CXCL12/CXCR4 display an inverse mRNA expression profile in gastric carcinoma that correlates with tumor progression

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Abstract. Chemokines and their receptors have been shown to contribute to tumor growth and metastatic spread in various gastrointestinal cancer entities. In the present study, the mRNA expression profiles and clinical significance of chemokine ligand CXCL12 and its corresponding receptor CXCR4 were investigated in patients with gastric cancer (GC). Using quantitative polymerase chain reaction, the expression profile of CXCL12/CXCR4 was analyzed in resection specimens from the patients with GC (n=66) and in corresponding normal gastric tissues. Upon investigating CXCL12/CXCR4 mRNA expression levels in the GC tissues, significant downregulation of CXCL12 expression was demonstrated (P<0.05), whereas CXCR4 mRNA expression was shown to be significantly upregulated (P<0.05). Likewise, in gastric carcinoma patients undergoing neoadjuvant chemotherapy, CXCR4 expression was found to be significantly upregulated (P<0.05), whereas in GC patients with lymph and vein infiltration, CXCL12 mRNA expression was significantly downregulated (P<0.05). These results demonstrate a significant inverse association between the development and progress of GC and CXCL12/CXCR4 mRNA expression. CXCR4 mRNA upregulation was promoted under the effect of neoadjuvant chemotherapy prior to surgery in GC patients, whereas higher tumor stages with lymph and vein infiltration negatively affected CXCL12 mRNA expression.

Introduction

Gastric cancer (GC) is one of the most common cancers in the world, constituting 8% of the all cancers. Although novel treatment techniques, such as neo- and/or adjuvant radiochemotherapy, are being applied and the incidence of gastric cancer (GC) has declined over the past decades, GC still has a poor prognosis and accounts worldwide for ~10% of all cancer-associated fatalities, with a five-year survival rate of 20-30% (1). To date, surgery is considered the only curative treatment, and >50% of the patients in whom radical surgical treatment is performed experience recurrence and metastasis (1,2).

Infection with *Helicobacter pylori* and subsequent mucosal inflammation is the best established risk factor for the development of GC. Besides a small percentage of hereditary cases, other risk factors for GC are smoking, the male gender and different nutritional factors (3). In total, >90% of all GCs are adenocarcinoma originating from the glandular epithelium of the gastric mucosa. In the following, the term GC will exclusively refer to gastric adenocarcinoma. According to the classification by Laurén, GC can be distinguished histopathologically into two major histological types, the diffuse and the intestinal types (4).

Metastasis is the main characteristic of a malignant tumor and the major cause of mortality for gastric cancers. As the metastatic forms and predilection sites depend very much on the features of the primary tumor, lymph node metastasis is the most common metastatic location of gastric cancer spread. While the molecular mechanisms involved in metastasis have not been fully established, chemokines have been suggested to contribute to the growth and metastatic spread of various cancer entities (5).

Chemokines constitute a subset of small secretory proteins that interact through their corresponding receptors to control and stimulate various cell types, including macrophages and lymphocytes, thus regulating leukocyte infiltration (6). In our previous studies, the role of certain chemokines was outlined in distinct gastrointestinal tumors, such as hepatocellular carcinoma, and pancreatic and colorectal cancer (7-9). Chemokine receptors are linked to seven-transmembrane heterotrimeric G proteins and divided into the chemokine (C-X-C motif) receptor (CXCR) and the chemokine (C-C motif) receptor (CCR), which mediate a range of pro- and anti-inflammatory responses through the interaction with the two major CXC and CC subfamilies of chemokines, respectively (10).

