Epithelial-mesenchymal transition induced by the stromal cell-derived factor-1/CXCR4 system in oral squamous cell carcinoma cells

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Abstract. Epithelial-mesenchymal transition (EMT) refers to critical events occasionally observed during tumor progression, including invasion and metastasis, by which cancer cells acquire a fibroblast-like phenotype. Since the stromal cell-derived factor-1 (SDF-1)/CXCR4 system can facilitate lymph node metastasis in oral squamous cell carcinoma (SCC), we have explored the possibility that this system might be involved in EMT. Oral SCC cells, B88 and HNt, which have functional CXCR4 and lymph node metastatic potential, were found to lose their epithelial cell morphology due to SDF-1. In this context, the downregulation of epithelial markers, cytokeratin, E-cadherin and ß-catenin, and the upregulation of mesenchymal marker, vimentin and snail were detected. Furthermore, upregulation of vimentin by treatment with SDF-1 was impaired by phosphatidylinositol 3 kinase (PI3K) inhibitor Wortmannin, but not by mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor U0126. In the type I collagen embedding culture, SDF-1-treated B88 cells formed protruding extensions, but the effect was impaired by treatment with Wortmannin. These results suggested that EMT induced by the SDF-1/CXCR4 system might be involved in the lymph node metastasis of oral SCCs via activation of PI3K-Akt/PKB pathway.

Introduction

Chemokines are a large family of small (7-15 kDa) structurally related heparin-binding proteins that have been identified as attractants of different types of blood leukocytes to sites of infection and inflammation (1,2). They are produced locally in the tissues and act on leukocytes through selective membrane-bound G-protein-coupled receptors, the two major subfamilies of which are designated as CCR and CXCR. Among these chemokines and their receptors, the stromal cell-derived factor-1 (SDF-1), also referred to as the CXCL12)/CXCR4 system, has been demonstrated to be involved in lymph node or distant metastasis of several types of cancer (3-9). We have also reported the involvement of this system in lymph node metastasis associated with oral squamous cell carcinoma (SCC) (10,11). Moreover, we have recently found that the expression of CXCR4 in oral SCC at the primary site was significantly associated with lymph node metastasis, the mode of invasion, tumor recurrence, and patient prognosis (12). On the other hand, recent investigations have suggested that the SDF-1/CXCR4 system is involved not only in metastasis but also in the growth and survival of the cancer cells (13-15), indicating that this system regulates the acquisition of various malignant phenotypes observed in cancer cells.

Epithelial-mesenchymal transition (EMT), i.e. the acquisition of a mesenchymal phenotype by epithelial cells, is known to take place during critical phases of embryonic development, including morphogenesis and tissue remodeling, in many animal species, and this process is characterized by the disassembly of cell-cell contacts, the reorganization of the actin cytoskeleton, and cell-cell separation (16). EMT is also a critical event that is occasionally observed during tumor progression, including invasion and metastasis, by which cancer cells acquire a more aggressive phenotype (17,18). Under these conditions, EMT is defined as the occurrence of a variable proportion of tumor cells that upregulate mesenchymal markers such as vimentin and snail, and that downregulate epithelial markers such as E-cadherin (17,18). The expression of these EMT markers is induced by a number of growth factor/receptor systems such as the hepatocyte growth factor (HGF)/c-met system (19-21) and the transforming growth factor (TGF)-ß/TGF-ß receptor system (22,23), both of which are associated with a more aggressive phenotype of cancer cells. However, little information is available regarding the type of EMT induced by chemokine system(s), including the SDF-1/CXCR4 system, despite abundant evidence of the diverse malignant behaviors of such systems in cancer cells. In this study, we investigated

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whether or not the SDF-1/CXCR4 system is associated with EMT in oral SCC cells.

Materials and methods

Cells and cell culture. B88 and HNt cells (10) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, and 100 U/ml penicillin in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C.

