CXCL1 promotes tumor growth through VEGF pathway activation and is associated with inferior survival in gastric cancer

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Abstract

The chemokine (C-X-C motif) ligand 1 (CXCL1) regulates tumor–stromal interactions and tumor invasion. However, the precise role of CXCL1 on gastric tumor growth and patient survival remains unclear. In the current study, protein expressions of CXCL1, vascular endothelial growth factor (VEGF) and phospho-signaling transducer and activator of transcription 3 (p-STAT3) in primary tumor tissues from 98 gastric cancer patients were measured by immunohistochemistry (IHC). CXCL1 overexpressed cell lines were constructed using Lipofectamine 2000 reagent or lentiviral vectors. Effects of CXCL1 on VEGF expression and local tumor growth were evaluated in vitro and in vivo. CXCL1 was positively expressed in 41.4% of patients and correlated with VEGF and p-STAT3 expression. Higher CXCL1 expression was associated with advanced tumor stage and poorer prognosis. In vitro studies in AGS and SGC-7901 cells revealed that CXCL1 increased cell migration but had little effect on cell proliferation. CXCL1 activated VEGF signaling in gastric cancer (GC) cells, which was inhibited by STAT3 or chemokine (C-X-C motif) receptor 2 (CXCR2) blockade. CXCL1 also increased p-STAT3 expression in GC cells. In vivo, CXCL1 increased xenograft local tumor growth, phospho-Janus kinase 2 (p-JAK2), p-STAT3 levels, VEGF expression and microvessel density. These results suggested that CXCL1 increased local tumor growth through activation of VEGF signaling which might have mechanistic implications for the observed inferior GC survival. The CXCL1/CXCR2 pathway might be potent to improve anti-angiogenic therapy for gastric cancer.

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Introduction

Gastric cancer (GC) is the fourth most prevalent cancer and the second common cause of cancer related death throughout the world [1–3]. It remains difficult to be cured because of limited effects of current chemotherapy and targeted therapy and a propensity for metastasis even in early stages of local tumor extent [4–6]. Previously we found that high expression of chemokine (C-X-C motif) ligand 1 (CXCL1) in lymphatic endothelial cells (LEC) promoted gastric cancer lymphatic metastasis by enhancing lymphatic endothelial cell migration and tube formation [7]. CXCL1, also termed GRO-1, was originally reported to be involved in carcinogenesis of melanoma [8]. CXCL1 binds specifically to the CXC chemokine receptor CXCR2, which is a member of the G protein–coupled receptor family [8]. Yang et al. reported that Ras-induced transformation of ovarian cells might depend largely on the activation of CXCL1 [9]. Cheng et al. also found that gastric cancer cells expressing CXCL1 exhibited an increase in migration and invasion, whereas CXCL1 or CXCR2 depletion significantly reduced migration and invasion [10]. Similar results were also reported in colitis associated cancer [11], spontaneous colorectal cancers [12], ovarian cancer [13] and pancreatic cancer [14]. Although the CXCL1/CXCR2 axis has been implicated in tumor–stromal interactions, its function in angiogenesis and local tumor growth in gastric cancer remains to be investigated.

Angiogenesis is a critical aspect of cancer biology and subject to regulation by multiple molecular pathways. Vascular endothelial growth factor (VEGF) is the major stimulator to angiogenesis [15]. The signal transducer and activator of transcription 3 (STAT3) is an important signaling molecule for many cytokines and growth factor receptors involved in angiogenesis [16]. STAT3 is activated...
constitutively in a number of human tumors where it promotes tumor progression and invasiveness [17]. In gastric cancer cells, constitutive expression of CXCL1 was shown to increase the expression and activation of STAT3 [10]. STAT3 has been identified as a direct transcription activator for the VEGF gene promoter and leads to angiogenesis in vivo [18]. Moreover, we performed STAT3–DNA binding ChIP assay and found that the STAT3–DNA can bind to the promoter of VEGF and activate VEGF transcription in GC cells [19]. Dysregulated STAT3 activation and VEGF overexpression in human GC tissues have been shown to contribute to gastric cancer development and progression [20]. Also, STAT3 mediated matrix metalloproteinase 2 (MMP2) and VEGF transcription has been reported in GC cells [21]. This supports a potential mechanism that CXCL1 activates STAT3 to enhance the motility and invasion of cancer cells [10]. STAT3 is activated by phosphorylation at Tyr705. Once activated, STAT3 translocates into the nucleus, binds to specific DNA promoter sequences and induces gene expression [17]. Panopoulos et al. found that STAT3 controls neutrophil chemotactic responses to natural ligands for CXCR2 and regulates the magnitude of chemotactic-induced actin polymerization [22]. Based on these findings, we hypothesize that the CXCL1/CXCR2 axis promotes angiogenesis and local tumor growth in GC through activation of VEGF signaling.