CXC ligand 12 (CXCL12) (also known as stromal-derived factor-1) is an important α-chemokine that primarily binds to the corresponding receptor, CXCR4; although in recent years, CXCL12 has also been shown to bind to CXCR7, thus

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regulating the trafficking of normal and malignant cells (11). The induction of intracellular signaling via the binding of CXCL12 to CXCR4 occurs when a number of divergent pathways initiate signals associated with chemotaxis, cell survival and/or proliferation. This binding also induces an increase in intracellular calcium and gene transcription. A range of cell types, including hematopoietic stem cells, lymphocytes, and endothelial, epithelial and cancer cells, express CXCR4. The two receptors have been shown to maintain critical roles in tumor metastasis in numerous cancer types, as well as functioning as biomarkers of tumor behavior and being potential therapeutic targets (12,13). Activation of the CXCL12/CXCR4/CXCR7 axis has been investigated with respect to regulating the pattern of tumor growth and metastatic spread to organs that express high CXCL12 levels for the development of secondary tumors (14). With regard to infectious disease, the human immunodeficiency virus (HIV) employs CXCR4 in order to obtain entry into cells (15). With respect to GC, several studies were able to show the upregulation of certain chemokines and their respective receptors in GC compared with normal gastric tissues (16). Ying *et al* examined the levels of CXCL12 and CXCR4 expression by immunohistochemical staining in primary gastric tumor tissues and metastatic lymph nodes. Positive staining for CXCR4 and CXCL12 were observed in primary gastric tumor tissues, and positive CXCR4 expression was positively association with lymph node metastasis, TNM staging and disease prognosis (17).

The aim of the present study was to investigate the mRNA expression profile of CXCL12/CXCR4 in GC patients and to evaluate the clinical significance.

Materials and methods

Materials. Surgical specimens and corresponding normal gastric tissue from the same samples were collected from patients who underwent surgical resection at the Department of General, Visceral, Vascular and Pediatric Surgery at the University of The Saarland (Homburg/Saar, Germany) between 2010 and 2012. Written informed consent was obtained from all patients for tissue procurement (approval no. 154/10) and publication of the present study. In addition, the study was approved by the Local Ethics Committee of the Medical Association of Saarland.

A total of 66 patients undergoing surgical resection for GC were enrolled in the present study. In every patient sample, the corresponding non-affected normal gastric tissue was also analyzed, adding up to a total sample size of 112. According to the Union for International Cancer Control tumor-node-metastasis (TNM) classification (18), cancers were classified as pT1 (n=2), pT2 (n=39), pT3 (n=19) and pT4 (n=6), with positive nodal involvement in 52 cases. In total, 10 patients had received neoadjuvant therapy prior to resection. The clinical data and patient characteristics were obtained from a prospective database and are summarized in Table I. Follow-up examinations for the analysis of the correlation between clinical data and molecular biological findings were performed for a mean time of 30 months (range, 2-92 month).

Table I. Clinical characteristics of gastric carcinoma patients (n=66).

Characteristics	Value
Gender, n	
Male	40
Female	26
Age, years	
Median	66
Range	37.5-88.5
Tumor type[a], n	
Intestinal type	33
Diffuse type	14
Mixed type	13
Signet-ring cell	6
Tumor category, n	
pT1	2
pT2	15
pT2a	1
pT2b	23
pT3	19
pT4	6
Lymph node metastases, n	
Positive	52
Negative	14
Peripheral metastases, n	
Positive	13
Negative	53
Lymphangiosis carcinomatosa, n	
Positive	23
Negative	43
Vascular permeation, n	
Positive	12
Negative	54
Surgical duration, min	
Median	237.7
Range	82-354
Perioperative blood loss, ml	
Median	312
Range	50-1500

[a]Laurén classification.

Tissue preparation. Tissue specimens were collected immediately after surgical resection, snap-frozen in liquid nitrogen and then stored at -80˚C until further processed under sterile conditions for RNA or protein extraction. Corresponding normal tissue consisted of adjacent macroscopically non-affected tissue from the same patients. All tissues obtained were reviewed by an experienced pathologist and examined for the presence of tumor cells.

