Chemokine (C-X-C motif) ligand 12 is associated with gallbladder carcinoma progression and is a novel independent poor prognostic factor

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Disclosures of Potential Conflicts of Interest

The authors disclose no conflicts.

Statement of translational relevance

Gallbladder carcinoma (GBC) is a lethal malignancy and is hard to cure by conventional therapies. Thus, new therapeutic strategies for GBC are urgently needed. Here we show that Chemokine (C-X-C motif) ligand 12 (CXCL12) is differentially expressed in patients with GBC. The expression of CXCL12 is significantly associated with a high histologic grade and nodal metastasis. Multivariate analyses clearly show that CXCL12 expression is an independent risk factor for patient survival. CXCL12 plays a crucial role in the progression of GBC by enhancing anchorage-dependent and -independent growth, migration, and invasion of GBC cells in vitro. Consistent with these results, overexpression of CXCL12 significantly promotes tumorigenicity of GBCs in a xenograft model. Our finding suggests that CXCL12 expression is associated with GBC progression, and may be a potential...
prognostic marker and therapeutic target for GBC.

Abstract

Purpose: Although recent studies have suggested that Chemokine (C-X-C motif) ligand 12 (CXCL12) is important in the progression of various malignancies, its role in gallbladder carcinoma (GBC) remains unknown. We investigated CXCL12 expression in GBC, and its biological and prognostic role in GBC tumorigenesis.

Experimental Design: We examined CXCL12 expression in tumor specimens from 72 GBC patients by immunohistochemistry and analyzed the correlation between CXCL12 expression and clinicopathologic factors or survival. The functional significance of CXCL12 expression was investigated by CXCL12 treatment and suppression of CXCR4, a major receptor of CXCL12, as well as by CXCL12 overexpression in \textit{in vitro} and \textit{in vivo} studies.

Results: CXCL12 was differentially expressed in GBC tissues. CXCL12 expression was significantly associated with a high histologic grade ($P = 0.042$) and nodal metastasis ($P = 0.015$). Multivariate analyses showed that CXCL12 expression (hazard ratio [HR] 8.675; $P = 0.014$) was an independent risk factor for patient survival. CXCL12 significantly increased anchorage-dependent and -independent growth, migration, invasion, adhesiveness, and survival of GBC cells \textit{in vitro}, and these effects were dependent on CXCR4. Consistent with these results, overexpression of CXCL12 significantly promoted GBC tumorigenicity in a xenograft model.

Conclusions: Our results indicate that GBC cells express both CXCL12 and its receptor CXCR4, and CXCL12 may have a role in GBC progression through an autocrine mechanism. In addition, CXCL12 is a novel independent poor prognostic factor in patients with GBC. Thus, targeting CXCL12 and CXCR4 may provide a novel therapeutic strategy for GBC treatment.
Introduction

Gallbladder carcinoma (GBC) is the most common malignancy of the biliary tract. Although occurrence is rare as compared with other gastrointestinal tract neoplasms, including gastric and colorectal cancers, GBC has a distinctly higher incidence in certain demographic groups and geographic areas (1, 2). The prevalence of GBC in China, Thailand, and northern India is much higher than in the United States and Europe (3). Surgical resection is currently the most effective and potentially curative treatment for GBC. However, many patients present late in the course of the disease when surgical intervention is no longer effective and most patients with advanced GBC experience frequent recurrence after surgery. Therefore, the overall survival rate remains poor (4).

Although the most important risk factor for the development of GBC is cholelithiasis (up to 95% of GBCs are associated with gallstones) (5), the molecular mechanisms involved in early carcinogenesis and the factors affecting disease progression remain unclear. Therefore, identification of factors crucial for GBC progression is necessary for the development of new therapeutic strategies.

Chemokines are 8- to 10-kilodalton chemoattractant cytokines that not only control leukocyte trafficking and homing (6-9), but also play a major role in the trafficking, migration, and metastasis of tumor cells released from the primary tumor expressing corresponding chemokine receptors to particular sites (10-12). Chemokine (C-X-C motif) ligand 12 (CXCL12) is mainly expressed in stromal fibroblasts of specific organs including the brain, breast, liver, bone, lung, and lymph nodes. CXCL12 activates its receptor CXCR4, which is involved in cell proliferation, migration, and invasion (13, 14), and thus promotes organ-specific localization of distant metastases of various carcinomas (15-17). Blocking the interaction between CXCR4 and CXCL12 by CXCR4 antagonists inhibits the number or size of tumor metastases (18-24). However, the mechanisms by which CXCL12 exerts its metastatic effects are unknown.

