Functional effects of SDF-1α on a CD44$^+$ CXCR4$^+$ squamous cell carcinoma cell line as a model for interactions in the cancer stem cell niche

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Abstract. Stromal cell-derived factor-1α (SDF-1α), also known as CXCL12, has variable effects on a plurality of cells. It is known to have selective effects on cell migration, morphology, survival and cell homing. As such the SDF-1-CXCR4 axis is postulated to be a crucial key pathway in the interaction between (cancer) stem cells and their surrounding supportive cells, the so-called (cancer) stem cell niche. We evaluated the expression of CD44 as a cancer stem cell (CSC) marker and the expression of CXCR4 in the head and neck squamous cell carcinoma (HNSCC) cell line UM-SCC 11A. In addition, we monitored proliferation, formation of podia and migration of UM-SCC 11A cells under the influence of SDF-1α. Whereas SDF-1α induced the formation of podia of CD44$^+$ CXCR4$^+$ UM-SCC 11A cells in a dose-dependent manner and the maximum number of cells exhibiting the formation of podia was observed under the influence of 10 ng/ml SDF-1α ($P=5.3x10^{-6}$), the highest number of migrating cells was noted using a concentration of 100 ng/ml (P=0.027). Proliferation and survival were not affected by SDF-1α. We showed that UM-SCC 11A cells could be a target for SDF-1α by CXCR4 expression and these cells also showed characteristics of HNSCC CSCs via CD44 expression. We demonstrated that SDF-1α is a chemoattractant for UM-SCC 11A cells, and a maximum directed migration was achieved under the influence of 100 ng/ml SDF-1α. Changes in cell morphology by presenting filopodia or a prominent uropod were noted following treatment of 10 ng/ml SDF-1α. The SDF-CXCR4 axis may play a crucial role in the interaction between CSCs and their supportive cells in the CSC niche. Understanding these interactions may help to gain further insight into the pathophysiology of the progression and recurrence of malignant diseases and thus help to develop novel strategies for therapy.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is a malignancy with a high rate of recurrence, largely because it is usually diagnosed at a late stage. Tobacco and alcohol exposure are the main risk factors and account for ~85% of HNSCC cases (1). Despite advances in surgical and non-surgical therapy, the mortality rate from this disease has remained constant over the last few years, mainly due to the development of therapy-resistant local and regional recurrences. Antineoplastic treatments such as chemotherapy or radiation can efficiently eradicate a majority of proliferating malignant cells within malignant tumors. However, there is increasing evidence that there is a subpopulation of resistant tumor cells that cannot be eradicated by these regimens. These cancer stem cells (CSCs) have distinct features of somatic stem cells such as self-renewal, extensive proliferation and differentiation. Therefore, these cells are essential and responsible for initiation, but also maintenance and recurrence of malignant disease. In recent years, the CSC hypothesis has been coined for HNSCC as well (2,3). Prince *et al* (2) showed that CD44$^+$ cancer cells, which typically comprise <10% of the cells in an HNSCC tumor, but not CD44$^-$ cancer cells, give rise to new tumors *in vivo*. Since then, CD44$^+$ cells in tumors of the head and neck are referred to as CSCs of HNSCC.

CD44 is an integral cell membrane glycoprotein and it comprises different isoforms that arise from alternative splicing of a region of variable exons. They differ in the primary amino acid sequence as well as the amount of N- and O-glycosylation (4), thereby its apparent molecular mass ranges from 85 to 250 kDa (5). At least 20 variants of CD44 have been reported due to the alternative splicing of 10 exons that encode the membrane's proximal portion of the extracellular domain (6-8). Originally, it was described as a receptor on circulating lymphocytes involved in cell homing, adhesion and migration (9,10).

In 1991 Günthert *et al* (11) showed that the expression of CD44 conferred metastatic potential to a non-metastatic cell

line in a rat carcinoma model (12). Since then, several analyses have indicated that there is a correlation between the expression of CD44 and progression, metastasis and prognosis of malignant disease. This has also been shown in different types of epithelial carcinoma, in addition to HNSCC, such as colorectal carcinoma (13,14), breast cancer (15) and certain types of gastric carcinoma (5,16). The in-depth analysis of expression markers such as CD44 in tissue samples of HNSCC patients may reveal their role as potential prognostic biomarkers or therapeutic targets, e.g., for antigen-directed immunotherapy.