Immunocytochemical staining. B88 cells were seeded on the FALCON culture slides (Becton Dickinson Labware, Franklin Lakes, NJ), followed by treatment with or without SDF-1α (R&D, Minneapolis, MN) under serum-starved conditions. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature 20 h later. After washing the cells with PBS, non-specific binding was blocked with 1% BSA in PBS for 1 h at room temperature. Cells were then incubated with the primary antibodies against vimentin (Dako Corporation, Carpinteria, CA) or E-cadherin (BD Biosciences, Franklin Lakes, NJ). Alexa 594-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR) was used for the detection. After staining with 2 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Roche Molecular Biochemicals, Mannheim, Germany) in PBS for 5 min, slides were mounted with ProLong Antifade (Molecular Probes), and fluorescence signals were observed under a fluorescence microscope (Nikon, Tokyo, Japan).

Quantitative RT-PCR. After stimulation of the cells with or without SDF-1α under serum-starved conditions, the preparation of total RNA and reverse transcription were performed as described previously (10). E-cadherin, vimentin, snail, matrix metalloproteinase (MMP)-2, MMP-7, urokinase type-plasminogen activator (uPA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were detected with Taqman® gene expression assay (Applied Biosystems, Foster City, CA), respectively. Gene-specific products were measured continuously by an ABI PRISM 7000 sequence detection system (Applied Biosystems). Experiments were performed three times.

Western blotting. Western blotting was performed as described previously (10). The nitrocellulose membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) was incubated with primary antibodies against vimentin, cytokeratin (Dako Corporation), ß-catenin (BD Biosciences), ß-actin (Sigma, St. Louis, MO) and phosphorylated- or total-ERK1/2 and Akt/PKB (Cell Signaling), followed by horseradish peroxidase-conjugated secondary antibodies. Detection was then performed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Collagen gel culture. Serum-free three-dimensional cultures of B88 cells were performed as described by Janda *et al* (22). Cells (in serum-containing medium), rat tail collagen (3-4 mg/ml) (Becton Dickinson) and 10% GF-reduced Matrigel solution (Becton Dickinson) were mixed rapidly at 0˚C (final collagen concentration, 1.5 mg/ml), and 200 μl droplets containing 3,000 cells were dispensed into 24-wells. After solidification on a level surface at room temperature for 30 min, the gels were incubated at 37˚C in a CO_2 incubator for another 1 h and overlaid with 500 μl of serum-free medium. The medium overlaying the gel was changed 1 day after seeding. After

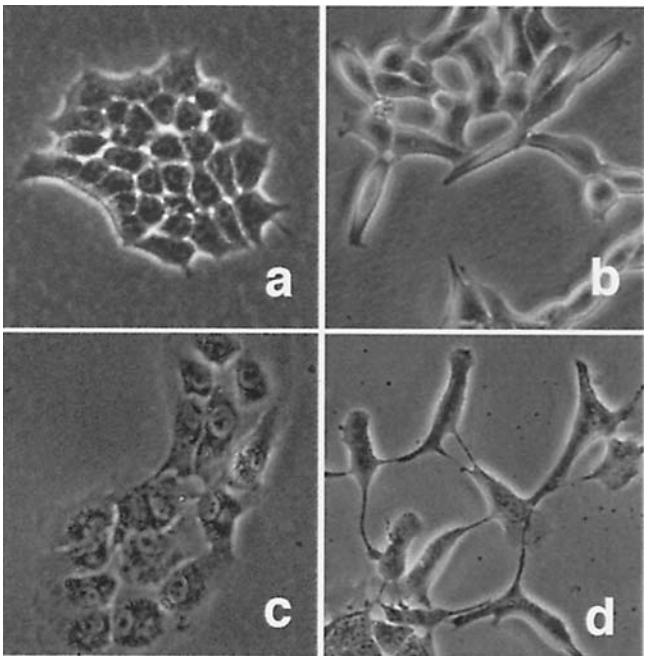

Figure 1. Morphology in SDF-1-treated B88 and HNt cells. B88 and HNt cells were treated with SDF-1 under serum-starved conditions for 72 h. The morphology of the cells was observed under a phase contrast microscope. B88 cells (a) without SDF-1 or (b) with SDF-1. HNt cells (c) without SDF-1 or (d) with SDF-1.

