Int J Clin Exp Pathol 2016;9(7):7202-7208
www.ijcep.com /ISSN:1936-2625/IJCEP0023559

Original Article

Expression of chemokine receptor CXCR5 in gastric cancer and its clinical significance

Qing Sun*, Lujun Chen*, Bin Xu, Qi Wang, Xiao Zheng, Peng Du, Dachuan Zhang, Changping Wu, Jingting Jiang

*Department of Tumor Biological Treatment, The Third Affiliated Hospital, Soochow University, Jiangsu Engineering Research Center for Tumor Immunotherapy, Changzhou, Jiangsu, China. *Equal contributors.*

Received January 8, 2016; Accepted March 22, 2016; Epub July 1, 2016; Published July 15, 2016

Abstract: The increased expression of chemokine receptor CXCR5 in cancers has been demonstrated. In order to characterize the expression pattern of CXCR5 in cell lines and tissues of gastric cancer and to assess clinical implications, the expression of CXCR5 mRNA in gastric cancer tissues and adjacent tissues was evaluated by real-time RT-PCR. Meanwhile, the expression of CXCR5 in cell lines of human gastric cancer was also analyzed by flow cytometry. Tissue microarray and immunohistochemistry were used to detect the protein expression of CXCR5 in human gastric cancer tissues and adjacent normal tissues. Flow cytometry results revealed the positive expression of CXCR5 in human gastric cancer cell lines such as BGC-823, SGC-7901 and HGC-27 cells. The immunohistochemistry results showed higher expression of CXCR5 in 52.87% of gastric cancer tissues. The expression of CXCR5 in patients with tumor size less than 2.8 cm subgroup was significantly lower than that in patients with tumor size larger than 2.8 cm subgroup (P = 0.0456). There was no significant correlation between the expression of CXCR5 and other clinical parameters in gastric cancer. Moreover, the survival analysis showed that the overall survival rate of the patients with higher CXCR5 expression was lower than that of the patients with lower CXCR5 expression (P = 0.0579, HR = 1.810, 95% CI: 0.9803-3.341). The results suggest that CXCR5 is involved in the oncogenesis and progression of gastric cancer.

Keywords: Gastric cancer, CXCR5, immunohistochemistry, microarray, flow cytometry

Introduction

Gastric cancer is the most common malignant tumor of upper digestive tract. Lymph node metastasis and abdominal implantation are the major causes of metastasis, which finally leads to cancer recurrence or cancer-related death [1]. Despite extensive studies on the identification of novel diagnostic and therapeutic agents, patients with advanced gastric cancer often suffer from a poor prognosis, and the treatment is still mainly dependent on conventional cytotoxic chemotherapy [2]. Thus, the advances in understanding novel factors involved in the progression of gastric cancer, and the development of prognostic and predictive markers, can potentially improve the therapeutic outcomes of this malignancy.

As a group of small molecule proteins, chemokines can bind target cells by chemotaxis through their receptors, which plays an essential role in the recruitment of leukocytes from the circulation system to local inflammatory sites [3]. Chemokines are able to regulate the infiltration of leukocytes in tumor, activate specific immune response, and stimulate the proliferation and migration of tumor cells by autocrine or paracrine models. The overwhelming evidences have supported that chemokine-chemokine receptor systems can regulate transformation, growth, neovascularization and metastasis of tumor cells [4].

The chemokine receptor CXCR5 expressed by B cells and certain T cells can control the migration of these cells into and within lymph nodes [5]. The abnormal expression of CXCR5 could be observed in many tumors and was closely associated with tumor progression [6]. However, the role of CXCR5 and its ligand in gastric cancer has not been investigated. The aim of the

Table 1. Correlation of clinical parameters in patients with the expression of CXCR5

