

CXCR4 expression is associated with lymph-node metastasis of oral squamous cell carcinoma

TOHRU ISHIKAWA, KOH-ICHI NAKASHIRO, SHINGO HARA, SEBASTIAN K. KLOSEK, CHUNNAN LI, SATORU SHINTANI and HIROYUKI HAMAKAWA

Department of Oral and Maxillofacial Surgery, Ehime University School of Medicine, 454 Shitsukawa, Toon, Ehime 791-0295, Japan

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Abstract. The support mechanisms that are involved in lymph-node metastasis of oral squamous cell carcinoma (OSCC) remain largely unknown. Recent studies have demonstrated that tumor cells express chemokine receptors and use chemokines to metastasize to the target organ in many malignancies in humans. In this study, we examined the expression and function of chemokines and their receptors in OSCC. The expression of chemokine receptors was assessed in eight OSCC cell lines. CXCR3 mRNA and protein were expressed in all the OSCC cell lines examined, while CXCR4 mRNA and protein were expressed only in HSC2, HSC3, and Ca9-22 cells. Treatment with the ligand for CXCR4, stromal cell-derived factor-1 (SDF-1), enhanced the motility and invasiveness of OSCC cells expressing CXCR4. However, the CXCR3 ligand, Mig, did not affect the migration or invasiveness of CXCR3-positive cells. We also evaluated the clinical significance of CXCR4 expression immunohistochemically. CXCR4 expression was detected in 27 (30%) of the 90 OSCC tissues tested, and was localized in the membrane and cytoplasm of cancer cells. There was a highly significant correlation between CXCR4 expression and lymph-node metastasis ($P=0.0035$). Collectively, these findings suggest that CXCR4 might be involved in the lymph-node metastasis of OSCC.

Introduction

Despite advances in surgery, radiation therapy, and chemotherapy, patients with oral squamous cell carcinoma (OSCC) spreading to the regional lymph nodes have a poor 5-year survival rate compared with patients without metastasis (1,2). The spread to regional lymph nodes in the neck (3,4), which can occur soon after the development of OSCC, is induced by rich lymphatic drainage from the oral cavity (5-7). Although a

number of molecules have been implicated in the metastasis of OSCC (8,9), the precise molecular mechanisms that determine the direction of migration and invasiveness of OSCC cells into the lymph nodes remain unclear.

Chemokines are a superfamily of small structurally related heparin-binding proteins, which have been identified as attractants that control the migration of leukocytes, especially during immune and inflammatory reactions. They are classified as CC, CXC, CX3C, or C chemokines, based on the pattern of four conserved cysteine residues (10-13). The specific effects of chemokines on their target cells are mediated by seven transmembrane-spanning G-protein-coupled receptors, the two major subfamilies of which are designated CCR and CXCR (14). Chemokines and their receptors are also important in dendritic cell maturation, B- and T-cell development, T-helper cell 1 (Th₁) and Th₂ responses, infections and angiogenesis (15). Moreover, recent studies have demonstrated that several types of cancer express chemokine receptors and use chemokines to metastasize to the target organ (16-18).

In the current study, we investigated the expression and function of chemokine receptors in OSCC, and considered their clinical significance.

Materials and methods

Cells and cell cultures. Eight human OSCC cell lines (HSC2, HSC3, HSC4, SAS, Ho-1-N1, Ca9-22, SCC4 and SCC9) were used in this study. All of the cell lines were obtained from the Japanese Collection of Research Bioresources (National Institute of Health Sciences, Tokyo, Japan) and were maintained in Dulbecco's modified Eagle's medium (D-MEM)/F-12 medium (Sigma-Aldrich Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 100 μ g/ml streptomycin, 100 U/ml penicillin and 0.25 μ g/ml amphotericin B (all from Invitrogen Corp., Carlsbad, CA). The cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Multiplex reverse-transcription polymerase chain reaction (MRT-PCR) analysis. Cells grown in monolayers were harvested at early confluence. Total RNA from the human OSCC cell lines was extracted using a TRIzol RNA extraction kit (Invitrogen Corp.) according to the manufacturer's instructions. A 1- μ g sample of total RNA and 300 pM of random

