

# Expression of CXCL12 and its receptor CXCR4 in esophageal squamous cell carcinoma

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**Abstract.** The chemokine CXCL12, also known as stromal cell-derived factor-1 and its receptor CXCR4 have been shown to play prominent roles in regulating the directional migration and proliferation of various types of cancer cells during the metastatic process. However, few researchers have examined the expression of CXCL12 and CXCR4 and their prognostic value in patients with esophageal squamous cell carcinoma (ESCC). We investigated immunohistochemically the relationship between CXCL12 and CXCR4 expression and clinicopathological factors including prognosis in surgical specimens of primary tumors in 214 patients with ESCC. The positive expression rate of CXCL12 was 53.7% and that of CXCR4 was 84.6%. Positive CXCL12 expression was significantly correlated with lymph node metastasis, tumor stage, gender and lymphatic invasion. The overall and disease-free survival rate was significantly lower in patients with positive CXCL12 expression than in those with negative CXCL12 expression. The expression of CXCR4 had no correlation with clinicopathological variables and prognosis. We showed that positive CXCL12 expression was related to a greater degree to tumor development, compared with CXCR4 expression. Evaluation of CXCL12 expression is useful for determining tumor properties, including nodal metastasis and prognosis in patients with ESCC.

## Introduction

Chemokines are soluble, small molecular weight, secreted proteins and are, to date, the largest known cytokine family.

Their receptors regulate a variety of immune responses to infection, inflammation and tissue repair. Besides controlling the trafficking of immune cells, chemokines also regulate the migration of several different cell types in embryogenesis. It has been established that cancer cells exploit signaling through seven transmembrane G-protein-coupled chemokine receptors for several steps in the initiation and progression of primary and metastatic cancer (1-5).

In particular, the chemokine CXCL12, also known as stromal cell-derived factor-1 and its receptor CXCR4 have prominent roles in common malignancies including breast, ovarian, prostate, kidney, brain and lung cancers (6-15).

There have been few studies focusing on the protein expression of CXCL12 and CXCR4 in cancer cells (16). The aims of this retrospective study were to examine the relationship between CXCL12 and CXCR4 expression and clinicopathological factors, and to evaluate whether their expression is useful for predicting clinical outcome in surgical specimens from patients with esophageal squamous cell carcinoma (ESCC).

## Materials and methods

**Patient characteristics.** The present study involved 214 consecutive patients with ESCC (196 men and 18 women) who underwent esophagectomy with lymph node dissection at the Kagoshima University Hospital between January 1987 and December 1998. The patients ranged in age from 36 to 92 years (mean, 64.1 years). Transthoracic esophagectomy by right thoracotomy and left thoracotomy was performed in 174 (81.3%) and 16 patients (7.5%), respectively. In addition, transhiatal esophagectomy without thoracotomy and the others were performed in 18 (8.4%) and 6 patients (2.8%), respectively. Three-field lymphadenectomy (cervical, mediastinal and abdominal regions) was performed in 93 patients (43.5%), 2-field lymphadenectomy (mediastinal and abdominal regions) was performed in 119 patients (55.6%) and 1-field (cervical or abdominal region) lymphadenectomy in the remaining 2 patients (0.9%). The median number of removed lymph nodes was 47 (range, 1-141). The number of patients with R0 and R1 resection was 182 and 32, respectively. None of these patients underwent endoscopic mucosal resection, preoperative chemotherapy and/or