Clinical parameters	Case	CXCR5		χ^2	*P*-value
		High	Low		
Gender					
Male	59	29 (49.2%)	30 (50.8%)	0.3020	0.5826
Female	28	12 (42.9%)	16 (57.1%)		
Age (years)					
< 60	33	17 (51.5%)	16 (48.5%)	0.4110	0.5215
≥ 60	54	24 (44.4%)	30 (55.6%)		
Tumor size (cm)*					
< 2.8	14	10 (71.4%)	4 (28.6%)	3.995	**0.0456**
≥ 2.8	71	30 (42.3%)	41 (57.7%)		
Tumor invasion depth (T)*					
T_1+T_2	11	7 (63.6%)	4 (36.4%)	1.200	0.2733
T_3+T_4	74	34 (45.9%)	40 (54.1%)		
Nodal metastasis (N)*					
Yes	22	10 (45.5%)	12 (54.5%)	0.0132	0.9083
No	64	30 (46.9%)	34 (53.1%)		
Distant metastasis (M)*					
No	76	35 (46.1%)	41 (53.9%)	0.6891	0.4065
Yes	10	6 (60%)	4 (40%)		
TNM stage*					
I+II	35	15 (42.9%)	20 (57.1%)	0.6893	0.4064
III+IV	50	26 (52%)	24 (48%)		

Values in bold were defined as $P < 0.05$. *Note: partial clinical data loss.

present study was to detect the expression of CXCR5 in gastric cancer tissues and to analyze its clinical significance.

Materials and methods

Patients and tissue microarray

Tissue microarray for human gastric cancer (HStm-Ade180Sur-05) was purchased from Shanghai Outdo Biotech Co., LTD (Shanghai, China). The tissue microarray included 90 cases of gastric cancer and adjacent normal tissues. All patients including 60 male and 30 female patients underwent surgical stomach resection between July 2006 and April 2007. The median age of these patients was 65 years and survival data were available, with follow-up to 2013 August. In addition, the gastric cancer tissues and adjacent normal tissues from 14 cases during the surgical resection were collected to determine the expression of CXCR5 mRNA. No patients received pre-operative chemotherapy or radiotherapy before surgical resection. The clinical parameters of these patients are shown in **Table 1**.

Total RNA extraction, reverse transcription and real-time PCR

In brief, total RNA was extracted from tissues using a total RNA purification kit (BiocolorBioScience and Technology Company, Shanghai, China). Complementary DNA was synthesized with cDNA synthesis kit (Fermantas, Vilnius, Lithuania), according to the manufacturer's instructions. The primers of CXCR5 and GAPDH were designed according to the National Center for Biotechnology Information (NCBI) database, and then synthesized by Sangon Biotech (Shanghai, China). The sequences of all primers used in this study are as follows: CXCR5-F: GGTCACCCTACCACATCGTC, CXCR5-R: GCCATTCAGCTTGCAGGTATTG, GAPDH-F: ACAACTTTGGTATCGTGGAAGG, GAPDH-R: GCCATCACGCCACAGTTTC. The mRNA level of CXCR5 was measured by real-time PCR using SYBR Green method on the Applied Biosystems 7500 real-time PCR system (Applied Biosystem, USA). All experiments were performed in triplicate. Data were normalized to the housekeeping gene GAPDH, and the relative abundance of transcripts was calculated by the comparative $^{\Delta\Delta}$CT method.

Immunohistochemistry

Immunohistochemical staining was performed using the EnVision™ method according to the manufacturer's instructions. The tissue microarray section was dewaxed in xylene, rehydrated and graded ethanol solutions. Antigen retrieval was conducted by heating tissue sections at 100°C for 30 min in EDTA (pH 9.0) solution. Then, the sections were immersed in a 3% hydrogen peroxide solution for 30 min to block endogenous peroxidase activity, rinsed in phosphate buffered saline (PBS) for 5 min, blocked with 3% BSA at room temperature for 30 min, andincubated with purified rabbit anti-human CXCR5 antibody (Millipore, USA) at 4°C over-

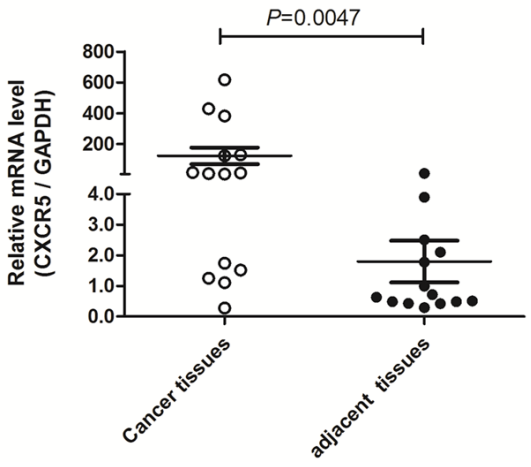

Figure 1. CXCR5 mRNA expression in gastric tissues. The expression level of CXCR5 mRNA in gastric cancer tissues was significantly higher than that in adjacent gastric tissues (P = 0.0047).