In the present study, the expression of CXCL1 and VEGF was determined by immunohistochemistry using archival tissues from 98 cases of resected human GC. The contribution of CXCL1 to VEGF expression and local tumor growth was evaluated in vitro and in vivo. Our goal was to determine whether CXCL1 can activate VEGF signaling and lead to tumor growth in GC.

Materials and methods

Human tissue specimens and patient information

Human tissue specimens and patient information were obtained from the GC tissue bank and database at the First Affiliated Hospital of Sun Yat-sen University. Ninety-eight patients with primary GC were obtained for this study. All patients had undergone gastric resection with lymph node dissection at the First Affiliated Hospital of Sun Yat-sen University between 2006 and 2007. The patients had prospective well-documented clinical history and follow-up information. None of them underwent preoperative chemotherapy and/or radiation therapy. Follow-up assessments were performed every 3 months for the first 2 years after surgery and then every 6 months until the patient’s death. The stage of gastric carcinoma was determined according to the TNM Classification of Malignant Tumors established by the International Union against Cancer 7th edition [23]. Tumor histology and the location of regional lymph nodes were described according to the Japanese classification of gastric carcinoma [24]. The survival status of all patients was ascertained in December 2012 and the mean and median follow-up time is 30.4 and 27.5 months. The study protocol was approved by the Ethical Review Committee of First Affiliated Hospital of Sun Yat-sen University (IRB No. 097).

Immunohistochemistry (IHC) was conducted as described [25]. Tissue sections were incubated with primary antibodies against CXCL1 (R&D System, Minneapolis, MN, USA; Catalog no. MAB275; dilution 1:100), p-STAT3 Tyr-705 (Abcam, Cambridge, MA, UK; Catalog no. ab176315 dilution 1:100), p-JAK2 Y1007 + 1008 (Abcam, Cambridge, MA, UK; Catalog no. ab21201 dilution 1:100) or VEGF (Abcam, Cambridge, MA, UK; Catalog no. ab52917 dilution 1:100) at 4 °C overnight. Tissue sections were also stained with antibodies against CD31 (Bioworld Technology, USA; dilution 1:1000) or ki-67 (Epitomics, Burlingame, CA, USA; dilution 1:200). Primary antibodies were then detected by DAKO EnVision-HRP (Dako Company, Denmark). Immunohistochemistry scoring (HistoScore) was performed using a modified scoring method previously reported by Nenutil et al. [26]. Briefly, total cells and cells with positive staining were counted and the percent positive cells in each high power field were calculated. The negative group consisted of cancer cells with no detectable (−) or only trace staining for CXCL1. The positive group consisted of cancer cells with moderate (+) or high levels (+1) of CXCL1 immunoreactivity. Two independent pathologists observed 5 random fields of each specimen and scored each sample without knowledge of patient outcome. The results were scored by multiplying the percentage of positive cells [1] by the intensity [1], according to the formula: H = P × I. An average value of the two scores was presented in the present study.

Human GC cells AGS, SGC-7901, HGC-27, MKN-28, BGC-823 and the normal gastric epithelium cell GES-1 were obtained from the cell bank of Sun Yat-sen University, Guangzhou, China. Both cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified incubator at 5% CO2. These cell lines were authenticated using short tandem repeat (STR) profiling and cell viability analysis. All cell lines used in this study were regularly screened for mycoplasma contamination and tested negative for the same. Recombinant human CXCL1 was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). AG490, a Jak/STAT3 specific inhibitor, was purchased from Selleck Chemical (Houston, TX, USA). SB225002 (CXCR2 inhibitor) and AF-293-NA (VEGF neutralizing antibody) were purchased from R&D System Inc. (Minneapolis, MN, USA).

