SDF1α/CXCR4 axis may be associated with the malignant progression of gastric cancer in the hypoxic tumor microenvironment

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Abstract. Stromal cell-derived factor 1α (SDF1α) and its receptor C-X-C chemokine receptor type 4 (CXCR4) have been reported to form an important chemokine signaling pathway. Our previous study reported that SDF1α from tumor stromal cells may stimulate the proliferation of gastric cancer (GC) cells through the CXCR4 axis in a hypoxic microenvironment. However, a limited number of studies have addressed the clinicopathological significance of the expression of SDF1α and CXCR4 in GC, particularly at hypoxic regions. Immunohistochemistry was used to investigate the expression levels of SDF1α, CXCR4 and the hypoxic marker carbonic anhydrase 9 (CA9) in 185 patients with stage II and III GC. The results demonstrated that CA9 was expressed on cancer and stromal cells in hypoxic lesions, CXCR4 was mainly expressed in cancer cells, and SDFα was mainly expressed in stromal cells. CXCR4 expression in cancer cells and SDFα expression in stromal cells were associated with the hypoxic regions with CA9 expression. The CA9 and CXCR4 expression in the cancer cells, and the SDF1α expression in the stromal cells (CA9/CXCR4/SDF1α) was significantly associated with macroscopic type 4 tumor (P=0.012) and the pattern of tumor infiltration into the surrounding tissue (P<0.001). The prognosis of the all CA9/CXCR4/SDF1α-positive patients was significantly poorer compared with that of patients with CA9-, CXCR4- or SDF1α-negative GC at Stage III (P=0.041). These results indicated that hypoxia may upregulate SDFα production in stromal cells and CXCR4 expression in cancer cells. The SDF1α/CXCR4 axis may serve an important role in the progression of GC.

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Introduction

Gastric cancer (GC) has the fifth highest cancer incidence and second highest rate of cancer-associated mortalities among all malignant neoplasms worldwide (1). Although curative resection (R0) with lymph node dissection and adjuvant chemotherapy has prolonged the survival of patients with GC, the recurrence rate of R0 cases remains at ~30% in patients with stage II/III GC (2). Peritoneal recurrence is the most frequent recurrence pattern in patients with GC following curative resection, and as such, peritoneal recurrence is the most common cause of subsequent cancer-associated mortality (3).

Stromal cell-derived factor 1α (SDF1α, also termed CXC ligand 12) and its receptor C-X-C chemokine receptor type 4 (CXCR4) have been known to serve a critical role in cancer cell migration and proliferation in solid tumors, including GC (4,5), breast (6), esophageal (7), prostate tumor (8), pancreatic cancer (9,10), melanoma (11), colon (12), ovarian (13) and lung cancer (14).

Various types of solid tumors, including GC have a heterogeneously hypoxic environment which is currently thought to be associated with aggressive tumor phenotypes (15-19). Clinical and experimental data on GC also provide evidence of an association between the hypoxic environment and a poor prognosis (16,18). Therefore, a hypoxic environment has been considered to be associated with aggressive tumor phenotypes of gastric carcinomas (20,21), including the metastatic ability of cancer cells (22).

Our recent study reported that the progression of GC may be recognized as the product of evolving crosstalk between the cancer cells and their surrounding tumor stroma (23,24). The results of our previous study reported that SDF1 from tumor stromal cells may stimulate the proliferation of GC cells through the CXCR4 axis in hypoxic microenvironments (4).

Certain studies also reported that the expression of CXCR4 in cancer cells has been upregulated under hypoxia (25,26). However, the clinical association between the expression of SDF1α/CXCR4 and hypoxic conditions in GC has been unclear. The present study investigated the clinicopathological significance of SDF1α and CXCR4 expression and a hypoxic environment in GC at stage II and III.

Materials and methods

Clinical materials. Human GC tissues were obtained from a total of 185 patients with stage II or III GC, who had undergone resection of a primary GC at Osaka City University Hospital. Patients with stage I or stage IV GC were excluded. None of the patients had undergone preoperative radiation and/or chemotherapy. The pathological diagnoses and classifications were made according to classified by the Japanese Classification of Gastric Carcinoma 3rd English edition (27) or the Union for International Cancer Control Tumor-Node-Metastasis classification of malignant tumors (28). Table I shows the clinicopathological characteristics of 185 patients with stage II and III GC. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki (29). The present study was conducted with the approval of the Ethical Committee of Osaka City University (reference number 924). Written informed consent was obtained from all patients prior to treatment.

