Tumor Microenvironment in Head and Neck Squamous Cell Carcinoma

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The tumor microenvironment (TME) of head and neck squamous cell carcinoma (HNSCC) is comprised of cancer-associated fibroblasts (CAFs), immune cells, and other supporting cells. Genetic changes in the carcinoma cells, such as alterations to TP53, NOTCH1, and specific gene expression profiles, contribute to derangements in cancer and microenvironment cells such as increased ROS, overproduction of cytokines, and epithelial to mesenchymal transition (EMT). CAFs are among the most critical elements of the TME contributing to proliferation, invasion, and metastasis. The adaptive immune response is suppressed in HNSCC through overexpression of cytokines, triggered apoptosis of T cells, and alterations in antigen processing machinery. Overexpression of critical cytokines, such as transforming growth factor-β (TGF-β), contributes to EMT, immune suppression, and evolution of CAFs. Inflammation and hypoxia are driving forces in angiogenesis and altered metabolism. HNSCC utilizes glycolytic and oxidative metabolism to fuel tumorgenesis via coupled mechanisms between cancer cell regions and cells of the TME. Increased understanding of the TME in HNSCC illustrates that the long-held notion of "condemned mucosa" reflects a process that extends beyond the epithelial cells to the entire tissue comprised of each of these elements.

Squamous cell carcinoma comprises more than 90% of cancers of the head and neck and arises from the squamous lining of the mucosal surfaces of the upper aerodigestive tract, including the oral cavity, pharynx, larynx, and sinonasal tract. Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, and only 50%–60% of patients are alive at 5 years after diagnosis.1,2 Treatment can be quite morbid and result in significant functional as well as aesthetic deficits, such as impairment of speech and swallowing and facial deformity. Treatment failure and locoregional recurrence are common and occur in up to 30% of patients and account for the majority of deaths.3 The high rate of local recurrence produced the long-held notion of "condemned mucosa" or "field cancerization" initially described in the 1950s.4 This concept underscores not only the difficulty in treating HNSCC but also denotes the complexity of the molecular conditions under which HNSCC develops and recurs. It is clear that the notion of the condemned mucosa reflects a "condemned tissue" composed of the cancerous cells, adjacent epithelial, stromal, and immune cells and their surrounding matrix. Together these elements comprise the tumor microenvironment (TME). In fact, this shift in thought from the concept that cancer is derived from a single cell type, to a disease occurring in a complex tissue, has led some investigators to suggest that the very definition of carcinoma be changed.5

Tumorigenesis requires multiple elements outlined by Hanahan and Weinberg: (1) limitless replicative potential, (2) self-sufficiency in growth signals, (3) insensitivity to anti-growth signals, (4) ability to evade apoptosis, (5) increased angiogenesis, and (6) invasion and metastasis.6 Knowledge of the mechanisms through which the cancer cells use the TME to execute these processes continues to evolve.7,8 There is great interest in the downstream paracrine interactions with...
the stroma, immune interactions, and metabolic changes and the role each plays in tumorigenesis.

HNSCC is genetically heterogeneous, but a number of pathways have been found to be commonly involved; the impact of several critical abnormalities on the TME is highlighted below. The cellular elements of the TME often coevolve with the tumor. Stromal fibroblasts, T cells, macrophages, and other cell types develop abnormal phenotypes in a disorganized response to the cancer (Figure 1). These non-cancerous cells provide many of the paracrine signals necessary to turn on the pleiotropic abilities of cancer cells. For example, fibroblasts become cancer-associated fibroblasts and secrete factors such as matrix metalloproteins (MMPs), contributing to tumor invasiveness. Furthermore, as the chronic inflammation of the TME remains unresolved, alterations in adaptive immune response such as apoptosis of cytotoxic T cells and activation of suppressor T cells occurs. Additionally, tumors reprogram their surroundings creating a metabolically fertile environment to meet their high energy and anabolic requirements. This process was aptly described by Paget as the “seed and soil” hypothesis. Fundamental tumornon-tumor microenvironmental interactions such as these represent potential points of intervention for therapeutic strategies. Many critical targets, such as nuclear factor-κB (NF-κB), hypoxia-inducible factor (HIF)-1α, and vascular endothelial growth factor (VEGF), have been, and continue to be, explored as therapeutic targets in the TME (Table 1).

IMPACT OF GENETIC AND EPIGENETIC CHANGES OF THE EPITHELIUM ON THE TME

The initiating genetic alterations in the epithelial cells of HNSCC are primarily the result of the carcinogenic properties of tobacco and alcohol, and in the oropharynx, oncogenic strains of the human papilloma virus (HPV). Classically, HNSCC has been thought of as a disease caused by tobacco and alcohol, yet tobacco-related cancers are decreasing in incidence. Over the past several decades, oncogenic strains of HPV have become apparent as an etiology for oropharyngeal squamous cell carcinoma (OPSCC). HPV-related OPSCC accounts for up to 60% of cases of oropharyngeal cancer in some regions; this has resulted in an increased incidence among younger nonsmokers, and has been equated to an epidemic by some investigators. Currently, this is the second most common malignancy caused by HPV. OPSCC is caused primarily by HPV16 (but also HPV18, HPV31, and others), via the E6 and E7 mechanisms established in cervical cancer.