Several studies have suggested that cytokine receptors and their corresponding ligands expressed in tumor cells contribute to distant metastatic spread of tumors via autocrine interactions (25-28). Inverse correlations between CXCL12 and long-term survival have been recognized in ovarian carcinoma (29), glioma (30), esophageal carcinoma (31), colorectal cancer (32), and gastric carcinoma.
A role for CXCL12 in the regulation of various carcinomas through an autocrine or paracrine mechanism is likely. However, little is known regarding cancer cells co-expressing CXCL12 and CXCR4 in patients. Kang et al. showed that CXCL12-positive breast cancer cells exhibited significant increases in invasiveness and migration as compared with CXCL12-negative breast cancer cells (34). High levels of CXCL12 expression in grade 3 and grade 2 tumors as compared with grade 1 tumors are presented in their work. However, there have not been any reports on CXCL12 expression and its role in gallbladder cancer cells.

In this study, we determined CXCL12 expression and its relationship with clinicopathological features and clinical outcomes in human GBC. We also clearly demonstrate that CXCL12 increased proliferation, migration, and invasion, as well as activates intracellular ERK, AKT, and FAK signaling in GBC cells in a CXCR4-dependent manner. To our knowledge, this is the first study showing the expression of CXCL12 in GBC cells and its role in GBC progression. Taken together, these findings suggest that CXCL12 may regulate tumor progression through an autocrine mechanism in GBC cells.

Materials and Methods

Cell line and cell culture

SNU-308 cells, established from a well-differentiated gallbladder adenocarcinoma, were grown in RPMI1640 media (Invitrogen) supplemented with 10% FBS. All cells were maintained at 37°C under 5% CO₂ and 95% relative humidity.

Patients and samples

We investigated 72 consecutive patients (33 men and 39 women) with GBC who underwent surgical resection between January 1998 and March 2009 at Chungnam National University Hospital. The median age of the patients was 68 years (range, 35-87). All specimens were routinely fixed in 10% formalin and embedded in paraffin. All tumors were diagnosed as adenocarcinoma and defined as a primary tumor arising from the gallbladder. Tumor stage was classified according to the 2002...
American Joint Committee on Cancer system. All protocols were approved by the Institutional Review Board.

**Immunohistochemistry**

Immunohistochemistry for CXCL12 was performed using the EnVision-HRP detection system (Dakocytomation, Carpinteria, CA) and a monoclonal CXCL12 antibody (MAB350; R&D Systems, Minneapolis, MN). Briefly, 3-µm-thick sections were cut from tumor tissue blocks mounted on slides and dried for 1 hour at 56°C. The sections were deparaffinized in xylene and rehydrated in graded alcohol. After antigen retrieval in a pressure cooker with target retrieval solution (DakoCytomation s1699) at full power for 4 minutes, tissue sections were treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Sections were incubated with the CXCL12 antibody diluted with background reducing diluent (1:50 dilution, DakoCytomation s3022) in a humid chamber at 4°C overnight. Slides were incubated with Envision reagent for 30 minutes followed by DAB chromogen for 5 minutes. Slides were then counterstained with Mayer’s hematoxylin and mounted. A mouse IgG1 control excluding the primary antibody was used as a negative control.

**Scoring of immunostaining**

Immunostaining was reviewed independently by two of the authors (JMK and HJL) who were blinded to the patient’s clinicopathologic information. Staining was scored according to the intensity and percentage of positive cells. Membranous and/or cytoplasmic staining of the tumor cells was considered as a positive. Tumors were classified into four grades by intensity: grade 0 (negative), no staining; grade 1, weak intensity (+); grade 2, moderate intensity (++); and grade 3, strong intensity (+++). In the case of heterogeneous staining, the higher score was accepted if more than 50% of the cells displayed the higher intensity of staining. The percentage of positive cells was scored as follows: score 0 (no positive cells, 0%); score 0.1 (percentage of positive cells, 1-9%); score 0.5 (percentage of positive cells, 10-49%); and score 1.0 (percentage of positive cells, more than 50%). For semiquantitative analysis of immunostaining, we used an H-Score. The H-Score was calculated by
multiplying the grade of the intensity and score of the positive staining cells. Thus, this score produces a continuous variable that ranges from 0 to 3.0. The median value of the H-Score was chosen \textit{a priori} as the cutoff point for separating positive from negative tumors (35).

\textbf{Quantitative real-time PCR}

Total RNA was isolated from GBC tissues using Ambion RecoverAll\textsuperscript{TM} Total Nucleic Acid Isolation Kit (Ambion Inc., Austin, TX) according to the manufacturer's protocol. Target RNA was converted to cDNA by treatment with 200 units of reverse transcriptase and 500 ng oligo (dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl\textsubscript{2}, 10 mM dithiothreitol, and 1 mM deoxynucleotide triphosphates for 1 hour at 42°C. The reaction was quenched by heating for 15 minutes at 70°C. Quantitative real-time PCR analyses were performed in a Rotor-gene 6000 thermocycler (Corbett Research) using 1× SYBR Green mix (Invitrogen).