Correspondence to: Dr Koh-ichi Nakashiro, Department of Oral and Maxillofacial Surgery, Ehime University School of Medicine, 454 Shitsukawa, Toon, Ehime 791-0295, Japan
E-mail: nakako@m.ehime-u.ac.jp

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primer (Invitrogen Corp.) in 20 μ l of 0.1% diethylpyrocarbonate (DEPC)-treated water (DEPC was from Sigma-Aldrich Co.) were heated at 65°C for 5 min, followed by cooling on ice for 1 min. cDNA synthesis was initiated with 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp.) under the conditions recommended by the manufacturer, and the reaction was allowed to proceed at 42°C for 1 h. The reaction was terminated by heating at 95°C for 5 min and was then frozen at -20°C until use. Subsequently, 1 μ l of the RT products was subjected to PCR amplification using MRT-PCR kits (Maxim Biotech Inc., San Francisco, CA) according to the manufacturer's instructions. All of the reactions were carried out in a DNA thermal cycler (PC707; Astec, Fukuoka, Japan) under the following conditions: 96°C for 1 min; 60°C for 4 min for 2 cycles and then 94°C for 1 min; 60°C for 3 min for 35 cycles; and a final extension step at 70°C for 10 min. Aliquots (10 μ l) of the MRT-PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide.

Western blot analysis. Cells grown in monolayers were harvested when subconfluent and lysed in phosphate-buffered saline (PBS) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 M NaF, 10 mg/ml leupeptin, 0.1 mg/ml aprotinin, and 50 mg/ml phenylmethylsulfonyl fluoride (PMSF; all reagents were from Sigma-Aldrich Co.). Following sonication and incubation on ice for 30 min, samples of the supernatants were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat dried milk (Wako, Osaka, Japan) in T-TBS containing 25 mM Tris-HCl, 125 mM NaCl and 0.1% Tween-20 (Sigma-Aldrich Co.) overnight at 4°C. They were then probed with primary antibodies against CXCR3 and CRX4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4°C, followed by re-probing with horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse immunoglobulin G (Amersham Biosciences, Piscataway, NJ) for 1 h. The immune complexes were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences) according to the manufacturer's protocol. As an internal control, the blots were stripped using 62.5 mM Tris-HCl (pH 6.8) buffer containing 0.7% 2-mercaptoethanol (Sigma-Aldrich Co.) and 2% SDS at 50°C for 30 min, and then re-probed with mouse anti- β -tubulin monoclonal antibody (BD Biosciences, San Jose, CA).

Cell invasiveness and motility assay. The invasion assay was performed using a Biocoat Matrigel invasion chamber (BD Biosciences, Bedford, MA) as previously described (19). Cells (5×10^4) were seeded on the upper chambers and medium supplemented with Mig or SDF-1 was added to the lower compartments. After 24-h incubation at 37°C and 5% CO₂, the cells on the upper surface at the filter were removed using a cotton-wool swab. Migrating cells on the lower surface were stained with Giemsa's solution. For each filter, the number of cells in 10 randomly chosen microscopic fields was determined and averaged. For neutralization studies, the cells were incubated with 20 μ g/ml of anti-CXCR4 antibody

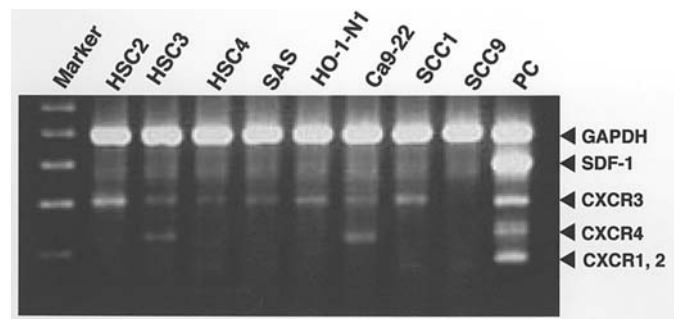


Figure 1. CXCR and SDF-1 mRNA expression in eight OSCC cell lines. The MRT-PCR protocol is described in the Materials and methods section.