The most widely identified mutation in non-HPVrelated HNSCC occurs in the tumor-suppressor gene TP53. This has been identified to occur in approximately 50% of HNSCCs and is likely an early event, as it is commonly found in premalignant lesions as well. Mutations also have been shown to correlate with aggression and poor outcomes; for example, p53 mutations have been found in 95% of radioresistant tumors. Histologically negative margins with p53 mutations have been shown to be associated with a greater incidence of local recurrence. Mutation of TP53 in tumor cells is associated with increased migration of cancer-associated fibroblasts (CAFs) to the TME, while intact TP53 inhibits migration. Loss of functional p53 increases reactive oxygen species (ROS) and reactive nitrogen species (RNS) and may drive carcinogenesis via NF-κB and other inflammatory-mediated mechanisms. Alterations in TP53 induce a DNA damage response (DDR) in adjacent non-tumor cells via production of ROS. This effect was recently demonstrated in esophageal SCC, and it increases with proximity to and size of the primary tumor, with effects being identified several centimeters from the tumor. TP53 mutations also have been linked to abnormal tumor metabolism, contributing to the Warburg effect through increased activity of glucose transporters and glycolytic enzymes furthering the production of an acidic environment and high levels of ROS toxic to normal cells (Figure 1).

**Figure 1.** Select elements and interactions of the TME. The tumor is shown here with the leading tumor edge and perivascular niche that commonly contain cancer stem cells and highly replicating tumor cells (blue). The more central compartment contains tumor cells that are glycolytic and less proliferative (orange). Peritumoral epithelium demonstrating DDR is shown between the leading edge tumor edge and normal epithelium. CAFs (purple) are shown in the tumor stroma adjacent to normal stroma and fibroblasts (yellow). CAFs express MT-MMP that interacts with extracellular metalloproteinase inducer (EMMPRIN) on cancer cells to activate MMP2. CSCs express CD144, which interacts with MMP9 to activate TGF-β. CAFs and tumor cells produce elements like VEGF, PGE2, and CXCL12 that trigger angiogenesis. Immune interactions between regulatory T cells (pink), cytotoxic T cells (red), M2 TAMs (green), and tumor-associated neutrophils (blue/green) are shown. TGF-β and IL-10 produced by TAMs and cancer cells suppress T-cell activity. TAMs also produce MIF that recruits neutrophils. Regulatory T cells induce tolerance by cytotoxic T cells. The Fas receptor on activated cytotoxic T cells in utilized by cancer cells to induce apoptosis. Tumor-associated neutrophils produce ROS, and also increase angiogenesis and invasion by production of MMP-9, VEGF, and HGF. CD34+ myeloid progenitor cells (yellow/orange) are recruited to the TME by GM-CSF produced by cancer cells which in turn induce immunosuppression through TGF-β.
**NOTCH1**, the second most commonly mutated gene in HNSCC occurring in approximately 15% of cases, functions as a tumor-suppressor.\(^{21}\) It encodes a transmembrane receptor that regulates cell differentiation and embryonic development.\(^{21,32}\) In HNSCC, it is dependent on intercellular signaling in the TME and contributes to proliferation and invasiveness through the pro-inflammatory cytokine, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)). This TNF-\(\alpha\) mechanism acts on Slug and Twist, two other important transcription factors that act as regulators of invasion and epithelial to mesenchymal transition (EMT).\(^{9,33}\) Evidence also
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Abbreviations: Rb, retinoblastoma gene; EGFR, epidermal growth factor receptor; CDKN2a, cyclin-dependent kinase inhibitor 2a; STAT 3, signal transducer and activator of transcription 3; PD-L1, programmed death ligand-1; FasL, Fas ligand; MMP, matrix metalloprotein; ALDH, aldehyde dehydrogenase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; IL, interleukin; GGF, fibroblast growth factor; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; PDGF, platelet-derived growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; EGF, epidermal growth factor; CSF-1, colony-stimulating factor-1; TCR, T-cell receptor; FoxP3, forkhead winged helix transcription factor; RNS, reactive nitrogen species; MCT, monocarboxylate transporter; PGE, prostaglandin; PD-1, programmed death-1; TOMM20, translocase of outer mitochondrial membrane 20; COX, cytochrome C oxidase complex; LDH-B, lactate dehydrogenase B.
has suggested that its activity is mediated through MMPs and the inflammatory transcription factor, NF-kB, among other critical mechanisms. EGFR is a membrane-bound tyrosine kinase receptor that binds epidermal growth factor (EGF) and TGF-α. It is the target for the effective and widely used monoclonal antibody, cetuximab. Mutation of the EGFR gene is only present in about 10% of cases, but gene amplification is present in about 30% of cases and overexpression has been identified in up to 90% of cases. Increased expression and gene copy number correlate with poor prognosis. After binding one of its ligands, EGFR triggers multiple intracellular signaling cascades that activate cell proliferation, survival, invasion, metastasis, and angiogenesis. It also allows for decreased response to radiotherapy by enhancing proliferation, DNA-repair, and hypoxic responses within the TME. Activation triggers increased interleukin-8 (IL-8) and VEGF production, promoting inflammation and angiogenesis. STAT3 is also central to maintaining self-renewal in cancer stem cells (CSCs). The STAT/JAK pathway is one of the critical targets of cetuximab. Numerous other abnormalities have been found to be prevalent in HNSCC, including DNA methylation, histone modification, microRNA interference, and small interfering RNA. Epigenetic regulation such as methylation of CDKN2a and other genes has been shown to occur. Methylation of death-associated protein kinase (DAPK) is associated with resistance to anti-EGFR agents, like cetuximab. Jung et al performed a combined analysis of the transcriptome, methylome, and miRNome of metastatic HNSCC and non-metastatic HNSCC and identified a signature that correlated with lower survival and metastatic phenotype. The pathways involved in this group were specifically related to cell–cell adhesion, EMT, immune response, and apoptosis. For example, they identified decreased expression of desmoglein 3 (DSG3), a component of desmosomes critical for cell–cell adhesion. Desmosomes also have been shown to have tumor-suppressor function, and decreased expression of DSG3 has been linked to a poor prognosis. They also identified several elements significant in EMT, including upregulation of vimentin and downregulation of cytokeratin intermediate fibers and activation of TGF-β–related EMT pathways. Analysis of miRNA demonstrated upregulation of pathways related to DDR and immune response.

