

The Cellular Microenvironment of Head and Neck Squamous Cell Carcinoma

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) tumors function much like organs with support from multiple cell lineages. Tobacco and alcohol abuse are strongly correlated with the disease. Environmental carcinogen exposure introduces genetic alterations not only in the epithelial cells but also in the surrounding stroma contributing to tumor initiation and progression [1]. Factors and cells that do not support tumor growth are commonly downregulated or mitigated in the tumor microenvironment. Several classes of stromal cells that exist in close proximity with HNSCC tumors have been identified. These include fibroblasts, immune cells and cells involved in vascular growth. Each of these cell types are involved in molecular cross-talk with the tumor resulting in tumor progression (Figure 1). Here we highlight each of the major cell types present in the HNSCC tumor microenvironment. Well characterized molecular markers have been used to identify the specific stromal cellular components (Table 1). There continues to be a tremendous need for improved understanding of the role of each of these cell types in tumor growth, dissemination and resistance to therapies. Tumor-associated stroma can support tumor cell proliferation, angiogenesis and invasion making them potential therapeutic targets. Since de novo acquisition of genetic mutations is not common in stromal cells they may be less prone to developing resistance to therapy via genomic instability. The synergistic relationship between stroma and tumor cells suggests that stroma targeted intervention may have a synergistic role in primary cancer therapy. However, fibrosis that follows surgery, chemotherapy and radiotherapy may trigger the release of stromal factors that support recurrence and metastasis. Thus stroma targeted therapies may emerge as important in adjuvant setting.

2. Tumor associated fibroblasts

Fibroblasts are important components of the mesenchymal stroma. Though they appear morphologically similar, fibroblasts show large differences in their functions and patterns of gene expression depending on their anatomical site of origin. Under normal physiological conditions, fibroblasts help maintain the boundary between the epithelial cells and the

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underlying tissue by functioning as a physical barrier. Fibroblasts play a major role in regulating and maintaining extracellular homeostasis. Tissue injury triggers fibroblast activation [2]. Activated fibroblasts are responsible for wound contraction, fibrosis, scarring and regulation of inflammatory reactions. Upon activation, fibroblasts transdifferentiate into motile cells with abundant endoplasmic reticulum, Golgi and α -SMA stress fibers [3]. These α -SMA positive fibroblasts termed myofibroblasts synthesize extracellular matrix components, and several proteinases, growth factors and cytokines. Myofibroblasts have a morphology much like muscle cells with highly contractile microfilaments. Tumors are frequently regarded as wounds that do not heal. HNSCC tumors are frequently associated with desmoplastic stromal myofibroblasts also known as tumor-associated fibroblasts (TAFs) or cancer associated fibroblasts.