(R&D systems, Minneapolis, MN). The cell motility was evaluated by a phagokinetic track assay. The cover slips were coated with colloidal gold, as described previously (20). Briefly, 9 ml of 14.5 mM AuCl₄H and 30 ml of 36.5 mM Na₂CO₃ were added to 55 ml of H₂O, and the mixture was heated in a glass beaker. Immediately after it had reached boiling point, the solution was removed from the heat and 9 ml of 0.1% formaldehyde solution was added. Colloidal gold formed within 1 min. The solution was then poured into 35-mm tissue-culture dishes that were coated with bovine serum albumin (BSA). After incubation, the solution was washed extensively with PBS. For the cell-motility assay, the cells were seeded on colloidal gold-coated dishes (at a density of 5×10^3 cells per dish) in 1 ml of serum-free medium and incubated for 24 h. We randomly selected 50 cells and measured the areas that were free of gold particles using the National Institutes of Health (NIH) image analysis program to evaluate cell motility.

Patients and tissue. OSCC tissue was obtained from 90 patients who underwent primary cancer resection at the Department of Oral and Maxillofacial Surgery, Ehime University School of Medicine, Japan. Clinical stage was defined according to the Tumor-Node-Metastasis (TNM) classification of malignant tumors (Union Internationale Contre le Cancer; 21) and tumor differentiation was graded using the criteria proposed by the World Health Organization (WHO; 22). Histological malignancy (i.e. the mode and stage of invasion) was determined on the basis of Anneroth's classification (23).

Immunohistochemistry. Tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin blocks of 4- μ m thickness were cut, and sections were deparaffinized with xylene and rehydrated in graded alcohol. Hydrogen peroxide (3%) was applied to block endogenous peroxidase activity. The sections were incubated overnight at 4°C with anti-human CXCR4 rabbit polyclonal antibody (diluted 1:100; Santa Cruz Biotechnology Inc.), and non-specific reactions were blocked with 5% dry milk after each primary antibody was applied. Immunostaining was performed using the Envision system (DakoCytomation, Carpinteria, CA) in accordance with the manufacturer's instructions. Peroxidase activity was visualized by applying diaminobenzidine chromogen containing 0.05% hydrogen peroxidase. The sections were then counterstained with hematoxylin, dehydrated, cleared

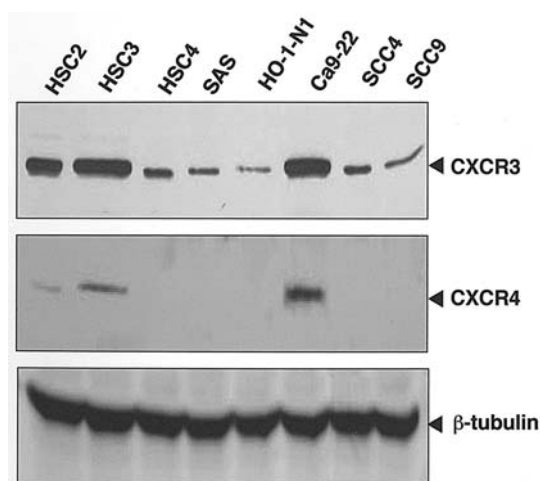


Figure 2. Western blot analysis of CXCR3 and CXCR4 in eight OSCC cell lines.