**CANCER-ASSOCIATED FIBROBLASTS**

Normal squamous mucosal lining of the upper aerodigestive tract is organized into distinct...
compartments: the upper layer of differentiated squamous or respiratory epithelial cells, a basal epithelial layer, the underlying basement membrane, and stromal layer. Fibroblasts are abundant in the stroma and are the primary element responsible for secretion of the basement membrane proteins. They secrete structural proteins such as type IV collagen and laminin and also produce numerous cytokines and paracrine signals. Accordingly, tumor-or cancer-associated fibroblasts (CAFs) are among the most critical cellular elements of the TME. CAFs are phenotypically altered fibroblasts, which are active participants in the process of tumorigenesis, promoting growth and metastasis.65

CAFs arise from the population of circulating fibroblasts and co-evolve with the tumor developing a distinct phenotype, and playing an active role in carcinogenesis.62–64 They produce a variety of contractile proteins, giving them an “active” phenotype. Frequently, they demonstrate ultrastructural accumulation of α-smooth muscle actin (α-SMA), characteristic of myofibroblastic (MF) differentiation.65–67 In HNSCC, CAFs frequently have this MF phenotype and are associated with dense collagen deposition and stromal desmoplasia.68,69 CAFs are also characterized by expression of integrin α6, which is critical to cell adhesion and surface signaling. It complexes to bind laminins, components of the extracellular matrix, and interacts with CDKN1A, altering cell cycle progression. Lim et al demonstrated that upregulation of α-SMA and integrin-α6 correlated with worsened prognosis in oral cancer.70

CAFs express a variety of factors critical to carcinogenesis, promoting cell motility by upregulation of cytokines, such as paracrine motility factor, hepatocyte growth factor (HGF), CXCL12, and TGF-β.71 HGF secreted by CAFs has been shown to promote invasion and angiogenesis in HNSCC and esophageal SCC.65,72–74 CXCL12 binds to CXCR4; this interaction plays a role in upregulation of MMP9, EMT, and HIF-1α expression.75 TGF-β is a critical element in the TME that serves numerous functions, including immunosuppression. Additionally, CAFs directly contribute to extracellular matrix remodeling by secreting MMPs.76,77

Marsh et al demonstrated that the MF phenotype seen in some oral carcinomas was strongly prognostic of a negative outcome.78 This study evaluated 282 oral HNSCC specimens and found that the presence of MF stroma was the strongest prognostic variable assessed, as compared to surgical margins, extracapsular spread, and stage, among others. MF stroma correlated with depth of invasion and with extracapsular spread in nodal metastasis. Interestingly, tumor-containing lymph nodes with extranodal spread were also surrounded with MF stroma. In oral and lingual carcinoma cell lines, Lin et al were able to demonstrate increased proliferation in association with CAFs.79,80 In a mouse model using heterotopic injection of HNSCC cells with normal fibroblasts or CAFs, Wheeler et al demonstrated that HNSCC cells with CAFs resulted in increased growth of the primary tumor and nodal and distant metastases compared to co-injection with normal fibroblasts.61

CAFs are also critical to tumor metabolism. Recent studies indicate that epithelial cancer cells may derive nutrients from the CAFs via a coupled metabolic mechanism. Cancerous cells induce glycolysis in adjacent stromal cells such as CAFs and then use their high-energy byproducts, such as lactate and pyruvate.81 This is somewhat contrarian to the long held belief of the Warburg effect, whereby tumors are thought to rely on aerobic glycolysis to produce energy for rapid growth. This has been labeled the “reverse Warburg effect” and has been shown to be a critical prognostic indicator in breast and other human cancers. There is some evidence that suggests this occurs in HNSCC as well.82