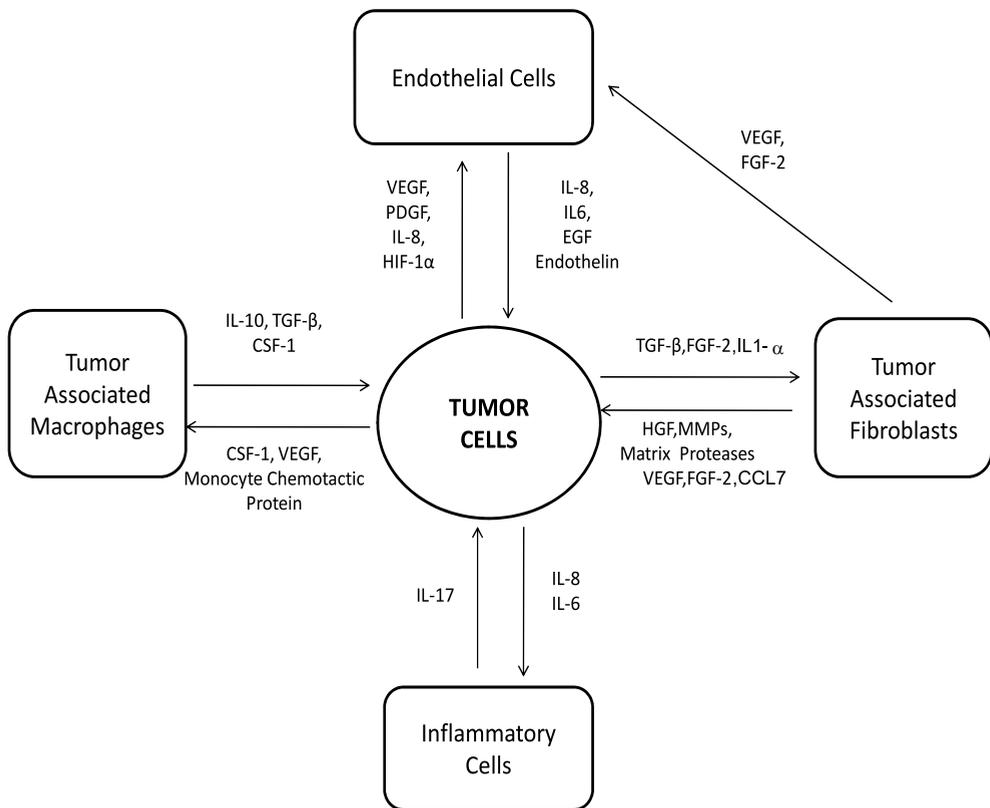


Fig. 1. Cross talk between HNSCC and stromal cellular components. Factors secreted by each cell type that influence target cells have been listed. Abbreviations include; VEGF- Vascular endothelial growth factor, PDGF-Platelet derived growth factor, IL-Interleukin, HIF-Hypoxia Inducible Factor, TGF-Transforming Growth Factor, CSF-Colony Stimulating Factor, EGF-Epithelial growth factor, HGF- Hepatocyte growth factor, MMP-Matrix metalloprotease, FGF- Fibroblast growth factor CCL7- Chemokine Ligand 7(C-C motif).

Cell Type	Molecular Marker
Tumor Associated Fibroblasts	α smooth muscle actin, Vimentin, Fibroblast activating protein
Tumor Associated Macrophages	CD-68, Macrophage inflammatory Protein-3 α
Tumor Infiltrating Lymphocytes	
T cells	CD-3 ⁺
NK cells	CD16/56 ⁺ CD3 ⁻
T helper cells	CD4 ⁺
Endothelial Cells	CD-31,CD34, VEGF-R1,VEGF-R2
Pericytes	α smooth muscle action
Mast Cell	Mast cell tryptase
Lymphatic Endothelial Cell	HIF-1 α , VEGF-C

Table 1. Molecular markers commonly used to identify cellular components of the stroma

TAFs constitute a major cellular component of the tumor associated stroma and are characterized by increased proliferation and aberrant expression of extracellular matrix components. They have been reported to change the phenotype of normal keratinocytes to that resembling squamous cell carcinoma [4]. In other tumor types including prostate, TAFs are reported to play a role in tumor initiation [5-7]. In addition, they play a role in tumor progression as evidenced by a correlation with tumor stage, metastasis and poor prognosis [8]. Although epithelial tumors undergo epithelial-to-mesenchymal transition to acquire a fibroblast-like morphology, they express epithelial cytokeratin markers that are otherwise not expressed on fibroblasts. Epithelial cells with mesenchymal characteristics are not included in these discussions. Several markers have been used to identify TAFs including α -smooth muscle actin, vimentin and fibroblast activating protein [3, 9]. However, these markers show only partially overlapping expression and no single marker consistently labels TAFs. TAFs in the tumor microenvironment are primed to facilitate HNSCC tumor invasion [8]. They are important modulators of tumor growth, invasion and metastasis producing extracellular matrix and angiogenic factors [10-12]. TAFs may be derived not only

from the fibroblasts in the locoregional vicinity of the tumor but also from circulating mesenchymal stem cells [13, 14]. TAFs are detected in both primary and metastatic HNSCC [15]. There are at least 4 possible explanations for the origin of the TAFs at metastatic sites; 1) they are derived from the stroma surrounding the metastatic site, 2) they co-metastasize along with the metastatic tumor cells from the primary tumor site or 3) they arrive at the metastatic site prior to the arrival of the tumor cells creating a metastatic niche permissible to the tumor growth or 4) they are derived from circulating mesenchymal stem cells. HNSCC stroma are either rich in TAFs dispersed throughout the tumor or have low levels of TAFs that are located at the periphery of HNSCC tumors or tumor islands [15]. TAFs are also commonly associated with the invasive margin of the tumor [10]. There is strong evidence to suggest that TAFs use protease and mechanical remodeling of the extracellular matrix to lay tracks along which HNSCC tumor cells invade [16]. They also influence the response of the tumors to conventional therapy [17]. Understanding the tumor microenvironment and the molecular mechanisms responsible for the highly invasive and metastatic nature of HNSCC tumors is vital in developing effective strategies to manage this disease.