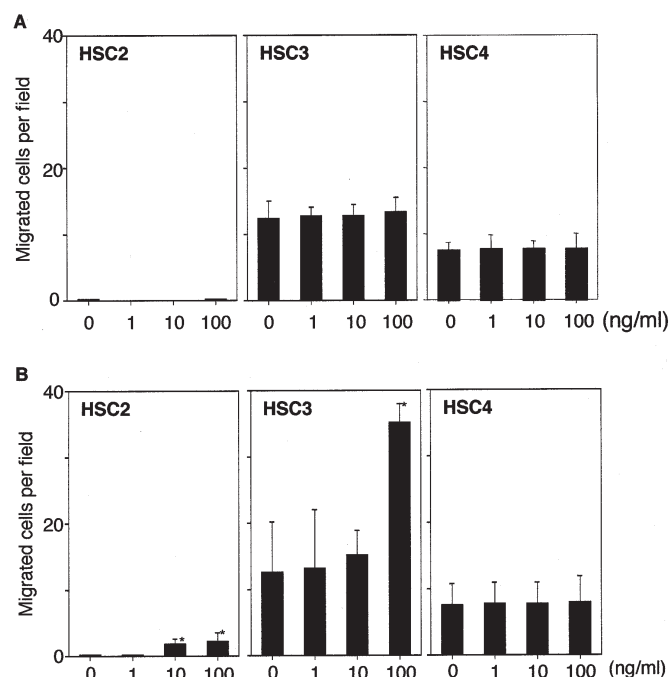


Figure 3. Chemokine-mediated invasion of OSCC cell lines. We used Matrigel-coated invasion chambers to test the effect of various concentrations of Mig (A) and SDF-1 (B) on the invasion of OSCC cells. The results are expressed as the mean number of migration cells per well. The bars denote the standard deviation (SD) of triplicate samples. * $P < 0.05$ compared with the respective untreated control culture.

and mounted. A negative control was obtained by omission of the primary antibody. Cases were considered to be CXCR4 positive if more than 10% of the cancer cells manifested cell membrane and cytoplasmic positivity.

Statistical analysis. All calculations were performed using the Statview 5.1 program (Avacus, NC). The results of the invasion assays were evaluated using the Student's t-test. The relationships between the expression of CXCR4 and the clinicopathological parameters were examined using Mann-Whitney U tests. Statistical significance was set at $P < 0.05$.

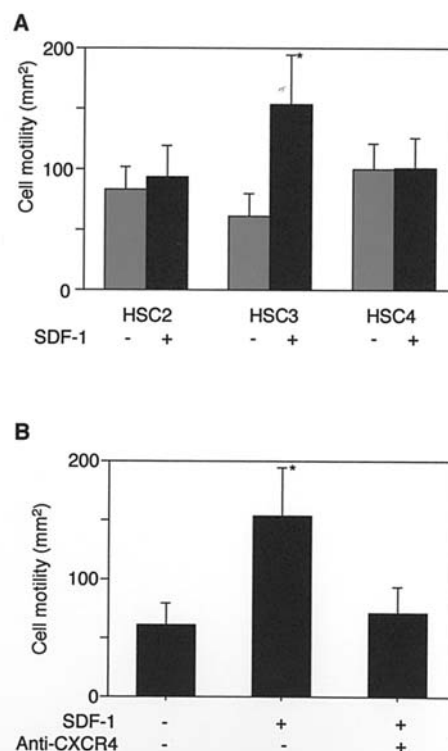


Figure 4. Chemokine-mediated migration of OSCC cell lines. The effect of SDF-1 (100 ng/ml) on cell migration was examined using the phagokinetic tract assay (A). We tested the effect of anti-CXCR4 neutralizing antibody on SDF-1 (100 ng/ml)-induced migration in HSC3 cells (B). The results are expressed as the mean area that is free of gold particles per cell. The bars denote the SD of triplicate samples. * $P < 0.05$ compared with the respective untreated control culture.

Results

Expression of CXCR and SDF-1 mRNAs in OSCC cell lines. The expression levels of chemokine receptors (CXCR1, CXCR2, CXCR3 and CXCR4) and chemokine (SDF-1) mRNAs in the eight OSCC cell lines were examined by MRT-PCR. The mRNA expression levels of the CXCR family members varied among these cells. CXCR3 mRNA was detected in all tested cell types, while the specific band for CXCR4 was found only in HSC2, HSC3 and Ca9-22 cells. By contrast, CXCR1, CXCR2 and SDF-1 mRNAs were not detected in any of the OSCC cell lines investigated (Fig. 1).

Expression of CXCR and SDF-1 proteins in OSCC cell lines. The expression levels of CXCR3 protein and the four other proteins (CXCR1, CXCR2, CXCR4, and SDF-1) in OSCC cells were evaluated by Western blot analysis. Similar to the results for expression at the mRNA level, all of the cell lines expressed CXCR3 protein, while CXCR4 protein was detected only in HSC2, HSC3, and Ca9-22 cells (Fig. 2).

