Upregulation of C-X-C chemokine receptor type 1 expression is associated with late-stage gastric adenocarcinoma

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Abstract. Chemokine receptors play multiple roles in the development and progression of various tumor types. The aim of this study was to examine C-X-C chemokine receptor type 1 (CXCR1) protein expression in gastric adenocarcinoma and to investigate the clinical implications of CXCR1 upregulation. Expression of CXCR1 protein in 83 specimens of sporadic gastric adenocarcinoma and their corresponding non-neoplastic mucosa obtained by gastrectomy was assayed using immunohistochemistry. The intensity of immunostaining in tumor tissue was considered strong when tumor tissue staining was more intense than in the corresponding non-neoplastic mucosa; the intensity was null when staining was weaker in the tumor than in the corresponding non-neoplastic mucosa; and the intensity was weak when staining was similar in both tissues. Microvascular density in tumor tissue and its corresponding non-neoplastic mucosa was measured using monoclonal antibody against CD34. A strong correlation was observed between elevated CXCR1 protein expression and tumor stage ($P<0.05$). T stage, N stage and overall stage positively correlated with CXCR1 protein expression. Microvascular density was higher in tumors with strong CXCR1 protein expression, but the correlation with CXCR1 was not linear ($P=0.07$). Multiple logistic regression analyses showed that, compared to no or weak expression, overexpression of CXCR1 protein was a significant risk factor for high N stage (N2, N3). These results indicate that CXCR1 may be considered as a new and promising target for gastric adenocarcinoma therapy.

Introduction

Gastric cancer is the second most common cause of cancer-related deaths worldwide, particularly in China, and the incidence is increasing yearly (1-3). Gastric adenocarcinoma accounts for the majority of gastric cancer cases. Despite substantial advances in treatment and effort in research over the past few decades, the outcome of gastric cancer remains unsatisfactory, and the overall 5-year survival rate of advanced gastric adenocarcinoma patients is low. Therefore, improvement in the therapy of gastric cancer now depends on improving our understanding of the complex molecular mechanisms governing the progression and aggressiveness of the disease. Invasion and metastasis are major prognostic factors for advanced gastric cancer (4). In addition to surgery, adjuvant chemotherapy is used to negate the effects of invasion and metastasis in gastric adenocarcinoma, but the survival benefit is only marginal. Thus, understanding the mechanism of invasion and metastasis is critical to develop new treatment strategies that contribute to improving the survival of patients with advanced gastric adenocarcinoma (5).

The aggressive nature of human gastric carcinoma is dependent on a number of events, including cell degradation of the basement membrane, cell migration through surrounding tissues, intravasation into lymphatic or blood vessels, cancer cells exiting from these vessels, cell survival and proliferation (5,6). Chemokine receptors are believed to be involved in these complicated processes. Chemokine receptors are divided into various families (7,8): CXC chemokine receptors, CC chemokine receptors, CX3C chemokine receptors and XC chemokine receptors, which correspond to the 4 distinct subfamilies of chemokines that they bind. Chemokine receptors are G protein-coupled receptors containing 7 transmembrane domains that are found predominantly on the surface of leukocytes. Previous studies have found that certain chemokine receptors are expressed in certain tumor cells, which, under the action of chemotactic substances, show directed chemotaxis and play a significant role in tumor angiogenesis, invasion and metastasis (9-18). Therefore, the association between chemokine receptors and tumor cell growth, progression, invasion and metastasis has attracted significant attention. Identification of such chemokine receptors not only leads to a better understanding of the carcinogenesis and progression of gastric adenocarcinoma, but also provides new strategies for developing targeted agents that specifically suppress the process.

CXCR1 is a receptor for interleukin 8 (IL-8), which binds to CXCR1 with high affinity and transduces the signal through

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a G-protein-activated second messenger system. CXCR1 is mainly expressed in neutrophils and is originally characterized by its ability to induce chemotaxis of leukocytes. CXCR1 has been shown to act on multiple cell types. Knockout studies in mice have indicated that this protein inhibits embryonic oligodendrocyte precursor migration in developing spinal cord. Moreover, it was found that CXCR1 overexpresses in many solid tumors, which shows a close correlation with drug-resistance, invasion, and metastasis (11,19-23). Although CXCR1 has been studied in several cancer types and a small number of studies have examined the role of CXCR1 in gastric adenocarcinoma specifically (24-29), the precise functional role of CXCR1 in gastric adenocarcinoma progression remains controversial and unclear. In our study, we investigated the level of CXCR1 protein expression in primary and sporadic gastric adenocarcinoma as well as in its corresponding non-neoplastic mucosa, and preliminarily discussed the clinical implications of our findings.

