

RESEARCH ARTICLE

CCR7 and CXCR4 Expression in Primary Head and Neck Squamous Cell Carcinomas and Nodal Metastases – a Clinical and Immunohistochemical Study

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Abstract

Background: Squamous cell carcinomas (SCCs) are common head and neck malignancies demonstrating lymph node LN involvement. Recently chemokine receptor overexpression has been reported in many cancers. Of particular interest, CCR7 appears to be a strong mediator of LN metastases, while CXCR4 may mediate distant metastases. Any relations between their expression in primary HNSCCs and metastatic lymph nodes need to be clarified. **Aims:** To investigate CCR7 and CXCR4 expression in primary HNSCCs of all tumor sizes, clinical stages and histological grades, as well as involved lymph nodes, then make comparisons, also with control normal oral epithelium. **Materials and Methods:** The sample consisted of 60 formalin-fixed, paraffin-embedded specimens of primary HNSCCs, 77 others of metastasi-positive lymph nodes, and 10 of control normal oral epithelial tissues. Sections were conventionally stained with H&E and immunohistochemically with monoclonal anti-CCR7 and monoclonal anti-CXCR4 antibodies. Positive cells were counted under microscopic assessment in four fields (X40) per case. **Results:** There was no variation among primary HNSCC tumors staining positive for CCR7 and CXCR4 with tumor size of for CCR7 with lymph node involvement. However, a difference was noted between primary HNSCC tumors stained by CXCR4 with a single as compared to more numerous node involvement. CXCR4 appear to vary with the clinical stage but no links were noted with histological grades. Staining for primary HNSCC tumors and metastatic lymph nodes correlated.

Keywords: CCR7- CXCR4- metastatic lymph nodes- HNSCC

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Introduction

CHEMOKINES are chemotactic cytokines that cause the directed migration of leukocytes, leukocytes express the appropriate chemokine receptor, and this migration occurs along a chemical gradient of ligand - known as the chemokine gradient - allowing cells to move towards high local concentrations of chemokines. Chemokines are induced by inflammatory cytokines, growth factors and pathogenic stimuli (Murphy et al., 2000; Rossi and Zlotnik, 2000) (Zlotnik and Yoshie, 2000).

The chemokine gradient that attracts infiltrating cells can be created by different cell populations in a tissue differs according to chemokine function (Balkwill, 2004a). Some chemokines are homeostatic in nature and are constitutively produced and secreted. These homeostatic proteins serve a variety of functions: For example, they direct the trafficking of lymphocytes to lymphoid tissues. They are also involved in immune surveillance and function to localize T or B cells with antigen (on the surface of antigen-presenting cells) in the lymphatic system (Rossi and Zlotnik, 2000). Other chemokines are

considered inflammatory and are only produced by cells during infection or a pro-inflammatory stimulus. The role of inflammatory chemokines is to induce the migration of leukocytes to the injured or infected site (Fernandez and Lolis, 2002).

In infections, the first cells that produce chemokines are probably tissue leukocytes, but fibroblasts, endothelial cells and epithelial cells (both normal and malignant) are all able to produce chemokines and generate a chemokine gradient (Balkwill, 2004a).

The small (8–10 kDa) chemokine proteins are classified into four highly conserved groups - CXC, CC, C and CX3C - based on the position of the first two cysteines that are adjacent to the amino terminus (FIG. 1). More than 50 chemokines have been discovered so far (FIG. 2) and there are at least 18 human seven-transmembrane-domain chemokine receptors. In general, these receptors, which belong to the G-protein-coupled receptor family, bind to more than one type of chemokine (FIG. 2). However, six receptors bind to only one cytokine: CXCR4, CXCR6, CCR6, CCR9 and CX3CR1 (Balkwill, 2004a).

Chemokine receptors are embedded in the lipid bilayer

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of the cell surface and possess seven-transmembrane domains (7TM) (Fig 3). These receptors have been designated CX3CR1 through 6, CCR1 through 11, XCR1, and CX3CR1 based on whether they bind chemokines from the CXC, CC, C, or CX3C chemokine subfamilies, respectively (Murphy et al., 2000). The prototypical GPCR, rhodopsin, has only recently been characterized by X-ray crystallography (Palczewski et al., 2000).

Activation of the chemokine receptor by binding its chemokine is followed by exchange of bound GDP for GTP in the α subunit of the G proteins. The G proteins disassociate from the receptor and activate several effector molecules downstream, which results in a cascade of signaling events within the cytoplasm of the cell (Mellado et al., 2001). This sequence of events results in diverse physiological processes including leukocyte migration and trafficking, leukocyte degranulation, cell differentiation, and angiogenesis or angiostasis (Luther and Cyster, 2001; Mackay, 2001; Szekanecz and Koch, 2001).

CCR7 and its ligands are essentially involved in homing of various subpopulations of T cells and antigen-presenting dendritic cells (DCs) to the lymph nodes. Within lymph nodes, T cells establish close physical contacts with DCs, which allow their antigen-specific activation. Although it is well established that these interactions are necessary for the optimal initiation of protective immunity, recent evidence demonstrates that the CCR7-dependent contacts of T cells and DCs are also essential for the induction of peripheral tolerance and the regulation of the immune response by CD4⁺CD25⁺ regulatory T (TReg) cells. Furthermore, a series of recent studies have shown that CCR7 is indispensable for the unperturbed thymic T-cell development and negative selection of self-reactive T cells (Förster et al., 2008).

CCL19 and CCL21 are the only ligands for CCR7

Unlike CCL19, CCL21 has a uniquely long C-terminal tail containing 32 amino acids of which 12 are basic

amino-acid residues that allow avid binding to glycosaminoglycans and other molecules. This binding may be required for efficient presentation of CCL21 on the surface of endothelial cells (Gunn et al., 1998; Yoshida et al., 1998; Stein et al., 2000) and other cells (Friedman et al., 2006). Podoplanin, a proteoglycan expressed by lymphatic endothelial cells, reticular stromal cells and other cell types might specifically present CCL21 (Kerjaschki, 2005), and the expression of podoplanin might regulate the availability of CCL21 at these sites.