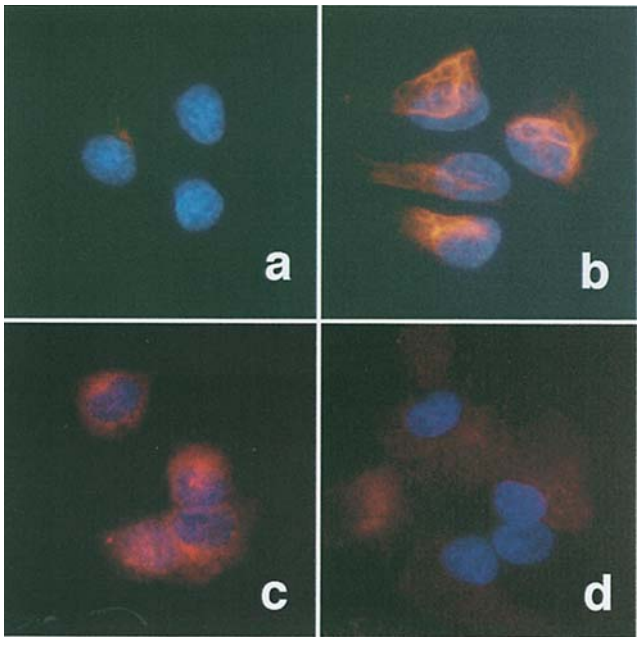

Figure 2. Expression of EMT markers in SDF-1-treated B88 cells. B88 cells were treated with or without SDF-1 under serum-starved conditions. After 72-h incubation, the cells were stained and observed under a fluorescence microscope. Expression of vimentin (red) in B88 cells without SDF-1 (a) or with SDF-1 (b). Expression of E-cadherin (red) in B88 cells without SDF-1 (c) or with SDF-1 (d). The nucleus was stained with DAPI (blue).