Immunohistochemical techniques. The GC tissue was preserved by fixing in a solution of 10% neutral-buffered formalin for ~24 h at room temperature. Immunohistochemical staining was performed on 4-μm sections of formalin-fixed paraffin-embedded tissue. The slides were deparaffinized in xylene and rehydrated in decreasing concentrations of ethyl alcohol. The sections were heated for 10 min at 105˚C by autoclave in Target Retrieval Solution (Dako; Agilent Technologies, Inc.). The sections were blocked for 10 min at room temperature with 10% normal goat serum (Histofine Simple Stain™ MAX-PO; Nichirei Biosciences Inc.) and subsequently incubated with 3% hydrogen peroxide to block endogenous peroxidase activity. Immunohistochemistry was performed using the following antibodies: Anti-CXCR4 (cat. no. ab124824; dilution 1:100; Abcam), anti-SDF1α (cat. no. MAB350; dilution 1:200; R&D Systems, Inc.), and a hypoxic marker, carbonic anhydrase 9 (CA9; clone; cat. no. M75; dilution 1:1,000; Novus Biologicals, LLC). The specimens were incubated with the antibodies at 4˚C overnight, followed by three washes with PBS. The slides were treated with streptavidin-peroxidase reagent and were incubated in PBS diaminobenzidine and 1% hydrogen peroxide vol/vol, followed by counterstaining with Mayer's hematoxylin for 1 min at room temperature and analysis of three fields per sample under a light microscope (magnification, x100).

Immunohistochemical determination of SDF1α, CXCR4 and CA9. Positive immunostaining was evaluated by two independent investigators who were blinded to patient outcomes and clinicopathological features. A numerical scoring system with two categories was used to assess the intensity and the extent of immunoreactivity. The proportion score was an estimate of the proportion of positive cells: 0, no immunoreactive cells; 1, <20% immunoreactive cells; 2, 20-50% immunoreactive cells; and 3, ≥50% immunoreactive cells. The intensity score estimates the average staining intensity of positive tumor cells: 0, no staining; 1, weak positive membrane staining; 2, moderate; and 3, strong staining. The two scores were multiplied together to give a final numerical score ranging between 0 and 9. The cases were considered positive if the score was 5 or more.

Statistical analysis. The χ^2 test or Fisher's exact test were used to determine the significance of the difference between the covariates. Survival curves were constructed using Kaplan-Meier survival analysis and compared using the log-rank test. The influence of each prognostic factor on patient survival was evaluated using Cox regression analysis. All analyses were performed using SPSS software version 22.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Association between clinicopathological features and CA9 expression, CXCR4 expression in cancer cells, and SDF1α expression in the stromal cells. Representative images of CA9, CXCR4 and SDF1α immunostaining are presented in Fig. 1. CA9 was heterogeneously expressed on stromal cells (arrows) and GC cells (arrowheads). SDF1α expression in stromal cells was observed primarily in the cytoplasm of fibroblast-like stromal cells (arrows). CXCR4 expression was observed primarily in cancer cells (arrowheads). CA9 expression was significantly associated with CXCR4 expression in the cancer cells and SDF1α expression in the stromal cells (P=0.001), and was significantly associated with macroscopic type 4 tumor (P=0.021), and a pattern of tumor infiltration into the surrounding tissue (P=0.005). CA9 expression, CXCR4 expression in the cancer cells, and SDF1α expression in the stromal cells (CA9/CXCR4/SDF1α) were significantly associated with macroscopic type 4 (P=0.012) and a pattern of tumor infiltration into the surrounding tissue (P<0.001; Table II).

Survival analysis. Fig. 2 shows the Kaplan-Meier survival curve for all 185 patients according to CXCR4 and SDF1α expression. The patients who were positive for all CA9, CXCR4, and SDF1α were defined as the CA9/CXCR4/SDF1α-positive group, whereas the those who were negative for CA9, CXCR4 or SDF1α were termed CA9/CXCR4/SDF1α-negative. The prognosis of the CA9/CXCR4/SDF1α-positive group tended to be poorer compared with that of the CA9/CXCR4/SDF1α-negative patients with stage II or III GC (Fig. 2A; P=0.0826). The prognosis of patients with stage II GC was not different between the all CA9/CXCR4/SDF1α-positive and -negative groups (Fig. 2B). By contrast, the prognosis of the CA9/CXCR4/SDF1α-positive group was significantly poorer compared with that of the CA9/CXCR4/SDF1α-negative patients with stage III GC (Fig. 2C; P=0.041).