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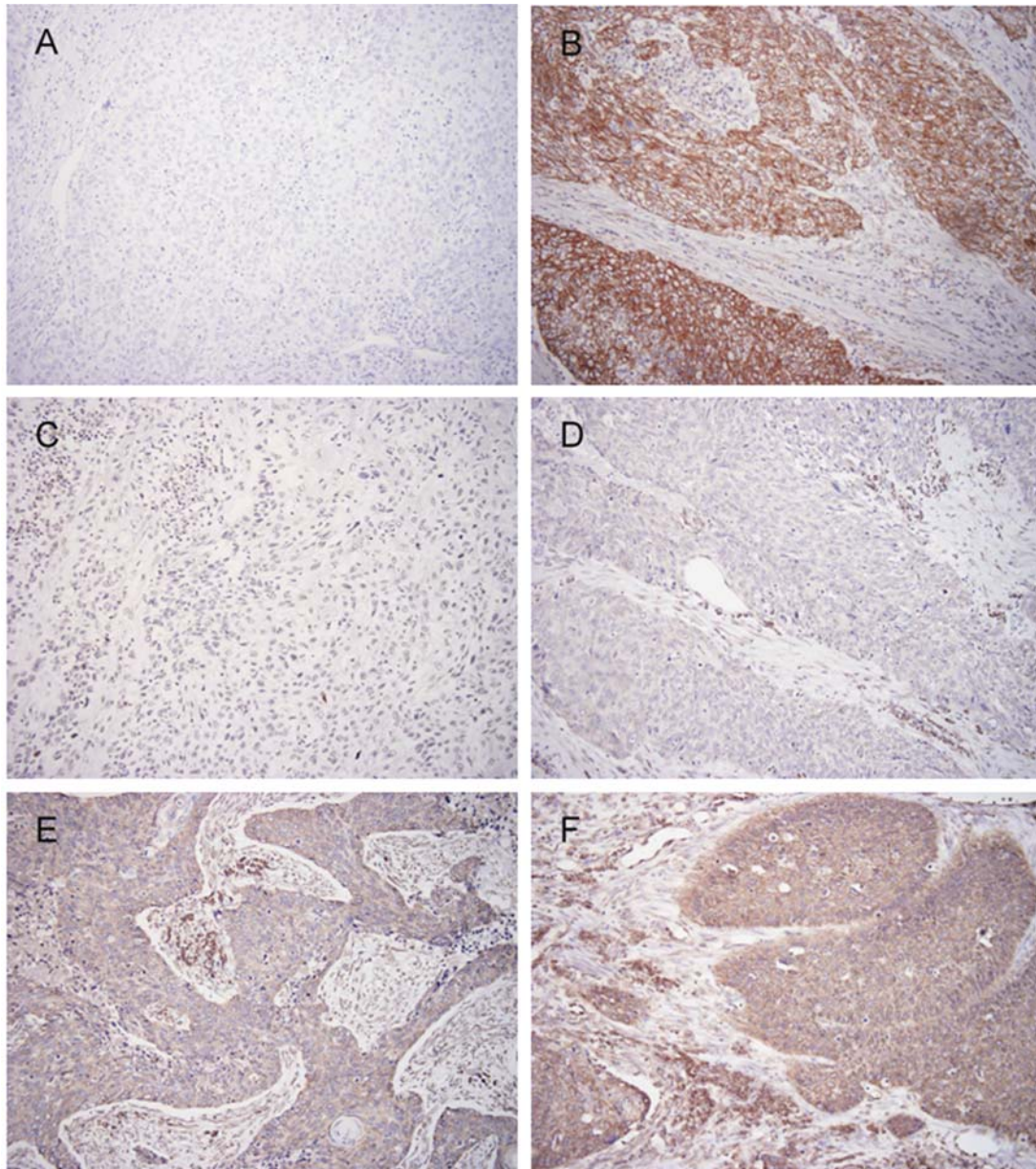


Figure 1. Immunohistochemical detection of CXCL12 and CXCR4 protein expression in ESCCs. (A) Negative expression of CXCL12, (B) positive expression of CXCL12, (C) negative expression of CXCR4, (D) positive expression of CXCR4 with weak intensity, (E) positive expression of CXCR4 with moderate intensity and (F) positive expression of CXCR4 with strong intensity (counterstaining, Mayer's hematoxylin; original magnification, x200).