TAFs differ in their phenotype, gene expression patterns and functionality from normal oral fibroblasts and normal-dermal fibroblasts derived from non-cancer patients [3, 4]. They are not contact inhibited and have a higher rate of proliferation than normal oral fibroblasts [3]. Somatic mutations such as in the *PTEN* and *TP53* tumor suppressor genes have been reported in TAFs derived from breast carcinoma [18]. There is extensive evidence to demonstrate that cross-talk between TAFs and HNSCC cells results in fibroblast activation and tumor promotion. Release of interleukin-1 α from HNSCC cell lines was reported to induce chemokine receptor ligand CCL7 from TAFs. CCL7 binds to its receptors on HNSCC cells promoting cancer cell migration [19]. Other cytokines released by HNSCC cells under the influence of fibroblasts include interleukin-1 β , -6, TNF- α and TGF- β [20, 21]. Several factors secreted by TAFs facilitate HNSCC invasion including MT1-matrix metalloprotease, [22]. Several aspects of the biology of TAFs suggest that targeting these cells may offer therapeutic benefits. Specific targeting of TAFs with CD8+ T-cells resulted in reduced growth and metastasis of colon and breast tumors [23]. Targeting galectin-1 expressed in TAFs reduced the secretion of monocyte chemoattractant protein-1 mitigating HNSCC migration and metastasis [24]. Several studies have demonstrated that TAFs express the hepatocyte growth factor which promotes the expression of angiogenic factors in HNSCC cells via the oncogenic c-Met receptor and its downstream effectors PI3 kinase and MEK [12, 25, 26].

3. Tumor associated macrophages

Monocytes are recruited by cytokine and chemokine gradients into tissues where further differentiation to macrophages is regulated by environmental signals. In neoplasms tumor associated macrophages (TAMs) represent a major component of the infiltrating leukocytes. The presence of TAMs can be beneficial for the growth of the tumor and sometimes they can cause the death of the tumor cells. For example it has been shown that the amount of TAMs in tumors can be associated with increased neoangiogenesis and worsened survival rates. TAMs also have potential for cytotoxicity towards tumor cells and some reports state an improvement in prognosis in relation to high number of TAMs in tumors. TAMs release various cytokines that cause further influx of monocytes in circulation into tumors. The cytokines released by the TAMs also play an important role in angiogenesis, lymphangiogenesis, invasion and metastasis. TAMs modulate the host immune response

against the tumor cell mass by releasing cytokines, chemokines, and enzymes that influence the function of antigen presenting cells and host lymphocytes.

In normal homeostasis, macrophages play an important role in immune surveillance and wound healing engulfing debris and dying cells. In addition they provide factors necessary for tissue matrix remodeling [27]. Depending on signals in the local microenvironment, macrophages mature into 3 distinct functional phenotypes namely classically, type I and type II activated. Macrophages induced by microbial products are classified as classically activated. Type 1 macrophages are antigen presenting cells capable of producing factors including cytokines, TNF α , reactive oxygen that trigger microbial and tumor cell kill [28]. In contrast, type II macrophages are anti-inflammatory, scavenge cell debris and promote angiogenesis, tissue remodeling and repair [29]. Macrophages develop into type 1 or type 2 phenotypes reversibly in response to changes in the microenvironment [30]. Tumor associated macrophages (TAMs) are typically type II cells reported to promote growth of various tumors including breast, prostate and lung [31]. CD68 stained TAMs are present at higher levels in HNSCC and modulate angiogenesis during tumor progression [32, 33]. Primary HNSCC tumor with high TAM infiltration is a strong predictor of lymph node metastasis, extracellular capsular spread and advanced HNSCC stage [34]. Further, expression of macrophage inflammatory protein-3 α was shown to promote oral SCC migration and invasion [35]. Thus, sufficient evidence exists to indicate that TAMs may be important therapeutic targets.

4. Tumor infiltrating lymphocytes

Pathologic examination of HNSCC demonstrates infiltration of cytotoxic T cell that are functionally inactive. Patients with stage 2 and stage 3 carcinoma of the glottis, tongue and hypo pharynx had significantly increased number of T lymphocytes compared to patients with stage 4 disease [36]. Further, increased T lymphocyte numbers at the margins of HNSCC tumors are associated with favorable prognosis. The T lymphocytes produce lymphokines and play an important role in the proliferation of cytotoxic effector cells, thereby play an important role in the local immune response in squamous cell carcinomas of head and neck.