As presented in Table III, the univariate analysis revealed that CA9/CXCR4/SDF1α, age, macroscopic type and tumor size were each significantly associated with a poor prognosis. The multivariate analysis revealed that macroscopic type was independent prognostic factor, whereas CA9/CXCR4/SDF1α expression was not.

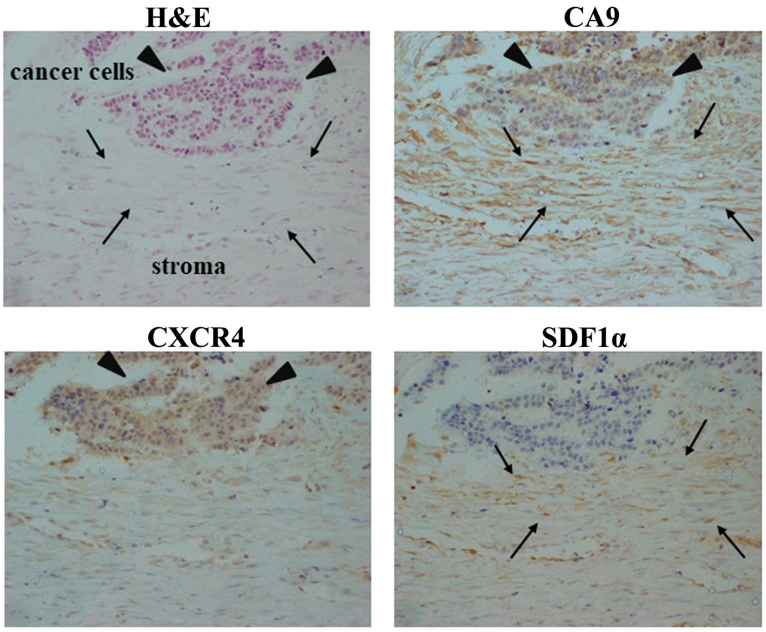

Figure 1. Immunohistochemical staining of CA9, CXCR4 and SDF1α. CA9 is expressed on cancer cells and stromal cells in hypoxic lesions. CXCR4 is primarily expressed in cancer cells (arrowheads) and SDFα is primarily expressed in stromal cells (arrows). Original magnification, x100.

Table I. Clinicopathological features of 185 patients with stage II or III gastric cancer.

Clinicopathological feature	n (n=185)
Sex	
Female	76
Male	109
Age, years	
<70	102
≥70	83
Macroscopic type	
Type 4	23
Other	162
Histological type	
Intestinal	85
Diffuse	100
Infiltration pattern	
a/b	123
c	58
Lymph node metastasis	
Negative	44
Positive	141
Stage	78
II	
III	107
Lymphatic invasion	
Negative	29
Positive	155
Venous invasion	
Negative	130
Positive	55

Discussion

CA9 is upregulated under hypoxic conditions through the upregulation and stabilization of hypoxia-inducible factor 1α (HIF-1α), which binds to the hypoxia-responsive element present in the promoter regions of CA9 (30). Therefore, CA9 was considered to indicate hypoxic loci, and was used as a hypoxic marker in the present study.

SDF1α was expressed in GC cells and stromal cells, as previously reported (31,32). In the GC microenvironment, SDF1α expression was observed mainly in the cytoplasm of fibroblast-like stromal cells, particularly frequently in the macroscopic type 4 or diffuse-type GC with abundant stromal cells. By contrast, the SDF1α expression on the cancer cells was observed primarily at the cell membrane. The SDF1α expression on the cancer cells was significantly associated with SDF1α expression on the stromal cells. SDF1α was first cloned from bone marrow-derived stromal cells (33) and was reported to be expressed on various stromal cells (34,35). These results suggested that SDF1α on the membrane of cancer cells may be derived from fibroblast-like stromal cells.

SDF1α was not expressed by any gastric or pancreatic cancer cell lines (36,37). Therefore, in the present study the SDF1α expression on stromal cells was investigated. Orimo *et al* (38) also demonstrated that SDF1α released by stromal fibroblasts directed the paracrine stimulation of tumor cells through CXCR4 expressed on breast cancer cells. SDF1α signaling may be associated with the malignant progression of cancer cells. It was also observed that the SDF1α expression on the tumor stromal cells was associated with the diffuse type, while that on the cancer cells was associated with the intestinal type (data not shown). SDF1α signaling may be different between the histological types of GC.

In the present study, CXCR4 expression on cancer cells was associated with macroscopic type 4, lymph node metastasis and peritoneal metastasis. It has been reported

Table II. Association between clinicopathological features and CA9/CXCR4/SDF1α expression in stage II and III gastric cancer.