Single-strand cDNA synthesis. Total RNA was isolated using RNeasy columns from Qiagen (Hilden, Germany) according

to the manufacturer's instructions. RNA integrity was confirmed spectrophotometrically and by electrophoresis on 1% agarose gels. For cDNA synthesis, 5 μg total RNA from each patient sample were reverse-transcribed in a final reaction volume of 50 μl containing 1X TaqMan RT buffer, 2.5 μM/l random primers, 500 μM/l of each dNTP, 5.5 mM/l $MgCl_2$, 0.4 U/μl RNase inhibitor and 1.25 U/μl Multiscribe Reverse Transcriptase. All reverse transcription polymerase chain reaction (RT-PCR) reagents were purchased from Applied Biosystems (Foster City, CA, USA). The reaction conditions were 10 min at 25˚C, 30 min at 48˚C and 5 min at 95˚C.

Quantitative (q)PCR. All RT-qPCR assays containing the primer and probe mix were purchased from Applied Biosystems and utilized according to the manufacturer's instructions. PCR was performed using 10 μl 2X Taqman PCR Universal Master Mix No AmpErase® UNG, 1 μl gene assay, 8 μl RNase-free water and 1 μl cDNA template (50 mg/l). The theoretical basis of the qRT assays has been described in detail in a previous study (19). All reactions were run in duplicates along with 'no template controls' and an additional reaction in which reverse transcriptase was omitted to assure no genomic DNA contamination in each RNA sample. For signal detection, the ABI Prism 7900 sequence detector was programmed for an initial step of 10 min at 95˚C, followed by 40 thermal cycles of 15 sec at 95˚C and 10 min at 60˚C, and the log-linear phase of amplification was monitored to obtain cycle threshold (CT) values for each RNA sample. Gene expression of all target genes was analyzed in relation to the levels of the slope matched housekeeping gene, CAPN2 (20). Results were analyzed using the ΔCT method.

Calculations and statistical analysis. The expression profiles of CXCL12 and CXCR4 in the different entities are presented as the mean ± standard error of the mean. All statistical calculations were performed with the MedCalc software package (MedCalc software, Mariakerke, Belgium) (21). The parametric Student's *t*-test was applied for a normal distribution, otherwise, the Wilcoxon's rank sum test was used. P<0.05 at a two-sided level of α<0.05 was considered to indicate a significant difference.

Results

CXCL12/CCR4 expression in GC. CXCL12/CXCR4 expression in GC tissues displayed an inverse mRNA expression profile. A significant downregulation of CXCL12 expression was demonstrated in the GC tissues with respect to the corresponding normal gastric tissues (P<0.05), as shown in Fig. 1. By contrast, CXCR4 mRNA expression was shown to be significantly upregulated in the GC tissues (P<0.05) (Fig. 1).

CXCL12 expression correlates inversely with lymph and vein infiltration in GC. In the GC patients with lymph and vein infiltration, CXCL12 mRNA expression was demonstrated to be significantly downregulated with respect to the GC patients without lymph and vein infiltration (both P<0.05), as shown in Fig. 2. Thus, CXCL12 mRNA expression correlates inversely with higher tumor stages in GC. CXCR4 mRNA expression showed no significant differences between GC patients with or without lymph and vein infiltration (P>0.05).

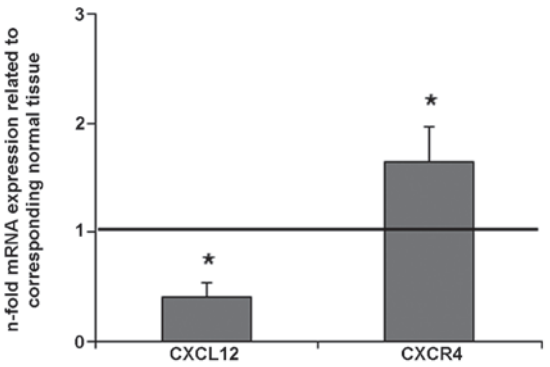

Figure 1. CXCL12/CXCR4 mRNA expression in gastric adenocarcinoma (GC) patients, as determined by RT-qPCR. RT-qPCR data are expressed as the mean ± standard error of the mean (n=66), *P<0.05 vs. unaffected adjacent tissues. Values <1 indicate CXCL12 mRNA downregulation and values >1 indicate CXCR4 mRNA overexpression in affected tissues with respect to unaffected neighboring tissues. CXCL12, chemokine (C-X-C motif) ligand 12; CXCR4, CXC receptor 4; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