T lymphocytes are the gatekeepers of autoimmune regulation. Failure of T lymphocytes to recognize and eradicate malignant cells contributes to tumor development [37, 38]. Tumors with a high infiltrate of lymphocytes are associated with improved prognosis [39-41]. HNSCC tumors are influenced by several classes of T lymphocytes including T helper cells, CD3, 4 or -8 positive T cells, natural killer cells, regulatory T cells and myeloid progenitor cells [42-45]. Depending up on the subtype of T cells infiltrating the tumor, the tumor experiences growth promotion or regression [46]. In Table 2 we list the tumor facilitating and tumor-promoting T cells. Myeloid-derived suppressor cells (MDSC) are reported to display antitumor effects or tumor promoting effects depending on the factors secreted in the tumor microenvironment [47]. In addition to modulating immune cells in its vicinity, HNSCC tumors actively recruit and trigger the production of tumor growth promoting interleukin-6 from CD34+ myeloid progenitor cells [48]. CD34+ progenitor cells differentiate into a variety of cell lineages including endothelial cells involved in angiogenesis [49]. Th17-T helper cells are characterized by the high levels of secreted pro-inflammatory cytokine interleukin-17. HNSCC tumor and draining lymph nodes are reported to be infiltrated with Th17 cells that are recruited by the tumor cells [45]. Interestingly, Th17 cells reduce HNSCC proliferation while increasing

angiogenesis. Natural killer cells on the other hand, are capable of profound antitumor effects. A deficiency in invariant CD1d-restricted natural killer cells was reported to predict a poor clinical outcome in HNSCC patients [42]. Dendritic cells and T regulatory (Treg) cells also play a role in HNSCC tumor suppression [43, 50]. Under normal physiological conditions these cells are responsible for antigen presenting and for discriminating between self and non-self-antigens, respectively. HNSCC use multiple mechanisms to evade immune surveillance including downmodulation of immunologic molecules, prevention of immune cell activation, inactivation or by triggering functional deficiencies in immune cells [51-54]. Immune evasion occurs not only in the primary HNSCC tumor but also during the process of metastasis allowing dissemination to regional lymph nodes and distant sites [55]. Reconstitution of immune cells with anti-tumor capabilities may be a feasible adjuvant immunotherapeutic strategy for HNSCC. Not all immune cells with anti-tumor activities are suppressed in HNSCC. Although the mechanisms remain unknown, in human-papillomavirus associated oropharyngeal carcinoma, large numbers of CD3 positive tumor-infiltrating lymphocytes correlate with higher overall survival and a decreased incidence of metastasis [44].

TILs Facilitating Tumor	TILs Antagonistic To Tumor
CD 34 +ve myeloid progenitor Cells	Cytotoxic T Cells
Th 17 cells (increasing angiogenesis)	Helper T Cells
	Natural Killer Cells
	Myeloid derived Suppressor Cells(MDSC)
	Dendritic Cells
	T regulatory Cells

Table 2. Tumor infiltrating lymphocytes that influence HNSCC tumors

5. Endothelial cells

Endothelial cells when stimulated by the growth factors form blood vessels that facilitate tumor growth and dissemination [56, 57]. HNSCC cells directly bind to endothelial cells through adhesion molecules including intercellular cell adhesion molecule-1, CD44, lymphocyte function-associated antigen-3, integrin chains $\alpha 6\beta 1$ and sialyl Lewis (x) [58]. Direct binding of HNSCC to endothelial cells is a prerequisite for penetration of and metastasis through the vasculature. In addition, direct interaction between HNSCC and endothelial cells trigger Notch-1 signaling in endothelial cells promoting capillary tubule formation [59]. Angiogenesis and neo-vascularization are complex processes involving cross-talk between multiple cell lineages in the vicinity [60]. HNSCC tumors and stromal cells secrete cytokines and growth factors including vascular endothelial growth factor

(VEGF), platelet-derived growth factor and interleukin-8 inducing angiogenesis [61]. VEGF plays an important role in endothelial cell survival [62, 63]. On binding to its receptor VEGFR2, VEGF induces expression of Bcl-2 and autocrine signaling through chemokines CXCL1 and CXCL8 facilitating proliferation of endothelial cells and sprouting of microvessels [64]. Global gene expression profiling revealed that HNSCC tumors induce angiogenesis by either expressing high levels of VEGF/fibroblast growth factor (FGF-2) and low levels of interleukin-8/CXCL8 or low levels of VEGF/FGF2 and high levels of interleukin-8/CXCL8 [65]. Tumor hypoxia also plays an important role in the release of angiogenic growth factors. Under hypoxic conditions stabilization of the hypoxia inducible factor 1 α (HIF-1 α) in tumor cells allows transcription of genes involved in angiogenesis and other critical aspects of tumor maintenance [66, 67]. Semaphorin 4D strongly induced by HIF-1 α , binds to plexin B1 on endothelial cells inducing migration [68]. In addition to the formation of new blood vessels, endothelial cells are also involved in a cross talk with squamous cell carcinoma cells resulting in a significant increase in tumor cell survival and migration [69]. Specifically, soluble factors secreted by endothelial cells including interleukin-8, interleukin-6, and epidermal growth factor induce phosphorylation of signal transducers and activators of transcription-3, extracellular-regulated kinase and Akt in HNSCC. Thus molecular targeting of endothelial cells may have tremendous therapeutic potential for HNSCC.