THE IMMUNE RESPONSE IN THE TME

The persistent unresolved inflammation associated with cancer results in a eventual decay and malfunction of the normal immune processes, which in turn contributes to tumorigenesis through immune tolerance and suppression and also to angiogenesis and production of ROS. Essentially, tumorigenesis is at least in part a byproduct of a failure of the immune system.10,12,83,84 The adaptive immune response contributes in a variety of ways to tumorigenesis through the immune interactions in the TME involving T lymphocytes, macrophages, dendritic cells, and others.85

T Lymphocytes

T lymphocytes are the central component of the anti-tumor response. They serve to initiate and regulate the adaptive immune response and to elicit the cytotoxic response to tumors.85 There is evidence that dysfunction occurs at the local, regional, and systemic levels in HNSCC. While a strong lymphocytic host presence at the tumor interface is indicative of an adaptive immune response and correlates with an improved survival,86–88 dysfunctional circulating T cells and tumor-infiltrating T cells have been identified in HNSCC, suggesting that tumors can suppress a previously intact local and systemic immune response.89 Moreover on a regional level, metastatic lymph nodes of HNSCC show significantly decreased levels of CD8+ lymphocytes.90,91 Common functional deficits of tumor-infiltrating T cells include: (1) absent or low expression of a key molecule in the signaling receptor
receptor chain (CD3ζ), (2) decreased proliferation in response to mitogens, (3) inability to kill tumor cell targets, (4) imbalance of their cytokine profile, and (5) evidence of profound apoptotic features. 84–99

Evasion of the adaptive response is executed through a variety of mechanisms such as decreased expression of major histocompatibility complexes (MHC I) or induction of apoptosis in T cells. Decreased expression of antigen-processing machinery such as MHC glycoprotein allows escape of subpopulations of tumor cells by avoiding activation of cell mediated immunity. 95–97 This mechanism has been demonstrated in HNSCC whereby tumor cells produce gangliosides, which downregulate MHC I. 98

Another means of evading detection is to induce apoptosis in cytotoxic T cells. The FasL receptor mechanism is expressed by activated cytotoxic T cells, which bind to FasL and typically result in triggering the cytotoxic response. However, this also predisposes the T cell to apoptosis. Oral SCC cells have been shown to contain membranous FasL-positive vesicles, which trigger induction of T-cell apoptosis, circumventing the cytotoxic response. 84,85,95

The cytotoxic response also can be dampened by suppression. Intratumoral cytotoxic CD8⁺ T cells in HNSCC show increased expression of programmed death-1 (PD-1), a marker of suppressed function. 87,99

Its ligand, programmed death receptor ligand-1 (PD-L1), is a surface protein that blocks function of T lymphocytes and is expressed on malignant oral SCC cells and also on CAFs. 100 Cho et al demonstrated that increased PD-L1 expression resulted in increased apoptosis of intratumoral CD8⁺ TILs. 101

Moreover, cytokines like, TGF-β, IL-10, and others allow local naive T cells to be triggered to become suppressor T cells, while also exploiting the suppressive functions of existing regulatory T cells. 102 PD-1 is of particular interest in HPV-associated HNSCC, as a lymphocytic infiltrate is one of the common features of HPV-related OPSCC. Infiltration of the TME by PD-1-positive T lymphocytes was correlated with improved prognosis. 103 While this is contrary to the above findings, in the case of HPV-related OPSCC, the PD-1-positive T lymphocytes, likely reflect an activated chronic immune response due to long-standing viral infection. 105

Langerhans cells are APCs located within the skin and mucous membranes of the upper aerodigestive tract. They detect antigens in the mucosa and then migrate to regional lymph nodes where they initiate a primary immune response. Some evidence suggests that greater infiltration of HNSCC tumor samples with Langerhans cells correlates with improved prognosis. 84,108–110

Tumor-associated macrophages (TAMs) are present with varying frequency in tumors, and are common in HNSCC. TAMs are classified into two varieties: proinflammatory (M1) and suppressive (M2). Accordingly, studies in various cancers have shown that TAMs can be associated with positive or negative prognosis. M1 TAMs contribute to the anti-tumor immune response via the production of proinflammatory cytokines IL-12, IL-23, and interferon-γ. 114,115 While the M2 TAMs appear to accumulate near blood vessels, promote angiogenesis, 114,115 and produce a variety of suppressive cytokines such as IL-10 and TGF-β. They also serve to promote tissue remodeling and inhibit anti-tumor cytotoxic effects of M1 TAMs. 84,111–115,116 Data in oral SCC suggest that TAMs are largely of the M2 type, as tumors with high levels of TAM infiltration correlate with higher stage, lymph node metastasis, and extracapsular spread. 114,117,118 Lago Costa et al demonstrated that macrophages were increased in the TME and the peripheral blood in HNSCC, and that samples with increased TAMs showed increased levels of TGF-β and its correlated immunosuppressive effects. 119 They produce ROS, RNS, and prostaglandins (PGs), all of which can contribute to inflammation and tumorigenesis. COX2 inhibitors and nitric oxide synthase inhibitors (iNOS) have been used to antagonize these inflammatory agents and their cytokines. 120,121 TAMs in HNSCC also produce significant levels of macrophage migration inhibitory factor (MIF), which is an inflammatory cytokine that stimulates neutrophils. MIF recruits neutrophils to the tumor via a CXCR2 mechanism and then by feedback mechanisms increases invasiveness of the tumor cells. 122 Neutrophils act on the tumor in a variety of ways: inducing genetic instability via ROS, increasing angiogenesis via MMP9 and VEGF, and increasing invasion via HGF. 123