\textbf{shRNA targeting CXCR4 mRNA and CXCL12 overexpression in GBC cells}

Knockdown of CXCR4 in GBC cells was achieved through lentivirus-mediated transduction via CXCR4 mRNA-specific shRNA interference using Mission RNAi system clones (Sigma). Overexpression of CXCL12 in GBC cells was achieved through retrovirus-mediated transduction of full-length human CXCL12 subcloned into a retroviral vector. To generate stable transfectants, lentiviral or retroviral vectors and packaging vectors were cotransfected into HEK293T cells with packaging vectors using Lipofectamine, according to the manufacturer's instructions (Invitrogen). The next day, virus harvested from the supernatant was added to SNU-308 cells along with 5 µg/mL polybrene. After 24 hours, the media was removed and replaced with fresh media containing 1 µg/mL puromycin. Puromycin-resistant clones were selected after culture for 1 week in the presence of puromycin. CXCL12 or CXCR4 expression levels were analyzed by RT-PCR and Western blot.

\textbf{Cell proliferation assay}

Cell proliferation was measured by using the WST-1 cell proliferation assay (Roche Diagnostics,
Basel, Switzerland) according to the manufacturer's instructions. Briefly, the cells were seeded in triplicate in flat-bottomed 48-well plates at a density of $2 \times 10^4$ cells per well and allowed to adhere for 24 hours in media supplemented with 10% FBS. Thereafter, the cells were treated with various concentrations of CXCL12 as indicated in reduced serum (3% FBS) media for 72 or 96 hours. After incubation with 30 μL WST-1 reagent for 4 hours, the absorption of the samples was measured at 450 nm (with a background control as a blank) using a microplate reader.

**Migration and invasion assays**

Migration and invasion of GBC cells was performed using Transwell plates (Corning Costar, Cambridge, MA) with 6.5-mm diameter polycarbonate filters (8-μm pore size). Briefly, the lower surface of the filter was coated with 10 μg gelatin for the migration assay and the upper side was coated with 12 μg reconstituted basement membrane substance for the invasion assay (Matrigel; BD Biosciences). Fresh media containing 5% FBS was placed in the lower wells. GBC cells were incubated for 24 hours in media containing 1% FBS, trypsinized, and suspended at a final concentration of $1 \times 10^6$ cells/mL in media containing 1% FBS. One hundred microliters of the cell suspension was loaded into each of the upper wells and the chamber was incubated at 37°C for 24 hours (migration) or 48-72 hours (invasion). Cells were fixed and stained with hematoxylin and eosin. Nonmigrating cells on the upper surface of the filter were removed by wiping with a cotton swab. Chemotaxis was quantified by counting the cells that migrated to the lower side of the filter with an optical microscope ($\times 200$). Eight random fields were counted for each assay.

**Soft agar colony forming assay**

Anchorage-independent growth assays were performed using the CytoSelect™ 96-well Cell Transformation assay kit (Cell Biolabs, San Diego, CA). Briefly, SNU-308 cells ($1 \times 10^5$ cells) were plated in Dulbecco’s modified Eagle’s medium containing 10% FBS in a cell suspension agar matrix between layers of base agar matrix. After 2 weeks, the agar matrix was solubilized and the cells were stained with MTT solution. Absorbance at 570 nm was measured using a microplate reader.
Cell adhesion assay

Ninety-six well culture plates were coated with purified collagen, laminin, fibronectin, or vitronectin at a concentration of 2 µg/mL each and incubated at 37°C for 18 hours. Wells were washed with PBS, incubated with $2 \times 10^4$ cells in serum-free RPMI1640 supplemented with 1% bovine serum albumin at 37°C for 30 minutes, and washed three times to remove unbound cells. The attached cells were measured by a WST-1 assay as described above.

Nude mice xenograft assays

Nude mice (6 weeks old) were obtained from Charles River Laboratories (Boston, MA). Mice were housed under specific pathogen free conditions and were used in accordance with the guidelines of the Animal Care Committee at the Korea Research Institute of Bioscience and Biotechnology. SNU-308 cells ($1.5 \times 10^7$) were inoculated subcutaneously into the right flank of each mouse. Tumor growth was monitored at 5-day intervals by measuring the length and width of the tumor with a caliper and calculating tumor volume based on following formula: volume = $0.523Lw^2$.

Western blot analyses

Cell lysates or immunoprecipitates from cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (Chemicon, Temecula, CA). The membranes were incubated with the indicated primary antibody followed by horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactive polypeptides were visualized using a chemiluminescent substrate (GE Life Sciences, Piscataway, NJ).