The analysis of the so-called CSC niche theory may provide information on cell trafficking and underlying mechanisms, such as tumor expansion and metastatic progression. The interaction between stromal cell-derived factor-1α (SDF-1α) and its receptor CXCR4 may play an important role in this field. SDF-1α is a multifunctional cytokine that is constitutively expressed and secreted by several types of tissues, including endothelium and stromal cells (17-19). It has a single open reading frame of 282 nucleotides encoding a polypeptide of 93 amino acids. SDF-1 consists of two forms, SDF-1α (amino acids 24-88) and SDF-1β (amino acids 24-93), by alternative splicing (18,20,21). SDF-1α is the only proven chemoattractant for primitive hematopoietic progenitor cells (HPCs), to date (18,22-24). Accordingly, SDF-1α is considered to be one of the key regulators for HPC trafficking between the peripheral circulation and the bone marrow (25). Faber *et al* (18) and others demonstrated that SDF-1α induces polarization and formation of podia of HPCs and leukemic cells (26), two properties that represent prerequisites for directed locomotion. SDF-1α alone showed a moderate effect on cell proliferation in $CD34^+$ cells (18,27), and its effect on survival or apoptosis of HPCs remains controversial (18,27-29). Furthermore the SDF-1-CXCR4 axis plays a crucial role in the regulation of cell homing and adhesion to the supportive cellular microenvironment in the hematopoietic stem cell niche (30).

The receptor for SDF-1α has been identified as the 7-transmembrane receptor CXCR4. SDF-1α/CXCR4 interaction was reported to play an important role during embryonic development, especially in hematopoiesis, vascular development and cardiogenesis. CXCR4 expression on bone marrow endothelial cells is important for internalization of circulating SDF-1α, resulting in its translocation into the bone marrow (17). CXCR4 is also expressed on primitive $CD34^+$ HPCs (27). Signal transduction pathways initiated by the binding of SDF-1α to CXCR4 are not fully understood. Mechanisms involved in CXCR4 signaling include Gi-protein-mediated activation of PI3K and the phospholipase C cascade (24,31). The function of SDF-1α can be mimicked by small peptide agonists (18,32). Such molecules have several advantages compared to the natural one such as the ease of manufacturing. Interference of the signal transduction pathways followed by the SDF-1-CXCR4 axis by these agonists/antagonists could open new possibilities for therapeutic intervention.

Here, we monitored the effects of SDF-1α on polarization, migration and proliferation of the $CD44^+$ $CXCR4^+$ HNSCC cell line UM-SCC 11A.

Materials and methods

Cell line and cell culture. The HNSCC cell line 11A (UM-SCC 11A) was obtained from Dr T.E. Carey (University of Michigan, Ann Arbor, MI, USA). It originated from a primary human HNSCC of the larynx of a male patient without prior treatment (33).

Cell cultures were carried out at 37˚C in a 5% CO_2 fully humidified atmosphere using Dulbecco's modified minimum essential medium (DMEM) (Fisher Scientific Co., Pittsburgh, PA, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics (Life Technologies, Inc., Gaithersburg, MD, USA).

Immunofluorescence labeling. To detect the expression of CD44 and CXCR4 in UM-SCC 11A, cells were incubated with $CD44^-$/$CXCR4^-$ antibody (mouse monoclonal, 1:100; Abcam, Cambridge, UK) for 1 h at 37˚C followed by incubation with a second biotinylated antibody (anti-mouse, 1:100) for 30 min. After further washing steps with PBS, cells were treated with streptavidin-Cy3 (1:1,000)/streptavidin-Alexia 488 (1:500) for 30 min at room temperature. Finally, cells were covered in FluorSave™ reagent and dried to be evaluated by fluorescence microscopy. Cell nuclei were stained with DAPI.

Proliferation assay. Proliferation of HNSCC cells was measured by the Alamar Blue® (Invitrogen, Darmstadt, Germany) proliferation assay. Proliferation was measured on Days 1, 2, 3 and 4 by measurement of the fluorescence at a wavelength of 540 nm (exitation) and 590 nm (emission). Absorbance was monitored at 590 nm. Three independent experiments were performed (n=3).

Microscopy. Analysis of cell morphology under the influence of SDF-1α was carried out as follows. $CD44^+$ HNSCC cells were seeded in DMEM (Fisher Scientific Co.) supplemented with 10% FCS and antibiotics and then incubated with SDF-1α (0, 10, 100 and 500 ng/ml) for 24 h.