6. Lymphatic cells, pericytes, mast cells and other cells in the tumor microenvironment

In addition to blood vessels, HNSCC are typically infiltrated by lymphatic vessels a process known as lymphangiogenesis. Lymph vessels are typically distributed throughout the tumor as well as in the peritumoral regions [70-72]. Metastasis to regional lymph nodes commonly occurs in HNSCC and correlates with poor prognosis [73, 74]. Due to the paucity of lymphatic endothelial cell line models, most of the data generated pertaining to lymphangiogenesis are based on immunohistochemical analysis of xenograft or patient tissues. HNSCC tumors secrete VEGF-C, a member of the VEGF family, which plays an important role in tumor lymphangiogenesis [75]. Increased tumor lymphatic vessel density correlates with metastasis to lymph nodes in HNSCC [76, 77]. HNSCC tumors expressing high levels of HIF-1 α and VEGF-C had high lymphatic vessel density and increased metastasis [78].

Pericytes are contractile stromal cells closely associated with vascular endothelial cells that stabilize the capillary walls [79-81]. In the absence of pericytes, blood vessels are unstable and undergo regression. [82]. Pericytes influence the proliferation, migration and maturation of endothelial cells [83]. In tumors, pericytes are loosely associated with endothelial cells resulting in increased capillary leakiness [84]. Very few studies have focused on pericytes in HNSCC. Majority of reports use markers such as α -smooth muscle actin to stain pericytes associated with endothelial cells via immunohistochemical analyses [85, 86].

Mast cells are white blood cells that directly associate with endothelial cells stimulating vascular tube formation [87]. As HNSCC progresses, there is an increase in mast cell numbers that correlated with angiogenesis suggesting a role in angiogenesis [88].

The oral cavity and associated areas of the head and neck region are exposed to several microorganisms. Metaproteomic analyses of human salivary microbiota revealed a large number of oral bacteria that are metabolically active and actively engaged in protein synthesis [89]. The role of the human oral microbiome in tumor pathogenesis remains

largely unknown. It is well known that bacteria associated with periodontitis a condition caused by chronic inflammation of the gums, poses an independent risk factor for HNSCC [90]. Human papilloma virus (HPV) infection is a major risk factor for oropharyngeal squamous cell carcinoma [91, 92]. A recent study demonstrated that stromal cells expressing high levels of carbonic anhydrase IX (a sensitive marker for hypoxia) significantly correlated with reduced survival in HPV-negative HNSCC patients [93].

Tumor associated stroma are complex and influence tumor growth in a coordinated manner. Further studies on their contribution to tumor recurrence and new primaries are needed. The identification of promising targets for stroma-directed therapy will pave the way for enhanced anti-tumor effects and improved HNSCC patient survival.

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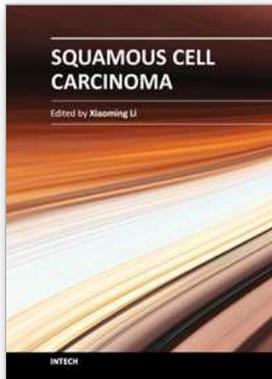
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This book points to some new areas for investigation on squamous cell carcinoma (SCC). Firstly, the features and management of some specific SCC is discussed to give the readers the general principles in dealing with these uncommon and sophisticated conditions. Some new concepts in adjuvant therapy including neoadjuvant therapy and gold nanoparticle-based photo dynamic therapy are introduced. Secondly, a detailed discussion of molecular aspects of tumor invasion and progression in SCC is provided with the emphasis on the roles of some important factors. The role of tumor microenvironment in head and neck SCC is specifically discussed. Thirdly, the roles of cancer stem cells (CSC) in cancer therapy of SCC are described. Molecular mechanisms involving therapeutic resistance and new therapeutic strategies targeting CSC are discussed in detail. Finally, other aspects concerning SCC are included, which involve the assessment, genetic manipulation and its possible clinical implications for the treatment of SCC.

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