In human and mouse secondary lymphoid organs, CCL21 is produced by fibroblastic reticular cells of the T-cell rich area and, in mice also, by high endothelial venules (HEVs) (Carlsen et al., 2005). In non-inflamed lymph nodes, fibroblastic reticular cells seem to be the only source of CCL19 production in both humans and mice (Link et al., 2007). As human DCs can also produce CCL19, it is possible that activated DCs that are recruited into lymph nodes under inflammatory conditions may serve as an additional source of CCR7 ligands (Sallusto et al., 1999b).

CCR7 is expressed by semi mature and mature DCs (Ohl et al., 2004), thymocytes during defined stages of

their development (Misslitz et al., 2004) (see later), naive B and T cells (Reif et al., 2002), (Sallusto et al., 1999a), TReg cells (Szanya et al., 2002) and a subpopulation of memory T cells known as central memory T (TCM) cells (Sallusto et al., 1999a). CCR7 is also expressed by different non-immune cells, most notably in various malignancies.

CXCR4 is a chemokine receptor for SDF-1 chemokine, which CXCR4 is its only receptor (Horuk, 2001). This fact already suggests that the SDF-1–CXCR4 axis may play an important and unique biological role.

The role of the SDF-1–CXCR4 axis was extensively investigated initially for hemato/lymphopoietic cells. Accordingly, it had been demonstrated that SDF-1 regulates trafficking of CD34⁺ hematopoietic stem/progenitor cells, pre-B- and T lymphocytes (Ma et al., 1998). However, in recent years, evidence has accumulated that functional CXCR4 is also expressed on the surface of several tissue committed stem/progenitor cells. Accordingly, in addition to hematopoietic stem cells (Rosu-Myles et al., 2000).

CXCR4 was also found to be expressed on the surface of primordial germ cells (Ara et al., 2003), skeletal muscle satellite progenitor cells (Pituch-Noworolska et al., 2002), neural stem cells (Lazarini et al., 2003), liver oval/stem cells (Hatch et al., 2002) and retinal pigment epithelium progenitors (Crane et al., 2000).

Similarly, it had been reported that the specific CXCR4 ligand, SDF-1, is expressed/secreted by several tissues/organs in the body. The most important sources of SDF-1 are bone marrow-, lymph node-, muscle- and lung-derived fibroblasts (Zou et al., 1998; Ratajczak et al., 2003). SDF-1 is also secreted by liver and kidney cells and in several regions of the central nervous system (Stumm et al., 2002).

CXCR4, CCR7 and tumor cells

Tumor cells from at least 23 different types of human cancers of epithelial, mesenchymal and hematopoietic origin express CXCR4 (Balkwill, 2004b). Not all cancerous cells in the primary tumor are CXCR4 positive. In ovarian and non-small-cell lung cancer, for instance, only a subpopulation of cells expresses this receptor (Scotton et al., 2001; Kijima et al., 2002). When it has been possible to study freshly isolated tumor cells - for example from leukemia and cells that have been isolated from ovarian cancer ascites - the CXCR4 receptor is functional and various signaling pathways are activated.

Activation of CXCR4 stimulates directed migration of cancer cells and increases their invasion through Matrigel and monolayers of endothelial cells, bone marrow stromal cells and fibroblasts (Koshiba et al., 2000; Libura et al., 2002; Scotton et al., 2002). If CXCR4 is associated with metastatic activity in vivo, expression of CXCR4 and/or its receptor CCL12 might be higher in metastases compared with primary tumors. This has been reported to be the case in two different cancer types. In a comprehensive series of more than 600 prostate cancer specimens (Sun et al., 2003), CXCR4 protein expression increased with tumor aggressiveness and levels of CXCL12 were higher in metastatic lesions than in the primary tumor.

High CXCR4- expressing breast tumors also produced more extensive nodal metastasis compared with low CXCR4-expressing tumors, but there was no significant correlation with blood-borne metastasis(Kato et al., 2003).

CCR7 has been found in breast, gastric, non-small-cell lung and esophageal squamous cancer, and chronic lymphocytic leukaemia (CLL) (Müller et al., 2001; Mashino et al., 2002) (Till et al., 2002; Ding et al., 2003; Takanami, 2003). CCR7 expression correlates with metastatic potential and poor prognosis, and its ligand CCL21 is found at high levels in the lymph nodes that drain many cancers.

Takanami et al., (2003) measured the expression of CCR7 in patients with nonsmall-cell lung cancer and found an excellent correlation between the expression of CCR7 and the ability of the cancer to spread to the lymph nodes. Shimansky et al.(Schimanski et al., 2005) reported that strong expression of CXCR4 in colorectal cancer is associated with lymphatic and distal dissemination in patients with this disease. Wang et al., (2004) found a correlation between expression of CCR7 and metastasis in squamous- cell carcinoma of head and neck cancer. Laverdiere et al., (2005) have observed an association between CXCR4 expression and metastasis/ poor prognosis in patients with osteosarcoma. Ghobrial et al., (2004) observed that CXCR4 and CCR7 expression correlates with disease progression in B cell chronic lymphocytic leukemia/small lymphocytic lymphoma.

Materials and Methods

Tissue Samples

Tissues were obtained from head and neck SCC specimens for 60 patients who underwent head and neck surgery in Department of Medical University Hospital AL-Muassah (Damascous-Syria) between 2012 and 2015.

Surgical excision for each cancer with lymph node dissection was performed

The whole sample was 60 primary tumor and 77 invaded lymph node, Specimens were fixed in a 10% formaldehyde solution and embedded in paraffin for immunohistochemical analysis.

Primary cancers of the head and neck were classified according to the pathological TNM classification (Barnes, 2005).

Immunohistochemical Staining

Immunohistochemical staining was performed using the Mouse/Rabbit PolyDetector HRP/DAB Detection System .

Tissue sections were deparafinized and rehydrated in water. tissues subjected to heated epitope retrieval using a retrieval solution in microwave for 15 minutes. Washed with 5 changes of PBS, slides Placed in Poly Detector Peroxidase Blocker for 5 min. then washed with 3 changes of PBS, then tissues were incubated with the Primary Antibody(CCR7 1/100 {abcam/England}), (CXCR4 1/500 {abcam/England}) at room temperature for 30 min. Sections were rehydrated, washed, and tissues Covered with PolyDetector HRP Label, incubate for 45

min at room temperature. After four rinses in PBS, tissues were Covered with prepared DAB substrate-chromogen solution, incubate for 10 min (DAB Prepared by adding one drop of PolyDetector DAB Chromogen per mL of PolyDetector DAB Buffer and mix.) tissues rinsed with 5 changes of PBS, tissues Counterstained and then dehydrated . then tissues coversliped.