Materials and methods

Patients and specimens. Our study was conducted on 83 primary and sporadic gastric adenocarcinoma tissue samples and their corresponding non-neoplastic mucosa specimens retrieved from the archives at the Department of Pathology of Xiang-ya Hospital of Central South Univesrsity between 2008 and 2010. All patients provided informed consent, and the protocol followed the ethical guidelines of the Declaration of Helsinki. None of the patients received chemotherapy or radiation therapy prior to tumor resection. Tissue blocks of non-neoplastic mucosa (>5 cm from the edge of the tumor) were obtained. Tumors stage was classified according to the AJCC staging system. Patient data and the histopathological characteristics of the tumors are shown in Table I.

Table I. Patient data and tumor characteristics.

Characteristic	n (%)
Total	83 (100)
Gender	
Male	61 (73.5)
Female	22 (26.5)
Median age, years (range)	55 (31-79)
TNM stage	
T stage	
T1	5 (6.0)
T2	17 (20.5)
T3	44 (53.0)
T4	17 (20.5)
N stage	
N0	26 (31.3)
N1	24 (28.9)
N2	18 (21.7)
N3	15 (18.1)
Overall stage	
IA, IB	10 (12.0)
II	39 (47.0)
IIIA	17 (20.5)
IIIB, IIIC, IV	17 (20.5)
Differentiation	
Good	21 (25.3)
Moderate	24 (28.9)
Poor	38 (45.8)

Detection of CXCR1 protein in specimens. Immunohistochemical staining for CXCR1 was performed on formalin-fixed and paraffin-embedded material using standard procedures. Sections (4 μm thick) were deparaffinized in turpentine and rehydrated in a series of graded alcohol. Microwave antigen retrieval was performed in citrate buffer (0.01 M, pH 6.0) for 2x10 min at 450 W. After cooling to room temperature, the specimens were rinsed three times for 3 min with phosphate-buffered saline. Endogenous peroxidase was blocked by pre-incubation of the slides with 3% hydrogen peroxide (H_2O_2), and non-specific binding was blocked with non-immune goat serum. Blocked sections were incubated in anti-CXCR1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight, and the antibody was used at a dilution of 1:100. The subsequent reaction was performed using the S-P kit (ZhongshanGoldenBridge Biotechnology Co., Beijing, China) according to the manufacturer's instructions. Finally, the immunoreaction was developed using diaminobenzidine (DAB) and counterstained with hematoxylin. IgG2b-stained sections were used as negative controls, and sections from tonsil were used as positive controls. Reddish-brown granules on the membrane and in the cytoplasm of tumor cells or in that of corresponding non-neoplastic mucosa epithelial cells indicated positive immunoreactivity. The intensity of immunostaining in tumor tissue was scored using corresponding non-neoplastic mucosa tissue as an internal control. Tumor tissue was considered to have strong expression if it showed stronger intensity than that of the corresponding non-neoplastic mucosa tissue. If the staining intensity was similar to that in the corresponding non-neoplastic mucosa tissue, we considered the sample to have weak expression. If the intensity was weaker than in corresponding non-neoplastic mucosa tissue, the samples were considered to have no expression. The samples were evaluated by two pathologists who were blinded to the patients' clinical data (5).

Detection of microvascular density in specimens. Immunohistochemical staining using monoclonal antibody to CD34 (Santa Cruz) was performed as described above to measure microvascular density (MVD) in the tumor tissue and corresponding non-neoplastic mucosa. Stained vessels were counted under high-power microscopic fields. The average number of vessels counted in the best-visualized area was recorded for each case (30).

Statistical analysis. Statistical analysis was performed using the Spearman correlation, when appropriate, to analyze the significance of the correlation between CXCR1 protein expression and tumor data, such as cancer cell differentiation, T stage, N stage,

Table II. CXCR1 expression and tumor status.