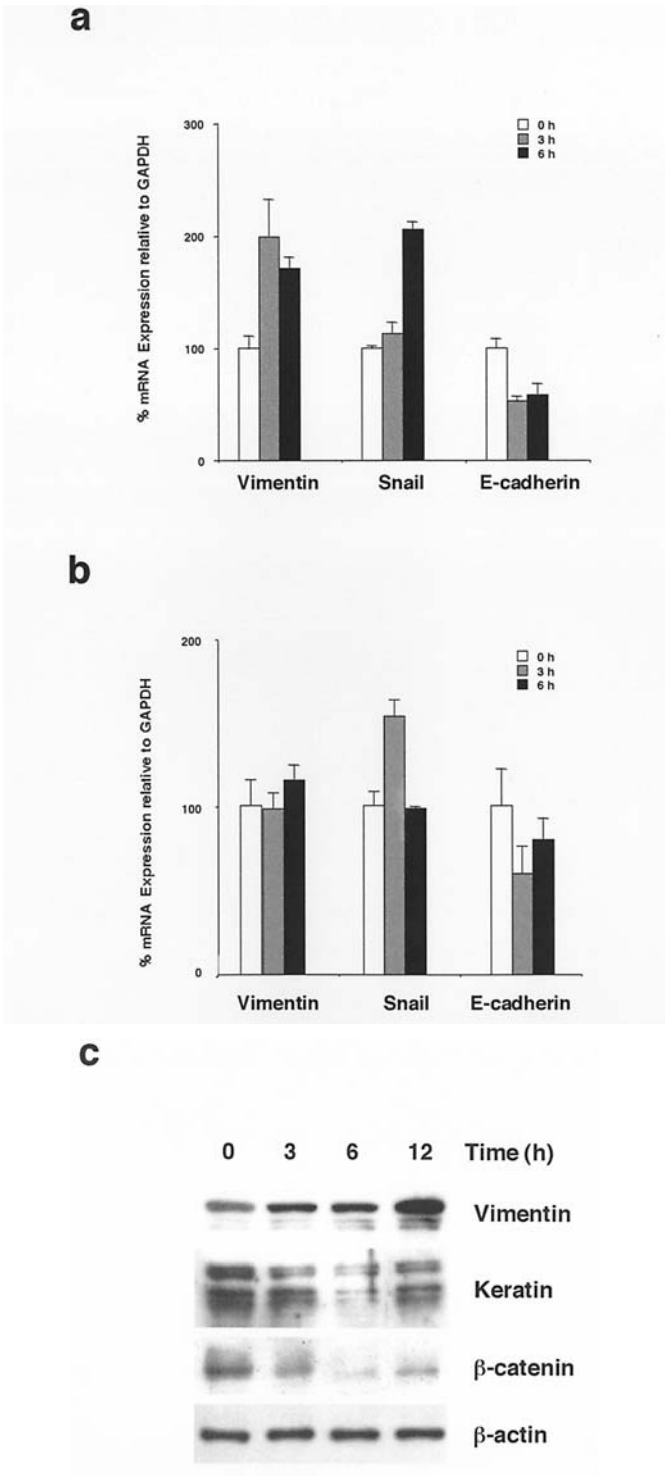

Figure 3. Expression of EMT markers in SDF-1-treated B88 and HNt cells. Expression of EMT marker mRNA (vimentin, snail, E-cadherin) in SDF-1-treated B88 cells (a) or HNt cells (b) was examined by quantitative PCR. The bars show the SD of triplicate samples. The data are representative of three separate experiments with similar results. (c) The expression of EMT marker proteins (vimentin, cytokeratin and ß-catenin) in SDF-1-treated B88 cells was examined by Western blotting. The data are representative of three separate experiments with similar results.

allowing structure formation of the cells for 4 days, 100 ng/ml SDF-1 or 5 ng/ml TGF-ß (R&D) were added upon medium change every other day, and the cells were further cultivated for 8 days. In some experiments, kinase inhibitor Wortmannin (50 nM) was added every other day.

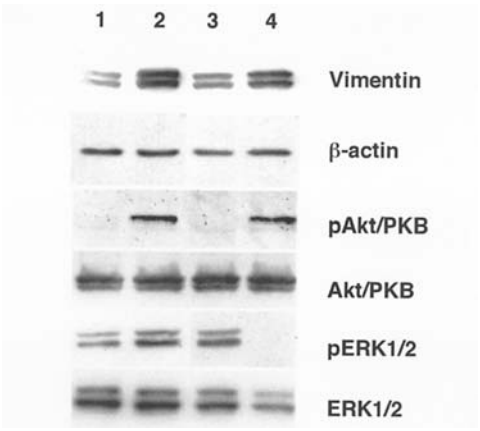

Figure 4. Effect of the kinase inhibitors on the expression of vimentin. Serum-starved B88 cells were incubated with 100 ng/ml SDF-1α for 12 h with or without 50 μM Wortmannin or 10 μM U0126, and cell lysates were probed with vimentin antibody. In case of detection of ERK1/2 or Akt/PKB protein, serum-starved B88 cells were incubated with the same concentration of Wortmannin or U0126 for 45 min and then stimulated with 100 ng/ml SDF-1α for 10 min. Cell lysates were probed with phosphorylated and total ERK1/2 or Akt/PKB Abs.

Results

Morphological changes induced by the SDF-1/CXCR4 system in B88 and HNt cells. Under conditions of cultivation in complete medium, B88 and HNt cells migrated toward SDF-1 (10), but cell scattering and morphological changes were equivocal. However, when cultivated under serum-starved conditions, some cells began to scatter after 48-h treatment with SDF-1 (12); most of the B88 cells (Fig. 1a and b) and 30% of the HNt cells (Fig. 1c and d) switched their morphology to fibroblast-like phenotype at 72 h. The morphological change effectively occurred in the low confluence of the cells.

Expression of EMT markers induced by the SDF-1/CXCR4 system in B88 and HNt cells. Under serum-starved conditions, SDF-1 was found to upregulate the mesenchymal marker, vimentin (Fig. 2a and b), but downregulate the epithelial marker, E-cadherin (Fig. 2c and d). Quantitative RT-PCR analysis revealed that SDF-1 downregulated E-cadherin mRNA, and upregulated the mesenchymal marker, vimentin as well as snail mRNA in B88 cells (Fig. 3a). The change of these EMT markers in HNt cells treated with SDF-1 was similar, but was slight (Fig. 3b). The expression of MMP-2, MMP-7 and uPA were not altered by treatment with SDF-1 in B88 cells (data not shown). At the protein level, the down-regulation of cytokeratin and ß-catenin was observed, as was the upregulation of vimentin (Fig. 3c). We observed similar changes of EMT proteins in the HNt cells (data not shown).