Cell morphology was assessed via light microscopy. At least 5 fields of view in each well were evaluated in each of 3 (n=minimum 3) independent experiments.

The ratio of polarized cells with filopodia or a prominent uropod compared to round cells was determined for the different concentrations of SDF-1α (10, 100 and 500 ng/ml).

Migration assay. Chemotaxis was assessed by an *in vitro* 2-chamber Transwell-assay. Different concentrations of SDF-1α (0, 10, 100 and 500 ng/ml) were added to the lower section of a Transwell chamber (8.0-μm pore size, 6.5-mm diameter inserts; Costar Inc.). Equal cell numbers of UM-SCC 11A were seeded in the upper chamber in medium without SDF-1α. After 24 h, the Transwells were removed, and the number of cells that had migrated through the micropores was calculated. As the cell line used (UM-SCC 11A) is adherent, cells turned out to migrate through the pores and stick to the bottom of the Transwell membrane. The width of the cell ring measured in nm served as a dimension for the number of cells that migrated (n=3).

Statistical analysis. All results were plotted as means ± standard deviation. To estimate the probability of differences, we adopted the Student's t-test (two-tailed distribution, 2-sample equal variance). Probability value of $P<0.05$ denoted statistical significance.

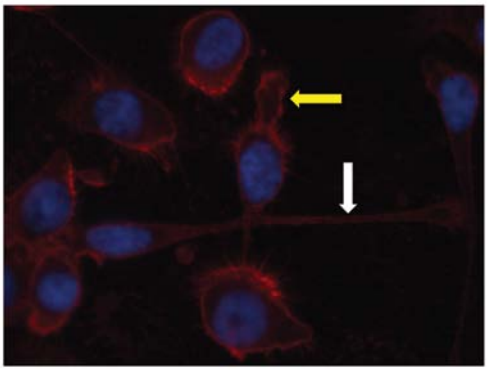

Figure 1. Expression of CD44 in UM-SCC 11A cells. CD44 was visualized in red color by immunofluorescence labeling via Cy3 in UM-SCC 11A. Cell nuclei were stained in blue by DAPI. High fluorescence intensity for CD44 was found in each samples stained (n=3). Every sample showed a surface staining pattern of CD44. Influence of SDF-1α on podia formation of UM-SCC 11A was evaluated (filopodia, white arrow; uropod, yellow arrow).

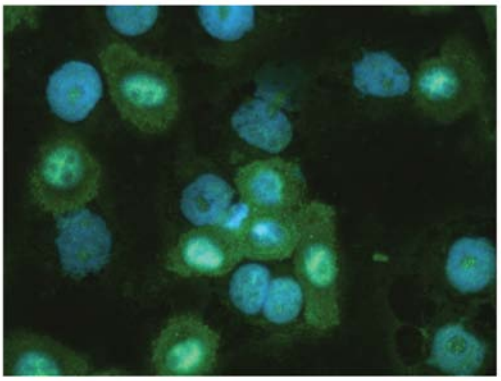

Figure 2. Expression of CXCR4 in UM-SCC 11A cells. CXCR4 was visualized in green color by immunofluorescence labeling via Alexia 188. Cell nuclei were stained in blue by DAPI. High fluorescence intensity for CXCR4 was noted in all samples stained (n=3) and a cytoplasmic staining pattern was exhibited.

Results

Expression of CD44 and CXCR4 in the HNSCC cell line. Immunofluorescence labeling of UM-SCC 11A cells was performed. CD44 was visualized in red color by immunofluorescence labeling via Cy3. CXCR4 was detected in green color by Alexia 488. In all cell lines an intense red fluorescence signal of all cells was detected by marking CD44 (Fig. 1). CD44 was mainly expressed on the cell surface in all samples stained. Most cells were also CXCR4$^+$. CXCR4 showed a cytoplasmatic staining pattern (Fig. 2).

Effects on cell proliferation. CD44$^+$ CXCR4$^+$ UM-SCC 11A cells were cultured at 37˚C in a 5% CO_2 fully humidified atmosphere using DMEM (Fisher Scientific Co.) supplemented with 10% FCS and antibiotics supplemented with SDF-1α in concentrations of 0, 10 or 100 ng/ml. Proliferation of HNSCC cell line under SDF-1α was measured by the Alamar Blue proliferation assay on Days 1, 2, 3 and 4 as described above. The addition of SDF-1α did not have any significant impact on the proliferation or viability of HNSCC cells (Fig. 3).