Transient transfection

Oligonucleotides were designed to amplify CXCL1 cDNA: forward 5’-CGCGGATCCATGGCCGCCGCTGCTT-3’ and reverse 5’-TGCTCTAGATCATGTTGGATTTGTCACTGTTCAGCATC-3’. CXCL1 cDNA was purchased from SB Company (Beijing, China). The CXCL1 cDNA was amplified by RT-PCR and cloned into the pcDNA3.1(Invitrogen China Limited, Beijing, China). Integrity and validity of SCC-7901 cells were added to the upper compartment and cultured with or without CXCL1. The STAT3 expression vector (EX-Z2835-M98) and empty control vector (EX-NEG-M98) were obtained from GeneCopoeia (Guangzhou, China). Transfection of plasmids was performed with Lipofectamine 2000 reagent (Invitrogen China Limited, Beijing, China) according to the manufacturer’s protocol. Expression of CXCL1 and STAT3 in transient cells was validated by Western blot assays.

RNA interference

The sequences for human VEGF siRNA were 5’-GACGUACCGCAUGAGAUCdTdT 3’ (forward) and 5’-GACUCUACGGGAGUUCUCdTdT 3’ (reverse) which were purchased from RiboIn (Guangzhou, China). Transfection was performed at about 50% confluence of AGS cells using Lipofectamine 2000 reagent (Invitrogen China Limited, Beijing, China) following the manufacturer’s protocol. Expression of VEGF antibody from Abcam, Cambridge, UK. After 48 hours of transfection, the cells were harvested for migration assay.

Migration assay

A cell migration assay was performed using Matrigel-coated transwell chambers (24-well insert; 8-µm pore size, BD Biosciences, Bedford, MA, USA), AGS and SGC-7901 cells were cultured in 24-well plates at a density of 5 × 10^4 cells per well. Cells were grown to 30% confluence and infected with lentivirus encoding CXCL1, Green fluorescent protein (GFP), sh-CXCL1 or control shRNA according to the manufacturer’s instructions. The cells were incubated for 48 h, and then the medium was replaced by 1% FBS RPMI-1640 medium with puromycin. After every 48 h incubation, the medium was changed and the supernatant was discharged. The expression level of CXCL1 was detected by Western blot.

Western blotting

Sub-confluent monolayers of cells were treated with CXCL1, SB225002, AG490, either alone or in combination. Cell lysates were obtained as previously described [27]. Supernatants were recovered by centrifugation at 13,000 rpm. Protein concentrations were measured and equal amounts of total protein were separated by SDS-PAGE. Proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA) and the membranes were blocked for 1 h in TBS-T with 5% BSA. The membranes were incubated overnight at 4 °C with the following antibodies: CXCL1 (R&D System, Minneapolis, MN, USA), VEGF (Abcam, Cambridge, UK), p-STAT3, total STAT3 and tubulin (Cell Signaling Technology, Beverly, MA, USA). The membranes were then incubated with the corresponding HRP-conjugated secondary antibodies (Pierce Biotechnologies, Santa Cruz, CA) for one hour. Specific bands were detected using the enhanced chemiluminescence reagent (ECL, Perkin Elmer Life Sciences, Boston, MA) on autoradiographic film.
Enzyme-linked immunosorbent assay

AGS cells were seeded in a 6-well plate with $5 \times 10^5$ cells per well. After serum starvation for 12 hours, cells were treated with CXCL1 (50 ng/mL) for a certain time period. Conditioned media were then harvested and centrifuged. VEGF concentrations in the supernatant were measured using VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MN, USA).

Subcutaneous tumor growth experiment

Animal experiments were approved by the Ethical Review Committee of First Affiliated Hospital of Sun Yat-sen University (Permit Number 2012-114). Six-week-old female Balb/c athymic mice were purchased from Guangdong Medical Laboratory Animal Center. Mice were randomized into two groups ($n = 5$ per group) and subcutaneously injected with $5 \times 10^6$ CXCL1-SGC-7901 cells (Study group) or GFP-SGC-7901 cells (Control group), respectively. The tumor size was measured twice a week and tumor volume ($V$) was calculated by using the formula $V = 0.5 \times \text{Length} \times \text{Width}^2$. After 25 days, all mice were euthanized with CO$_2$ and tumors were removed, weighed and processed for immunohistochemical and Western blot analyses.