ANTIGEN-PRESENTING CELLS AND TUMOR-ASSOCIATED MACROPHAGES

Dendritic cells are specialized antigen-presenting cells (APCs) common in the TME of HNSCC. 84,98,104 They have a high a capacity for antigen capture and also stimulate T-cell maturation. In contrast, when exposed to TGF-β and IL-10, they can promote immune tolerance and differentiation of CD4⁺ T cells into suppressive regulatory T cells. 84,105–107

THE BASEMENT MEMBRANE, INVASION, AND MATRIX METALLOPROTIENASES

The basement membrane is barrier to tumor progression, and its degradation facilitates tumor invasion and metastasis. For this to occur, cancer cells must (1) develop motility, (2) alter cell–cell adhesion, and (3) remodel the ECM. 124 The basement membrane not only serves as a structural framework for the overlying epithelial cells but also provides paracrine signals that
affect their behaviors such as differentiation and migration. Many of the key elements of the basement membrane, including collagen type IV and fibronectin, have been shown to be disregulated in HNSCC. MMPs are most important group of proteolytic enzymes used by cancer to degrade the ECM. MMPs in normal tissues are expressed in balance with their inhibitors to maintain a well-organized system. MMPs are upregulated by NOTCH1 pathways, EGFR, TGF-β, HGF, and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are commonly overexpressed in HNSCC. Among the most commonly identified metalloproteinases in HNSCC are MMP-2, MMP-9 and membrane-bound MMP (MT-MMP). MMP-2 and MMP-9 are gelatinases and degrade collagen type IV, the most critical step in degrading the BM. Increased levels of MMP-2 and MMP-9 correlate with increased nodal metastasis and poor prognosis. MMP-9 is the most structurally complex and can degrade numerous elements of the TME, including elastin, fibrillin, laminin, gelatin, and types IV, V, XI, and XVI collagen. MMPs were initially thought to be produced solely by the tumor cell, but further investigation has shown production also by the CAFs and surrounding inflammatory cells. CAFs are primarily responsible for the increased production of MMP-2 in co-culture experiments. MT-MMP is critical in activating MMP-2. There are numerous other significant MMPs, such as MMP-13, which participates in angiogenesis increasing the level of VEGF at the invasive front.

Importantly, the functions of MMPs extend beyond protein degradation and invasion, as they target growth factors, growth factor receptors, and cytokines. For example, MMP-9 also produces a tolerogenic effect on dendritic APCs and also on regulatory T cells. Release of MMP-9 results in endothelial cell invasion and vessel formation. MMPs impact differentiation and maturation of bone cells into osteoclasts, which is critical to the process of bony invasion. HNSCC CSCs are characterized by expression of CD44; CD44 is a surface protein that functions as a receptor for hyaluronic acid and also is the docking receptor necessary for MMP-9 function. Given the broad significance of MMPs they represent a possible target for therapy directed at the TME. Interestingly, quercitin, a flavonoid isolated from onions, inhibits MMP-2 and -9 pathways.

TGF-β AND EPITHELIAL–MESENCHYMAL TRANSITION

A number of chemokines and cytokines provide critical paracrine signaling in the TME; here we focus on TGF-β, as it broadly impacts many cellular behaviors in the TME. TGF-β has both growth-promoting and -suppressive effects on cells, and for some time the role of TGF-β in malignancy had been controversial. It typically inhibits epithelial cell proliferation and promotes secretion of matrix proteins and proteases. Currently it is understood to act as a tumor-suppressor early in tumorigenesis, then in later phases it enhances the malignant phenotype. TGF-β primarily acts through the SMAD family of transcription factors and works in concert with mitogen-activated protein kinases (MAPKs), which regulate diverse cellular activities such as mitosis, differentiation, proliferation, cell survival, and apoptosis. Dysregulation of TGF-β in malignancy occurs through several mechanisms, including loss of response to its ligand, defects in the transduction pathway, and others. Oral SCC has been shown to be resistant to the suppressive effects of TGF-β, secondary to downregulation of TGF-β receptor II (TBRRII). TGF-β is a primary factor triggering EMT in HNSCC. EMT contributes to invasion allowing for enhanced mobility via expression of a protein expression patterns more characteristic of a mesenchymal phenotype. Once established, nests of metastatic tumor can transition back to a phenotype recapitulating the original tumor in a distant site. EMT is mediated through disruption of epithelial cell junctions, remodeling of the actin cytoskeleton, and upregulation of mesenchymal markers like vimentin and fibronectin. TGF-β pathways as well as those triggered by the inflammatory cytokines TNF-α and IL-6 converge upon STAT3, upregulating it. STAT3 proteins are commonly overexpressed in HNSCC. STAT3 interacts with Twist, Snail, and Slug (Snail2), transcription factors that contribute to EMT in various cancers. Twist increases expression of N cadherin, a marker of a mesenchymal motile phenotype, and decreases expression of E cadherin, a marker of an epithelial phenotype. Slug also decreases expression of E-cadherin. Loss of E-cadherin and gained expression of N-cadherin is critical to invasion and is referred to as cadherin switching. Prime et al were able to demonstrate morphologic evidence of EMT and cadherin switching after several days exposure to TGF-β. Emerging evidence suggests that EMT is fundamental to gaining “stemness” or the transition of cancer cells to becoming CSCs. CSCs are thought to serve as a fountainhead for tumors as they give rise to the remaining population of tumor cells, and contribute to treatment resistance. CSCs accumulate at the invasive front and perivascular spaces and are demarcated by expression of markers such as CD133 and CD44, and by aldehyde dehydrogenase activity. On the surface of CSCs, CD44 interacts with MMP-9 and this allows for proteolytic activation of TGF-β.