Evaluation of Immunostaining

All counts were performed in four alternate microscopic high-power fields ($\times 400$) using an Nikon microscope, Germany). Immunostaining was evaluated by two authors blinded to patient outcome and other clinical findings by considering The percentage of positive tumor cells from all tumor cells.

Results

Immunohistochemical Staining Analysis of CCR7 and CXCR4 in Head and Neck SCC

Staining of the CCR7 and CXCR4 proteins were identified in the cytoplasm and cell membrane of cancer cells but were not detected in the normal oral epithelium (Figure 9,11).

54 of the 60 cases were positive for CXCR4 protein (90 %)(Figure 8).six cases were completely negative (10%), In addition to primary tumors, 65 regional lymph node metastasis were positive for CXCR4 expression in the cases (84.5%), 12 cases were completely negative (15.5%).

56 of the 60 cases were positive for CCR7 protein (93.3%) (Fig 10).Four cases were completely negative (6.7%). In addition to primary tumors,73 cases from 77 regional lymph node metastasis were positive for CCR7 expression in the cases (94.9%), 4 cases were completely negative (5.1%).

CCR7, CXCR4 IHC expression in primary tumor

- Correlation between the CCR7, CXCR4 IHC expression of each one according to tumor depth in primary tumor:

By one-way ANOVA test and LSD test and we found there are no difference between CCR7, CXCR4 IHC expression of each one according to tumor depth in primary tumor Table 1, 2 Figure 4.

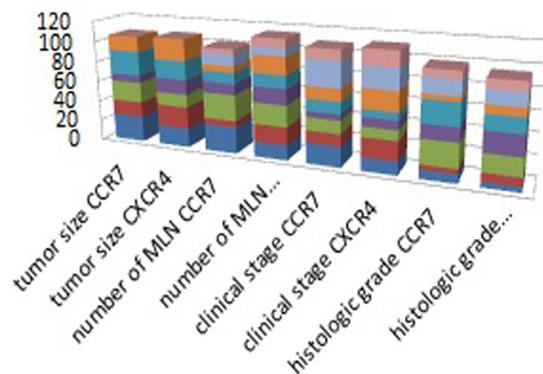


Figure 1. The Percentages of CCR7 and CXCR4 in the Positive and Negative Cells in the Primary Tumours

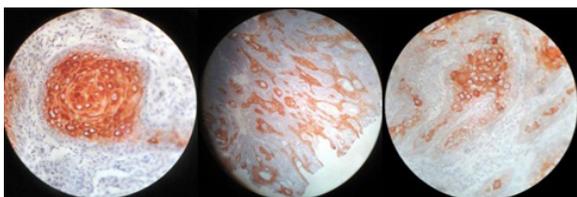


Figure 2. CXC4 Expression

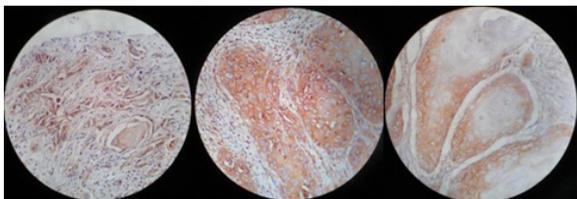


Figure 3. CCR7 Expression

- Correlation between the CCR7, CXC4 IHC expression of each one in primary tumor and number of metastatic lymph nodes:

By one-way ANOVA test we found no differences between CCR7 IHC expression in primary tumor according to number of metastatic lymph nodes, while there are difference between CXC4 IHC expression in primary tumor according to number of metastatic lymph nodes (Table 3) and (Figure 5).

By LSD test we found there is difference CXC4 IHC expression in primary tumor according to between one lymph node metastases and 3 lymph node metastases, and between 3 lymph node metastases and 5 lymph node metastases (Table 4).

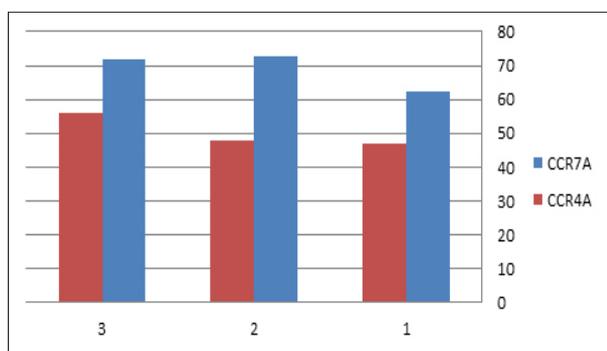


Figure 4. Percentage Positive Cells Expression According to Tumor Size

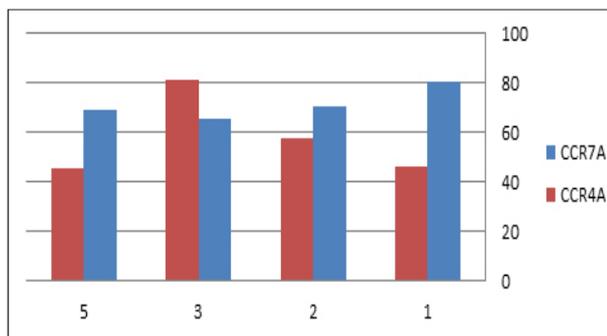


Figure 5. Percentage Positive Cells Expression According to Number of Metastatic Lymph Nodes