night. The section omitting primary antibody was used as the negative control. The horseradish peroxidase (HRP)-labeled goat anti-mouse/rabbit secondary antibody used in immunohistochemical staining was purchased from Dako (Glostrup, Denmark). Diaminobenzene was used as the chromogen and hematoxylin as the nuclear counterstain. Sections were dehydrated, cleared and mounted.

Evaluation of CXCR5 immunostaining

Immunohistochemical staining was assessed using the *H-score* method as described in our previous study [7]. *H-score* = (% tumor cells unstained × 0) + (% tumor cells stained weak × 1) + (% tumor cells stained moderate × 2) + (% tumor cells stained strong × 3). The H-scores ranged from 0 (100% negative tumor cells) to 300 (100% strong staining tumor cells). Results from two pathologists were averaged and used in the statistical analysis.

Cell culture

Human gastric cancer cell lines such as BGC-823, SGC-7901 and HGC-27 cells were purchased from Shanghai Academy of Sciences Library (Shanghai, China). Fetal bovine serum (FBS) and RPMI 1640 medium were purchased from Gibco Company (CA, USA), All cell lines were cultured in RPMI1640 medium supplemented with 10% FBS. The cell lines were incubated instandard culture conditions (5% CO_2, 37°C).

Intracellular staining and flow cytometry analysis

The expression of CXCR5 in cells was examined by flow cytometry using IntraPrepPermeabilization Reagent. Human gastric cancer cells such as BGC-823, SGC-7901 and HGC-27 cells were cultured in a standard condition (5% CO_2, 37°C). Harvested cells and dispersed cells (5×10^5~1×10^6) were washed with PBS and aliquoted into 100 mL of IntraPreppermeabilization reagent 1 (Immunotech, Marseille, France) for 15 min at room temperature. After washing once with 1 mL of Hanks' buffered saline solution (HBSS) with 2% FBS, the cells were permeabilized for 5 min with IntraPreppermeabilization reagent 2. The cytoplasmic expression of CXCR5 was detected by flow cytometry (FACSCanto II, BD, USA), and the data were analyzed by using FlowJo10.0.6.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0. The T-test was used to analyze the difference in the expression of CXCR5 protein between cancer tissues and adjacent normal tissues. The χ^2 tests were used to analyze the relationship between CXCR5 expression and pathological parameters. Overall survival rate of patients with different clinic-pathological parameters was compared by log-rank survival analysis. A statistically significant difference was considered at $P < 0.05$.

Results

Expression of CXCR5 mRNA in gastric tissues

As shown in **Figure 1**, the mRNA of CXCR5 could be observed in gastric tissues, and the mRNA expression level of CXCR5 in gastric cancer tissues was significantly higher than that in adjacent normal gastric tissues (P = 0.0047).

Expression of CXCR5 in gastric cancer cell lines, tumor and adjacent normal gastric tissues

As shown in **Figure 2**, high expression of CXCR5 was detected in BGC-823, SGC-7901 and HGC-27 gastric cancer cells. Immunostaining results

