Association between CXCR2 and IL-22BP expression indicate a poor outcome for gastric adenocarcinoma progression

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Received March 14, 2015; Accepted April 1, 2016

DOI: 10.3892/ol.2016.4790

Abstract. C-X-C motif chemokine receptor type 2 (CXCR2), a key regulatory protein, has been associated with multiple roles in the progression of numerous tumors, including gastric adenocarcinoma (GA). However, the mechanism of CXCR2 in the development of tumors remains controversial and unclear. In a previous study, the expression of CXCR2 and interleukin-22 receptor 2 (IL-22BP) was observed in GA. This promoted the present study, which aimed to explore the association between the two proteins, and to further analyze their roles in GA. CXCR2 and IL-22BP protein expression was analyzed by immunohistochemistry and reverse transcription-quantitative polymerase chain reaction assays in gastric cancer (GC) tissue, additionally confirmed via western blotting and immunocytochemical analysis in the MKN-45, BGC-823 and SGC-7901 cell lines. The association between expression levels and clinicopathological characteristics was evaluated by the Mann-Whitney U and Kruskal-Wallis tests. Using Kaplan-Meier plots and Cox proportional hazard models, overall survival (OS) was analyzed. Compared with non-cancerous tissue, CXCR2 and IL-22BP were over expressed (P<0.001 and P<0.001, respectively), and were observed mainly in the cytoplasm (P=0.022 and P=0.014, respectively) in GA. The associated protein and messenger RNA levels were analyzed, and coexpression was identified. Increased expression and more positive cases of CXCR2 and IL-22BP were observed with advanced pathological tumor-node-metastasis (p-TNM) stage in GC (P<0.001 and P<0.001, respectively), as well as the presence and absence of lymph node metastasis (LNM) (P=0.003 and P=0.041, respectively) and deep or superficial muscular invasion (P=0.002 and P=0.004, respectively). In addition, an association between IL-22BP and tumor diameter was indicated (P=0.021). In a Kaplan-Meier analysis, compared with negative expression, the two proteins identified a group of patients with the shortest OS. Cox proportional hazard models revealed that the two proteins, in addition to p-TNM stage, LNM and depth of invasion, predicted a short time to OS. The coexpression of CXCR2 and IL-22BP was demonstrated in GA, which may indicate that CXCR2 is involved in more complex mechanisms and roles, and indicate a poor outcome in GA progression.

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Key words: C-X-C motif chemokine receptor type 2, interleukin-22 receptor 2, gastric adenocarcinoma, outcome, MKN-45, BGC-823, SGC-7901

Introduction

Gastric cancer (GC) is the second most frequent cause of cancer-associated mortality around the world (1). Although a number of studies have been performed to analyze a variety of molecules associated with GC development, only a few molecular mechanisms has been revealed and translated into clinical application (2-4). Despite making advances in treatment and increasing efforts into research over the past 10 years, the outcome of gastric cancer remains unsatisfactory. Increasing evidence indicates that tumors are promoted and sustained by inflammatory signals from the tumor microenvironment, which is important for tumor progression (5-7), including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and interleukin-8 (IL-8).

Previously, studies on the mechanisms and roles of C-X-C motif chemokine receptor type 2 (CXCR2) in GC were performed (8,9). CXCR2 belongs to the chemokine receptor family, which consists of G-protein-coupled receptors containing 7 transmembrane domains (10-12). The structure of the protein family is unique: The C-X-C motif chemokine receptor type 1 (CXCR1) and CXCR2 proteins have a single polypeptide chain that is 350, 355 or 360 amino acids in length; the protein share 76% amino acid identity to one another, with the highest homology over the membrane-spanning regions; and the receptor N- and C-termini structure also display high homology (11,12). However, CXCR1 and CXCR2 vary considerably in ligand specificity and affinity. CXCR2, with a high affinity for binding to numerous ligands, interacts with a wide range of chemokines, including C-X-C motif chemokine ligand (CXCL)8, CXCL1, CXCL2, CXCL5 and granulocyte chemotactic protein 2 (GCP-2/CXCL6), whereas the binding specificity for CXCR1 is limited to CXCL8 and CXCL6 with low affinity (13-15). In a previous study, certain functions of CXCR2

in GC development were identified, which revealed that CXCR2 may be involved in important signals that promote proliferation and invasion and be associated with cancer angiogenesis, metastasis and drug resistance in the tumor (16). Although numerous molecular mechanisms in certain tumors, including GC and in particular GA, were elaborated in the study, the expression, distribution and roles of CXCR2 were explored in little detail.