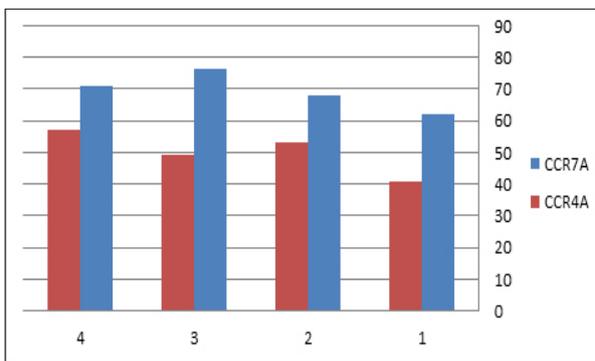


Figure 6. Percentage Positive Cells Expression According to Clinical Stage

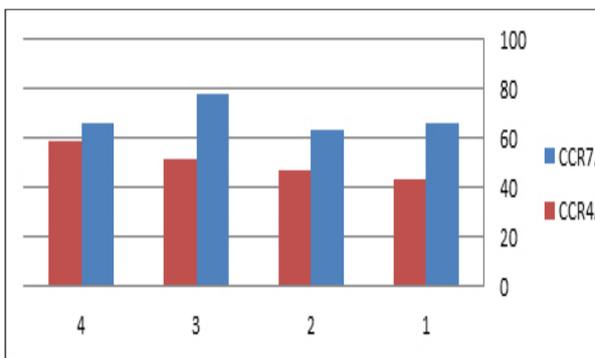


Figure 7. Percentage Positive Cells Expression According to Histological Grade

Correlation between the CCR7, CXC4 IHC expression of each one and clinical stage in primary tumor:

By one-way ANOVA test we found difference between CCR7, CXC4 IHC expression in cancer samples and normal samples (Table 5) (Figure 6).

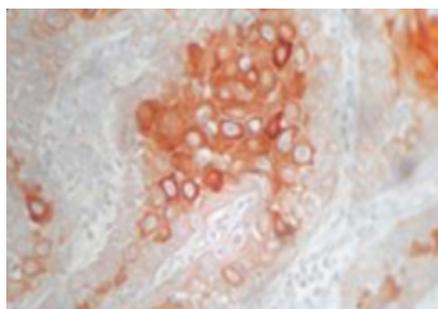


Figure 8. CXC4 IHC Expression in HNSCC Cancer Cells



Figure 9. CXC4 IHC Expression in Normal Macosa Cells

Table 1. CCR7, CXCR4 IHC Expression According to Tumor Depth in Primary Tumor

| | Tumor Size | CCR7 | | CXCR4 | | Lymph node number | CCR7 | | CXCR4 | | Histologic Grade | CCR7 | | CXCR4 | | Clinical stage | CCR7 | | CXCR4 | |
|--------------------|------------|-------|----------------|-------|----------------|-------------------|-------|----------------|-------------|----------------|------------------|-------------|----------------|-------|----------------|----------------|-------|----------------|-------|----------------|
| | | Mean | Std. Deviation | Mean | Std. Deviation | | Mean | Std. Deviation | Mean | Std. Deviation | | Mean | Std. Deviation | Mean | Std. Deviation | | Mean | Std. Deviation | Mean | Std. Deviation |
| arithmetic mean | normal | 0.00 | 0.00 | 0.00 | 0.00 | 1 | 80.31 | 46.09 | normal | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | normal | 0.00 | 0.00 | 0.00 | 0.00 |
| Standard deviation | | 0.00 | 0.00 | 0.00 | 0.00 | | 28.04 | 17.36 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | | 0.00 | 0.00 | 0.00 | 0.00 |
| Standard error | | 0.00 | 0.00 | 0.00 | 0.00 | | 9.92 | 6.14 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | | 0.00 | 0.00 | 0.00 | 0.00 |
| Cases number | | 10.00 | 10.00 | 10.00 | 10.00 | 2 | 8.00 | 8.00 | | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | | 10.00 | 10.00 | 10.00 | 10.00 |
| arithmetic mean | 1 | 62.40 | 46.83 | 2 | 70.83 | 57.53 | 70.83 | 57.53 | SCC grade 1 | 66.25 | 43.31 | SCC stage1 | 62.11 | 40.88 | 40.88 | | 62.11 | 40.88 | 40.88 | 40.88 |
| Standard deviation | | 34.61 | 29.50 | | 21.21 | 24.06 | 21.21 | 24.06 | | 36.40 | 25.42 | | 33.82 | 29.27 | 29.27 | | 33.82 | 29.27 | 29.27 | 29.27 |
| Standard error | | 7.07 | 6.02 | | 7.07 | 8.02 | 7.07 | 8.02 | | 12.87 | 8.99 | | 7.76 | 6.71 | 6.71 | | 7.76 | 6.71 | 6.71 | 6.71 |
| Cases number | | 24.00 | 24.00 | | 9.00 | 9.00 | 9.00 | 9.00 | | 8.00 | 8.00 | | 19.00 | 19.00 | 19.00 | | 19.00 | 19.00 | 19.00 | 19.00 |
| arithmetic mean | 2 | 72.81 | 47.89 | 3 | 65.63 | 80.94 | 65.63 | 80.94 | SCC grade 2 | 63.52 | 47.26 | SCC stage 2 | 68.18 | 53.39 | 53.39 | | 68.18 | 53.39 | 53.39 | 53.39 |
| Standard deviation | | 20.99 | 26.02 | | 44.36 | 7.17 | 44.36 | 7.17 | | 31.72 | 22.62 | | 30.84 | 25.08 | 25.08 | | 30.84 | 25.08 | 25.08 | 25.08 |
| Standard error | | 5.25 | 6.50 | | 22.18 | 3.59 | 22.18 | 3.59 | | 6.76 | 4.82 | | 9.30 | 7.56 | 7.56 | | 9.30 | 7.56 | 7.56 | 7.56 |
| Cases number | | 16.00 | 16.00 | | 4.00 | 4.00 | 4.00 | 4.00 | | 22.00 | 22.00 | | 11.00 | 11.00 | 11.00 | | 11.00 | 11.00 | 11.00 | 11.00 |
| arithmetic mean | 3 | 72.00 | 56.08 | 5 | 68.75 | 45.31 | 68.75 | 45.31 | SCC grade 3 | 77.65 | 51.03 | SCC stage 3 | 76.39 | 49.31 | 49.31 | | 76.39 | 49.31 | 49.31 | 49.31 |
| Standard deviation | | 28.80 | 25.85 | | 33.26 | 23.06 | 33.26 | 23.06 | | 21.86 | 30.13 | | 28.75 | 18.89 | 18.89 | | 28.75 | 18.89 | 18.89 | 18.89 |
| Standard error | | 6.44 | 5.78 | | 16.63 | 11.53 | 16.63 | 11.53 | | 5.30 | 7.31 | | 9.58 | 6.30 | 6.30 | | 9.58 | 6.30 | 6.30 | 6.30 |
| Cases number | | 20.00 | 20.00 | | 4.00 | 4.00 | 4.00 | 4.00 | | 17.00 | 17.00 | | 9.00 | 9.00 | 9.00 | | 9.00 | 9.00 | 9.00 | 9.00 |
| Mean Difference | Total | 58.61 | 43.03 | Total | 72.70 | 55.66 | 72.70 | 55.66 | SCC grade 4 | 65.77 | 58.31 | SCC stage 4 | 70.71 | 57.33 | 57.33 | | 70.71 | 57.33 | 57.33 | 57.33 |
| Standard deviation | | 36.40 | 30.81 | | 28.20 | 22.58 | 28.20 | 22.58 | | 30.56 | 32.51 | | 25.69 | 28.73 | 28.73 | | 25.69 | 28.73 | 28.73 | 28.73 |
| Std. Error | | 4.35 | 3.68 | | 5.64 | 4.52 | 5.64 | 4.52 | | 8.48 | 9.02 | | 5.61 | 6.27 | 6.27 | | 5.61 | 6.27 | 6.27 | 6.27 |
| Cases number | | 70.00 | 70.00 | | 25.00 | 25.00 | 25.00 | 25.00 | | 13.00 | 13.00 | | 21.00 | 21.00 | 21.00 | | 21.00 | 21.00 | 21.00 | 21.00 |
| value F | | 18.29 | 11.62 | | 0.29 | 3.09 | 0.29 | 3.09 | | 13.91 | 8.81 | | 13.51 | 9.67 | 9.67 | | 13.51 | 9.67 | 9.67 | 9.67 |
| Sig | | 0.00 | 0.00 | | 0.83 | 0.04 | 0.83 | 0.04 | | 0.00 | 0.00 | | 0.00 | 0.00 | 0.00 | | 0.00 | 0.00 | 0.00 | 0.00 |