Effects of chemokines on OSCC cell invasiveness and motility. To examine the effects of chemokines on the invasiveness of OSCC cells, we carried out an assay using Matrigel-coated invasion chambers. The Mig ligand for CXCR3 had no effect on cell invasiveness, regardless of the level of CXCR3 expression (Fig. 3A). By contrast, SDF-1, which is a ligand for CXCR4, significantly enhanced the invasiveness of HSC2 and HSC3

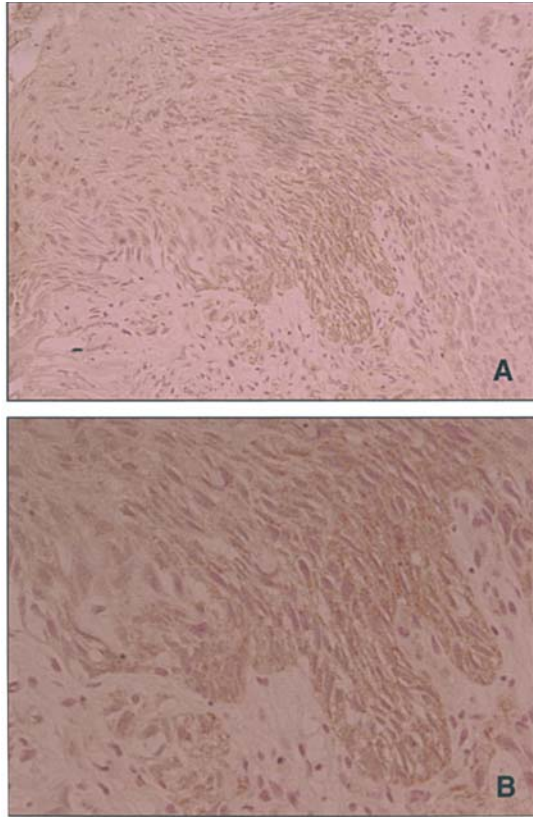


Figure 5. Immunohistochemical staining of CXCR4 in OSCC tissue. Tumor cells showed diffuse membrane and cytoplasmic staining for CXCR4. Original magnification, x100 (A) and x400 (B). The immunoreactivity of CXCR4 was upregulated at the infiltrating tumor edge.

cells expressing CXCR4 (Fig. 3B). Anti-CXCR4 neutralizing antibody significantly inhibited the stimulatory effect of SDF-1 (data not shown). We subsequently tested the effect of SDF-1 on cell motility and found that it markedly stimulated the motility of HSC3 cells expressing CXCR4 at a high level (Fig. 4A). Blockade of SDF-1/CXCR4 interactions with neutralizing CXCR4 antibody almost completely suppressed SDF-1-induced cell motility in this cell line (Fig. 4B). These data suggest that SDF-1/CXCR4, but not the Mig/CXCR3 system, activates the invasion and motility of OSCC cells.

Immunohistochemical staining of CXCR4 in OSCC tissue. In OSCC tissue, CXCR4 protein was detected in both the cell membranes and the cytoplasm. The number of CXCR4-positive cells ranged from only a few up to almost one-half of the total number of tumor cells. CXCR4 expression was frequently upregulated at the infiltrating tumor front (Fig. 5). In the CXCR4 immunohistochemical examination, 27 cases (30%) showed positive staining. Lymph-node involvement was significantly associated with CXCR4 protein expression (Table I). However, there was no significant correlation between CXCR4 expression and the T stages, tumor differentiation, or the mode of invasion.

Discussion

Tumor cells at metastatic sites express chemokine receptors in several types of carcinoma, including breast (16), ovary

Table I. Clinicopathological profiles of OSCC patients and CXCR4 immunoreactivity.

	No.	CXCR4 positive	P-value
T stage			0.2331
T1 and T2	62	21 (34)	
T3 and T4	28	6 (21)	
Lymph nodes			0.0035
Negative	51	9 (18)	
Positive	39	18 (46)	
Differentiation			0.0541
Well	44	9 (20)	
Moderate/poor	46	18 (39)	
Pattern of invasion			0.3610
Grade 1/2	26	6 (23)	
Grade 3/4	64	21 (33)	
Clinical stage			0.3262
Stage I/II	37	9 (24)	
Stage III/IV	53	18 (33)	

(17), and prostate (18). Chemokines and their receptors are known to play important roles in the processes of leukocyte trafficking and homing, especially at sites of inflammation, infection, tissue injury, cell damage and malignant tumor growth (15). A recent study suggests that these proteins can also regulate non-leukocyte cell functions, such as cell migration (24). We used MRT-PCR, Western blot analysis and immunohistochemical staining to verify the expression of chemokine receptors in OSCC cell lines and tissue. Our results demonstrated that CXCR4 mRNA and protein were expressed in several of the cell lines tested. Furthermore, immunohistochemical staining showed that the expression of CXCR4 protein in cancer cells was associated with lymph-node metastasis. These results suggest that CXCR4 might play an important role in promoting metastasis to the cervical lymph nodes in OSCC.