At present, two interleukin-22 (IL-22) receptors, interleukin-22 receptor 1 (IL-22R1) and interleukin-22 receptor 2 (IL-22R2/IL-22BP), have been reported. The IL-22/IL-22R expression pattern makes IL-22 an important cytokine for mediating the cross-talk between leucocytes and epithelia, including tumor cells (17,18). Compared with IL-22R1, fewer studies have been performed on IL-22BP to examine its structure and biological functions in certain tissue tumors (19-21). Previously, reports demonstrated that IL-22R1 and IL-22R2 share limited similar biological roles, and that the significant differences identified between their biological functions may be partly explained by the varied affinity of the two proteins binding to IL-22 (22,23). In a pre-experiment of the present study, CXCR2 and IL-22BP were indicated to be overexpressed in GA, which may provide another reason for exploring the mechanism of CXCR2 further.

The present study aimed to reveal the functions of CXCR2 and IL-22BP in GA, and any association between the two. The notable results indicate that the two proteins may be used as a novel clinical treatment possibility for GA.

Materials and methods

Human tissue samples and reagents. The tissues of 112 patients with primary GA, who underwent thoracic surgical procedures at The First Affiliated Hospital of Sun-Yat Sen University (Guangzhou, China) between January 2009 and July 2014 and had received no radiation treatment, chemotherapy or other anticancer treatments, were recruited to a tissue bank. The protocol of the present study was approved by the Sun-Yat Sen University Institutional Review Board, and included keeping all tissues frozen, and collecting 42 non-cancerous tissue samples that were located >5 cm away from the primary tumor edge site. The stage and histological type of the tumor were defined according to the 7th edition of the American Joint Committee on Cancer tumor-node-metastasis (TNM) staging system (24). The relevant clinicopathological data collected included age, gender, pathological tumor-node-metastasis (p-TNM) stage, lymph node metastasis (LNM), differentiation, depth of invasion and follow-up to overall survival (OS). The MKN-45, BGC-823 and SGC-7901 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Anti-CXCR2 (mouse monoclonal; dilution, 1:200; catalog no., MAB331) and anti-IL-22BP (polyclonal goat; dilution, 1:200; catalog no., AF1087) primary antibodies, which were shown to be highly specific in western blot, immunohistochemistry (IHC) and immunocytochemistry assays in pre-experiments, were purchased from R&D Systems Europe, Ltd., Abingdon, UK (data not shown).

IHC. IHC was performed as previously described (25,26). Tissue samples were fixed in 4% paraformaldehyde for 24 h at 4˚C, and were then dehydrated by graded alcohol and paraffin-embedded. The tissues were subsequently stored at 4˚C and were dewaxed and rehydrated in phosphate-buffered saline (PBS). The sections were subjected to antigen retrieval in sodium citrate buffer (pH 6.0) for 10 min at 100˚C and were washed with PBS. Endogenous peroxidases were quenched in PBS containing 3% H_2O_2 and 10% methanol for 30 min. Anti-CXCR2 and anti-IL-22BP antibodies were used at a dilution of 1:200 and 1:50, respectively, with overnight incubation at 4˚C. Experiments with no primary antibody were used as a negative control. Slides were then washed with PBS and incubated for 1 h with the horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig)G secondary antibody (dilution, 1:1,000; catalog no., S2023; Dako North America, Inc., Carpinteria, CA, USA). The immunohistochemical expression was independently assessed by two pathologists (Department of Pathology, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China). The Allred scoring system was applied to evaluate the entire slide, as previously described (27). The expression of CXCR2 and IL-22BP were defined as positive if distinct staining of the cytoplasm, cytomembrane or nuclear was observed in at least 10% of tumor cells.