Effect of the kinase inhibitors on the expression of vimentin. In our previous study, B88 cells migrated toward SDF-1α via activation of both ERK1/2 and Akt/PKB signaling pathways (10). To study the contribution of these kinases, we examined the effect of an MEK inhibitor, U0126, or a PI3K inhibitor, Wortmannin, on the expression of vimentin induced by SDF-1. Although ERK1/2 and Akt/PKB were rapidly activated by SDF-1α, the activation was completely blocked by treatment with each kinase inhibitor (Fig. 4). Moreover, upregulation

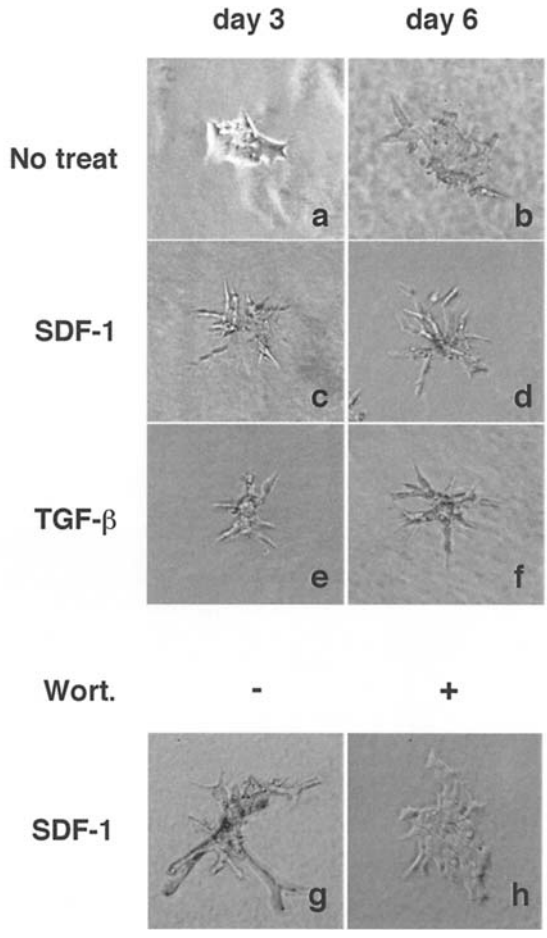

Figure 5. SDF-1 induced EMT in the collagen gel. B88 cells were embedded in rat tail collagen and 10% GF-reduced Matrigel solution. After allowing structure formation of the cells for 4 days, 100 ng/ml SDF-1 (c and d) or 5 ng/ml TGF-ß (e and f) were added for a further 3 or 6 days. SDF-1-treated cells were incubated (g) without or (h) with kinase inhibitor Wortmannin (wort.) for 6 days.

of the vimentin protein was inhibited by treatment with Wortmannin, but not by U0126 (Fig. 4). In the collagen embedding culture of B88 cells, SDF-1 induced the production of protruding extensions that invade the basement membrane gel, as did TGF-ß. However, the protrusion of B88 cells by SDF-1 was impaired by the use of Wortmannin, indicating that PI3K-Akt pathway was critical for the SDF-1-induced EMT.

Discussion

In this study, we investigated the EMT induced by the SDF-1/CXCR4 system in oral SCC cells. The findings obtained from the present series of experiments were as follows. First, SDF-1 induced fibroblast-like morphological changes in CXCR4-expressing B88 and HNt cells. Second, the down-regulation of epithelial markers and the upregulation of mesenchymal markers were also detected. Third, induction of mesenchymal marker, vimentin, and protruding extensions in the B88 cells with the treatment of SDF-1 was inhibited by treatment with PI3K inhibitor Wortmannin. These results suggest that the SDF-1/CXCR4 system induces EMT via activation of PI3K-Akt/PKB pathway.

Few common carcinoma cell types with a well-defined epithelial phenotype can complete EMT *in vitro*, perhaps because EMT is very sensitive to culture conditions, including substrates and the presence of serum (18). In the present study, oral SCC cells, both B88 and HNt cells changed morphology to a fibroblast phenotype by treatment with SDF-1; however, the alterations were dependent on culture conditions, i.e. serum starvation and low confluence. Although these reasons were unclear, factor(s) contained in the serum or molecule(s) recruited by a cell-cell tight contact might inhibit EMT by the SDF-1/CXCR4 system.