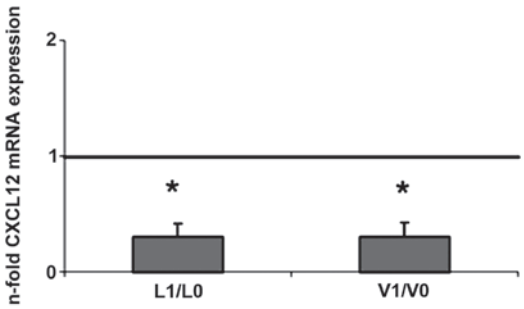

Figure 2. CXCL12 mRNA expression in gastric carcinoma patients with lymph and vein infiltration, as determined by RT-qPCR. RT-qPCR data are expressed as the mean ± standard error of the mean, *P<0.05 for L1 vs. L0 and V1 vs. V0 tissues. n=52 (L1) and n=14 (L0), and n=12 (V1) and n=54 (V0). Values <1 indicate CXCL12 mRNA downregulation in L1/V1 tissues with respect to L0/V0 tissues. CXCL12, chemokine (C-X-C motif) ligand 12; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

CXCR4 mRNA expression under the influence of neoadjuvant chemotherapy. In the GC patients who underwent neoadjuvant chemotherapy, CXCR4 mRNA expression was shown to be significantly upregulated with respect to the untreated GC patients (P<0.05) (Fig. 3). This indicates that CXCR4 mRNA upregulation is significantly promoted under the effect of neoadjuvant chemotherapy prior to surgery in GC patients. However, no significant difference in CXCL12 mRNA expression was observed in GC patients who underwent neoadjuvant chemotherapy compared with those that did not undergo neoadjuvant chemotherapy (P>0.05).

Discussion

In the last decade, a number of chemokines have been indicated to possess important functions in tumor progression and metastasis. In this respect, the binding of CXCL12 to CXCR4 was shown to stimulate the activation of several downstream signaling pathways that regulate the progression and metastasis of various tumors. The mitogen-activated protein kinase

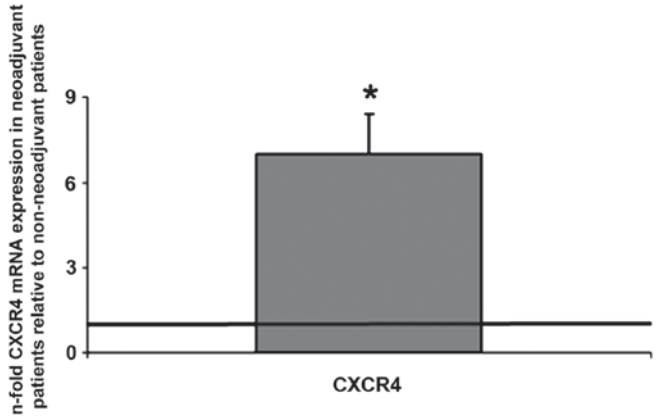

Figure 3. CXCR4 mRNA expression in neoadjuvant chemotherapy-treated patients with gastric carcinoma, as determined by RT-qPCR. RT-qPCR data are expressed as the mean ± standard error of the mean (n=10), *P<0.05 vs. non-neoadjuvant chemotherapy-treated patients. Values >1 indicate CXCR4 mRNA overexpression in the neoadjuvant chemotherapy-treated patients with respect to the non-treated patients. CXCR4, chemokine (C-X-C motif) receptor 4; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