Western blot analysis. Proteins were extracted from frozen tissues (including adenocarcinoma and non-cancerous tissues) in 100 mmol/l Tris (pH 7.5), 300 mmol/l NaCl, 4 mmol/l ethylenediaminetetraacetic acid, 2% NP40, 0.5% sodium deoxycholate and 1 mmol/l sodium orthovanadate. The protein was quantified, laemilli buffer was added to 10 μg protein, and samples were boiled for 5 min. Proteins were resolved on 4-15% gradient sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked and stained with the primary antibodies in PBS, 5% milk and 0.1% Tween 20, and washed in PBS and 0.1% Tween 20. Blots were incubated at 4˚C for >12 h with anti-CXCR2 (1:400), anti-IL-22BP (1:200) and anti-β-actin (clone AC-15; mouse monoclonal; dilution, 1:10,000; catalog no., A5441; Sigma-Aldrich, St. Louis, MO, USA). Following further washing with PBS, blots were incubated with horseradish peroxidase-conjugated goat anti mouse IgG (dilution, 1:1.000; Dako North America, Inc.). The cells were homogenized in protein lysis buffer (radioimmunoprecipitation assay buffer) containing 10% protease inhibitor (Sigma-Aldrich), and the protein concentrations were then quantified using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunocytochemistry. MKN-45, BGC-823 and SGC-7901 cells ($3x10^5$ at 50% confluency) were harvested and dissolved by the addition of SDS-containing lysis buffer. The lysate was used for SDS-polyacrylamide gel electrophoresis on a 75 g/l gel. The proteins were electrophoretically transferred from the gel to a nitrocellulose membrane. The transferred membrane was treated with anti-CXCR2 (dilution, 1:200) and anti-IL-22BP (dilution, 1:200) antibodies, followed by detection with peroxidase-conjugated goat anti-mouse IgG (dilution, 1:1.000). The two proteins were detected by immunocytochemistry. The normal gastric tissue cells were used as control.