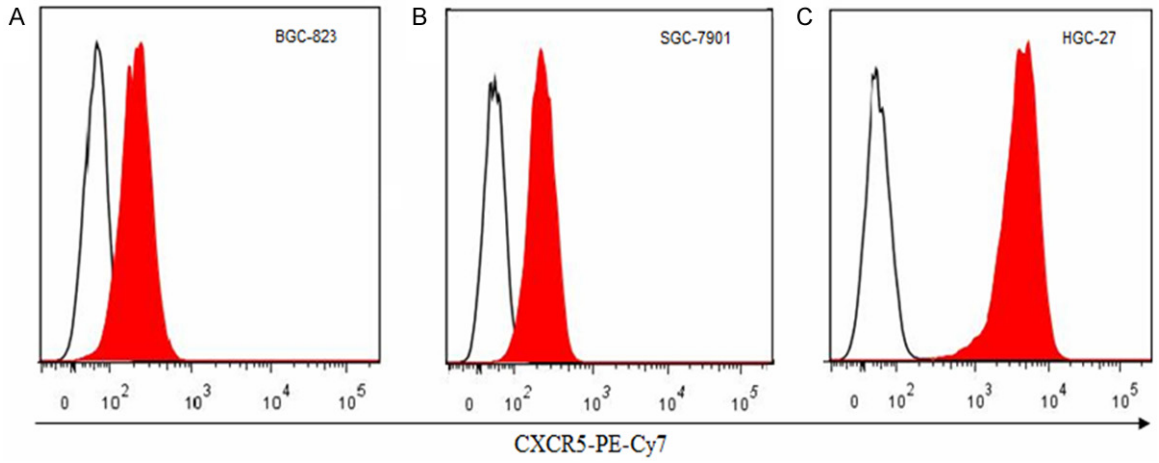

Figure 2. A. CXCR5 was overexpressed in gastric cancer cell line, BGC-823; B. CXCR5 was over-expressed in gastric cancer cell line, SGC-7901; C. CXCR5 was over-expressed in gastric cancer cell line, HGC-27.

showed that CXCR5 was mainly localized on the membrane of tumor cells, and adjacent normal gastric tissues. In addition, there was no significant difference in CXCR5 expression between 87 cases of gastric cancer patients and adjacent normal mucosa tissues (**Figure 3**).

Relationship of CXCR5 expression and clinical parameters

All patients were divided into two groups according to the staining intensity: HS-score < 5 (41cases) and HS-score ≥ 5 (46 cases). The results of tissue microarray and immunohistochemistry showed that the ratio of higher CXCR5 expression in gastric cancer tissues was 52.87%. The expression of CXCR5 differed significantly according to tumor size in patients ($P < 0.05$). Nevertheless, no significant correlation was observed between CXCR5 and other parameters such as gender, age, pathological grade, tumor location and TNM staging ($P > 0.05$, **Table 1**).

Prognostic value of CXCR5 expression in gastric cancer tissues

As shown in **Figure 4**, 90 cases of gastric cancer patients with follow-up data were included for survival analysis. The log-rank survival analysis showed that patients with high CXCR5 expression had poorer overall survival rate than that of the patients with low CXCR5 expression ($P = 0.0579$, HR = 1.810, 95% CI: 0.9803-3.341).

Discussion

Chemokines are small molecular polypeptides with molecular weights of 8-10 kDa, and can lead to the infiltration of inflammatory cells in tumor tissues, and promote the angiogenesis, tumor cell proliferation, alive and dissemination [4]. Recent studies have demonstrated that chemokines and their receptors play an important role in organ-specific metastasis of malignant tumor [8, 9]. Chemokines and their receptors are highly expressed in certain tumor cells and tissues. The expression level is closely correlated with many clinical parameters. For example, higher expression of CXCR1, CXCR2 and CXCR4 with corresponding ligands of CXCL5, CXCL8 and CXCL12 is highly associated with tumor angiogenesis, metastasis, and poor survival of the patients with lung cancer [10, 11].

CXCR5, one of the members for CXC chemokine receptor family, is mainly distributed in mature B cells and a few T cell subsets. CXCR5 plays a significant role in B cell homing to secondary lymphoid tissue [12]. It is a transmembrane receptor containing 7 transmembrane domains coupled with guanosine triphosphate 2 (GTP2) [13]. CXCR5 contributes to the returning of B lymphocytes to lymph node follicles, and promotes lymph follicle assembly as one of the important lymphnode factors. CXCR5 is expressed primarily in mature, recirculating B cells and small subsets of $CD4^+$ and $CD8^+$ T cells. The migration of these leukocytes into