	CA9 expression			CA9/CXCR4/SDF1α expression		
Factors	Positive n=96 (%)	Negative n=89 (%)	P-value	Positive n=20 (%)	Negative n=165 (%)	P-value
Age, years						
≥70	41 (49.4)	42 (50.6)	0.632	9 (10.8)	74 (89.2)	1.000
<70	54 (52.9)	48 (47.1)		11 (10.8)	91 (89.2)	
Sex						
Female	32 (42.1)	44 (57.9)	0.036	10 (13.2)	66 (86.8)	0.391
Male	63 (57.8)	46 (42.2)		10 (9.2)	99 (90.8)	
Macroscopic type						
Type 4	17 (73.9)	6 (26.1)	0.021	6 (26.1)	17 (73.9)	0.012
Other	78 (48.1)	84 (51.9)		14 (8.6)	165 (91.4)	
Tumor size, mm						
≥50	60 (52.6)	54 (47.4)	0.659	10 (8.8)	104 (91.2)	0.258
<50	35 (49.3)	36 (50.7)		10 (14.1)	61 (85.9)	
Histological type						
Diffuse	58 (58.0)	42 (42)	0.050	14 (14)	86 (86)	0.508
Intestinal	37 (43.5)	48 (56.5)		6 (7.1)	79 (92.9)	
[a]Infiltration pattern						
INF a/b	53 (43.1)	70 (56.9)	0.005	7 (5.7)	116 (94.3)	<0.001
INF c	38 (65.5)	20 (34.5)		12 (20.7)	46 (79.3)	
Stage						
II	35 (44.9)	43 (55.1)	0.132	8 (13.0)	70 (87.0)	0.836
III	60 (56.1)	47 (43.9)		12 (26.8)	95 (73.2)	
Lymph node metastasis						
Positive	73 (51.8)	68 (48.2)	0.837	15 (10.6)	126 (89.4)	0.727
Negative	22 (50.0)	22 (50.0)		5 (11.4)	39 (88.6)	
Lymphatic invasion						
Positive	76 (49.0)	79 (51.0)	0.103	15 (9.7)	140 (90.3)	0.230
Negative	19 (65.5)	10 (34.5)		5 (17.2)	24 (82.8)	
Venous invasion						
Positive	28 (50.9)	27 (49.1)	0.938	5 (9.1)	50 (90.9)	0.624
Negative	67 (51.5)	63 (48.5)		15 (11.5)	115 (88.5)	
CXCR4/SDF1α expression						
Positive	20 (83.3)	4 (16.7)	0.001			
Negative	75 (46.6)	86 (53.4)				

[a]INF, pattern of tumor infiltration into the surrounding tissue. The predominant pattern of infiltrating growth into the surrounding tissue is classified as follows; INF a, the tumor shows expanding growth and a distinct border with the surrounding tissue; INF b, this category is between INF a and INF b; INF c, the tumor shows infiltrating growth and an indistinct border with the surrounding tissue. SDF1α, stromal cell-derived factor 1α; CXCR4, C-X-C chemokine receptor type 4.

that CXCR4 expression was associated with lymph node or liver metastasis in GC (34,35), and was a prognostic factor in GC (39-41). In the present study, patients with CXCR4 and SDF1α expression exhibited significantly poorer prognoses. The results of the present study suggested that the SDF1α/CXCR4 axis may serve an important role in the progression of cancer, and that the expression of these molecules may be a useful prognostic factor for patients with stage III GC.

Hypoxia is thought to be associated with aggressive tumor phenotypes of gastric carcinomas (42,43), including the metastatic ability of cancer cells (44,45). Clinical and experimental data have also provided evidence of an association between the hypoxic environment and a poor prognosis (45,46). In the present study, CA9, which was used to investigate the hypoxic cells, was demonstrated to be expressed heterogeneously in a gastric tumor, and it was found that the CA9 expression was significantly associated with the CXCR4 expression on the

Table III. Univariate and multivariate analysis with respect to overall survival in gastric cancer.

	Univariate analysis			Multivariate analysis		
Variables	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
CA9/CXCR4/SDF1α						
Either negative vs. all positive	1.860	1.094-3.163	0.022	1.583	0.925-2.710	0.094
Age, years						
>70 vs. <70	1.659	1.050-2.622	0.030	1.379	0.852-2.233	0.191
Sex						
Female vs. male	1.179	0.733-1.896	0.497			
Macroscopic type						
Type 4 vs. other types	3.779	2.219-6.434	<0.001	2.685	1.475-4.886	0.001
Tumor size, mm						
<50 vs. ≥50	2.385	1.414-4.024	<0.001	1.593	0.894-2.837	0.114
Histological type						
Intestinal vs. diffuse	1.341	0.840-2.141	0.219			
Lymphatic invasion						
Negative vs. positive	1.779	0.816-3.880	0.147			

CI, confidence interval; CXCR4, C-X-C chemokine receptor type 4; SDF1α, stromal cell-derived factor 1α.

