representative candidate biomarker to be validated by ELISA. As expected, ELISA assay results were in complete concordance with that of antibody microarray analysis, On the one hand, this proves the reliability of our experimental data, and on the other hand, it provides a solid foundation and guarantee for our further research.

To further explore the expression sites of CCL5 in colorectal cancer, we investigated CCL5 expression in radical resected colorectal cancer specimens by immunohistochemistry. Positive CCL5 expression was detected in cytoplasm of colorectal cancer cells and some infiltrated inflammatory cells, while no positive staining were detected in mesenchymal cell. Based on semi-quantitative immunohistochemical study, CCL5 expression was positive correlated to advanced primary tumor stages, the more advanced tumor stage, and the higher staining scores. CCL5 expression was higher in CRC accompanied with lymph node metastasis than in those without. These results suggested that the CCL5 expression in colorectal cancer might promote tumor invasion and lymph node metastasis. Our findings are consistent with Cambien et al [8], who have found that CCL5 promoted migration of colorectal cancer cells in vitro and promoted the growth of tumor cells in dose dependent manner.

The exact function of CCL5 in CRC biology remains unclear. CCL5 is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites [2]. Similarly, CCL5 expressed by tumor cells could recruit the white blood cells such as T lymphocytes, macrophages inside the tumor or to the junction with normal tissue. In colorectal cancer tissues, the increased CCL5 level was thought to be positively correlated with the quantity and activity of CD8+ cytotoxic T cells [11]. CD8 + T cells is supposed to inhibit tumor growth and metastasis [11,12]. This might infer that the expression of CCL5 in colorectal cancer tissues has an antitumor effect. However, our studies suggest that CCL5 may have promoting effect in the development and lymph node metastasis of colorectal cancer. It has been shown that CCL5 could recruit regulatory T cells(Treg), and CCL5′s natural receptor CCR5 was highly expressed by human CRC infiltrating Treg, which may help the tumor cells to escape from the host immune response [13,14], and result in immunological tolerance in pancreatic tumors [8]. Therefore in colorectal cancer CCL5 could recruit not only CD8 + T cells but also regulatory T cells into tumor sites according to previous studies [11,13,14]. Treg cells can prevent CD8 + T cell differentiation into effector T cells. Moreover, CCL5 could enhance the killing ability of regulatory T cells on CD8 + T cells [15], which may induce immune suppression and increase the possibility of tumor progression and metastasis. The activity of CCL5 is mediated mainly by binding to its receptor CCR5 [16]. CCR5 was highly expressed by human CRC infiltrating Treg [17]. So we speculate that CCL5 produced by tumor cell allowed it to recruit CCR5 +Treg from blood into CRC. On the other hand, binding of CCL5 to its ligand CCR5 activates a series of downstream effects, which induces tumor cell proliferation and metastasis [18]. Moreover CCL5 could also induce macrophages secrete MMP9 and promote tumor metastasis [19].

In summary, our data demonstrated that CCL5 immunostaining positive correlated with node metastasis and advanced tumor stage in CRC cancers. CCL5 recruits different inflammatory cells into tumor site, under complex interaction of cytokines an immunosuppressive state was development in tumor microenvironment. On the other hand, tumor cells may promote their growth through autocrine mechanism by the binding of CCL5 to its ligands. The expression of CCL5 in tumor cells may provide a rationale for targeting CCL5/CCR5 axis in advanced CRC, and worth further studies both in vitro and in vivo.

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