Table 2. Primary Tumor and Lymph Nodes Metastasis According to CCR7, CXCR4 IHC Expression

| FAC | CCR7 IHC expression | | | | CXCR4 IHC expression | | | |
|-----------------------|----------------------------------|----------------|--------------------|-----------------------|--|----------------|--------------------|-----------------------|
| | Mean | Std. Deviation | Std. Error of Mean | FAC | Mean | Std. Deviation | Std. Error of Mean | FAC |
| Primary | 74.91 | 22.83 | 4.24 | Primary | 56.82 | 24.38 | 4.53 | Primary |
| Lymph node Metastasis | 60.89 | 29.56 | 5.80 | Lymph node Metastasis | 37.46 | 24.50 | 4.80 | Lymph node Metastasis |
| T-TEST | 2.227 | | | T-TEST | 2.933 | | | T-TEST |
| SIG | 0.03 | | | SIG | 0.005 | | | SIG |
| | Significant difference | | | | Significant difference | | | |
| Pearson's test | 0.4108 (sig = 0.033) Significant | | | | Pearson's test 0.402 (sig = 0.046) Significant | | | |

Table 3. Correlation between the CCR7, CXCR4 IHC Expression of Each One in Primary Tumor and Number of Metastatic Lymph Nodes One-Way ANOVA Test?

| Lymph node number | | CCR7A | CXCR4A |
|-------------------|--------------------|-------|--------|
| 1 | arithmetic mean | 80.31 | 46.09 |
| | Standard deviation | 28.04 | 17.36 |
| | Standard error | 9.92 | 6.14 |
| | Cases number | 8.00 | 8.00 |
| 2 | arithmetic mean | 70.83 | 57.53 |
| | Standard deviation | 21.21 | 24.06 |
| | Standard error | 7.07 | 8.02 |
| | Cases number | 9.00 | 9.00 |
| 3 | arithmetic mean | 65.63 | 80.94 |
| | Standard deviation | 44.36 | 7.17 |
| | Standard error | 22.18 | 3.59 |
| | Cases number | 4.00 | 4.00 |
| 5 | arithmetic mean | 68.75 | 45.31 |
| | Standard deviation | 33.26 | 23.06 |
| | Standard error | 16.63 | 11.53 |
| | Cases number | 4.00 | 4.00 |
| Total | arithmetic mean | 72.70 | 55.66 |
| | Standard deviation | 28.20 | 22.58 |
| | Standard error | 5.64 | 4.52 |
| | Cases number | 25.00 | 25.00 |
| value F | | 0.29 | 3.09 |
| Sig | | 0.83 | 0.04 |

Then we examined LSD test and we found there is no difference between clinical stages of tumor samples according to CCR7 IHC expression, while there is difference between clinical stage 1 and 4 of tumor samples according to CXCR4 IHC expression (Table 6)

Correlation between the CCR7, CXCR4 IHC expression of each one and histologic grade in primary tumor

By one-way ANOVA test we found difference between

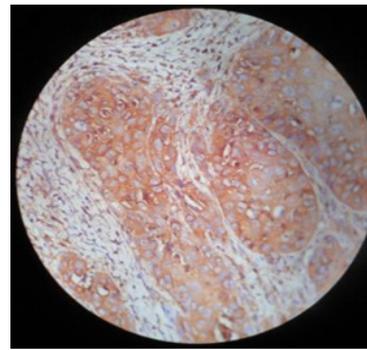


Figure 10. CCR7 IHC Expression in HNSCC Cancer Cell

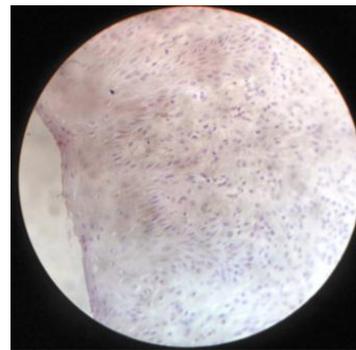


Figure 11. CCR7 IHC Expression in Normal Mucosa Cells

CCR7, CXCR4 IHC expression in cancer samples and normal samples (Table 7) and (Figure7).