Statistical analyses

Group comparisons of categorical variables were evaluated using the $\chi^2$ test, or linear by linear association. Comparisons of average means were performed with an independent samples T-test or one-way ANOVA. Cancer-specific survival was defined from the date of surgery to the date of death.
Results

Expression of CXCL12 in GBC

To analyze the function of CXCL12 in GBC progression, we first evaluated CXCL12 expression by immunohistochemical analyses in tumor specimens from 72 GBC patients. CXCL12 was located in the membrane and/or cytoplasm and the intensity of the immunohistochemical staining was variable: negative staining intensity (grade 0), 4 cases; weak staining intensity (grade 1), 18 cases; moderate staining intensity (grade 2), 22 cases; and strong staining intensity (grade 3), 28 cases (Fig. 1A). The percentage of positive cancer cells on each slide was variable, ranging from 10% to 90%. The median H score for GBC was 1.5. Based on these data, the patients were divided into CXCL12-positive (CXCL12 > 1.5) or CXCL12-negative (CXCL12 ≤ 1.5) expression groups. Of the 72 tumors examined, 58 (81%) were classified as CXCL12-positive and 14 (19%) were classified as CXCL12-negative (Table 1). In addition, quantitative evaluation of CXCL12 mRNA expression in GBC tissues (grade 0-3) by real-time PCR also showed that the mRNA levels from the grade 2 or 3 groups were markedly higher than those for grade 0-1 groups (Fig. 1B).

Correlation between CXCL12 expression and clinicopathologic factors

We next analyzed the correlation between CXCL12 expression and various clinicopathologic factors that can affect the prognosis of patients with GBC. The results are summarized in Table 1. There was no significant difference in age, gender, pathologic T stage, distant metastasis, perineural
invasion, or lymphatic invasion between the CXCL12-negative group and the CXCL12-positive group. However, CXCL12 expression was significantly associated with lymph node metastasis ($P = 0.015$) and histologic grade ($P = 0.042$).

**Correlation between CXCL12 expression and survival**

To further investigate the clinical relevance of CXCL12 expression in GBC, we compared cancer-specific survival according to CXCL12 expression (Fig. 1C). The median survival time of patients with GBC was 13.5 (range, 1-117) months. Remarkably, the 10-year survival rates in the CXCL12-positive and -negative groups were 10 and 85.7%, respectively ($P = 0.005$), indicating a crucial impact of CXCL12 expression on clinical outcome in patients.

Next, to estimate the clinical significance of various prognostic factors that might influence survival in the study population, univariate analyses were performed. As summarized in Table 2, advanced pathologic T stage ($P = 0.002$), nodal metastasis ($P = 0.003$), distant metastasis ($P = 0.045$), differentiation ($P = 0.023$), lymphatic invasion ($P = 0.001$), and CXCL12 expression ($P = 0.012$) were statistically significant risk factors affecting overall survival of patients with GBC. To further determine the independent prognostic impacts of these various factors, multivariate analyses were performed using the Cox’s proportional-hazards model. These analyses showed that CXCL12 expression (HR, 8.675; 95% CI, 1.554-48.413; $P = 0.014$) was an independent risk factor predicting cancer-specific survival in patients with GBC (Table 2). Taken together, our findings indicate that CXCL12 expression may be a useful marker to predict the survival of patients with GBC.

**CXCL12 promotes GBC cell growth and tumorigenicity**

To investigate whether CXCL12 plays an important role in the progression of GBC, we selected a GBC cell line, SNU-308, that expresses a low level of CXCL12 (Fig. 2A) and treated these cells with CXCL12 for the indicated times. As shown in Fig. 2B, phosphorylation of intracellular signals such as extracellular signal-regulated kinase (ERK), AKT, and focal adhesion kinase (FAK) was significantly induced in a time-dependent manner. In addition, CXCL12 increased anchorage-dependent or -
independent growth of the cells (Fig. 2C-2D). To further confirm the effect of CXCL12 on the growth of GBC cells, SNU-308 cells were stably transfected with full-length human CXCL12 and the cell lines showing high level expression of CXCL12 were selected. Elevated CXCL12 levels were confirmed by Western blot analyses (Fig. 2E). Consistently, CXCL12 overexpressing cells showed significantly enhanced cell growth compared with the control mock-transfected cells (Fig. 2E). Next, to evaluate the role of CXCL12 in tumor growth in vivo, CXCL12-overexpressing cells were injected into the right flank of nude mice and tumor formation was monitored. As shown in Fig. 2F, an increase in tumor growth was observed in mice with CXCL12-overexpressing cell xenografts as compared to those injected with mock-transfected GBC cells. Remarkably, all mice inoculated with CXCL12-overexpressing cells formed tumors, whereas mice injected with mock-transfected cells did not develop tumors. These results suggest that CXCL12 promotes the growth and tumorigenicity of GBCs.

**CXCL12 increases migration, invasion, adhesiveness, and survival of GBC cells.**

It has been shown that CXCL12 is closely involved in motility, invasiveness, adhesiveness, and survival of several types of tumors (13, 22, 27). To address this issue, we also examined the effect of CXCL12 on motility and invasiveness of GBC cells using Transwell filter assays. Treatment of GBC cells with various concentrations of CXCL12 enhanced cell motility and invasiveness in a dose-dependent manner (Fig. 3A-3D). Consistently, CXCL12-overexpressing cells displayed significantly increased migration and invasion as compared with control cells (Supplementary Fig. 1A-1B). In addition, CXCL12 increased the adhesion of GBC cells to matrix components such as fibronectin, collagen, laminin, and vitronectin (Fig. 3E).