radiotherapy, and none of them had synchronous or metachronous multiple cancers in other organs. Seventy-three patients had relapsed disease in the follow-up period. Classification of the specimens was determined according to the International Union Against Cancer Tumor-Node-Metastasis Classification System (17). All of the M1 tumors had distant lymph node metastases. All patients were followed up after discharge with a chest X-ray every 1 to 3 months, computed tomography every 3 to 6 months, and ultrasonography every 6 months. Bronchoscopy and endoscopy were performed when necessary. Follow-up data after surgery were available for all patients with a median follow-up period of 42 months (range, 1-181 months). The study was approved by the institutional review board of the hospital, and informed consent was obtained from the patients.

**Immunohistochemical analysis.** Tumor samples were fixed with 10% formalin in phosphate-buffered saline (PBS), embedded in paraffin, and sectioned into 4- $\mu$ m slices. They were deparaffinized in xylene and dehydrated with a series of graded ethanol. For antigen retrieval, sections were autoclaved in 10 mM citrate buffer solution for 10 min at 120°C. The endogenous peroxidase activity of specimens was blocked by immersing the slides in a 3% hydrogen peroxide solution in methanol for 30 min at room temperature. After washing with PBS, the sections were treated with 1% bovine serum albumin for 30 min to block nonspecific reactions at room temperature. The blocked sections were incubated with primary antibody CXCL12 (MAB350, 1:200; R&D Systems, Minneapolis, MN) or CXCR4 (MAB171, 1:200; R&D Systems) diluted in PBS at room temperature for 45 min,



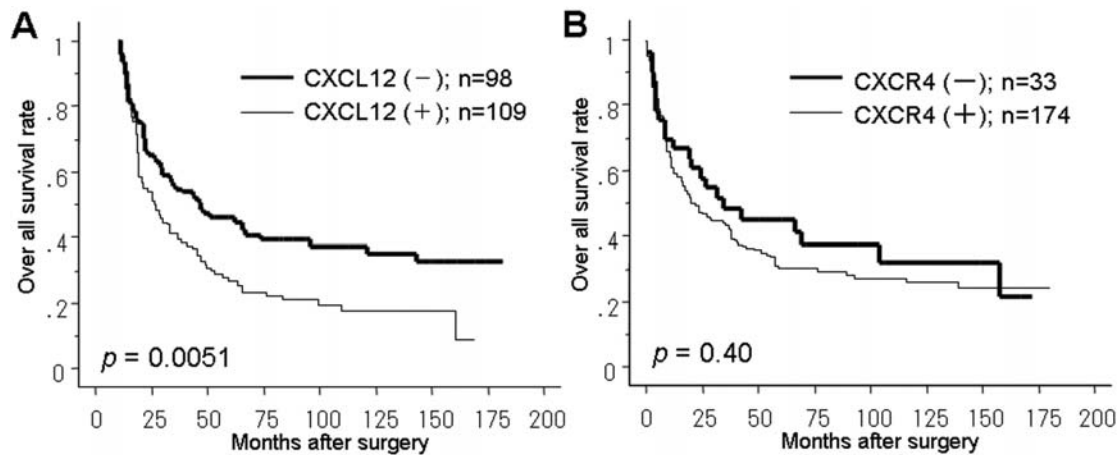


Figure 2. The postoperative overall survival curves according to the expression of CXCL12 (A) and CXCR4 (B) proteins. There was a significant difference in survival between the patients with positive (+) and negative (-) expression of CXCL12 ( $P=0.0051$ ). There was no significant difference in survival between the patients with positive (+) and negative (-) expression of CXCR4 ( $P=0.40$ ).