CXCR4 is constitutively expressed in a broad range of tissues, including lymphatic tissue and the thymus, brain, spleen, stomach and small intestine (25). The functions of CXCR4 have not previously been confirmed, although it seems to mediate leukocyte development and trafficking, human immunodeficiency virus (HIV) infection, vascularization, central nervous system development, and possibly cell division, tumorigenesis and growth (26-28). Tumor metastasis of SCC and a number of other malignancies are associated with increased cell migration (29). In a recent study, CXCR4 was shown to be highly expressed in malignant breast tumors and metastases (16). The interaction of CXCR4 with its specific ligand, SDF-1, was associated with actin polymerization and pseudopodia formation, and subsequently induced motile and invasive responses (16). In our current study, SDF-1 significantly induced cell invasion and motility in OSCC cells expressing CXCR4. SDF-1 is continuously expressed

in bone marrow stromal cells as well as various organs, including the brain, heart, lung, kidney, thymus, spleen and liver. It might also play a role in immune surveillance, B-cell-progenitor proliferation, B-cell migration, monocyte chemotaxis, testing T lymphocytes and primitive hematopoietic cells (25,30). As none of the eight OSCC cell lines tested expressed significant amounts of SDF-1 mRNA, this ligand did not appear to function as an autocrine factor in OSCC. Recent reports suggest an important role for the SDF-1/CXCR4 system later in the cancer sequence, in targeting metastasis of breast cancer cells that strongly express CXCR4 both to the liver and to other organs that have relatively high SDF-1 expression (16). *In vivo*, a blockade of the interactions of SDF-1 and CXCR4 significantly impairs the metastasis of breast cancer cells to regional lymph nodes (16). Uchida and colleagues also demonstrated that SDF-1 was expressed in the submandibular lymph nodes, mainly in stromal cells adjacent to the cancer cells, and that its extracts promoted the chemotaxis of OSCC cell lines (31).

CXCR4 was detected on the cell membrane or in the cytoplasm; in addition, intense membrane and cytoplasmic staining of carcinoma cells was observed along the invasive border and in individual infiltrating carcinoma cells within our series. CXCR4 might be upregulated during the malignant process. Indeed, in breast cancer, its expression was seen in malignant areas but not in normal tissue (16). Similar findings have been reported in thyroid cancer (32), glioma (33), and pancreatic cancer (34). In the current study, we examined the relationships between CXCR4 expression and clinicopathological factors in OSCC. There was no correlation between CXCR4 expression and T classification, tumor differentiation or mode of invasion. However, CXCR4 expression was significantly correlated with lymph-node metastasis. CXCR4 is a serpentine transmembrane protein that is reportedly present either on the cell membrane or in the cytoplasm (35). Its activity is mediated via its interaction with SDF-1. It could be speculated that if CXCR4 expression is deficient on the cell surface, its ability to respond to the signal provided by SDF-1 will be inhibited, thereby decreasing metastasis. Zeelenberg and colleagues reported that the loss of CXCR4 expression on the cell membrane of T-cell hybridoma reduced metastasis (36). In another study, Kollet and co-workers reported that hematopoietic stem cells exhibiting a loss of transmembrane CXCR4 were unable to migrate in bone marrow (37). These studies highlight the fact that the loss of CXCR4 expression on the cell surface is associated with decreased homing of both cancer and normal cells.

In conclusion, OSCC cells expressing CXCR4 and its specific ligand, SDF-1, induce signaling circuits that regulate cell invasiveness and motility, leading to metastasis. CXCR4 expression was significantly correlated with lymph-node metastasis in OSCC tissue. Our findings might contribute to the future development of therapeutic interventions for the treatment of OSCC metastasis.

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