Then we examined LSD test and we found there is no difference between histologic grade of tumor samples according to CCR7 and CXCR4 IHC expression (Table 8).

Correlation between the CCR7, CXCR4 IHC expression of each one in primary tumor and lymph nodes metastasis

By two independent T student test and Pearson's test we found differences between primary tumor and lymph nodes metastasis according to CCR7, CXCR4 IHC expression (Table 9).

Table 4. Correlation between the CXCR4 IHC Expression of Each One in Primary Tumor and Number of Metastatic Lymph Nodes LSD Test

| Dependent Variable | (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Sig. |
|--------------------|--------------|--------------|-----------------------|-------|
| CXCR4 | 1 | 2 | -11.43 | 0.255 |
| | | 3 | -34.84(*) | 0.01 |
| | | 5 | 0.78 | 0.95 |
| | 2 | 1 | 11.43 | 0.255 |
| | | 3 | -23.41 | 0.066 |
| | | 5 | 12.22 | 0.324 |
| | 3 | 1 | 34.84(*) | 0.01 |
| | | 2 | 23.41 | 0.066 |
| | | 5 | 35.62(*) | 0.021 |
| | 5 | 1 | -0.78 | 0.95 |
| | | 2 | -12.22 | 0.324 |
| | | 3 | -35.62(*) | 0.021 |

Table 5. Correlation between the CCR7, CXCR4 IHC Expression of Each One and Clinical Stage in Primary Tumor One-Way ANOVA Test

| | CCR7A | CCR4A |
|---------|-------|-------|
| value F | 13.51 | 9.667 |
| Sig | 0 | 0 |

And there is a positive correlation between primary tumor and lymph nodes metastasis according to CCR7, CXCR4 IHC expression (Tab10).

Discussion

Tumor cells at metastatic sites express chemokine receptors in several types of carcinoma, including breast , ovary , and prostate (Table 11). Chemokines and

Table 6. Correlation between the CCR7, CXCR4 IHC Expression of Each One and Clinical Stage in Primary Tumor LSD test?

| | | (I) DS | (J) DS | Mean Difference (I-J) | Std. Error | Sig. |
|--------|-----|-----------|--------|-----------------------|------------|-------|
| CCR7A | LSD | 0 | 1 | -62.105(*) | 10.827 | 0 |
| | | | 2 | -68.182(*) | 12.109 | 0 |
| | | | 3 | -76.389(*) | 12.733 | 0 |
| | | | 4 | -70.714(*) | 10.648 | 0 |
| | | 1 | 0 | 62.105(*) | 10.827 | 0 |
| | | | 2 | -6.076 | 10.499 | 0.565 |
| | | | 3 | -14.284 | 11.214 | 0.207 |
| | | | 4 | -8.609 | 8.775 | 0.33 |
| | | 2 | 0 | 68.182(*) | 12.109 | 0 |
| | | | 1 | 6.0765 | 10.499 | 0.565 |
| | | | 3 | -8.207 | 12.456 | 0.512 |
| | | | 4 | -2.532 | 10.315 | 0.807 |
| | | 3 | 0 | 76.389(*) | 12.733 | 0 |
| | | | 1 | 14.284 | 11.214 | 0.207 |
| | | | 2 | 8.207 | 12.456 | 0.512 |
| | | | 4 | 5.675 | 11.041 | 0.609 |
| 4 | 0 | 70.714(*) | 10.648 | 0 | | |
| | 1 | 8.609 | 8.775 | 0.33 | | |
| | 2 | 2.532 | 10.315 | 0.807 | | |
| | 3 | -5.675 | 11.041 | 0.609 | | |
| CXCR4A | LSD | 0 | 1 | -40.881(*) | 9.821 | 0 |
| | | | 2 | -53.386(*) | 10.983 | 0 |
| | | | 3 | -49.305(*) | 11.550 | 0 |
| | | | 4 | -57.333(*) | 9.658 | 0 |
| | | 1 | 0 | 40.881(*) | 9.821 | 0 |
| | | | 2 | -12.504 | 9.524 | 0.194 |
| | | | 3 | -8.424 | 10.172 | 0.411 |
| | | | 4 | -16.451(*) | 7.959 | 0.043 |
| | | 2 | 0 | 53.386(*) | 10.983 | 0 |
| | | | 1 | 12.504 | 9.524 | 0.194 |
| | | | 3 | 4.081 | 11.298 | 0.719 |
| | | | 4 | -3.947 | 9.356 | 0.675 |
| | | 3 | 0 | 49.305(*) | 11.550 | 0 |
| | | | 1 | 8.424 | 10.172 | 0.411 |
| | | | 2 | -4.081 | 11.298 | 0.719 |
| | | | 4 | -8.028 | 10.015 | 0.426 |
| 4 | 0 | 57.333(*) | 9.658 | 0 | | |
| | 1 | 16.452(*) | 7.959 | 0.043 | | |
| | 2 | 3.947 | 9.356 | 0.675 | | |
| | 3 | 8.028 | 10.015 | 0.426 | | |

Table 7. Correlation between the CCR7, CXCR4 IHC Expression of Each One and Histologic Grade in Primary Tumor One-Way ANOVA Test

| sample | | CCR7A | CCR4A |
|-------------|--------------------|-------|-------|
| normal | arithmetic mean | 0.00 | 0.00 |
| | Standard deviation | 0.00 | 0.00 |
| | Standard error | 0.00 | 0.00 |
| | Cases number | 10.00 | 10.00 |
| SCC grade 1 | arithmetic mean | 66.25 | 43.31 |
| | Standard deviation | 36.40 | 25.42 |
| | Standard error | 12.87 | 8.99 |
| | Cases number | 8.00 | 8.00 |
| SCC grade 2 | arithmetic mean | 63.52 | 47.26 |
| | Standard deviation | 31.72 | 22.62 |
| | Standard error | 6.76 | 4.82 |
| | Cases number | 22.00 | 22.00 |
| SCC grade 3 | arithmetic mean | 77.65 | 51.03 |
| | Standard deviation | 21.86 | 30.13 |
| | Standard error | 5.30 | 7.31 |
| | Cases number | 17.00 | 17.00 |
| SCC grade 4 | arithmetic mean | 65.77 | 58.31 |
| | Standard deviation | 30.56 | 32.51 |
| | Standard error | 8.48 | 9.02 |
| | Cases number | 13.00 | 13.00 |
| total | arithmetic mean | 58.61 | 43.03 |
| | Standard deviation | 36.40 | 30.81 |
| | Standard error | 4.35 | 3.68 |
| | Cases number | 70.00 | 70.00 |
| value F | | 13.91 | 8.81 |
| Sig | | 0.00 | 0.00 |

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 Rossi and Zlotnik, 2000

CCR7 is a chemokine receptor, which is expressed on lymphocytes, such as T cells and dendritic cells and it plays an important role in the mediation of migration of those cells toward lymph nodes which express the CCR7 ligand, CCL21 (Förster et al., 2008).