followed by staining with a streptavidin-biotin peroxidase kit (Nichirei, Tokyo, Japan). The sections were washed in PBS, and the immune complex was visualized by incubating the sections with diaminobenzidine tetrahydrochloride. The sections were rinsed briefly in water, counterstained with hematoxylin and mounted. Normal human tonsil and spleen tissues were used as positive controls for CXCL12 and CXCR4, respectively. Negative controls were created by replacing the primary antibodies with PBS. Evaluation of immunohistochemistry was independently carried out by two investigators (K.S. and S.I.) who were unaware of the clinical data or disease outcome. In cases in which the results of immunohistochemical expression differed between the two observers, slides were evaluated by a third observer (S.N.). Positive expression of CXCL12 was defined as detectable immunoreaction in membrane and cytoplasmic lesions of >10% of the cancer cells. CXCR4 expression was divided into two groups according to intensity as follows: CXCR4 negativity was defined as no expression of CXCR4, and CXCR4 positivity was defined as weak to strong intensity of CXCR4 expression. To evaluate expression of CXCL12 and CXCR4, ten fields (within the tumor and at the invasive front) were selected, and expression in 1000 tumor cells (100 cells/field) was evaluated using high-power (x200) microscopy.

**Statistical analysis.** Statistical analysis of group differences was carried out using the  $\chi^2$  and t-tests. The Kaplan-Meier method was used for survival analysis, and differences in survival were estimated using the log-rank test. Prognostic factors were examined by univariate and multivariate analyses (Cox proportional hazards regression model).  $P<0.05$  was considered to be statistically significant. All statistical analyses were performed with the software package JMP 5 for Windows (SAS Institute, Inc., Cary, NC).

## Results

**Expression of CXCL12 and CXCR4 in esophageal squamous cell carcinoma.** The positive expression of CXCL12 and

CXCR4 was detected in both the membrane and the cytoplasm of ESCC. The expression of CXCL12 was observed in 53.7% of samples, and the expression of CXCR4 was observed in 84.6% of samples (Fig. 1).

**Relationship between the expression of CXCL12 and CXCR4 and clinicopathological findings.** The expression of CXCL12 was associated with the following clinicopathological parameters: lymph node metastasis, tumor stage, gender and lymphatic invasion. The tumors with positive CXCL12 expression had more lymph node metastasis and greater lymphatic invasion than those with negative CXCL12 expression ( $P=0.0004$  and  $P=0.0166$ , respectively). The expression of CXCR4 had no correlation with the clinicopathological variables. The expression of CXCL12 and CXCR4 had a weak correlation ( $P=0.072$ ) (Table I).

**Relationship between clinical outcome and expression of CXCL12 and CXCR4.** Seven of the patients died of postoperative complications within 30 days of the beginning of the study period, leaving 207 patients for survival analysis, and 32 patients underwent palliative resection. The number of palliative resection patients with stage IIA, III, IVA and IVB were 1, 11, 3 and 17, respectively. There was a significant difference in overall survival rates between negative and positive expression of CXCL12 ( $P=0.0051$ ; Fig. 2A). On the other hand, no significant difference was found between overall survival rates and CXCR4 expression (Fig. 2B). There was a significant difference in disease-free survival rates between negative and positive expression of CXCL12 ( $P=0.0036$ ; Fig. 3A). On the other hand, no significant difference was found between disease-free survival rates and CXCR4 expression (Fig. 3B).

**Univariate and multivariate analyses of survival.** Univariate analysis showed that the following factors were significantly related to postoperative survival: gender, tumor depth, lymph node metastasis, distant metastasis, stage, lymphatic invasion, venous invasion and CXCL12 expression. Multivariate

Table I. CXCL12 and CXCR4 expression in relation to clinicopathological findings.