Characteristic	No expression (n=24), n (%)	Weak expression (n=46), n (%)	Strong expression (n=13), n (%)	P-value
Male:female	18:6	34:12	9:4	P>0.05
Age (years)	55.6±24.6	52.3±20.0	56.0±27.3	P>0.05
Cancer cell differentiation				P>0.05
Good	7 (29.2)	11 (23.9)	3 (23.1)	
Moderate	5 (20.8)	15 (32.6)	4 (30.8)	
Poor	12 (50.0)	20 (43.5)	6 (46.1)	
T stage				P<0.05[a]
T1	4 (16.7)	1 (2.2)	0 (0.0)	
T2	7 (29.2)	9 (19.6)	1 (7.7)	
T3	11 (45.8)	25 (54.3)	8 (61.5)	
T4	2 (8.3)	11 (23.9)	4 (30.8)	
N stage				P<0.05[a]
N0	13 (54.2)	12 (26.1)	1 (7.7)	
N1	8 (33.3)	16 (34.8)	0 (0.0)	
N2	2 (8.3)	14 (30.4)	2 (15.4)	
N3	1 (4.2)	4 (8.7)	10 (76.9)	
Overall stage				P<0.05[a]
IA, IB	7 (29.2)	3 (6.5)	0 (0.0)	
II	14 (58.3)	24 (52.2)	1 (7.7)	
IIIA	2 (8.3)	14 (30.4)	1 (7.7)	
IIIB, IIIC and IV	1 (4.2)	5 (10.9)	11 (84.6)	

[a]P<0.05 by Spearman correlation.

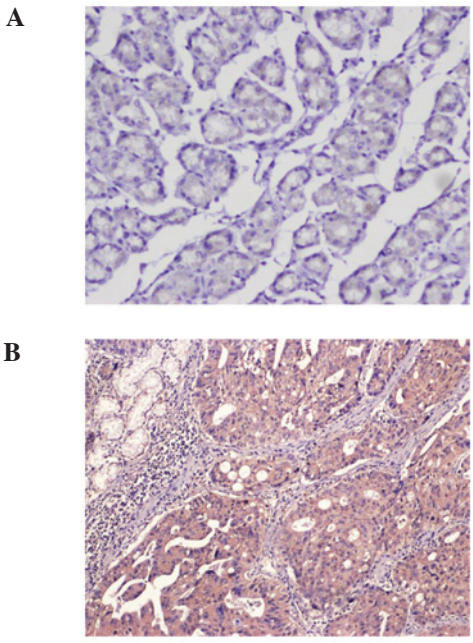

Figure 1. Representative immunostaining for CXCR1 in corresponding non-neoplastic mucosa epithelial and tumor cells. (A) Corresponding non-neoplastic mucosa epithelial cells. (B) Tumor tissue with strong expression.

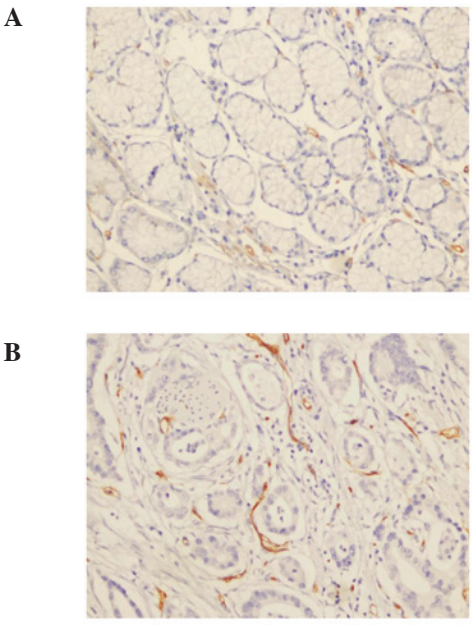

Figure 2. Representative immunostaining for CD34 to calculate MVD in corresponding non-neoplastic mucosa epithelial and tumor cells. (A) Corresponding non-neoplastic mucosa epithelial cells. (B) Tumor tissue with strong expression.

overall stage and MVD. Multivariate logistic regression analysis was performed to determine factors associated with tumor stage. The SPSS13.0 software system was used and a P-value <0.05 was considered to indicate statistical significance.

Table III. Microvascular density (MVD) and tumor status.

Characteristic	MVD	P-value
CXCR1 expression		P>0.05
No expression	14.5±12.8	
Weak expression	16.5±14.7	
Strong expression	17.8±12.2	
T stage		P>0.05
T1	11.0±7.2	
T2	14.4±11.5	
T3	17.3±14.6	
T4	16.5±14.1	
N stage		P<0.05[a]
N0	13.7±11.8	
N1	16.3±14.3	
N2	18.0±14.0	
N3	18.0±14.8	
Overall stage		P<0.05[a]
IA, IB	12.0±11.5	
II	14.8±12.0	
IIIA	20.5±13.7	
IIIB, IIIC and IV	17.4±15.1	

[a]P<0.05 by Spearman correlation.