One important feature of tumor cells is the ability to regulate their survival. Therefore, we addressed whether CXCL12 contributes to the survival of GBC cells. To test this, CXCL12-overexpressing cells or mock-transfected cells were cultured in serum free media and cell viability was evaluated after 96 or 120 hours using trypan blue exclusion assays. Serum deprivation decreased cell viability to approximately 55% of control cells after 120 hours. However, the viability of
CXCL12-overexpressing cells was significantly increased (up to 75%, Fig. 3F).

CXCL12-induced proliferation, migration, and invasion of GBC cells is inhibited by abrogation of CXCR4

To determine whether changes in CXCR4 expression affect GBC progression in response to CXCL12 treatment, we generated GBC cell clones with stable knockdown of CXCR4. Specific depletion of CXCR4 in these cells was accomplished by lentivirus-mediated transduction and expression of a CXCR4 mRNA–specific shRNA. Cell clones stably expressing low levels of CXCR4 were selected, and CXCR4 expression was confirmed by RT-PCR and Western blot (Fig. 4A). Stable depletion of CXCR4 expression in GBC cells inhibited CXCL12-induced ERK, AKT, and FAK activation (Fig. 4B). In addition, suppression of CXCR4 in GBC cells caused significant decreases in growth, migration, and invasion with CXCL12 stimulation. Interestingly, depletion of CXCR7, a new receptor for CXCL12, did not significantly affect CXCL12-induced cellular activities (Supplementary Fig. 2A-2C). These results suggest that the CXCR4/CXCL12 system plays an important role in the growth, migration, and invasion of GBC cells.

Discussion

In the present study, we demonstrate for the first time that CXCL12 is expressed differentially in GBCs and is associated with GBC progression. In addition, CXCL12 expression is an independent poor prognostic marker for survival in patients with GBC who underwent surgical resection. These results may have important clinical implications for risk stratification and the planning of postsurgical surveillance, as well as for the potential of CXCL12 as a therapeutic target.

We investigated and clarified the clinical significance of CXCL12 expression in GBC. Recently, data showed that CXCL12 expression is upregulated and associated with tumor progression and poor prognosis in several types of cancer (30-33, 36, 37), suggesting that tumor-derived CXCL12 plays a role in tumorigenesis. Ishigami et al. reported that CXCL12 positivity was significantly associated with lymph node metastasis, depth of invasion, lymphatic invasion, tumor diameter, and higher stage
in gastric cancer (33). In addition, CXCL12-positive patients showed significantly poorer oncologic outcomes than did the CXCL12-negative patients, suggesting CXCL12 is an independent prognostic factor in gastric cancer. Salmaggi et al. also showed that CXCL12 expression was correlated with a significantly shorter time to progression in glioma as an independent predictor (30). A similar pattern has been reported for colon cancer (32), esophageal cancer (31), malignant glioma (36), and nonsmall cell lung cancer (37). However, CXCL12 expression and its clinical relevance in GBC have not yet been investigated. In this study, we observed overexpression of CXCL12 in GBC. Moreover, we demonstrated that CXCL12 expression was significantly associated with a high histologic grade and lymph node metastasis and is an independent poor risk factor for predicting survival in patients with GBC. Our results suggest that CXCL12 could be involved in GBC tumorigenesis and be useful as a potential prognostic biomarker for GBC patients.

Next, we investigated and clarified the functional role of CXCL12 in GBC progression. CXCL12 directly impacts cancer cell proliferation through interactions with its cognate receptor, CXCR4 (25, 26). When GBC cells were treated with CXCL12, SNU-308 cells, which express an endogenously high level of CXCR4, showed increased anchorage-dependent and -independent growth. Furthermore, xenograft mice injected with CXCL12-overexpressing cells showed a significant increase in tumor growth as compared with controls. These observations suggest that CXCL12 promotes the growth and tumorigenicity of GBC through interaction with CXCR4. CXCL12 is involved in cell migration, invasion, and adhesiveness in several types of cancer (13, 22, 23, 27). In this study, GBC cells showed enhanced motility and invasiveness in response to CXCL12. Furthermore, CXCL12 increased the adhesiveness of GBC cells to extracellular matrix components and significantly reduced serum-deprived apoptosis, indicating that CXCL12 is deeply involved in motility, invasiveness, adhesiveness, and survival of GBC cells expressing CXCR4. Taken together, these observations indicate that CXCL12 may affect biologic behaviors in GBC via CXCR4 activation.

Finally, we sought to confirm whether changes in CXCR4 expression influences GBC progression by CXCL12 using GBC cell clones with stable knockdown of CXCR4 because several recent reports indicate that CXCL12 also binds to another seven-transmembrane span receptor called CXCR7 (38,
Indeed, with CXCR7 identified as a new receptor for CXCL12, the role of the CXCL12/CXCR4 axis in regulating biological processes in cancer development and progression has become more complex (40). In the present study, stable depletion of CXCR4 in GBC cells caused a significant decrease in growth, migration, and invasion upon CXCL12 treatment. Interestingly, depletion of CXCR7 had no significant effect on CXCL12-induced cellular activities (Supplementary Fig. 2A-2C). These observations suggest that the CXCL12/CXCR4 axis plays a critical role in the tumorigenesis of GBC.