Characteristics	Total n=214	CXCL12 expression		P	CXCR4 expression		P
		Positive n=115 (53.7%)	Negative n=99 (46.3%)		Positive n=181 (84.6%)	Negative n=33 (15.4%)	
Mean age $\pm$ SD		65.1 $\pm$ 9.7	62.9 $\pm$ 8.2	0.21	64.0 $\pm$ 9.4	64.3 $\pm$ 6.4	0.58
Gender							
Male	196 (91.6)	110 (95.7)	86 (86.9)	0.020	165 (91.2)	31 (93.9)	0.55
Female	18 (8.4)	5 (4.4)	13 (13.1)		16 (8.8)	2 (6.1)	
Tumor location							
Upper	34 (15.9)	23 (20.0)	11 (11.1)	0.20	31 (17.1)	3 (9.1)	0.42
Middle	107 (50.0)	54 (47.0)	53 (53.5)		88 (48.6)	19 (57.6)	
Lower	73 (34.1)	38 (33.0)	35 (35.4)		62 (34.3)	11 (33.3)	
Histology							
Well	80 (37.4)	35 (30.4)	45 (45.5)	0.075	71 (39.2)	9 (27.3)	0.40
Moderate	98 (45.8)	58 (50.4)	40 (40.4)		81 (44.8)	17 (51.5)	
Poor	36 (16.8)	22 (19.1)	14 (14.1)		29 (16.0)	7 (21.2)	
pT							
pT1	56 (26.2)	25 (21.7)	31 (31.3)	0.14	45 (24.9)	11 (33.3)	0.28
pT2	31 (14.5)	19 (16.5)	12 (12.1)		24 (13.3)	7 (21.2)	
pT3	88 (41.1)	45 (39.1)	43 (43.4)		79 (43.7)	9 (27.3)	
pT4	39 (18.2)	26 (22.6)	13 (13.1)		33 (18.2)	6 (18.2)	
pN							
pN0	81 (37.9)	31 (27.0)	50 (50.5)	0.0004	65 (35.9)	16 (48.5)	0.18
pN1	133 (62.2)	84 (73.0)	49 (49.5)		116 (64.1)	17 (51.5)	
pM							
pM0	152 (71.0)	76 (66.1)	76 (76.8)	0.084	126 (69.6)	26 (78.8)	0.27
pM1	62 (29.0)	39 (33.9)	23 (23.2)		55 (30.4)	7 (21.2)	
pStage							
I	37 (17.3)	14 (12.2)	23 (23.2)	0.0076	29 (16.0)	8 (24.2)	0.32
IIA	37 (17.3)	13 (11.3)	24 (24.2)		30 (16.6)	7 (21.2)	
IIB	25 (11.7)	15 (13.0)	10 (10.1)		19 (10.5)	6 (18.2)	
III	53 (24.8)	34 (29.6)	19 (19.2)		48 (26.5)	5 (15.2)	
IVA	17 (7.9)	13 (11.3)	4 (4.0)		16 (8.8)	1 (3.0)	
IVB	45 (21.0)	26 (22.6)	19 (19.2)		39 (21.6)	6 (18.2)	
Lymphatic invasion							
Positive	137 (64.0)	82 (71.3)	55 (55.6)	0.017	120 (66.3)	17 (51.5)	0.11
Negative	77 (36.0)	33 (28.7)	44 (44.4)		61 (33.7)	16 (48.5)	
Venous invasion							
Positive	74 (34.6)	42 (36.5)	32 (32.3)	0.52	65 (35.9)	9 (27.3)	0.33
Negative	140 (65.4)	73 (63.5)	67 (67.7)		116 (64.1)	24 (72.7)	
CXCR4 expression							
Positive	181 (84.6)	102 (88.7)	79 (79.8)	0.072			
Negative	33 (15.4)	13 (11.3)	20 (20.2)				

regression analysis indicated that depth of invasion and distant lymph node metastasis were independent prognostic factors but not CXCL12 expression (Table II).