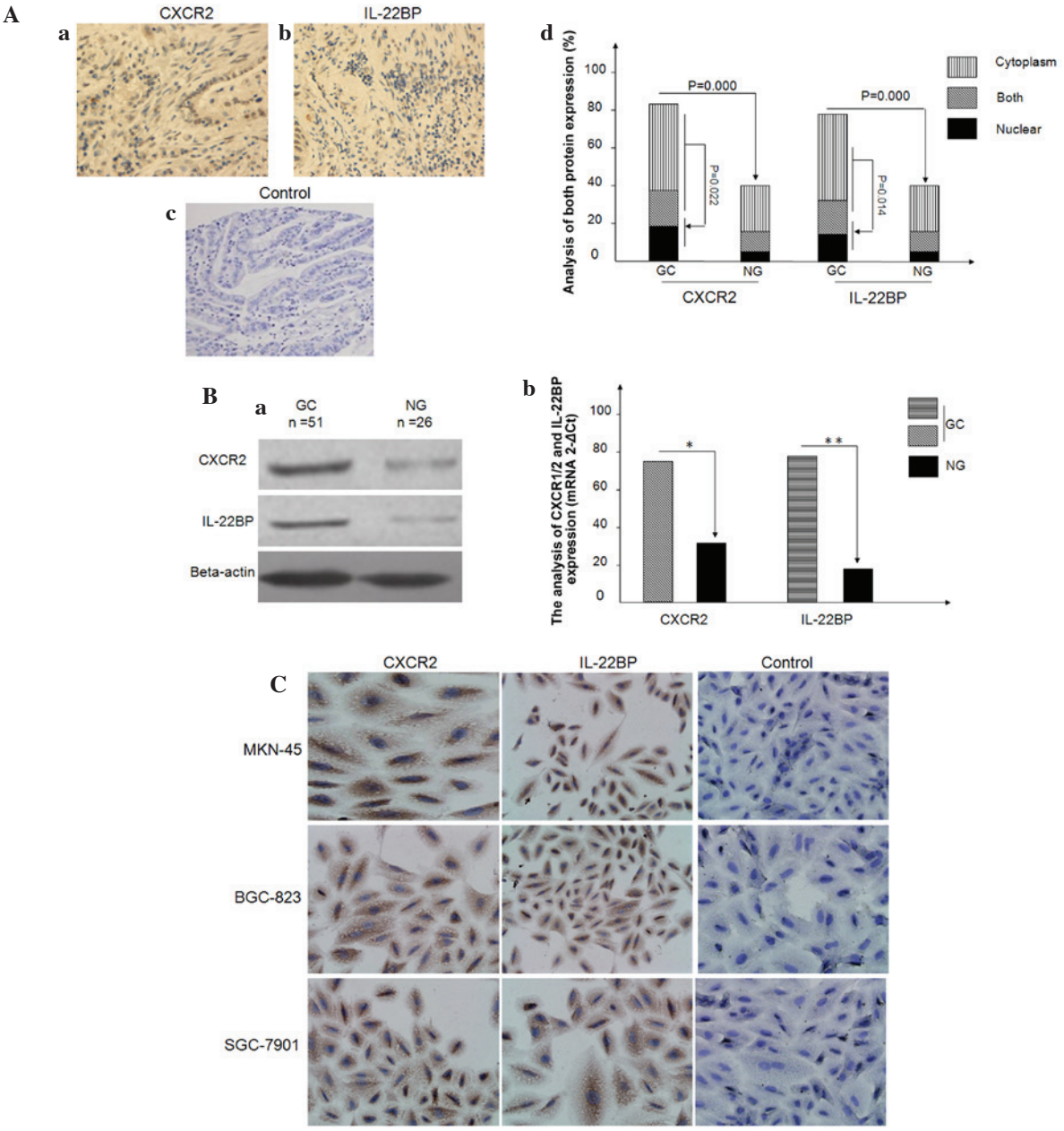

Figure 1. Expression of CXCR2 and IL-22BP proteins in GA via immunohistochemistry, western blotting and immunocytochemistry. Expression two proteins were defined as positive if distinct staining of the cytoplasm, cytomembrane or nuclear was observed in at least 10% of tumor cells. (Aa) CXCR2 and (Ab) IL-22BP expression in GA, compared with (Ac) non-cancerous tissue (magnification, x200). (Ad) Compared with non-cancerous tissue, the expression of CXCR2 and IL-22BP in GA was significantly increased, and the proteins were mainly located in the cytoplasm. Using western blotting, (Ba) CXCR2 and (Bb) IL-22BP expression was detected in frozen GA tissue, which confirmed the overexpression of the two proteins in GA. *P=0.013 and **P=0.008. (C) CXCR2 and IL-22BP expression was detected in the MKN-45, BGC-823 and SGC-7901 cell lines via immunocytochemistry (magnification, x400). CXCR2, C-X-C motif chemokine receptor type 2; IL-22BP, interleukin-22 receptor 2; GA, gastric adenocarcinoma.

RNA extraction and reverse transcription-quantitative polymerase chain reaction. Total RNA was extracted using the RNAeasy mini kit (Qiagen GmbH, Hilden, Germany) with additional purification by centrifugation through QIAshredder spin columns (Qiagen GmbH). RNA concentration and purity was calculated using the Nanodrop 2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Pittsburgh, PA USA). Complementary DNA (cDNA) from 100 ng of total RNA was amplified by PCR using the following primers and conditions: CXCR2 sense, 5'-ACT TTTCCGAAGGACCGTCT-3' and antisense, 5'-GTAACA GCATCCGCCAGTTT-3'; IL-22BP sense, AGGGTACAA TTTCAGTCCCGA and antisense, CGGCGTCATGCTCCA TTCTGA; incubated at 55˚C for 40 cycles. Levels of genes of interest were normalized to β-actin.