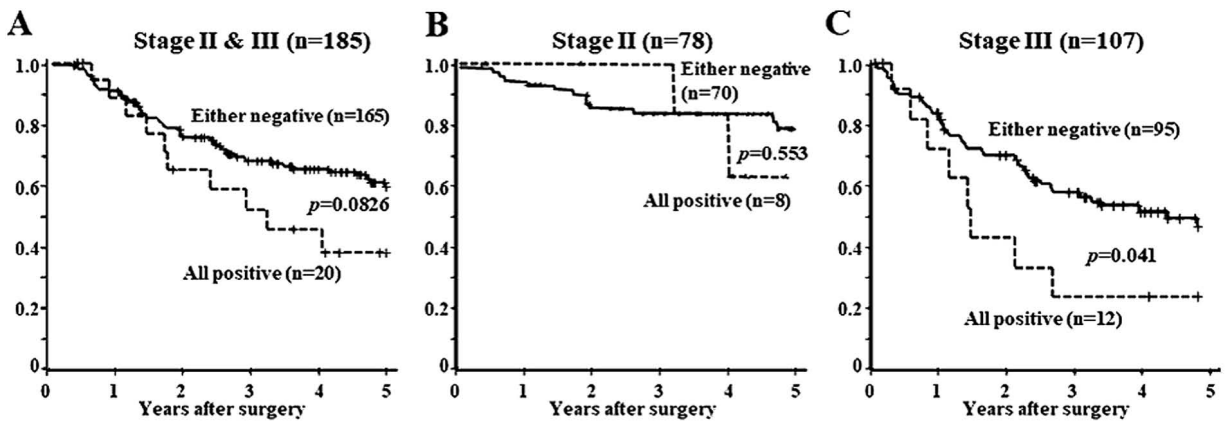

Figure 2. Survival curves for the 185 patients according to CA9, SDF1α and CXCR4 expression. (A) The prognosis of patients with CA9/CXCR4/SDF1α-positive GC tended to be poorer compared with that of patients with CA9/CXCR4/SDF1α-negative stage II or III GC. (B) No significant difference in prognosis was identified between patients with CA9/CXCR4/SDF1α-positive and CA9/CXCR4/SDF1α-negative GC at stage II. (C) The prognosis of patients with CA9/CXCR4/SDF1α-positive GC was significantly poorer compared with that of patients with CA9/CXCR4/SDF1α-negative stage III GC. All positive, patients who were positive for all CA9, CXCR4 and SDF1α; either negative, patients who were negative for CA9, CXCR4 or SDF1α.

cancer cells and the SDF1α expression on the stromal cells. These results suggested that hypoxia, which was evaluated by CA9 staining, may induce SDF1a and CXCR4. Recent studies have demonstrated that SDF1α is upregulated in fibroblasts to fulfill its role in cell protection against hypoxia (4,32). These results suggested that the heterogeneous hypoxic environment in cancer may be one of the reasons for cancer heterogenicity, which is associated with tumor resistance for various types of therapy (15,47,48).

SDF1α may serve as a protective factor to promote cell repair following hypoxic injury via its main receptor, CXCR4 (49). Our previous study demonstrated that the hypoxic condition affected the expression level of certain receptors of cancer cells (17,18,50). The results of our present study suggested that these results indicated that hypoxia may upregulate SDFα production from stromal cells and CXCR4 expression in cancer cells. Therefore, the SDF1α/CXCR4 axis may serve an important role in the progression of GC cells in hypoxia.

In conclusion, the SDF1α/CXCR4 axis may be involved in the progression of GC at stage II and III, particularly under hypoxic conditions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MY and HK designed, performed the experiments and co-wrote the manuscript. GT, TF, YY, TS, ST and AS prepared the samples. SN, SK, MY and TT accumulated the data. TS, KK, ST and MO sampled the material and MO reviewed manuscript. All authors read and approved the final manuscript.

Ethics statement and consent to participate

The present study was conducted with the approval of the Ethical Committee of Osaka City University (reference no. 924). Written informed consent was obtained from all patients prior to treatment.

Patient consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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