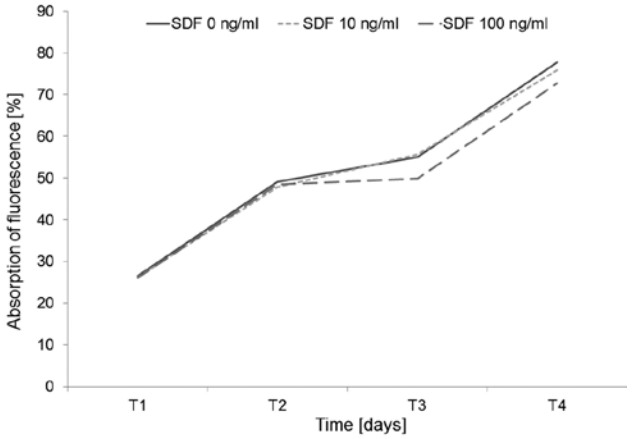

Figure 3. Effect of SDF-1α on the proliferation of CD44$^+$ CXCR4$^+$ UM-SCC 11A cells. The influence of SDF-1α at increasing concentrations on the proliferation of CD44$^+$ CXCR4$^+$ UM-SCC 11A cells was evaluated via the Alamar Blue proliferation assay (T, time in days). Proliferation was measured on Days 1, 2, 3 and 4 (n=3, example shown). The addition of SDF-1α did not have significant impact on the proliferation of UM-SCC 11A cells, even when the concentration of SDF-1α was increased (0, 10 and 100 ng/ml SDF-1α).

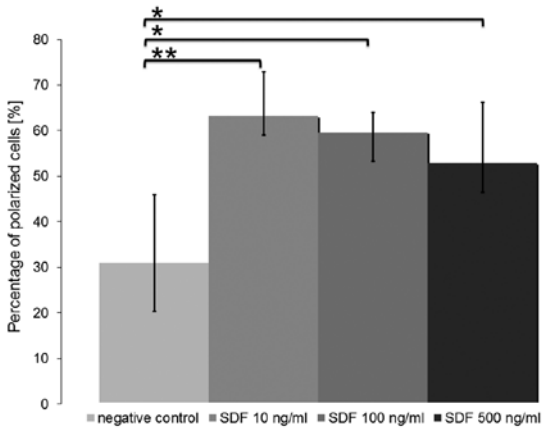

Figure 4. Effect of SDF-1α on the formation of podia of CD44$^+$ CXCR4$^+$ UM-SCC cells. Formation of filopodia and prominent uropodia was evaluated via microscopy following a 4-h treatment with SDF-1α at increasing concentrations (0, 10, 100 and 500 ng/ml). The percentage of polarized cells increased under the influence of SDF-1α in a concentration-dependent manner (means: 0 ng/ml SDF-1α, 31.01±14.97 (10.68%); 10 ng/ml SDF-1α, 63.20±9.73 (4.15%), P=5.3x10^{-6}; 100 ng/ml SDF-1α, 59.36±4.35 (6.39%), P=0.0017; 500 ng/ml SDF-1α, 52.88±13.33 (9.75%). P=0.00022; *P<0.01, **P<0.0001).

Effects on the formation of podia. Polarization and formation of podia are prerequisites for directed locomotion of cells. We analyzed podia formation of CD44$^+$ CXCR4$^+$ HNSCC cells following a 4-h treatment with SDF-1α. In our control experiments (0 ng/ml SDF-1α) 31.01±14.97 (10.68%) of the cells demonstrated an elongated morphology with a prominent uropod of filopodia. The percentage of polarized cells increased following treatment with SDF-1α in a concentration-dependent manner (up to 63.20±9.73 (4.15%) at 10 ng/ml SDF-1α; P=5.3x10^{-6}). Notably, the highest increase in the formation of podia was not achieved by the highest concentration tested (500 ng/ml SDF-1α). Thus the main impact on HNSCC cells