Next, to evaluate the clinical significance of CXCR4 expression in GBC, we also examined CXCR4 expression in GBC by immunohistochemistry as in CXCL12 expression, patients were classified into CXCR4-positive and -negative groups (Supplementary Fig. 3A). Of the 72 tumors examined, 47 (65%) were classified as CXCR4-positive and 25 (35%) were classified as CXCR4-negative. However, we did not find any correlation between CXCR4 expression and various clinicopathologic factors or patient survival (Supplementary Table 1 and Supplementary Fig. 3B). These results suggest that CXCL12 expression may play an important functional role in GBC progression whereas CXCR4 alone may not.

Several signaling molecules related to the activation of CXCR4 by CXCL12 have been identified and include AKT/PKB, ERK1/2 MAPK, PI3K, Stat3, paxillin, Crk, and NF-κB. The JAK and FAK system has also been involved in the CXCL12/CXCR4 signaling cascades (28, 41, 42). However, these signaling pathways are strictly regulated by cell type. We found that CXCL12 activated ERK, AKT, and FAK in GBC cells expressing a high level of CXCR4 in a time-dependent manner. In addition, stable depletion of CXCR4 in GBC cells, which express a high endogenous level of CXCR4, inhibited CXCL12-induced ERK, AKT, and FAK activation. Our findings suggest CXCL12-induced intracellular signal activations are down-stream effectors of CXCR4. These observations are also compatible with the changes in biological responses, such as cell growth, migration, and invasion, to CXCL12 treatment that we demonstrated using GBC cell clones with knockdown of CXCR4.

However, the precise mechanism by which tumor-derived CXCL12 contributes to tumor development and progression is not clear. One potential explanation is that CXCL12 is engaged in...
tumorigenesis in an autocrine and/or paracrine manner. The concomitant expression of CXCL12 and its receptor CXCR4 in brain tumor cells has been characterized as an autocrine and/or paracrine mechanism of cancer cell stimulation, resulting in aggressive behavior (43, 44). Subsequently, autocrine/paracrine mitogenic activity of CXCL12 was reported in glioblastoma multiforme in vitro (26, 45). Barbieri et al. also demonstrated that CXCL12 overexpression induced autocrine/paracrine cell proliferation in pituitary tumor cells (43). Collectively, these reports support our data. Another possible mechanism is that CXCL12 may play a role in attracting endothelial cells to the tumor microenvironment. Pathologically induced CXCL12 secretion by brain tumor cells increases the recruitment of circulating endothelial progenitors (46). Furthermore, inhibition of the CXCL12/CXCR4 axis reduced recruitment of vascular progenitors and decreased tumor growth through suppression of angiogenesis (47, 48). Lastly, CXCL12 has also been demonstrated to repel tumor-specific effector T-cells and to recruit immunosuppressive cells at the tumor site, suggesting that CXCL12 is involved in immune evasion of cancer cells (49).

In conclusion, our results indicate that CXCL12 is overexpressed in patients with GBC and is significantly associated with oncogenic properties and tumor progression. Furthermore, CXCL12 expression is a novel independent poor prognostic factor for predicting survival in GBC patients. In vitro and in vivo experiments using GBC cells clearly demonstrated that CXCL12 induced cell growth, migration, invasion, adhesiveness, and tumor formation through interaction with CXCR4. Collectively, we postulate that tumor-derived CXCL12 may be involved in tumorigenesis of GBC in an autocrine and/or paracrine manner via the CXCL12/CXCR4 axis.
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Figure Legends

Figure 1. Expression of CXCL12 in GBC. A. Representative photomicrographs of immunohistochemical staining for CXCL12 in human GBC tissues: isotype control, normal gallbladder tissue, negative staining intensity, weak staining intensity, intermediate staining intensity, and strong staining intensity. (Original magnification: ×400). B. Quantitative evaluation of CXCL12 expression in GBC tissues (grade 0-3; n=10 per group) by real-time PCR. Three independent experiments were performed in duplicate. Data are expressed as mean ± SD; *p < 0.05, **p < 0.01 versus grade 0-1 group. C. Correlation between CXCL12 expression and survival in patients with gallbladder carcinoma. Cancer-specific survival curve according to CXCL12 expression (P = 0.005).