Table IV. Results of the multivariate analysis regarding high N stage.

Characteristic	P-value	Exp (B)	95% CI for Exp (B)
T stage (1, 2 vs. 3, 4)	0.799	1.230	(0.250-6.042)
Cancer cell differentiation (good vs. poor)	0.896	0.895	(0.169-4.729)
MVD	0.716		
CXCR1 expression			
No expression	0.002[a]	0.019	(0.001-0.246)
Weak expression	0.113	0.151	(0.015-1.564)
Strong expression		1	

MVD, microvascular density. [a]P<0.05 by logistic regression analysis.

Results

Association between CXCR1 overexpression and late-stage tumors. Non-neoplastic gastric mucosal epithelium expressed CXCR1 in a heterogeneous manner. In all cases, immunoreactivity was observed in the membrane and in the cytoplasm of the tumor cells (Fig. 1). Demographic characteristics and tumor status were analyzed according to CXCR1 expression levels (Table II). In this study, we grouped the tumor stages as follows: IA and IB as I, II as II, IIIA as III, and IIIB, IIIC and IV as IV. As tumor expression of CXCR1 increased, so did the overall tumor stage (P<0.05). Of 13 tumors with strong CXCR1 expression, 11 (84.6%) were stage IV, but only 1 (4.2%) of 24 tumors and 5 (10.9%) of 46 tumors with no or weak CXCR1 expression, respectively, were stage IV. N stage positively correlated with CXCR1 expression, as 1 (4.2%) of 24 tumors with no expression and 4 (8.7%) of 46 tumors with weak expression were at the N3 stage, compared to 10 (76.9%) of 13 tumors with strong expression (P<0.05). CXCR1 expression also correlated with T stage (P<0.05). However, we observed no correlation between cancer cell differentiation and CXCR1 expression.

No correlation between CXCR1 expression and MVD. MVD was calculated as the number of vessels per high-power microscopic field (Fig. 2). According to the statistical analysis, MVD correlated with N stage and overall tumor stage (P<0.05), but not with T stage or CXCR1 expression (Table III). MVD for stages I, II, III and IV was 12.0±11.5, 14.8±12.0, 20.5±13.7, and 17.4±15.1, respectively. MVD positively correlated with overall stage (P<0.05). The average MVD was 14.5±12.8 for tumors with no CXCR1 expression, 16.5±14.7 for tumors with weak CXCR1 expression, and 17.8±12.2 for tumors with strong expression. MVD tended to be higher in tumors with higher T stage and marked CXCR1 expression, but the correlation with T stage and CXCR1 expression levels was not linear (P>0.05).

Factors associated with tumor stage. N2- and N3-stage tumors were considered high-N-stage tumors. T3- and T4-stage tumors served as high-T-stage tumors. Based on univariate analysis, cancer cell differentiation, T stage, MVD, and CXCR1 levels were significantly associated with high N stage, and cancer cell differentiation, N stage, MVD, and CXCR1 levels were significantly associated with high T stage. However, multivariate logistic regression analysis with cancer cell differentiation, T stage, MVD, and CXCR1 levels showed that CXCR1 was the only factor significantly associated with high N stage (Table IV), but CXCR1 was not a factor significantly associated with high T stage. Poorly differentiated cancer cells were associated with high N stage, but this finding was not statistically significant. Strong CXCR1 expression had a 52.3- and 6.6-fold higher risk for high N stage compared to no and weak CXCR1 expression, respectively (P<0.05 and P>0.05, respectively). Multivariate analysis showed 78.8% sensitivity and 90.0% specificity for predicting high N stage.

Discussion

Currently, despite advances in early diagnosis and treatment that have improved the survival of patients with gastric adenocarcinoma, this malignancy has retained a high mortality rate (5). To further improve survival, treatments based on a better understanding of cancer progression are necessary (31). CXCR1 protein, a receptor for interleukin 8 (IL-8), is a member of the G-protein-coupled receptor family, which binds to IL-8 with high affinity and transduces the signal through a G-protein-activated second messenger system. Previous studies have found that CXCR1 expression shows a close correlation with drug-resistance, invasion and metastasis in a number of

solid tumors (11,19-23,32). Taken together, these observations indicate that CXCR1 may play a role in the development and progression of certain tumors by interacting with IL-8.