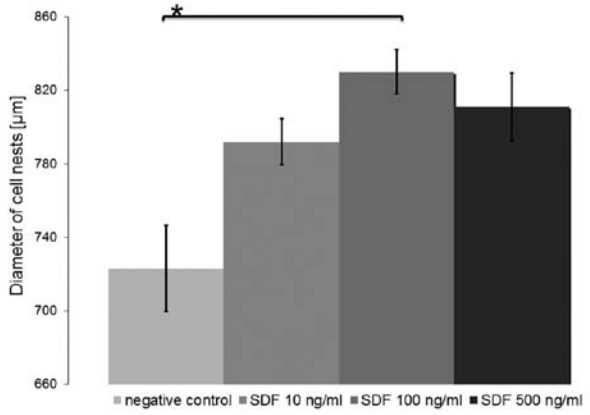

Figure 5. Effect of SDF-1α on migration of CD44$^+$ CXCR4$^+$ UM-SCC 11A cells. Chemotaxis of CD44$^+$CXCR4$^+$ UM-SCC 11A towards a gradient of SDF-1α was assessed in a Transwell migration assay. Induction of significant migration was observed following treatment with all concentrations tested (means: 0 ng/ml SDF-1α, 734±23.5 μm; 10 ng/ml SDF-1α, 792±12.5 μm, P=0.094; 100 ng/ml SDF-1α, 830±12 μm, P=0.027; 500 ng/ml SDF-1α, 812±18.5 μm, P=0.067; *P<0.05).

might be observed under the influence of lower concentrations than those tested in our experiments. The concentration-dependent effect on the formation of podia in UM-SCC 11A cells is shown in Fig. 4 (means: 0 ng/ml SDF-1α, 31.01±14.97 (10.68%); 10 ng/ml SDF-1α, 63.20±9.73 (4.15%), P=5.3x10^{-6}; 100 ng/ml SDF-1α, 59.36±4.35 (6.39%), P=0.0017; 500 ng/ml SDF-1α, 52.88±13.33 (9.75%), P=0.00022).

Effects on migration. Chemotaxis of CD44$^+$ CXCR4$^+$ UM-SCC 11A cells towards a gradient of SDF-1α was assessed in a Transwell migration assay (Fig. 5). The number of migrating cells increased continuously with increasing concentrations of SDF-1α up to 100 ng/ml, but decreased with concentrations higher than that (500 ng/ml). However, induction of significant migration was observed under the influence of all concentrations tested (means: 0 ng/ml SDF-1α, 734±23.5 μm; 10 ng/ml SDF-1α, 792±12.5 μm, P=0.094; 100 ng/ml SDF-1α, 830±12 μm, P=0.027; 500 ng/ml SDF-1α, 812±18.5 μm, P=0.067).

Discussion

To examine the potential role of SDF-1α in HNSCC, we monitored its effect on directed migration, the formation of podia and proliferation of CD44$^+$ CXCR4$^+$ UM-SCC 11A. We showed that the HNSCC cell line UM-SCC 11A may be a target for SDF-1α by expressing CXCR4 (34) and also shows characteristics of head and neck squamous cell carcinoma cancer stem cells by expressing CD44 (2,35). It should be mentioned that 'the real cancer stem cells' in HNSCC are defined by a combination of membrane markers that have been found to be characteristic for cells that have the capability to build an entire new bulk of a tumor (36,37), e.g., in mouse models (2). These cells are said to have the properties of self-renewal, differentiation and unlimited proliferation. The cancer stem cell theory postulates these cells to be resistant to available therapeutical options to date, such as chemotherapy and radiation (38). Unfortunately, the marker used as a CSC marker in our experiments (CD44) is also expressed in ordinary cells (36). It is the current challenge of research to find a combination of surface markers that identifies more precisely the subgroup of potent CSCs from the bulk of the tumor. CD44 cannot be sufficiently used as a defining CSC marker alone, since as a cell surface glycoprotein it takes part in many cell-cell interactions (36,39) and in humans it is present in a multitude of splice variants (36) and can be found ubiquitously. Approaches for the investigation of the 'real cancer stem cell' in HNSCC requires research using human tumor material with isolation of a small subpopulation of cells out of the bulk of the tumor using complicated cell separation steps including recurring washing steps, lysis by DNAses and FACS sorting. Based on the current knowledge, a combination of lineage markers must be negative in order to separate CSCs out of the entire population of HNSCC cells (CD3, CD3, CD10, CD18, CD31, CD64 and CD140) (2,35,36). Other markers have been postulated as CSC markers yet only a small amount of cells carrying these markers are said to be able to initiate entirely new HNSCC tumors, e.g., in mouse models (CD44, ALDH1) (2,40). The remaining cell amount after separation as described above is often quite small and remaining cells are often not useful for further experiments. Thus, it is often necessary to revert to cell lines to perform experiments.