Statistical analysis. The Mann-Whitney U, Kruskal-Wallis test and Spearman rank correlation coefficient were used for statistical analysis using SAS 9.12 (SAS Institute, Inc., Cary, NC, USA). Associations with OS were analyzed initially by Kaplan-Meier plots (Log-rank test). Cox multivariate proportional hazards regression models were used to assess the OS power of these parameters. A P-value of <0.05 was considered to indicate a statistically significant difference.

Table I. Association between CXC2R and IL-22BP in gastric adenocarcinoma and non-cancerous tissues.

	Tumor tissue			Non-cancerous tissue		
Protein	No. of patients	Positive expression, %	P-value	No. of patients	Positive expression, %	P-value
mRNA			0.023			0.142
CXCR2	64	57.14		12	28.57	
IL-22BP	60	53.57		10	23.80	
Protein			0.021			0.023
CXCR2	82	73.21		15	35.71	
IL-22BP	85	75.89		13	30.95	

CXCR2, C-X-C motif chemokine receptor type 2; IL-22BP, interleukin-22 receptor 2; mRNA, messenger RNA.

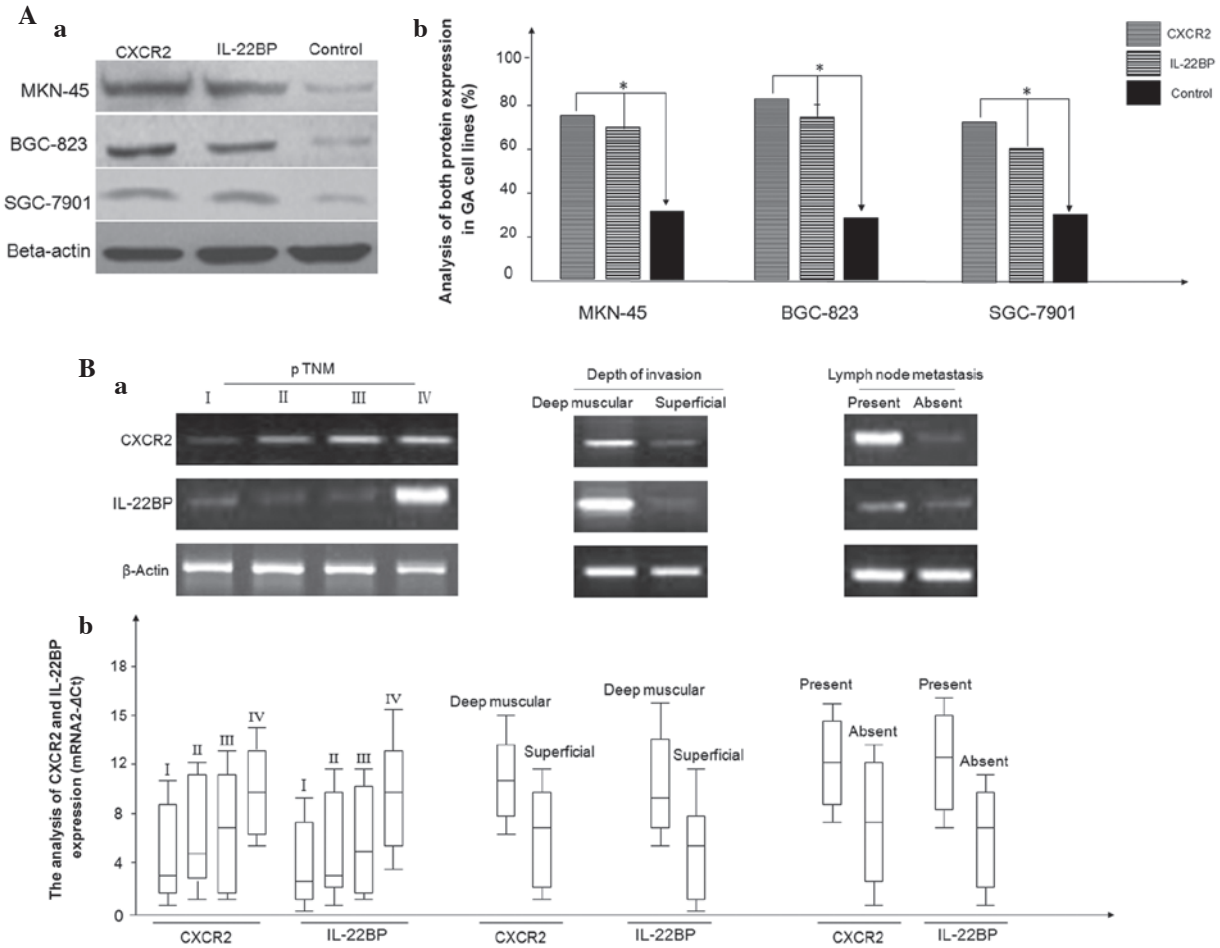

Figure 2. Analysis of CXCR2 and IL-22BP expression via western blotting and RT-qPCR. Expression of CXCR2 and IL-22BP in the MKN-45, BGC-823 and SGC-7901 cell lines via (Aa) western blotting and (Ab) quantification. Using (Ba) RT-qPCR and (Bb) additional quantification, the results revealed that the mean level of CXCR2 and IL-22BP expression was increased with advanced clinical stage of disease, increased depth of tumor invasion and lymph node metastasis. CXCR2, C-X-C motif chemokine receptor type 2; IL-22BP, interleukin-22 receptor 2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; p-TNM, pathological tumor-node-metastasis. *P<0.05.

Results