TGF-β extends beyond the epithelial cancer cells of a tumor, and many of the effects have been described above. Lewis et al showed that TGF-β produced at the
invasive leading edge of the tumor induced a MF phenotype in primary fibroblasts. They also showed that this effect resulted in secretion of HGF by myofibroblasts, which in turn promoted invasion through the basement membrane. TGF-β serves to inhibit TH1 lymphocytes and cytotoxic T lymphocytes and the functions of natural killer cells.\textsuperscript{84}

**ANGIOGENESIS, INFLAMMATION, AND HYPOXIA**

Small tumor deposits of 1–3 mm can be supplied by diffusion of nutrients from the surrounding tissue; beyond this, the tumor is dependent on angiogenesis to supply its needs.\textsuperscript{149} A number of studies have shown that angiogenesis is correlated with tumor aggression.\textsuperscript{48,150–154} HNSCC often has large hypoxic areas of tumor necrosis where growth exceeds angiogenesis.\textsuperscript{155–157} Hypoxic response and inflammation are driving forces in angiogenesis.\textsuperscript{12,158} Moreover, CSCs in HNSCC appear to be concentrated along the invasive front of the tumor and in the perivascular niche, an area within 100 μm of the microvasculature. A variety of factors in the TME, such as VEGF, NF-κB, and HIF-1α play central roles in this process.

VEGF enhances endothelial growth, migration of endothelial precursors, and their differentiation. High VEGF expression in oral SCC has been correlated with a poor prognosis, and a recent meta-analysis suggested that VEGF overexpression could be a useful prognostic marker.\textsuperscript{159} VEGF binds to its receptor, VEGFR1 in tumor cells, and induces expression of Bcl-2, inducing chemokines like CXCL1 and CXCL8. CXCL1 and CXCL8 promote endothelial cell proliferation and survival.\textsuperscript{160} Endothelial cells in turn produce factors like EGF, which significantly increase tumor cell survival and migration.\textsuperscript{161} VEGF and other angiogenic factors such as IL-6 and IL-8 are increased by a number of chemokines such as CXCL12, which binds to chemokine receptors CXCR2 and CXCR4. High CXCR2 and CXCR4 levels have been shown to be associated with increased microvessel density within tumors.\textsuperscript{55,77,162}

Chronic inflammation of the TME contributes to tumor progression through a variety of mechanisms, including production ROS and angiogenic factors. NF-κB is an inflammatory signal transcription factor playing a variety roles in invasion, proliferation, and angiogenesis. Constitutive activation NF-κB results in overexpression of a variety of factors, including IL-6, IL-8, and VEGF.\textsuperscript{153}

There are many downstream inflammatory markers expressed as a result of NF-κB and other mechanisms, such as cyclooxygenases like COX-2.\textsuperscript{165} COX enzymes catalyze the production of PGs and likely are the rate-limiting step in their synthesis. COX-2 is usually overexpressed in inflammation and preneoplastic lesions. PGs are increased in HNSCC, and PGE\textsubscript{2} promotes invasion and angiogenesis and inhibits apoptosis of cancer cells. COX-2 acts on VEGF, fibroblast growth factor, and MMPs and is also pro-angiogenic. COX-2 levels have been found to be prognostic and selective COX-2 inhibitors have been shown to increase the efficacy of radiotherapy in vitro.\textsuperscript{51,164} NF-κB is the target of many therapeutic interventions, such as curcumin, n-acetyl cysteine (NAC), epigallocatechin gallate (EGCG), and others.\textsuperscript{5}