To investigate whether CXCR1 is associated with the invasion and metastasis of gastric adenocarcinoma and performs certain biological functions, we examined the expression of CXCR1 protein in primary gastric carcinoma and its corresponding non-neoplastic mucosa using immunohistochemistry. Our study showed that the expression level of CXCR1 was higher in primary gastric adenocarcinoma than in its corresponding non-neoplastic mucosa in certain cases. Our experimental results revealed a marked association between overexpression of CXCR1 and late gastric adenocarcinoma stage. CXCR1 expression was significantly associated with high N stage, as demonstrated using multivariate analysis. Tumors with strong CXCR1 expression exhibited higher risk of high N stage compared to no and weak CXCR1 expression. CXCR1 expression was also correlated with T stage and overall stage. These findings suggest that CXCR1 may be involved in gastric adenocarcinoma invasion and metastasis, and the association between strong CXCR1 expression and late-stage gastric adenocarcinoma may contribute to its association with high N stage. A number of studies have postulated an association between CXCR1 expression and cancer cell invasion and metastasis in certain cancer types (11,19-23); our findings further support this hypothesis.

It is believed that through various mechanisms, chemokine receptors play multiple roles in the development and progression of a number of tumor types (32-36). CXCR1 regulates cell motility and angiogenesis and cell migration and invasion, in which various intracellular pathways are involved, and motility can be activated by chemokine receptors (37-42). CXCR1 and its related pathways may become potential targets for cancer treatment, thus it is important to clarify the mechanistic roles of CXCR1 and whether CXCR1 plays a major role in cancer progression. IL-8 binding to CXCR1 is a strong neutrophil attractant. In non-cancerous conditions, neutrophils recruited by IL-8 binding to CXCR1 cause tissue damage. One study has suggested that increasing amounts of tumor-infiltrating neutrophils in advanced gastric cancer are associated with reduced mortality (43). However, neutrophils can either eliminate tumor cell populations or contribute to their invasive potential (44,45). Neutrophils may enable tumor cells to migrate through the extracellular matrix, helping them to enter the vasculature (46). Based on our results, neutrophil recruitment by CXCR1 binding to its ligand IL-8 may aid gastric adenocarcinoma cells in metastasizing to lymph nodes. Although neutrophil infiltration was not analyzed and the role of neutrophil in tumors is controversial, this may be another hypothesis supporting our results. Another possibility is that overexpression of CXCR1 in gastric adenocarcinoma cell binding to its ligand IL-8 results in tumor cell migration. It has been reported that *Helicobacter* infection is associated with chemokine IL-8 and its receptor CXCR1 (47,48), and moreover that it is associated with gastric cancer; however, we are currently unable to conclude that *Helicobacter* infection contributed to gastric cancer via CXCR1.

A promising recent study found that CXCR1 expression subdivides cancer stem cell populations. The IL-8/CXCR1 axis may be involved in the regulation of cancer stem cell proliferation, self-renewal and drug-resistance, which leads to tumor cell invasion and metastasis (22). Further studies are warranted to determine whether this finding is applicable to gastric adenocarcinoma.

Studies on malignant melanoma and breast cancer suggest that expression of CXCR1 *in vivo* and *in vitro* is associated with poor prognosis; these studies indicate that CXCR1 is associated with tumor growth and enhanced angiogenesis (22,23,49,50). Angiogenesis is another essential step for tumor growth and metastasis, and expression of CXCR1 and VEGF can provide a positive feedback loop (51); this hypothesis is supported by our immunohistochemistry results. In our study, CXCR1 expression and microvessel count were evaluated on 83 sporadic gastric adenocarcinoma tissue sections to observe a correlation between CXCR1 expression and MVD within a certain area of the tumor. Notably, tumor samples with strong CXCR1 expression had a high MVD, but the correlation between CXCR1 expression and MVD was not linear. Furthermore, there may be more than one pathway regulating angiogenesis (52). However, we cannot exclude the possibility that CXCR1 promoted tumor cell survival by supplying blood vessels to late-stage gastric adenocarcinoma.

In conclusion, this is, to our knowledge, the first relatively clear report that overexpression of CXCR1 is associated with advanced gastric adenocarcinoma stage, specifically high N stage. Through multiple mechanisms, CXCR1 may be involved in the invasion and metastasis of gastric adenocarcinoma cells and late-stage gastric adenocarcinoma progression. Therefore, the novel expression and function of CXCR1 not only adds to our knowledge of CXCR1, but also elucidates the pathogenesis of gastric adenocarcinoma. Further studies are required to confirm and understand this observation and to determine whether CXCR1 may serve as a new and promising therapeutic target for gastric adenocarcinoma treatment.

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