Statistical analysis

SPSS 17.0 (SPSS Inc, Chicago, IL, USA) software was used for Student’s t test, $\chi^2$ analysis or analysis of variance. The Chi-square test was utilized to assess the associations of the expression of CXCL1, p-STAT3, VEGF with clinicopathological features. The relationship among the three proteins’ expression was assessed by Spearman rank correlation testing. Survival curves and overall survival comparisons were established according to the Kaplan–Meier and log-rank methods. Cox regression model with forward conditioned selection was used for multivariable analysis. Differences with a p value of < 0.05 were considered to be statistically significant.

Results

CXCL1 is upregulated in gastric cancer tissues and cancer cell lines and correlates with tumor stage and patient survival

We first examined mRNA and protein levels of CXCL1 in 4 patients (paired gastric tumor tissues and matched normal mucosa) and in human gastric cell lines (GES-1, AGS, SGC-7901, HGC-27, MKN-28 and BGC-823). For all the 4 patients examined, we found that protein and mRNA levels of CXCL1 were higher in gastric tumors than in the adjacent non-cancerous gastric tissues from the same patient (Fig. 1A). CXCL1 was also expressed in all gastric cancer cell lines. The expression was higher in AGS and SGC-7901 gastric cancer cells than in normal gastric epithelial GES-1 cells (Fig. 1B).

Ninety-eight cases were analyzed, representing all stages and histological types of malignant GC. The study included tissue samples from 62 men and 36 women. The mean patient age was 55 years.

Fig. 1. CXCL1 expression is increased in tumor tissues and is a prognostic factor for patients with gastric cancer. A. Expression of CXCL1 was analyzed by western-blotting and qRT-PCR in tumor and normal gastric tissues. B. Expression of CXCL1 was analyzed by western-blotting and qRT-PCR in gastric cancer cell lines. C. Expression of CXCL1 in gastric cancer tissues by IHC analysis. D. Patients with positive CXCL1 expression had a worse survival than those with negative expression of CXCL1 ($p = 0.002$). E. Expression of VEGF and p-STAT3 in representative tissues from gastric cancer patients.
Proximal cancer localization was observed in 24 cases. Clinicopathological data are shown in Table 1. CXCL1 expression was found to be positive in 41 cases (41.4%) and negative in 57 cases (58.6%) (Fig. 1C). Significant differences of CXCL1 expression were detected among different subgroups of tumor differentiation as well as pathological T, N and M categories, but not in distribution of gender, age, tumor location or tumor diameter subgroups (Table 1). The CXCL1 positive group (n = 41) had a lower survival rate than the negative group (n = 57) (p = 0.002, Fig. 1D). Median survival time was 20.6 months in the CXCL1 positive group and 36.3 months in the CXCL1 negative group.

**CXCL1 expression is associated with VEGF expression and p-STAT3 phosphorylation in human gastric tumor tissues**

Expressions of p-STAT3 and VEGF were evaluated by immunohistochemistry in the primary cancer tissue of 98 patients (Fig. 1E). Protein expression was positive in 70 cases (70.7%) for p-STAT3 and 59 cases (59.6%) for VEGF. Expression of p-STAT3 was mainly nuclear in cancer cells, while VEGF and CXCL1 were cytoplasmic. CXCL1 expression was moderately correlated with VEGF expression and STAT3 phosphorylation (Fig. 1C). Significant differences of CXCL1 expression were detected among different subgroups of tumor differentiation as well as pathological T, N and M categories, but not in distribution of gender, age, tumor location or tumor diameter subgroups (Table 1). The CXCL1 positive group (n = 41) had a lower survival rate than the negative group (n = 57) (p = 0.002, Fig. 1D). Median survival time was 20.6 months in the CXCL1 positive group and 36.3 months in the CXCL1 negative group.