Intratumoral hypoxia is a key characteristic of HNSCC, and is a negative prognostic factor, contributing to both chemotherapy and radiotherapy resistance. Intratumoral hypoxia is generally accepted to be a pO\textsubscript{2} < 10 mm Hg, and intratumoral pO\textsubscript{2} levels ≤2.5 mm Hg correlate with a worsened prognosis, as does the overall volume of hypoxic tumor at the primary site.\textsuperscript{165} HIF-1α is the most important factor induced in adaptive response to hypoxia, and elevated expression is also directly associated with a poor prognosis.\textsuperscript{166} This transcription factor interacts with more than 100 genes to alter expression of VEGF, CA9, lysyl oxidase, and many others.\textsuperscript{48,167} It has been shown to alter cellular metabolism, and to increase lymphatic vessel density and blood vessel density in oral SCC.\textsuperscript{168,169} CA9 functions to regulate pH homeostasis and alter the uptake of chemotherapeutic drugs, and also is purported to play a role in proliferation and cell adhesion.\textsuperscript{165,170} Lysyl oxidase catalyzes the crosslinking of collagens and elastins, and overexpression increases microvascular density.\textsuperscript{171,172} Agents such as resveratrol, EGCG and others may act by promoting degradation of HIF-1α.\textsuperscript{173–175} Resveratrol has been shown to decrease expression of HIF-1α and VEGF in vitro.\textsuperscript{176}

**METABOLISM IN THE TME**

Cancer cells have high bioenergetic requirements needed to maintain tumor growth. Tumor cells in culture have long been demonstrated to rely heavily on glycolysis with decreased, dysfunctional, or absent mitochondrial OXPHOS. Reliance on glycolysis in the presence of oxygen is referred to as the Warburg effect.\textsuperscript{177} This results in the generation of less ATP than OXPHOS and yields high levels of pyruvate and lactate. This is somewhat counterintuitive as there is such a high bioenergetics requirement, yet OXPHOS is a more efficient means of energy generation than glycolysis. Thus it is unclear why tumor cells would thrive with a less efficient mechanism. It has been hypothesized that glycolysis may confer a growth advantage.\textsuperscript{178–180} Some normal, highly proliferative cells, such as lymphocytes, favor aerobic glycolysis over oxidative metabolism, providing a rationale for
the Warburg effect. Many cancer cells have defects in critical components of the OXPHOS pathway, such as the mitochondrial B-catalytic subunit of H\textsuperscript{+}-ATP synthase. Furthermore, when glycolytic flux is high, the ATP yield can exceed that produced by OXPHOS. Additionally, the intermediates of glycolytic metabolism can provide substrates for amino acid, fatty acid, and nucleotide synthesis. The metabolic pressures induced by hypoxia in the setting of rapid growth may then in turn select for tumor cells which favor glycolytic metabolism even in the presence of oxygen, as is suggested by the frequent overexpression of HIF-1\textalpha in many cancers. Hypoxic induction of HIF-1\textalpha favors this process specifically inducing pyruvate dehydrogenase kinase (PDK) and lactate dehydrogenase a (LDH-A). PDK inactivates pyruvate dehydrogenase preventing import of pyruvate to the mitochondria. LDH-A restores NAD positivity and also uses pyruvate in the cytosol, which together can reduce electron flow though OXPHOS and also reduce oxidative stress. Additionally, glycolytic metabolism results in the acidic efflux into the TME that assists in breakdown of the ECM and kills non-adapted normal cells.

However, this is not likely the whole picture: much recent evidence suggests that a metabolic symbiosis exists within tumors cell between different populations. Feron has likened this to the coupling between fast and slow-twitch muscle fibers. Fast-twitch glycolytic fibers release lactate that is then taken up and utilized by slow twitch fibers. MCT1 is a high-affinity transporter of lactate, which mediates influx into the cell; MCT4 is a low-affinity transporter of lactate, which primarily mediates efflux of lactate from cells. These transporters couple cancer cells, so that hypoxic cells maintain functioning glycolytic metabolism while aerobic tumor cells recycle and utilize lactate and other high-energy substrates produced by them. A similar process in cancer would allow for an efficient intratumoral metabolic coupling mechanism between oxygenated cells and hypoxic cells.

Additional evidence favors multicompartmental metabolism between the cancer cells and CAFs. Numerous co-culture experiments and in situ tumor analyses have demonstrated this effect in breast and other cancers. This work has brought to light a "reverse Warburg effect", where oxidative stresses exerted by tumor cells induce aerobic glycolysis and autophagy in CAFs. This, in turn, results in increased levels of intermediate catabolites such as lactate, glutamine, and ketone bodies. These catabolites are released into the TME and used for OXPHOS in carcinoma cells. This metabolically enriches the TME and creates an environment that favors growth, apoptosis resistance, invasion, and metastasis. This is Paget's seed and soil hypothesis, a phenomenon that may have been unnoticed in previous homotypic culture experiments.

Most studies on HNSCC cellular metabolism suggest that the carcinoma cells are highly glycolytic with high l-lactate generation, yet recent studies suggest that metabolic heterogeneity and metabolic coupling occur. Most HNSCC cells generate significantly higher levels of lactate compared with normal human oral keratinocytes (NHOK), although several cell lines generate significantly lower lactate levels than NHOKs. It has been postulated that the cells with decreased lactate production have increased lactate uptake via MCTs, allowing them to utilize OXPHOS. When some HNSCC cell lines that are typically glycolytic are supplemented with excess pyruvate, some of the effects were reversed, which suggests that OXPHOS is important to support HNSCC cell proliferation in the presence of a catabolite-rich microenvironment. High tumor lactate concentrations in HNSCC are associated with subsequent nodal and distant metastases.