Expression pattern of CXCR2 and IL-22BP in GA. Immunohistochemistry assays revealed that CXCR2 and IL-22BP were overexpressed in GA (73.21%, 82/112, P<0.001 and 75.89%, 85/112, P<0.001; respectively) (Fig. 1Aa-1Ac; Table I). In addition, the expression of the two proteins was identified in the GA frozen tissue via western blot analysis (Fig. 1B) and the MKN-45, BGC-823 and SGC-7901 cell lines via immunocytochemistry analysis (Fig. 1C; Table I). CXCR2 and IL-22BP were also indicated to be mainly located in the cytoplasm (P=0.022 and P=0.014, respectively; Fig. 1Ad).

Table II. Association between the immunohistochemical expression of CXCR2 and IL-22BP and the clinicopathological parameters of gastric cancer.

	CXCR2 expression			IL-22BP expression		
Parameters	+	-	P-value	+	-	P-value
Gender			0.968			0.148
Male	55	20		60	15	
Female	27	10		25	12	
Age, years			0.909			0.150
<50	40	15		45	10	
≥50	42	15		40	17	
p-TNM stage			<0.001			<0.001
I	5	12		4	13	
II	11	8		12	7	
III	32	6		36	2	
IV	34	4		33	5	
Tumor size			0.231			0.021
<5 cm	46	13		50	9	
≥5 cm	36	17		35	18	
Differentiation			0.293			0.076
Well/moderate	40	18		40	18	
Poor	42	12		45	9	
Lymph node metastasis			0.003			0.041
Present	42	6		41	7	
Absent	40	24		44	20	
Depth of invasion			0.002			0.004
Superficial	31	21		33	19	
Deep muscular	51	9		52	8	

CXCR2, C-X-C motif chemokine receptor type 2; IL-22BP, interleukin-22 receptor 2; p-TNM, pathological tumor-node-metastasis.

The increased expression of the two proteins was observed in all three GA cell lines via western blot analysis (P<0.05; Fig. 2A).

Messenger RNA quantification was performed using RT-qPCR, and the results were compared with β-actin as the internal reference gene. CXCR2 and IL-22BP expression levels ranged between 0.308 and 14.362 (median, 7.204) and between 0.227 and 15.154 (median, 7.132), respectively. The expression levels of CXCR2 and IL-22BP increased gradually with advanced p-TNM stage (Fig. 2B), invasion depth (superficial to deep muscular) and LNM (absent to present). By adopting cut-off values according to the median expression levels, compared with non-cancerous tissues, a significantly increased expression of the two proteins was observed (Fig. 2B; Table I).