Table 1

| Clinicopathological features in 98 gastric cancer specimens and expression of CXCL1, VEGF and p-STAT3. |
|---|---|---|---|---|---|---|---|---|---|
| n | CXCL1 expression | VEGF expression | p-STAT3 expression |
|---|---|---|---|---|---|---|---|---|---|
| Male | 62 | 38 | 24 | 0.410 | 17 | 45 | 0.740 | 27 | 35 |
| Female | 36 | 19 | 17 | | 13 | 23 | 0.208 | 12 | 24 |
| Age (years) | | | | | | | | | |
| >60 | 36 | 24 | 12 | 0.193 | 13 | 23 | | 71 | 19 |
| ≤60 | 62 | 33 | 29 | | 15 | 47 | | 22 | 40 |
| Tumor location | Upper | 19 | 13 | 6 | 0.313 | 6 | 13 | 0.747 | 10 | 9 |
| Middle/lower | 79 | 44 | 35 | | 22 | 57 | | 29 | 50 |
| Tumor diameter (cm) | ≤5 | 46 | 30 | 16 | 0.183 | 14 | 32 | 0.701 | 21 | 25 |
| >5 | 52 | 27 | 25 | | 14 | 38 | | 18 | 34 |
| Tumor differentiation | | | | | | | | | |
| I/II | 25 | 22 | 3 | <0.001 | 12 | 13 | 0.013 | 16 | 9 |
| III/IV | 73 | 35 | 38 | | 16 | 57 | | 23 | 50 |
| pT categories | T1 | 9 | 9 | 0 | 0.009 | 7 | 3 | | 8 | 1 |
| T2/T3/T4 | 89 | 48 | 41 | | 64 | 60 | | 31 | 58 |
| pN categories | N0 | 20 | 16 | 4 | 0.026 | 11 | 9 | | 12 | 8 |
| N1/N2/N3 | 78 | 41 | 37 | | 17 | 61 | | 27 | 51 |
| M categories | M0 | 78 | 54 | 24 | <0.001 | 28 | 50 | | 39 | 39 |
| M1 | 20 | 3 | 17 | | 0 | 20 | | 0 | 20 |
| pTNM stage | I/II | 42 | 36 | 6 | <0.001 | 22 | 20 | <0.001 | 30 | 12 |
| III/IV | 56 | 21 | 35 | | 6 | 50 | | 9 | 47 |

**Effect of CXCL1 on cell proliferation and migration**

To study the effect of CXCL1 on GC cell proliferation, we assessed AGS and SGC-7901 cell proliferation in vitro. No difference in cell proliferation index was observed for AGS or SGC-7901 cells when treated with or without CXCL1 (Supplementary Fig. S2). We also studied the effects of CXCL1 on cell migration by transwell assays (Fig. 2A and B and Supplementary Fig. S3). Compared to the control group, CXCL1 (50 ng/ml) significantly increased AGS (2.23 ± 0.119 fold increase, p < 0.001) and SGC-7901 (1.912 ± 0.074 fold increase, p < 0.001) cell migration. For each cell line, the CXCR2 inhibitor SB225002 (50 nM) blocked CXCL1-induced migration (Fig. 2A and B). When VEGF expression was down-regulated in AGS cells with siRNA, CXCL1 treatment caused no effect on VEGF expression and cell migration (Fig. 2C and D). The VEGF neutralizing antibody (10 μg/ml) also blocked CXCL1-induced migration (Fig. 2D).

**CXCL1 increases expression of VEGF and p-STAT3**

To further explore the potential relationship among CXCL1, VEGF and p-STAT3, in vitro experiments were performed on AGS cells. When AGS cells were treated with recombinant human CXCL1 (50 ng/ml), mRNA of VEGF was increased at the transcriptional level.
and then also at the protein level within cells. The mRNA of VEGF was highest at 8 hours after treatment (Fig. 3A) and VEGF protein was highest (about 3.2-fold increase from baseline) at 12 hours (Fig. 3B). After these peak time points, both mRNA and protein began to decline. Concentration of secreted VEGF in the supernatant was measured by ELISA and increased with the treatment of CXCL1 (Fig. 3C). We also measured phosphorylation of STAT3 by Western blot in AGS cells with or without CXCL1 treatment. Phosphorylated STAT3 was elevated by about 2-fold from baseline after cells had been treated for 0.5 hour with CXCL1 (Fig. 3D). Interestingly, the highest level of p-STAT3 protein occurred 4 hours after treatment with CXCL1, which was earlier than that of VEGF expression (Fig. 3E). Overexpression of CXCL1 in AGS cells resulted in elevated expression of VEGF and increased p-STAT3 and a slight increase in t-STAT3 compared to control transfected cells (Fig. 4A-C). A stable CXCL1 overexpressing cell line CXCL1-7901 was constructed and validated by Western blot and showed similar results (Fig. 4D-F).