In the present study, both oral SCC cells, B88 and HNt, changed morphology to a fibroblast phenotype, which is associated with the alteration of EMT markers; however, the alterations are weak in HNt cells. HNt cells express functional CXCR4 (10), but the expression is 7.5-fold lower than that in B88 cells (12). Moreover, since HNt cells are somewhat heterogeneous, it might be considered that the expression of CXCR4 in each cell is different. Thus, in HNt cells, morphological changes associated with intracellular signaling by SDF-1 might be inadequate for the alteration of EMT markers. In contrast, Zhang *et al* reported that the autocrine HGF/c-met system was critical for the mesenchymal transition of mammary epithelial cells transformed by the expression of activated M-Ras (24). Therefore, B88 cells, but not HNt cells, might have critical pathway(s) for the enhancement of EMT.

Oral SCCs are characterized by a high degree of local invasiveness and a high rate of metastasis to the cervical lymph nodes. We have previously reported that oral SCC cells produce a large amount of matrix degrading enzyme, such as MMPs or uPAs (25), which might contribute to invasiveness and lymph node metastasis. During EMT, the involvement of MMPs or uPAs has been also suggested (26-28); however, we could not find significant alteration of MMP-2, MMP-7 and uPA by treatment with SDF-1. Scotton *et al* showed that the invasion of ovarian cancer cells through Matrigel stimulated by SDF-1 inhibited the presence of the MMP inhibitor, marimastat, but that no MMPs or tissue inhibitors of metalloproteinases (TIMPs) appeared to be regulated by SDF-1 (29). Therefore, they speculated that the ovarian cancer cells which they used produce a variety of matrix-degrading enzymes, and that SDF-1 merely gives them a direction in which to move. In the present study, B88 cells, which express several types of MMP (12), invaded the collagen gel, but could not form EMT change without treatment with SDF-1. Thus, MMPs or uPAs play a critical role in the invasion of oral SCC cells, but they might not contribute to the SDF-1-induced EMT.

During EMT, epithelial cells acquire fibroblast-like properties and exhibit reduced cell-cell adhesion and increased motility. The plasticity afforded by EMT is central to the complex remodeling of embryo and organ architecture during gastrulation and organogenesis. In pathological processes such as oncogenesis, EMT may endow cancer cells with enhanced motility and invasiveness. Indeed, oncogenic transformation may be associated with signaling pathways promoting EMT (30), in which numerous studies have been conducted to investigate the ras-ERK or Akt/PKB pathways (23,31,32). For example, Ellenrieder *et al* reported that TGF-ß1

treatment induced EMT in TGF-ß-responsive pancreatic cancer cells with constitutive Ki-Ras mutations (31). Moreover, Grille *et al* demonstrated that oral SCC cell lines engineered to express constitutively active Akt underwent EMT, as characterized by the downregulation of the epithelial markers desmoplakin, E-cadherin, and ß-catenin, and by the upregulation of the mesenchymal marker vimentin (32). Chan *et al* reported that oncogenic ras and src, both inducers of EMT, also activate PI3K (33). Our group previously demonstrated that the src family kinases are involved in the SDF-1/CXCR4 mediated-lymph node metastasis in oral SCC (10). Thus, it is possible that the activation of the PI3K-Akt pathway via src family kinases might be involved in the establishment of EMT by the SDF-1/CXCR4 system.

We have previously reported the involvement of the SDF-1/CXCR4 system in lymph node metastasis associated with oral squamous cell carcinoma (SCC) (10-12). During the process of lymph node metastasis, oral SCC cells must alter their morphology by use of EMT in order to intravasate into lymphatics through lymphatic valve (34). In the present study, oral SCC cells B88 and HNt were found to lose their epithelial cell morphology due to SDF-1, but the alterations could be recovered by the withdrawal of SDF-1. Thus, for the intravasation into lymphatics, oral SCC cells might use the SDF-1/CXCR4 system transiently to alter their shape as an 'EMT force'. When the cancer cells complete the intravasation into lymphatics, they might use the SDF-1/CXCR4 system as an 'attractive force' for the establishment of lymph node metastasis. The expression of CXCR4 in oral SCC might be used as a molecular target of diagnosis, not only for lymph node metastatic potential, but also for EMT potential. Furthermore, CXCR4 inhibitors or antagonists might be potential anticancer agents to suppress both EMT and lymph node metastasis.

Acknowledgments

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