Figure 2. CXCL12 promotes the growth and tumorigenicity of GBCs. A. CXCL12 expression was examined by RT-PCR and ELISA in GBC cell line, SNU-308. B. SNU-308 cells were treated with 50 ng/mL of CXCL12 for the indicated times. The phosphorylation of FAK, AKT, and ERK were determined. Blots are representative of three independent experiments. Relative phosphorylation levels of FAK were quantified by densitometry. Data are expressed as means ± SD; *p < 0.05 versus control time (0 minute). C. The effect of CXCL12 on anchorage-dependent growth in GBC cells. SNU-308 cells were seeded at a density of 2 × 10^4 cells/well in flat-bottomed 48-well plates. After 24 hours, the cells were treated with various concentrations of CXCL12 as indicated for 72 or 96 hours. Cell proliferation was measured by using the WST-1 cell proliferation assay as described in Materials and Methods. Three independent experiments were performed in triplicate. Data are expressed as means ± SD; **p < 0.01 versus PBS. D. The effect of CXCL12 on anchorage-independent growth in GBC cells. Soft agar colony forming assay was performed as described in Materials and Methods. Three independent experiments were performed in triplicate. Data are expressed as means ± SD; **p < 0.01 versus PBS. E. SNU-308 cells were stably transfected with CXCL12 as described in Materials and Methods. The expression of CXCL12 was determined by Western blot analysis (upper panels). Stable transfectants were seeded in 6-well plates at a density of 2.0 × 10^5 cells/well in 2 mL RPMI
media containing 3% FBS. Viable cells were counted after incubation for 72 hours or 96 hours. 
Three independent experiments were performed in duplicate. Data are expressed as mean ± SD; **p < 0.01 versus mock. F. CXCL12 overexpression confers tumorigenicity of GBC cell xenografts in nude mice. SNU-308 cells transfected with mock or CXCL12 were injected into the right flank of 7-week-old nude mice. Tumor growth was monitored on the indicated days. Results represent mean tumor volume ± SD (n = 7 animals). **p < 0.01 versus mock group.

Figure 3. CXCL12 increases migration, invasion, adhesiveness, and survival of GBC cells.

Migration (A and B) and invasion (C and D) were analyzed as described in Materials and Methods. Three independent experiments were performed in triplicate. Data are expressed as means ± SD; **p < 0.01 versus PBS. E. The effect of CXCL12 on adhesion of SNU308 cells to extracellular matrix proteins. Attachment of SNU308 cells by CXCL12 to plastic plates coated with fibronectin (Fibro), collagen (Coll), laminin (Lam), or vitronectin (Vitro) was determined using a WST-1 assay as described in Materials and Methods. Three independent experiments were performed in triplicate. Data are expressed as means ± SD; **p < 0.01 versus PBS. F. CXCL12-overexpressing or control mock-transfected cells were seeded in 6-well plates at a density of 4.0 × 10^5 cells/well in 2 mL serum free media and cell viability was assayed by trypan blue exclusion after 96 or 120 hours. Three independent experiments were performed in triplicate. Data are expressed as means ± SD; **p < 0.01 versus mock.

Figure 4. CXCL12-induced proliferation, migration, and invasion of GBC cells were inhibited by abrogation of CXCR4. SNU-308 cells were stably transfected with CXCR4 shRNA using lentivirus as described in Materials and Methods. A. The expression of CXCR4 was determined by RT-PCR and Western blot analysis. B. Stable transfectants were treated with CXCL12 for 10 minutes. The phosphorylation of FAK, AKT, and ERK was determined. Blots are representative of three independent experiments. C. The effect of CXCR4 on CXCL12-induced anchorage-dependent growth
of GBC cells. Stable transfectants were treated with 50 ng/mL of CXCL12 for 96 hours. WST-1 cell proliferation assay was performed as described in Figure 2C. Three independent experiments were performed in triplicate. Data are expressed as means ± SD; **p < 0.01 versus untreated control in scramble. D and E. The effect of CXCR4 on CXCL12-induced migration (D) and invasion (E) of GBC cells. Three independent experiments were performed in triplicate. Data are expressed as means ± SD; **p < 0.01 versus untreated control with scrambled shRNA.
Figure 1

A. Isotype control

B. Normal gallbladder tissue

(Grade 0)

Negative staining intensity

(Grade 1)

Weak staining intensity

(Grade 2)

Moderate staining intensity

(Grade 3)

Strong staining intensity

B. Relative mRNA expression

C. Overall survival

Expression group

Months after surgery

CXCL12 negative group

CXCL12 positive group

P = 0.005
Figure 2

A. mRNA level

- CXCL12
- GAPDH

B. Protein level

- P-ERK
- ERK
- P-AKT
- AKT
- P-FAK
- FAK

C. OD 450nm

- PBS
- CXCL12 (10 ng/ml)
- CXCL12 (20 ng/ml)
- CXCL12 (50 ng/ml)
- CXCL12 (100 ng/ml)