To further investigate the effect of STAT3 overexpression on VEGF expression in CXCL1-silenced cells, we employed short hairpin RNA (shRNA) to knock down CXCL1 in SGC-7901 cells, which was confirmed by Western blot (Supplementary Fig. S4A). As shown in Supplementary Fig. S4B, overexpressing STAT3 significantly augmented VEGF expression in shCXCL1-7901 cells.

CXCL1/CXCR2 and JAK/STAT3 pathway inhibitors block CXCL1-induced VEGF expression

AGS cells were pretreated with CXCR2 inhibitor SB225002 or JAK/STAT3 pathway inhibitor AG490 for 1 hour and then cultured in serum-free medium with or without CXCL1 (50 ng/ml) for 8 hours. CXCL1 increased expression of VEGF and p-STAT3 which was blocked by either SB225002 or AG490 (Fig. 4C). The intensity of the bands was quantitated by densitometry (Boilrad). Similar results were found in CXCL1-7901 cells (Fig. 4F). Pharmacologic inhibition of CXCL1/CXCR2 and JAK/STAT3 also blocked CXCL1-induced cell migration.
These indicate that CXCL1 induces VEGF expression in a STAT3-dependent manner in GC cells.

**CXCL1 increases local tumor growth and tumor angiogenesis**

The effect of CXCL1 on local tumor growth was evaluated in a human tumor xenograft model. CXCL1-7901 and GFP-7901 cells were subcutaneously injected into BALB/C nude mice (n = 5). Tumor growth was measured for 25 days. Tumors grew faster in the CXCL1-7901 group compared to the control GFP-7901 group (Fig. 5A). Mean tumor weight was significantly higher in the CXCL1-7901 group than that in the GFP-7901 group (1.916 ± 0.584 g vs. 1.164 ± 0.349 g, p = 0.037) (Fig. 5B).

Mechanisms of CXCL1 in local tumor growth were further examined in tumor tissues obtained from CXCL1-7901 and GFP-7901 xenografts. A significant increase in the expression of VEGF, p-JAK2 and p-STAT3 (Fig. 5D) was observed in CXCL1-7901 xenograft tumors compared to GFP-7901 xenograft tumors by immunohistochemistry. Concomitant with VEGF expression, intratumoral microvessel density was also significantly higher in CXCL1-7901 xenografts compared to controls based on the CD-31 positive vessel numbers (p < 0.001) (Fig. 5C). Further, Ki67 expression-based intratumoral proliferative index increased by 90.7% (p < 0.001) compared to controls (Fig. 5D).

**Discussion**

We previously reported that secretion of CXCL1 from LEC promoted GC metastasis and expression of CXCR2 correlated with GC tumor metastasis [7]. In the present study, tumoral CXCL1 expression correlated with advanced tumor stage and poor patient survival and forced expression of CXCL1 increased angiogenesis and local tumor growth. CXCL1 expression is associated with VEGF expression and STAT3 phosphorylation in clinical tumor tissues and CXCL1 increased VEGF and p-STAT3 expression in vitro and in vivo. CXCL1/CXCR2 and JAK/STAT3 inhibitors blocked VEGF expression induced by CXCL1 and inhibited CXCL1-stimulated GC cell migration. Our findings support a novel mechanism for GC growth by which CXCL1 increases the expression of VEGF to promote angiogenesis and local tumor growth resulting in inferior survival.

Overexpressed CXCL1 was found in 41.4% GC tumors and significantly correlated with advanced tumor stage and inferior patient prognosis. These results were consistent with a recent report [10] in which the upregulation of CXCL1 was one of the independent
prognostic factors for GC patients’ survival. CXCL1 increased GC cell migration but had little effect on cell proliferation in vitro. Moreover, CXCL1 increased cell proliferation, angiogenesis and tumor growth in vivo. These results demonstrated that CXCL1 promotes not only tumor metastasis but also tumor growth and then leads to poor patient survival in GC.