## Discussion

Many studies of the CXCR4/CXCL12 pathway have been reported, mainly in the field of immunology and infection, in

areas such as hematopoiesis, lymphocyte homing and HIV infection. In 2001, Muller *et al* showed that CXCR4 was expressed more highly in breast cancer tissue than in normal breast tissue and that CXCL12 was expressed in many organs, such as lymph nodes, bone marrow and lungs, in which breast cancer metastasis was often found, but not expressed in kidneys, where metastasis hardly occurred. In addition, studies *in vivo* showed that injecting an antibody which neutralizes

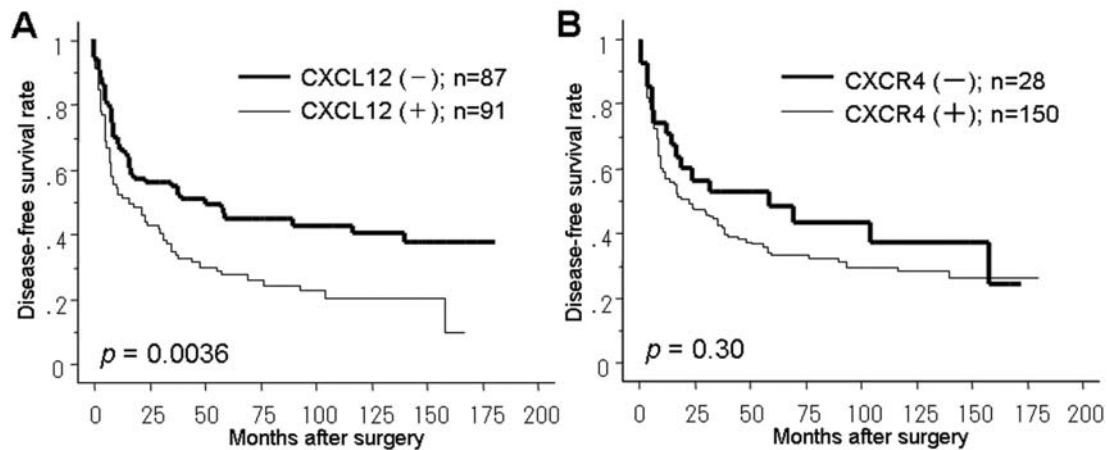


Figure 3. The postoperative disease-free survival curves according to the expression of CXCL12 (A) and CXCR4 (B) proteins. There was a significant difference in disease-free survival between the patients with positive (+) and negative (-) expressions of CXCL12 ( $P=0.0036$ ). There was no significant difference in disease-free survival between the patients with positive (+) and negative (-) expression of CXCR4 ( $P=0.30$ ).

Table II. Univariate and multivariate analyses of prognostic factors in ESCC.

Independent factors	Univariate P	Multivariate P	Hazard ratio	95% confidence interval
pT (pT1, 2/pT3, 4)	<0.0001	<0.0001	2.317	1.590-3.431
pN (pN0/pN1)	<0.0001	0.2530	1.349	0.808-2.271
pM (pM0/pM1)	<0.0001	0.0009	1.946	1.312-2.898
Lymphatic invasion (negative/positive)	<0.0001	0.4980	1.186	0.727-1.954
Venous invasion (negative/positive)	0.0020	0.7570	1.061	0.730-1.537
CXCL12 (negative/positive)	0.0056	0.2620	1.216	0.865-1.722
Gender (female/male)	0.0229	0.1200	1.670	0.884-3.582

CXCR4 activity led to the inhibition of metastasis to bone marrow and lungs (15). Subsequently, researchers began to become interested in the role of the CXCR4/CXCL12 pathway in tumors. We report for the first time, to the best of our knowledge, that CXCL12 was expressed in ESCC and that its expression was associated with overall survival and the clinicopathological parameters; nodal metastasis, lymphatic invasion, tumor stage and gender. There have been no reports disclosing the clinical implications of chemokine positivity in tumor cells, except for some reports on CXCL12 expression in glioma cells (18), epithelial ovarian cancer cells (16) and oral squamous cell carcinoma (19). CXCL12 expression significantly correlated well with prognosis in glioma, but not in epithelial ovarian cancer cells and oral squamous cell carcinoma. Although the positive expression of CXCL12 was not an independent prognostic factor upon multivariate

analysis in this study, its expression correlated well with nodal metastasis and lymphatic invasion. Thus, these results suggest that positive CXCL12 expression has the potential to become an important predictor of lymphogenous metastasis and poor prognosis.