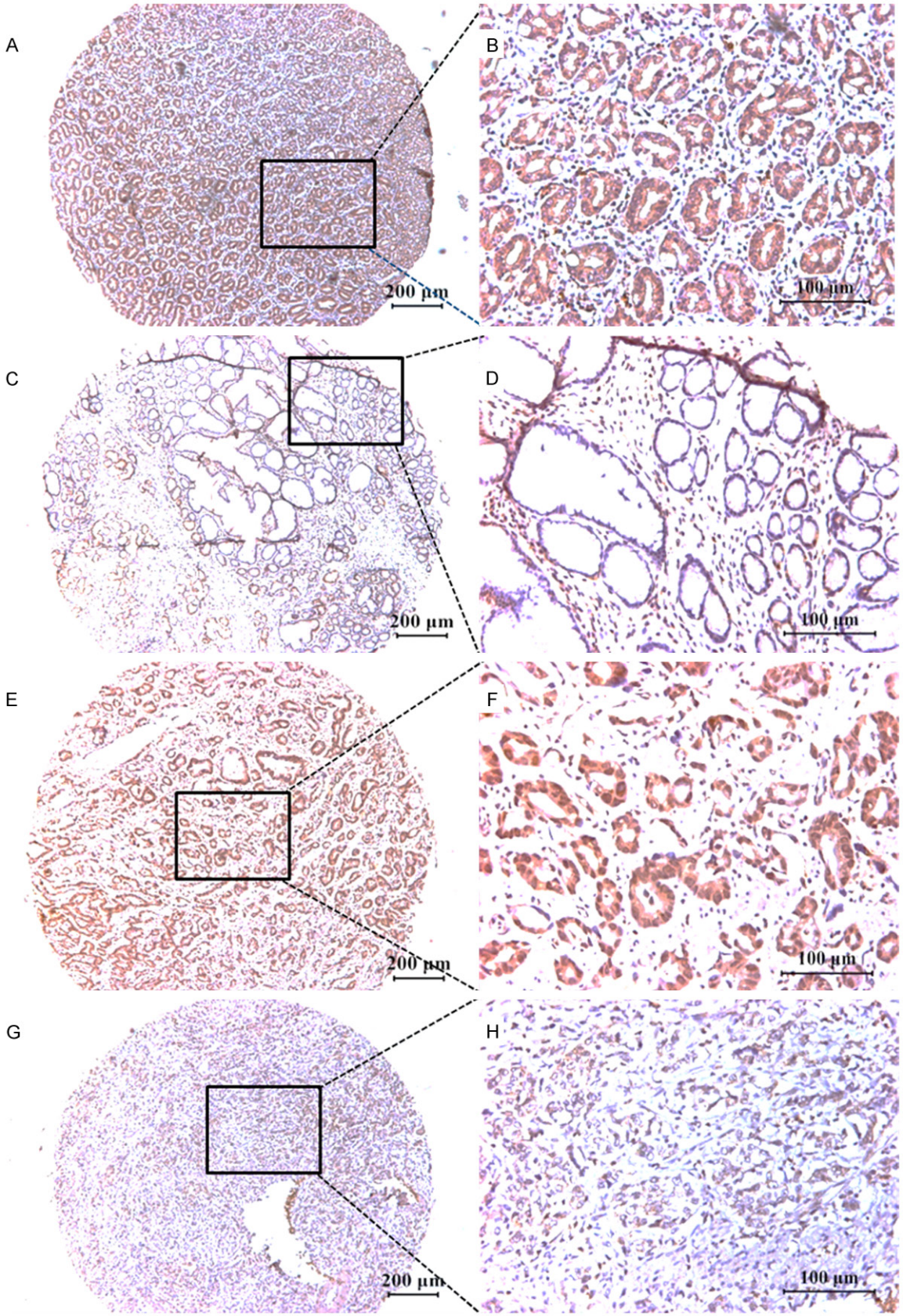

Figure 3. Immunohistochemical staining of CXCR5 in gastric cancer and adjacent normal mucosa tissues. A, B. The high expression of CXCR5 in adjacent normal mucosa tissues; C, D. The low expression of CXCR5 in adjacent

normal mucosa tissues; E, F. The high expression of CXCR5 in gastric cancer; G, H. The low expression of CXCR5 in gastric cancer.