CXCL1/CXCR2 has been implicated in the haptotactic migration and angiogenic effects of melanoma cells [28–31]. Miyake et al. [32] reported that secretion of CXCL1 from epithelial cells induced angiogenesis. CXCL1 increased VEGF expression and cell migration in vitro which was blocked by VEGF-siRNA and VEGF neutralizing antibody. These results indicate that CXCL1-induced cell migration is VEGF dependent. Zhao et al. found that VEGF promotes GC invasiveness via integrin α5β6 [33]. In bladder cancer, effects of VEGF on cell invasion were also reported [34]. Within an in vivo xenograft study, CXCL1 significantly increased angiogenesis, cell proliferation and local tumor growth. VEGF is a primary stimulant of angiogenesis and has been reported to be expressed in 48% of GC patients [35]. In our study, VEGF was positively expressed in 59.6% of GC patients and correlated with poor prognosis. CXCL1 also increased VEGF expression and microvessel density in vivo. Thus, CXCL1 can activate VEGF signaling in GC which increases angiogenesis.

Cheng et al. [10] observed that STAT3 might be a putative downstream effector of the CXCL1/CXCR2 pathway. In this study, we found that STAT3 was phosphorylated in GC cells after treatment with CXCL1 and followed immediately by elevated VEGF expression. Increased expression of VEGF and p-STAT3 can be blocked by either CXCR2 or STAT3 blockade. Migration of GC cells enhanced by CXCL1 was also reversed by concurrent treatment with either CXCR2 or STAT3 inhibition. We reported previously that STAT3 can activate VEGF transcription in AGS cells [19]. Our clinical data also implied some internal interactions among these three proteins. Xenograft tumor tissue analysis by immunohistochemistry confirmed that CXCL1 increased expression of VEGF and p-STAT3. These results indicate that STAT3 is phosphorylated after CXCL1 binding to its receptor CXCR2 which then in turn leads to up-regulation of VEGF. Similar results were reported by Burger et al. in KSHV-GPCR and CXCR2 transfected NIH 3T3 cells where the JAK2-STAT3 pathway was constitutively activated and VEGF was up-regulated [36]. STAT3 has also been found to take part in the neutrophil chemotactic responses to natural ligands for CXCR2 [22]. STAT3 has recently been linked to poor prognosis and tumor metastasis in GC [37–39], as we found in our current study.

CXCL1/CXCR2 signaling regulated tumor–stromal interactions and inhibiting tumor–stromal interactions with CXCR2 inhibitor might be a promising therapeutic strategy [14]. Recently, CXCR2 inhibition was found to be effective in breaking the CXCL1/2-S100A8/9 loop that caused chemoresistance, augmenting the efficacy of
Chemotherapy against breast tumors and particularly against metastatic activity [40]. Knockdown of CXCR2 was also found to enhance the antitumor activity of paclitaxel in an in vivo mammary tumor model [41]. Anti-VEGF therapy is in clinical use for the treatment of GC. [42,43]. However, anti-angiogenic therapy is often insufficient to control tumor growth especially for increased invasion and metastasis [44]. The novel mechanism in GC that CXCL1 increases VEGF expression through activation of the JAK2-STAT3 signaling suggests that targeting CXCR2 therapy has the potential to improve anti-angiogenic therapy and chemotherapy.

In conclusion, CXCL1 increased tumor growth and correlated with poor survival in GC. CXCL1 enhanced VEGF expression through activation of STAT3 and thus promoted angiogenesis and tumor growth of GC. The CXCL1/CXCR2 pathway represents potentially promising targets for chemoresistance and anti-angiogenic therapy in GC.

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**Authors’ contributions**

Zhang CH, Wei ZW, Xia GK and He YL designed the research. Xia GK, Wu Y, Wei ZW, Xiang Z and Chen W performed the research. Schwarz RE, Brekken RA, Awasthi N, He YL and Zhang CH analyzed the data and wrote the paper.

**Conflict of interest**

All authors have no conflicts of interest to disclose regarding this manuscript.

**Appendix: Supplementary material**

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.01.033.

**References**