In our previously published work on oral SCC, we demonstrated evidence of this multicompartment model of metabolism. We have suggested that there may be three metabolic compartments in HNSCC, where the leading tumor edge relies on OXPHOS and the deeper layers of the tumor are more glycolytic (aerobic or anaerobic) and tumor stroma represents a third compartment undergoing aerobic glycolysis (Figure 2). This was demonstrated through high expression of MCT4 in the stroma and deeper tumor, while MCT1 was more highly expressed by the leading tumor edge. We also confirmed OXPHOS in the leading tumor edge with assays for TOMM20 and LDHb, both functional markers for mitochondrial metabolism. This pattern of metabolic coupling was demonstrated in a subset of our oral SCC patients, and correlated with aggressive behavior including a worsened disease-free survival and perineural invasion. Interestingly, it also correlated with increased specific uptake values (SUV) on positron emission tomography/computed tomography. We further tested this metabolic coupling theory with a squamous cell carcinoma line co-culture experiment. Using immortalized squamous cell lines we were able to generate two divergent SCC populations, one RAS-dependent and another NF-kB-dependent. These cell lines were each able to induce metabolic reprogramming of CAFs via oxidative stress. This resulted in a lactate shuttling process that feeds the cancer cells fueling anabolic growth via and MCT1/ MCT4 metabolic couple between the tumor and the stroma. Interestingly, this model also demonstrated that the CAFs protected the cancer cells against oxidative stress by reducing oxidative stresses within the carcinoma cells. RAS-transformed cells were able to reprogram adjacent epithelial cells, as well as...
fibroblasts, suggesting that cancer cells can subjugate either group.

MCT4 may represent a possible target for metabolic interruption and uncoupling of the tumor and stroma. In an animal model, we were able to demonstrate that NAC was able to selectively inhibit MCT4 induction in CAFs, halting mitochondrial biogenesis in cancer cells but not in normal epithelial cells. This may allow targeted therapy that selectively starves cancer cells. MCT1 also has been proposed as a possible target to prevent uptake of lactate, forcing aerobic cells to use glucose and depriving or decreasing availability to hypoxic cells.\(^\text{167}\) In fact, the MCT1 inhibitor, a-cyano-hydroxycinnamate has been shown to slow tumor growth and potentiate the effect of radiotherapy in MCT1-expressing tumors in mice.\(^\text{179,184}\)

Hypoxia contributes to chemotherapy and radiotherapy resistance.\(^\text{155}\) Inhibition of HIF-1α can prevent the induction of the hypoxic response blocking angiogenesis.\(^\text{190}\) Zhang et al inhibited HIF-1α with siRNA and oligonucleotides, which increased apoptosis in oral SCC.\(^\text{191}\) The EGFR inhibitor cetuximab blocks downstream signaling activated by EGFR; this triggers G1 phase arrest and can also trigger apoptosis.\(^\text{192}\) In addition, it has been shown to downregulate HIF-1α; this, in turn, downregulates their LDH-a and glycolytic potential. This inhibition of

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**Figure 2.** Metabolic coupling in HNSCC. The leading edge of the tumor relies on OXPHOS while the inner compartment and CAFs rely on glycolytic metabolism. Higher expression of MCT1 is seen in the leading tumor edge, while higher expression of MCT4 is seen in the central compartment and stromal CAFs.
glycolytic potential leads to inhibition of proliferation. Metformin is a commonly used antihyperglycemic drug in type 2 diabetics and has been proposed as a potential anticancer therapy also that may impact tumor metabolism in the TME. Metformin has been shown to inhibit cancer cell proliferation in several human cancers, such as gastric, medullary thyroid, breast, and pancreatic cancers. Epidemiologic studies also have shown significant effects from metformin use in diabetics, lowering the risk of cancer incidence and mortality. In oral SCC, Luo et al demonstrated that metformin blocked cell cycle progression at the G0/G1 phase and induced apoptosis. Metformin triggered alterations in multiple other pathways as well: increasing activation of the adenosine monophosphate (AMP) kinase pathway, suppressing the mammalian target of rapamycin (mTOR) pathway, decreasing cyclin D1 levels and retinoblastoma (Rb) phosphorylation, and downregulating Bcl 2. They also were able to demonstrate in vivo evidence of increased apoptosis in a xenograft model. While this study demonstrated various effects on the cell cycle; the metabolic effects of metformin on cancer have yet to be investigated.

CONCLUSION

Many elements of the TME beyond the cancerous epithelial cells impact progression of HNSCC. Genetic alterations induced by tobacco and alcohol or the HPV virus initiate the sequence of events that trigger transformation of stromal cells, immune suppression, and chronic inflammation. In turn, unchecked growth, invasion, and metastasis prevail. The complexity of these processes reveals that the long-held notion of "condemned mucosa" actually reflects a "condemned tissue" comprised of many cell types which have co-evolved during tumorigenesis.

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