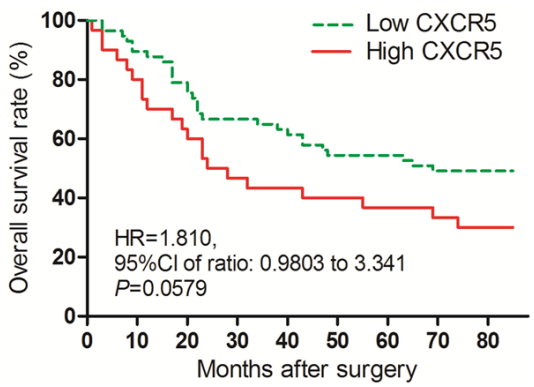

Figure 4. Prognostic values of CXCR5 in human gastric cancer. The log-rank survival analysis showed poorer overall survival rate of the patients with high CXCR5 expression than that of the patients with low CXCR5 expression (P = 0.0579, HR = 1.810, 95% CI: 0.9803-3.341).

lymph nodes is controlled by CXCR5/CXCL13 interaction [14]. It has been demonstrated that the CXCR5/CXCL13 axis is involved in the development and the progression of solid tumors, such as breast and neuronal cancers [15, 16]. Moreover, the clinical and biological relevance of CXCL13/CXCR5 pathway has been demonstrated to have a critical role in invasion and migration of prostate cancer [17].

The increasing evidences suggest that multiple chemokines and their corresponding receptors are involved in the progression of gastric cancer and play significant roles in metastatic process. So far, there are few reports regarding the expression of CXCR5 in gastric cancer tissues. In the present study, the expression of CXCR5 in gastric cancer tissues was detected, and the role of CXCR5 in the development of gastric cancer, and its prognostic value in patients with gastric cancer were explored. We found that the expression level of CXCR5 mRNA in gastric cancer tissues was significantly higher than that in adjacent normal gastric tissues, indicating that over-expression of CXCR5 might be involved in the progression of gastric cancer. Meanwhile, CXCR5 expression was positively associated with tumor size. In contrast, no significant correlation of CXCR5 with gender, age, pathological grade, tumor location and TNM stage was observed. In addition, CXCR5 was highly expressed in gastric cancer cell lines. Given the expression of CXCR5 in a substantial proportion of pancreatic carcinomas, this option may be particularly interesting for this aggressive and incurable tumor type [18]. The exact mechanism for the regulation of abnormal expression of CXCR5 in human gastric cancer and the potential biological function need to be further explored in the future.

Acknowledgements

This study was supported by the grants from the National Science and Technology Support Projects (2015BAI12B12), The National Natural Science Foundation of China (No. 31570877, 31570908, 81201741, 81301960, 31428005 and 81171653), Natural Science Foundation of Jiangsu Province (BK2011246, BK2011247), the project of Six Batch of Major Talent Summit (BRA2010037), the Key R&D Project of Science and Technology Department of Jiangsu Province (BE2015633 and BE2015634), and the Innovative Talents Training Project of Changzhou Health Bureau.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jingting Jiang, Department of Tumor Biological Treatment, The Third Affiliated Hospital of Soochow University, Changzhou 213003, Jiangsu, China. Tel: +86 519-68870978; Fax: +86 519-66621235; E-mail: jiangjingting@suda.edu.cn

References

[1] Zhang M, Liu L, Wang J, Lu K, Shu Y, Wang R and Liu P. Effects of high-intensity focused ultrasound for treatment of abdominal lymph node metastasis from gastric cancer. J Ultrasound Med 2015; 34: 435-440.

[2] Lee HJ, Song IC, Yun HJ, Jo DY and Kim S. CXC chemokines and chemokine receptors in gastric cancer: from basic findings towards therapeutic targeting. World J Gastroenterol 2014; 20: 1681-1693.

[3] Qi XW, Xia SH, Yin Y, Jin LF, Pu Y, Hua D and Wu HR. Expression features of CXCR5 and its ligand, CXCL13 associated with poor prognosis of advanced colorectal cancer. Eur Rev Med Pharmacol Sci 2014; 18: 1916-1924.

[4] Franciszkiewicz K, Boissonnas A, Boutet M, Combadiere C and Mami-Chouaib F. Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response. Cancer Res 2012; 72: 6325-6332.