CXCR4, acts as a receptor specific for SDF-1 and plays roles in cell migration and proliferation.

A recent study suggests that these proteins can also regulate non-leukocyte cell functions, such as tumor cell migration (wang et al., 1998)

CCR7 expression in tumor tissue specimens has recently been reported to be associated with lymph node metastases by immunohistochemical analyses in various carcinomas ((Müller et al., 2001))

Tumor cells from at least 23 different types of human cancers of epithelial, mesenchymal and hematopoietic origin express CXCR4 (Balkwill, 2004b).

However, there are no studies to investigate this correlation between CCR7 and CXCR4 in primary and

lymph node metastases in clinical stages and histological grades.

In this study, we found that CCR7 ,CXCR4 expression was detected in HNSCC tissues, but not detected in normal oral mucosa .we explained this result that one of tumor cells strategy is mimicking the movement cells , we are agree with(Xia et al., 2015) for CCR7 at SCC in tongue and agree with (Katayama et al., 2005) and (Teng et al., 2009) for CXCR4.

we found there is no differences between CCR7, CXCR4 IHC expression of each one according to tumor depth in primary tumor ,and that because CCL19 chemokine is more than CCL21 in tumor stroma and the complex CCL19-CCR7 protect cancer cells against apoptosis and amplify proliferative activity Tsuzuki et al., 2006 who's results showed that the staining score of proliferating cell nuclear antigen (PCNA) in SCCs was correlated with that of CCR7, however no reference connect CCR7 with MMPs, therefor the cancerous cells spread had no relation with CCR7 expression. we agree with Oliveira-Neto et al., 2013 we disagree with Ueda et al., 2010, Shang et al., 2009, Ding et al., 2003, while CXCR4-SDF1 complex has a relation with MMPs however cancer stromal macrophage's cytokines inhibit SDF1 and make its gradient far from cancer cell therefor the cancerous cells spread had no relation with CXCR4 expression we agree with (Almofti et al., 2004) Yin and Gao, 2007,Ishikawa et al., 2006,and disagree with (Teng et al., 2009), (Ueda et al., 2010)

we found no differences between CCR7 IHC expression in primary tumor according to number of metastatic lymph nodes, while there are differences between CXCR4 IHC expression in primary tumor according to number of metastatic lymph nodes: we explained that CCR7 has a correlation with lymph node metastasis from its early stage while CXCR4 has a correlation with lymph node metastasis from its late stage, we agree with Tsuzuki et al., 2006, (Ding et al., 2003),Shang et al., 2009 for CCR7 and with Kato et al., 2003 for CXCR4.

we found there is no difference between clinical stages of tumor samples according to CCR7 IHC expression, while there are difference between clinical stage 1 and 4 of tumor samples according to CXCR4 IHC expression so we can predict CCR7 expression in primary tumor as a diagnostic and prognostic factor, and we can predict CXCR4 as a prognostic factor ,we agree with (Oliveira-Neto et al., 2013) for CCR7 and with (Uchida et al., 2003; Almofti et al., 2004; Ishikawa et al., 2006; Yin and Gao, 2007; Oliveira-Neto et al., 2008) (Delilbasi et al., 2004; Albert et al., 2012) (Zhang et al., 2005; Tan et al., 2008) for CXCR4.

we found there is no difference between histologic grade of tumor samples according to CCR7 and CXCR4 IHC expression , that because CCR4, CXCR4 have no relation with cancer cells differentiation ,we agree with Ding et al., 2003 , Ishida et al., 2009 and disagree with (Ueda et al., 2010) ,(Xia et al., 2015) for CCR7 and agree with (Katayama et al., 2005) ,Ishikawa et al., 2006, Yin and Gao, 2007 for CXCR4 and agree with.

we found differences between primary tumor and

Table 8. Correlation between the CCR7, CXCR4 IHC Expression of Each One and Histologic Grade in Primary Tumor LSD Test