Association between CXCR2 and IL-22BP expression and clinical characteristics. The associations between CXCR2 and IL-22BP expression and a range of standard clinicopathological parameters were tested. The Mann-Whitney U and Kruskal-Wallis tests indicated a significant association between CXCR2 and IL-22BP expression and p-TNM stage (P<0.001 and P<0.001, respectively), LNM (P=0.003 and P=0.041, respectively) and depth of invasion (P=0.002 and P=0.004, respectively) (Table II). In addition, the percentage of cells with positive CXCR2 and IL-22BP expression increased with advanced p-TNM stage. IHC and RT-qPCR also indicated an association between the expressions of the two proteins (P=0.021 and P=0.023, respectively; Table I). Following completion of the Kruskal-Wallis test, an association was indicated between tumor diameter and IL-22BP expression, but not with CXCR2 expression (Table II).

Association of CXCR2 and IL-22BP expression with OS. Kaplan-Meier survival analysis was used to determine survival with respect to CXCR2 and IL-22BP. The increased expression of CXCR2 and IL-22BP was indicated to predict a significantly shorter OS (Log-rank 3.652, P=0.015 and log-rank 3.443, P=0.025, respectively). Similar results were observed from other clinical characteristics, including p-TNM stage (log-rank 7.051, P=0.032), depth of invasion (log-rank 6.281, P=0.014) and LNM (log-rank 4.821, P=0.017).

A Cox regression analysis was performed in order to assess whether the expression of the two proteins may be prognostic of survival, independently from other variables.

Table III. Kaplan-Meier (univariate) and Cox multivariate proportional hazard analysis.

	Univarivate analysis		Multivariate analysis	
Factors	Log-rank	P-value	Hazard ratio (95% CI)	P-value
Age, years <50 ≥50	4.042	0.351	6.82 (3.03-8.36)	0.213
CXCR2 expression Negative Positive	3.652	0.015	3.70 (0.32-7.51)	0.008
IL-22BP expression Negative Positive	3.443	0.025	3.30 (1.23-6.22)	0.025
p-TNM stage I-II III-IV	7.051	0.032	2.41 (1.45-5.13)	0.015
Depth of invasion Superficial Deep muscular	6.281	0.014	2.79 (2.11-6.24)	0.018
Lymph node metastasis Present Absent	4.821	0.017	4.96 (2.37-7.04)	0.034

CXCR2, C-X-C motif chemokine receptor type 2; IL-22BP, interleukin-22 receptor 2; p-TNM, pathological tumor-node-metastasis; CI, confidence interval.

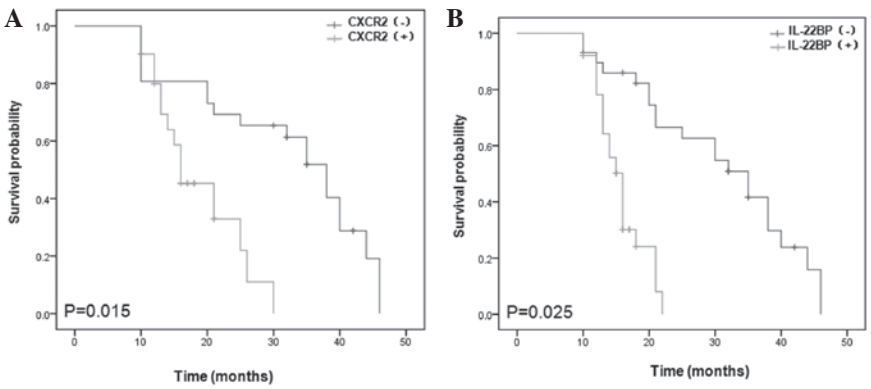

Figure 3. Evaluation of (A) CXCR2 and (B) IL-22BP as a predictor for OS by Kaplan-Meier plots. Association between CXCR2 and IL-22BP expression and OS was observed, and indicated that positive CXCR2 and IL-22BP expression is associated with shorter OS time. Through analyzing the results and data (Table III), CXCR2 and IL-22BP show potential role as a predictor for OS in gastric adenocarcinoma progression. OS, overall survival.