and phosphoinositide 3-kinase pathways are the two most significant downstream pathways that are regulated by the CXCL12-CXCR4 interaction. CXCR4 expression in malignant epithelial cells and those cells from a number of hematopoietic malignancies indicates that the CXCL12/CXCR4 pathway may affect cancer biology and possess an essential function in directing $CXCR4^+$ tumor cell metastasis to organs expressing CXCL12 (22). Various CXCR4-expressing tumors metastasize to the bones and lymph nodes in a CXCL12-dependent manner, where the bone marrow in particular is able to provide a protective environment for the tumor cells (23). CXCL12 has also been shown to bind to CXCR7, and similar to CXCR4, CXCR7 expression has also been shown to be expressed and involved in the progression of various tumor entities. In bone sarcomas, the involvement of the CXCL12/CXCR4/CXCR7 axis was demonstrated in tumor growth and metastasis, and the targeting of this axis in preclinical studies was shown to affect tumor growth (14). The CXCL12/CXCR4 axis was also demonstrated to play a major role in cell survival, proliferation, the promotion of angiogenesis and the migration of tumor cells into metastatic sites in various other cancer entities, including breast, renal and prostate cancer (24-26). CXCR4 expression has been shown to be associated with tumor progression in a number of gastrointestinal malignancies, but particularly in esophageal, gastric, pancreatic, hepatocellular and colorectal cancer. In hepatocellular cancer, high CXCR4 expression was associated with locally advanced primary tumors and lymphogenic metastasis (27), and in pancreatic cancer progression, the CXCL12/CXCR4 axis has a significant function in tumor cell migration and angiogenesis (28). CXCR4 was previously indicated as a risk factor for the development of colon carcinoma micrometastases (29). Moreover, CXCR4 was demonstrated to increase the risk for recurrence and poor survival in colorectal cancer patients (30), while in rectal cancer following chemoradiotherapy, CXCR4 and CXCL12 expression was shown to be associated with distant recurrence and a poor prognosis (31).

Furthermore, recent results of our previous studies indicated that a correlation existed between CXCR4 expression and colorectal liver metastasis development (32). CXCR4 mRNA silencing was also shown to abrogate the CXCL12-induced migration of colorectal cancer cells (33).

Additionally, in GC, CXCR4 expression was shown to play a role with respect to the prediction of lymph node status, including micrometastasis (34). Moreover, a significant correlation was found between CXCR4-expressing primary GCs and the development of peritoneal carcinomatosis and malignant ascites, which contained high CXCL12 concentrations (35).

While Ying *et al* examined the expression levels of CXCL12 and CXCR4 by immunohistochemical staining in primary gastric tumor tissues and metastatic lymph nodes (17), the present study intended to investigate the expression profile of CXCL12/CXCR4 on the mRNA level in GC patients and to evaluate the clinical significance. Ying *et al* identified positive staining for CXCR4 in the majority of the primary gastric tumor tissues under investigation and demonstrated that the intensity of CXCR4 staining in these tissues was positively associated with lymph node metastasis, TNM staging and disease prognosis. In concordance with these findings, the present study also demonstrated significant upregulation of CXCR4 mRNA expression in the GC tissues. Moreover, it was shown that in GC patients who underwent neoadjuvant chemotherapy, CXCR4 mRNA expression was significantly upregulated with respect to untreated GC patients. Therefore, the present study concluded that CXCR4 mRNA upregulation was significantly promoted under the effect of neoadjuvant chemotherapy prior to surgery in GC patients. In contrast to the immunohistochemical data presented by Ying *et al* demonstrating positive staining for CXCL12 in the majority of the primary gastric tumor tissues, the present study observed a significant downregulation of CXCL12 in the GC tissues with respect to the corresponding normal gastric tissues. Moreover, in the GC patients with lymph and vein infiltration, CXCL12 mRNA expression was shown to be significantly downregulated with respect to the GC patients without lymph and vein infiltration. It was therefore concluded that CXCL12 mRNA expression correlates inversely with higher tumor stages in GC.

In summary, the present findings have demonstrated the relevance of CXCR4 expression for the tumor progression of GC. Thus, the results may suggest CXCR4 as a potential therapeutic target for this aggressive disease.

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