| | | (I) DS | (J) DS | Mean Difference (I-J) | Std. Error | Sig. |
|--------|-----|--------|--------|-----------------------|------------|-------|
| CCR7A | LSD | 0 | 1 | -66.250(*) | 13.058 | 0 |
| | | | 2 | -63.523(*) | 10.499 | 0 |
| | | | 3 | -77.647(*) | 10.971 | 0 |
| | | | 4 | -65.769(*) | 11.579 | 0 |
| | | 1 | 0 | 66.250(*) | 13.058 | 0 |
| | | | 2 | 2.727 | 11.365 | 0.811 |
| | | | 3 | -11.397 | 11.803 | 0.338 |
| | | | 4 | 0.481 | 12.370 | 0.969 |
| | | 2 | 0 | 63.523(*) | 10.499 | 0 |
| | | | 1 | -2.727 | 11.365 | 0.811 |
| | | | 3 | -14.124 | 8.889 | 0.117 |
| | | | 4 | -2.246 | 9.630 | 0.816 |
| | | 3 | 0 | 77.647(*) | 10.971 | 0 |
| | | | 1 | 11.397 | 11.803 | 0.338 |
| | | | 2 | 14.124 | 8.889 | 0.117 |
| | | | 4 | 11.878 | 10.142 | 0.246 |
| | | 4 | 0 | 65.769(*) | 11.579 | 0 |
| | | | 1 | -0.481 | 12.370 | 0.969 |
| | | | 2 | 2.246 | 9.630 | 0.816 |
| | | | 3 | -11.878 | 10.142 | 0.246 |
| CXCR4A | LSD | 0 | 1 | -43.312(*) | 12.125 | 0.001 |
| | | | 2 | -47.261(*) | 9.749 | 0 |
| | | | 3 | -51.029(*) | 10.187 | 0 |
| | | | 4 | -58.308(*) | 10.752 | 0 |
| | | 1 | 0 | 43.312(*) | 12.125 | 0.001 |
| | | | 2 | -3.949 | 10.554 | 0.709 |
| | | | 3 | -7.717 | 10.960 | 0.484 |
| | | | 4 | -14.995 | 11.487 | 0.196 |
| | | 2 | 0 | 47.261(*) | 9.749 | 0 |
| | | | 1 | 3.949 | 10.554 | 0.709 |
| | | | 3 | -3.768 | 8.255 | 0.65 |
| | | | 4 | -11.046 | 8.942 | 0.221 |
| | | 3 | 0 | 51.029(*) | 10.187 | 0 |
| | | | 1 | 7.7169 | 10.960 | 0.484 |
| | | | 2 | 3.768 | 8.255 | 0.65 |
| | | | 4 | -7.278 | 9.418 | 0.442 |
| | | 4 | 0 | 58.308(*) | 10.752 | 0 |
| | | | 1 | 14.995 | 11.487 | 0.196 |
| | | | 2 | 11.046 | 8.942 | 0.221 |
| | | | 3 | 7.278 | 9.418 | 0.442 |

Table 9. Correlation between the CCR7 IHC Expression of Each One in Primary Tumor and Lymph Nodes Metastasis T- Student Test and Pearson's test

| FAC | Mean | Std. Deviation | Std. Error of Mean |
|-----------------------|----------------------------------|----------------|--------------------|
| Primary | 74.914 | 22.830 | 4.239 |
| Lymph node Metastasis | 60.890 | 29.558 | 5.797 |
| T-TEST | 2.227 | | |
| SIG | 0.03 | | |
| | Significant difference | | |
| Pearson's test | 0.4108 (sig = 0.033) Significant | | |

Table 10. Correlation between the CXCR4 IHC Expression of Each One in Primary Tumor and Lymph Nodes Metastasis T- Student Test and Pearson's Test

| FAC | Mean | Std. Deviation | Std. Error of Mean |
|-----------------------|---------------------------------|----------------|--------------------|
| Primary | 56.819 | 24.385 | 4.528 |
| Lymph node Metastasis | 37.462 | 24.501 | 4.805 |
| T-TEST | 2.933 | | |
| SIG | 0.005 | | |
| | Significant difference | | |
| Pearson's test | 0.402 (sig = 0.046) Significant | | |

Table 11. Examples of Chemokines Receptors in Cancers

| Referances | Receptor expressed | Cancer |
|--|--------------------------|------------------------------|
| (Müller et al., 2001) | CXCR4, CCR7 | Breast |
| (Li et al.,2004) | | |
| (Milliken et al., 2002) | CXCR4 | Ovarian |
| (M darash-yahana et al.,2004) | CXCR4 | Prostate |
| (F Marchesi et al.,2004) | CXCR4 | Pancreas |
| (Müller et al., 2001) | CXCR4, CCR10, CCR7, CCR9 | Melanoma |
| Letsch et al.,2004,Scala et al.,2005) | | |
| (kaifi et al.,2005, Ding et al., 2003) | CXCR4 | Esophageal |
| (pillips et al.,2003 , Takanami, 2003) | CXCR4, CCR7 | Lung (NSCLC) |
| Katayama et al., 2005(| CXCR4, CCR7, CXCR5 | Head and Neck |
| (Muller et al., 2006)) | | |
| (Eisenhardt et al.,2005) | CXCR4 | Bladder |
| (Kim et al.,2005 , Gunther et al.,2005 , Schimanski et al.,2005) | CXCR4, CCR7 | Colorectal |
| (Laverdiere et al., 2005Laverdiere et al., 2005) | CXCR4 | Osteosarcoma |
| Russell et al.,2004 | CXCR4 | Neuroblastoma |
| Corcione et al.,2006 | CXCR4, CXCR3 | Acute-lymphoblastic leukemia |
| (Burger et al.,2002, Trentin et al.,2004) | CXCR4, CXCR5, CXCR3 | Chronic-myelogenous leukemia |
| Mashino et al., 2002 | CCR7 | Stomach cancer |

lymph nodes metastasis according to CCR7, CXCR4 IHC expression And there is a positive correlation between primary tumor and lymph nodes metastasis according to CCR7, CXCR4 IHC expression.

we agree with (Issa et al., 2009) who supposed that a relation between VEGF-C and CCR7 cancer cell's expression that enhances lymph node metastasis , plus VEGF-C plays a positive role in CCL21 lymphatic induction so it considered as an enhancer for cancer cells chemotaxis and migration. we agree with Ding et al., 2003 that supposed increase CCR7primary tumor expression with lymphatic invasion. Tsuzuki et al., 2006 also correlated CCL21 induced in lymph node with cancer cell migration.

we agree with (Kijowski et al., 2001; Shen et al., 2001) that CXCR4 increase the ability of cells which express to cell adhesion by controlling many surface integrines, we agree with (Katayama et al., 2005) who found that Exogenous SDF-1 promoted cell migration as well as proliferation in a dose dependent manner in CXCR4-positive cells but never in CXCR4-negative cells. His results showed that CXCR4 plays a role in cell

proliferation in response to SDF-1. These contradictory findings suggest that cell proliferation role for CXCR4 may vary in tumor types and/or sites ,he found strong SDF-1 expressions in stromal tissues surrounding CXCR4-expressing cancer nests in metastatic lymph nodes but hardly detected SDF-1 expression in stromal tissues surrounding primary cancer nests.

In conclusion the high expression of CCR7 in the cancer cells was clearly associated with early lymph node metastases expression of CXCR4 involved in cell migration at late lymph node metastasis in HNSCC.

Our results may provide an insight into future therapeutic agent that inhibits tumor metastasis and progression via down-regulating CXCR4 and CCR7 expression in patients with HNSCC.

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