D. OD 570nm

- CXCL12 (ng/ml) 0 20 50 100

E. OD 450nm

- Mock
- CXCL12

F. Tumor volume (mm³)

- Mock
- CXCL12

Days after injection

0 10 15 20 25 30 35

72h 96h

** **
Figure 3
Figure 4

A

B

C

D

E
Table 1. Relationship of CXCL12 expression and clinicopathological characteristics of gallbladder carcinoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total n=72</th>
<th>CXCL12 Positive (n=58)</th>
<th>CXCL12 Negative (n=14)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 65</td>
<td>21</td>
<td>17 (29.3%)</td>
<td>4 (28.6%)</td>
<td>1.000*</td>
</tr>
<tr>
<td>≥ 65</td>
<td>51</td>
<td>41 (70.7%)</td>
<td>10 (71.4%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33</td>
<td>29 (50.0%)</td>
<td>4 (28.6%)</td>
<td>0.232*</td>
</tr>
<tr>
<td>Female</td>
<td>39</td>
<td>29 (50.0%)</td>
<td>10 (71.4%)</td>
<td></td>
</tr>
<tr>
<td>Pathologic T stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>13 (22.4%)</td>
<td>4 (28.6%)</td>
<td>0.350†</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>26 (44.8%)</td>
<td>7 (50.0%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>16 (27.6%)</td>
<td>3 (21.4%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3 (5.2%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Nodal metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abscent</td>
<td>54</td>
<td>40 (69.0%)</td>
<td>14 (100%)</td>
<td>0.015*</td>
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<tr>
<td>Present</td>
<td>18</td>
<td>18 (31.0%)</td>
<td>0 (0%)</td>
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<tr>
<td>Distant metastasis</td>
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<tr>
<td>Abscent</td>
<td>68</td>
<td>54 (93.1%)</td>
<td>14 (100%)</td>
<td>0.580*</td>
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<td>Present</td>
<td>4</td>
<td>4 (6.9%)</td>
<td>0 (0%)</td>
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</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>G 1</td>
<td>12</td>
<td>6 (10.3%)</td>
<td>6 (42.9%)</td>
<td>0.042†</td>
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<tr>
<td>G 2</td>
<td>37</td>
<td>32 (55.2%)</td>
<td>5 (35.7%)</td>
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</tr>
<tr>
<td>G 3</td>
<td>19</td>
<td>16 (27.6%)</td>
<td>3 (21.4%)</td>
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<td>-------</td>
</tr>
<tr>
<td></td>
<td>G 4</td>
<td>4</td>
<td>4 (6.9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Perineural invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abscent</td>
<td>35</td>
<td>28 (48.3%)</td>
<td>7 (50.0%)</td>
<td>0.908*</td>
</tr>
<tr>
<td>Present</td>
<td>37</td>
<td>30 (51.7%)</td>
<td>7 (50.0%)</td>
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<tr>
<td>Lymphatic invasion</td>
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<tr>
<td>Abscent</td>
<td>26</td>
<td>19 (32.8%)</td>
<td>7 (50.0%)</td>
<td>0.228*</td>
</tr>
<tr>
<td>Present</td>
<td>46</td>
<td>39 (67.2%)</td>
<td>7 (50.0%)</td>
<td></td>
</tr>
</tbody>
</table>

* P values were calculated by pairwise comparisons from χ² test.
† P values were calculated by comparisons of four groups from linear-by-linear associations.
Table 2. Univariate and multivariate analyses of the association of prognosis with clinicopathological parameters and CXCL12 expression in patients with gallbladder carcinoma

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate</th>
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<th>Multivariate</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
<td>HR (95% CI)</td>
<td>P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, (years, ≥ 65 vs. &lt;65)</td>
<td>1.470 (0.660–3.277)</td>
<td>0.346</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Gender (women vs. men)</td>
<td>0.869 (0.430–1.756)</td>
<td>0.696</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathologic T stage (T3,4 vs. T1,2)</td>
<td>3.169 (1.574–6.379)</td>
<td>0.002</td>
<td>1.532 (0.672–3.491)</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodal metastasis (yes vs. no)</td>
<td>3.124 (1.462–6.676)</td>
<td>0.003</td>
<td>1.331 (0.532–3.331)</td>
<td>0.541</td>
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</tr>
<tr>
<td>Distant metastasis (yes vs. no)</td>
<td>3.516 (1.028–12.028)</td>
<td>0.045</td>
<td>1.410 (0.354–5.607)</td>
<td>0.626</td>
<td></td>
<td></td>
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<tr>
<td>Differentiation (G3,4 vs. G1,2)</td>
<td>1.602 (1.067–2.405)</td>
<td>0.023</td>
<td>1.506 (0.795–2.851)</td>
<td>0.209</td>
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<tr>
<td>Perineural invasion (yes vs. no)</td>
<td>2.031 (0.957–4.311)</td>
<td>0.065</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Lymphatic invasion (yes vs. no)</td>
<td>5.346 (1.858–15.376)</td>
<td>0.001</td>
<td>3.661 (0.988–13.566)</td>
<td>0.052</td>
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<tr>
<td>CXCL12 expression (positive vs. negative)</td>
<td>6.487 (1.500–28.050)</td>
<td>0.012</td>
<td>8.675 (1.554–48.413)</td>
<td>0.014</td>
<td></td>
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</tr>
</tbody>
</table>
Chemokine (C-X-C motif) ligand 12 is associated with gallbladder carcinoma progression and is a novel independent poor prognostic factor


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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.