The multivariate analysis indicated a significant effect and revealed that increased CXCR2 and IL-22BP expression (P=0.008 and P=0.025, respectively), p-TNM stage (P=0.015), depth of invasion (P=0.018) and LNM (P=0.034) are independent prognostic factors for survival (Fig. 3; Table III).

Discussion

Increasing numbers of studies regarding novel treatments for GA are being reported due to the gradually rising incidence of gastric diseases, worldwide (28,29). GA is the most common neoplastic tumor of the stomach, and is, therefore, the focus of the present study. The present study investigated the expression patterns of the CXCR2 and IL-22BP proteins and their functions in GA. As a result, the clinical value of CXCR2 and IL-22BP was identified. In addition, through evaluating the OS of patients, CXCR2 and IL-22BP were indicated to demonstrate potential as important molecular prognostic factors.

CXCR2, a member of the G-protein-coupled receptor family, is an important receptor for proteins including CXCL1,

CXCL8 and CXCL6 (14,15). Previous studies report that, in the progression of certain solid tumors, CXCL8-mediated activation of the CXCR2 biological axis is important for the regulation of the phosphoinositide 3 kinase/Ras/Raf pathway (30-32). The differences between CXCR1 and CXCR2 were previously identified, and the latter showed a high affinity for binding to numerous ligands, including CXCL8, CXCL1, CXCL2, CXCL5 and GCP-2 (13-15,30,31,33-35). Yang *et al* (32), reported that the Ras-induced transformation of ovarian tumors may depend largely on the roles of CXCL1 binding to the CXCR2 axis. Although the biological functions of CXCR2 have been investigated widely, the role of CXCR2 for GA tumorigenesis has not been clarified in detail.

IL-22BP, the second IL-22 receptor, is a soluble-secreted receptor that belongs to the IL-10 cytokine family (36,37). Compared with the first receptor, IL-22R1, certain studies reported that the binding of IL-22 to IL-22BP has a 20-1,000-fold higher affinity (22,23); therefore the structure and biological functions of IL-22BP are of value for additional studies. One study revealed that colon tumor development was strongly accelerated and the diameter of tumors was increased in IL-22BP-/- using a colitis-associated colon cancer mice model, and showed that low expression of IL-22BP may be important in colon tumor progression (36). These results suggest that IL-22BP may perform important, complex functions in tumor development, but its role in GA is less understood.

In the present study, coexpression between CXCR2 and IL-22BP was observed in GA via IHC, immunocytochemistry assays, western blot analysis and RT-qPCR experiments. In addition, CXCR2 and IL-22BP were indicated to be mainly located in the cytoplasm, and demonstrated increased expression with GA advanced p-TNM stage, depth of invasion and LNM, which may reveal that the two proteins are important for promoting GA progression. The present study investigated the association between CXCR2 and IL-22BP expression and revealed the importance of the two proteins in GA, and the results for CXCR2 was in agreement with previous reports (16). Although the tumor tissues are varied, the biological effect on tumor development showed similarity, which may indicate that IL-22BP has similar biological mechanisms.

The data in present study showed that CXCR2 and IL-22BP proteins may be a powerful predictor of OS in GA. In Kaplan-Meier plots, compared with negative expression, either CXCR2 or IL-22BP positive expression showed significant association with poor OS. In addition, a COX regression analysis was performed, which revealed that the increased expression of either CXCR2 or IL-22BP protein may be an independent prognostic factor. These results were agreement with the expected results of the study.

The results revealed that CXCR2 and IL-22BP proteins may take part in GA progression, and explored the mechanisms of CXCR2 on regulating tumor development. Although *in vitro* experiments with CXCL1, CXCL8 or other ligands were not performed, the close association in GA progression between CXCR2 and IL-22BP proteins has sufficient clinical value to be explored further. Although the mechanisms for tumor progression remain unclear, the functions of the two proteins in GA development are notable and may be considered as a novel pathway for GA treatment.

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