SDF-1α acts on the CXCR4 receptor and we demonstrated that the functional effects of SDF-1α varied with concentration. The maximum formation of podia in UM-SCC 11A cells was achieved at a concentration of 10 ng/ml SDF-1α, while most cells showed directed migration against a gradient of 100 ng/ml SDF-1α. Generally, the formation of podia is considered to be a pre-stage condition to directed locomotion (18). It is possible that the concentration of SDF-1α that transforms elongated cells into migrating cells is found between 10 and 100 ng/ml SDF-1α. It is also possible that a maximum percentage of elongated UM-SCC 11A cells could be found using lower concentrations of SDF-1α than those tested in our experiments. Faber *et al* (18) used hematopoietic progenitor cells, and various concentrations of the SDF-1α analogue (CTCE-0214) in the range of μl/ml (18). CD34$^+$ cells showed an effect on the formation of podia and directed migration under these conditions, which was comparable to SDF-1α in a range of ng/ml (18).

Although a marked effect of SDF-1α was noted in UM-SCC 11A cell morphology and directed migration, we did not note any effect on cell proliferation and survival. Despite the fact that SDF-1α acts on the same CXCR4 receptor, it is possible that its effects are not only increased in a dose-dependent manner but also depend on the influence of a particular dose of the agent. These results also suggest that the signal cascade induced by SDF-1α is not a monocausal succession (18) but rather a complex network. In previous experiments we showed that the binding of SDF-1α analogues has selective effects on hematopoietic progenitor cells (18). This indicates that the signal cascade induced by SDF-1α is not a monocausal succession (SDF-1α binding to CXCR4 activating G-proteins further activating downstream mediators) but rather a complex network (18,41,42). Analysis of the downstream targets in SDF-1α signal cascades such as G-protein-associated activation of intracellular Ca-stores (42,43) or Akt-triggered

activation of IL-8 secretion (41,42) are only a few examples for the complexity of intracellular SDF-1α signaling.

It is well-known that SDF-1α is a powerful chemoattractant for primitive hematopoietic cells (18). Its effect as a chemoattractant has also been shown in cholangiocarcinoma (44) and breast cancer (45). The SDF-CXCR4 axis is involved in several aspects of tumor progression, such as angiogenesis, metastasis and survival (42,46). The microenvironment of the bone marrow has been said to support survival, differentiation and proliferation of hematopoietic progenitor cells (47), but also malignant progenitor cells of the hematopoietic system, e.g., B-cell acute lymphoblastic leukemia (B-ALL) (47,48). The pathway including the SDF-1-CXCR4 axis is postulated to be responsible for retention of lymphoid and myeloid leukemia cells in the bone marrow (42,49,50). The importance of the SDF-1-CXCR4 axis has been well discussed in the hematopoietic system, but this is the first time that we provide evidence that the SDF-1-CXCR4 axis may also play a crucial role in the development and particularly the progression, invasion and metastasis of HNSCC.

In this study, we showed that polarization and formation of filopodia and a prominent uropod were increased in $CD44^+$ $CXCR4^+$ UM-SCC 11A cells in a dose-dependent manner by SDF-1α. This effect can probably be attributed to cytoskeleton rearrangements of actin-containing protrusions (51,52) and this also might be influenced by extracellular factors such as matrix metalloproteinases (52-54). In general, the formation of podia is said to be concomitant with cell adhesion to the microenvironment, especially the cellular microenvironment surrounding them (18). If there is evidence that the formation of podia and adhesion to their cell cancer stem cell niche also play a role in HNSCC $CD44^+$ cells, understanding of these interactions might offer insights into new strategies of cancer-directed therapy in HNSCC. Thus, small-molecule agonists or antagonists (18) could be used to influence the cancer stem cell niche of HNSCC resulting in inhibition or ideally attenuation of tumor invasion and metastasis. Further experiments must be carried out in human tissue to expand and focus our insight into the cell-cell-interactions in the so-called cancer stem cell niche of solid tumors. Thus, it may be possible to develop therapeutic strategies specifically aimed at CSCs or targeted to the SDF-1-CXCR4 axis, thus affecting the interaction of CSCs with their cancer stem cell niche.

Acknowledgements

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