[5] Meijer J, Zeelenberg IS, Sipos B and Roos E. The CXCR5 chemokine receptor is expressed by carcinoma cells and promotes growth of colon carcinoma in the liver. Cancer Res 2006; 66: 9576-9582.

[6] Charbonneau B, Wang AH, Maurer MJ, Asmann YW, Zent CS, Link BK, Ansell SM, Weiner GJ, Ozsan N, Feldman AL, Witzig TE, Cunningham JM, Dogan A, Habermann TM, Slager SL, Novak AJ and Cerhan JR. CXCR5 polymorphisms in non-Hodgkin lymphoma risk and prognosis. Cancer Immunol Immunother 2013; 62: 1475-1484.

[7] Chen L, Sun J, Wu H, Zhou S, Tan Y, Tan M, Shan B, Lu B and Zhang X. B7-H4 expression associates with cancer progression and predicts patient's survival in human esophageal squamous cell carcinoma. Cancer Immunol Immunother 2011; 60: 1047-1055.

[8] Rainczuk A, Rao J, Gathercole J and Stephens AN. The emerging role of CXC chemokines in epithelial ovarian cancer. Reproduction 2012; 144: 303-317.

[9] Yang L, Gao L, Chen Y, Tang Z, Zhu Y, Han P, Li X and Wei Q. The Differential Expression and Function of the Inflammatory Chemokine Receptor CXCR5 in Benign Prostatic Hyperplasia and Prostate Cancer. Int J Med Sci 2015; 12: 853-861.

[10] Gillard-Bocquet M, Caer C, Cagnard N, Crozet L, Perez M, Fridman WH, Sautes-Fridman C and Cremer I. Lung tumor microenvironment induces specific gene expression signature in intratumoral NK cells. Front Immunol 2013; 4: 19.

[11] Singh R, Gupta P, Kloecker GH, Singh S and Lillard JW Jr. Expression and clinical significance of CXCR5/CXCL13 in human nonsmall cell lung carcinoma. Int J Oncol 2014; 45: 2232-2240.

[12] Moser B. CXCR5, the Defining Marker for Follicular B Helper T (TFH) Cells. Front Immunol 2015; 6: 296.

[13] Jiang J, Karimi O, Ouburg S, Champion CI, Khurana A, Liu G, Freed A, Pleijster J, Rozengurt N, Land JA, Surcel HM, Tiitinen A, Paavonen J, Kronenberg M, Morre SA and Kelly KA. Interruption of CXCL13-CXCR5 axis increases upper genital tract pathology and activation of NKT cells following chlamydial genital infection. PLoS One 2012; 7: e47487.

[14] Zhu Z, Zhang X, Guo H, Fu L, Pan G and Sun Y. CXCL13-CXCR5 axis promotes the growth and invasion of colon cancer cells via PI3K/AKT pathway. Mol Cell Biochem 2015; 400: 287-295.

[15] Panse J, Friedrichs K, Marx A, Hildebrandt Y, Luetkens T, Barrels K, Horn C, Stahl T, Cao Y, Milde-Langosch K, Niendorf A, Kroger N, Wenzel S, Leuwer R, Bokemeyer C, Hegewisch-Becker S and Atanackovic D. Chemokine CXCL13 is overexpressed in the tumour tissue and in the peripheral blood of breast cancer patients. Br J Cancer 2008; 99: 930-938.

[16] Tang X. Tumor-associated macrophages as potential diagnostic and prognostic biomarkers in breast cancer. Cancer Lett 2013; 332: 3-10.

[17] Singh S, Singh R, Sharma PK, Singh UP, Rai SN, Chung LW, Cooper CR, Novakovic KR, Grizzle WE and Lillard JW Jr. Serum CXCL13 positively correlates with prostatic disease, prostate-specific antigen and mediates prostate cancer cell invasion, integrin clustering and cell adhesion. Cancer Lett 2009; 283: 29-35.

[18] Kim SJ, Ryu KJ, Hong M, Ko YH and Kim WS. The serum CXCL13 level is associated with the Glasgow Prognostic Score in extranodal NK/T-cell lymphoma patients. J Hematol Oncol 2015; 8: 49.