Although the relationship between CXCR4 expression and malignant potentiality has been investigated in various types of cancer, the clinical significance is still controversial (19-22). The role of CXCR4 in cancer metastasis is still debated, because CXCR4 mRNA expression was not found to be significantly different between cancer and noncancerous tissues in the colon, esophagus and stomach (23). According to studies in which CXCR4 expression was immunohistochemically examined in esophageal and gastric cancers, no correlation was found between CXCR4 expression and prognosis (24,25). These results were in agreement with ours.

In several types of cancer cells, the function of CXCR4 in cell migration in response to CXCL12 has been reported (6-15). Although the mechanism by which the CXCL12/CXCR4 pathway enhances cell migration has not been fully evaluated, several investigators have shown that CXCL12/CXCR4 interaction induces matrix metalloproteinases in megakaryocytes (26), rhabdomyosarcoma cells (27) and CD34<sup>+</sup> cells (28). CXCL12/CXCR4 interaction stimulates the phosphatidylinositol-3-kinase pathway that subsequently activates the protein kinase Akt (PI3K/Akt pathway) (8,29,30), mitogen-activated protein kinase pathway (MAPK pathway) (31), extracellular signal-related kinase-1/2 (ERK1/2 pathway) (6,13,29), and Janus kinase/signal transducer and activator of transcription pathways (JAK/STAT pathway) (2) in carcinoma cells. Furthermore, CXCL12/CXCR4 interaction induces the tyrosine phosphorylation of focal adhesion kinase and of RAFTK/Pyk2 (32). CXCL12/CXCR4 also promotes adhesion to components of the extracellular matrix, including collagen and fibronectin, through integrins  $\alpha_2$ ,  $\alpha_4$ ,  $\alpha_5$  and  $\alpha_1$  (33). On the other hand, the Slit-Robo pathway was reported to be a negative regulator of CXCL12/CXCR4 functions (2), and Katayama *et al* showed that INF- $\gamma$  down-regulated both gene and cell surface protein expression of CXCR4 in certain head and neck squamous cell carcinoma cells (34).

CXCL12/CXCR4 has been widely implicated in the promotion of angiogenesis. CXCR4 has been found to be expressed on human intestinal microvascular endothelial cells, and CXCL12 can stimulate chemotaxis and proliferation of these cells in addition to promoting endothelial tube formation (35). CXCL12 was also found to increase the expression of VEGF by endothelial cells, and in a positive feedback loop, VEGF was found to up-regulate CXCR4 on endothelial cells (36). Additionally, the inhibition of the CXCL12/CXCR4 pathway was found to decrease the growth of gastrointestinal tumors through the suppression of angiogenesis (37). On the other hand, CXCL12/CXCR4 was found to mediate metastasis, but not angiogenesis, in kidney, breast and non-small cell lung cancer (6,7,15). We also examined the relationship between CXCL12/CXCR4 expression and microvessel density in 86 submucosal ESCCs; close relationships were not found (38). Further studies are needed to clarify the role of the CXCL12/CXCR4 pathway in cancer cells since a number of factors were correlated with this pathway.

In the present study, we found that the examination of CXCL12 expression is useful to predict lymph node metastasis and clinical outcome. Alternatively, this pathway could be targeted by blocking the expression of CXCR4 in tumor cells or the secretion of CXCL12 by tumor cells and stromal cells in the tumor microenvironment. Various blocking agents against CXCR4 have been developed (39,40), and these agents should be used to validate this chemokine receptor as a molecular target for the treatment of patients with ESCC who have not received toxic systemic treatment.

In conclusion, cancerous CXCL12 expression played a more significant role in properties associated with malignancy compared with CXCR4 expression in this study. Evaluation of CXCL12 expression is useful for determining tumor properties, including nodal metastasis and prognosis in patients with ESCC.

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