Mechanisms of Invasion in Head and Neck Cancer

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Abstract

Context—The highly invasive properties demonstrated by Head and Neck Squamous Cell Carcinoma (HNSCC) is often associated with locoregional recurrence and lymph node metastasis in patients, and is a key factor leading to an expected 5-year survival rate of approximately 50% for patients with advanced disease. It is important to understand the features and mediators of HNSCC invasion so that new treatment approaches can be developed.

Objective—To provide an overview of the characteristics, mediators and mechanisms of HNSCC invasion.

Data Sources—A literature review of peer-reviewed articles in PubMed on HNSCC invasion.

Conclusions—Histological features of HNSCC tumors can help predict prognosis and influence clinical treatment decisions. Cell surface receptors, signaling pathways, proteases, invadopodia function, epithelial-mesenchymal transition, microRNAs, and tumor microenvironment are all involved in the regulation of the invasive behavior of HNSCC cells. Identifying effective HNSCC invasion inhibitors has the potential to improve outcomes for patients by reducing the rate of spread and increasing responsiveness to chemoradiation.

The clinical relevance of invasion

The ability of tumors to invade into surrounding tissues is a “hallmark of cancer”¹ and is arguably the property that most greatly impacts morbidity and mortality. The head and neck region is very complex, with a dense organization of fascial planes, bony and cartilaginous scaffolding, and abundant neurovascular structures. On one hand, this complex anatomy poses several barriers for an invasive tumor to overcome. On the other hand, these structures, when breached, can provide insidious pathways for cancer cells to travel, cause damage, and evade treatment. From a clinical perspective, levels of invasion impact prognosis and are therefore considered both in the current cancer staging system and in the decision-making process for determining treatment.

Head and neck squamous cell carcinoma (HNSCC) is an epithelial cancer, arising from the mucosa of the upper aerodigestive tract. Therefore, an invasive cancer, by definition, must first invade the basement membrane of the native epithelium. This property is achieved
during the process of carcinogenesis as multiple genetic insults are accumulated and in turn the acquisition of invasive capabilities allows invasion through the basement membrane. This primary event of invasion differentiates carcinoma in situ from invasive carcinoma. In oral tongue cancer, as this layer of connective tissue is breached, the invading tumor encounters the underlying tongue musculature. Depth of invasion (DOI) for these tumors is directly associated with patient outcome, and as the depth approaches 2–4 mm of invasion, the rate of lymph node metastasis increases greatly and prognosis worsens²–⁴. Therefore, elective neck dissection is recommended for oral tongue cancers with a depth of invasion of more than 2–4 mm, even when no clinical evidence of lymph node spread exists. A modified staging system has been developed which integrates DOI into the T category of the currently accepted American Joint Committee on Cancer (AJCC) TNM staging³,⁵. DOI was significantly associated with disease-specific survival in oral squamous cell carcinoma (OSCC)³.

In addition to depth, analysis of histological parameters in resected tumors of patients has indicated that invasion of tumor cells into the normal host tissue can predict patient outcome. The invasive front of an invading tumor has been shown to be an important indicator of patient prognosis²,⁶–¹⁰. Bryne et al. developed an invasive front grading system (IFG) of tumors that incorporated four parameters: the pattern of invasion, the degree of keratinization, nuclear polymorphism, and host response (inflammatory cell infiltration)⁹,¹⁰. The scores for each parameter were combined to yield a total malignancy score for the whole tumor. The worst part of the tumor (most invasive) was used for the assessment. A higher malignancy score meant a poorer prognosis. Sandu et al. used the invasive front grading system described by Bryne et al. and found that patients with a higher IFG score had significantly lower disease-free survival². Brandwein-Gensler et al. found the histological parameter, the pattern of invasion, to predict patient outcome in OSCC patients as part of their risk assessment model⁶,⁸. The worst pattern of invasion was significantly associated with overall survival, as well as local recurrence⁶,⁸. Li et al. demonstrated that this risk model predicted local recurrence and disease-specific survival in an independent study for patients with low-stage OSCC⁷.

Bone and cartilage are ensheathed in a dense layer of connective tissue. Therefore, the mandible has a natural barrier to tumor invasion. If a mandible cancer is able to breach the mandibular periosteum and invade the bony cortex, the relatively uninhibited spread of tumor in the bone marrow space can lead to extensive involvement and ill-defined tumor margins with poor prognosis¹¹–¹⁴. The presence of mandibular invasion places a tumor in an advanced primary stage (T4a) and overall stage (IV) according to the AJCC staging system¹¹. Similar to invasion of OSCCs into surrounding normal tissue, the histological pattern of invasion of OSCC into bone can also be assessed and correlated with outcome. Invasion into bone displaying a broad pushing front and a sharp interface between tumor and bone is categorized as an erosive pattern. Projections of tumor cells along an irregular front with residual bone islands within the tumor are categorized as an infiltrative pattern¹¹–¹⁴. A retrospective study of patients with mandibular invasion by OSCC found that patients with an infiltrative pattern of invasion into the bone had a four-fold increased risk of death with disease compared to the patients who displayed erosive pattern of invasion¹³. The infiltrative pattern of invasion into bone tissue is associated with more aggressive tumor behavior.
increased likelihood of positive margins, recurrence, death with disease, and shorter disease-free survival\textsuperscript{11–13}. Tumors with mandibular spread are generally treated aggressively, most often with segmental mandible resection, followed by postoperative radiation or chemoradiation. Similarly, the laryngeal cartilage poses a barrier to tumor spread. A cancer of the larynx that exhibits frank cartilage destruction is deemed a T4/stage IV cancer by AJCC criteria. If extensive cartilage invasion is present, often an organ-sparing approach with chemoradiation is not feasible, and laryngectomy with postoperative adjuvant therapy is the treatment of choice.

Another feature that HNSCC can exhibit is a propensity for invasion into neural structures. The head and neck region is populated by a rich network of innervation. The hypoglossal nerve extensively innervates the fine musculature of the tongue, while the lingual nerve provides a network for tongue sensation, and carries distal fibers from the chorda tympani to provide special sensory taste fibers. The mandible carries the V3 branch of the trigeminal nerve, which innervates the teeth and transmits to the mental foramen and provides sensation to the buccal mucosa of the lower lip and the skin of the chin. Similarly, the pharynx and larynx are innervated by vagus nerve fibers and branches of the glossopharyngeal nerve. When tumor cells invade perineurium and nerve sheaths, the cranial nerves can essentially provide a direct route of spread toward the base of the skull and intracranial extension. This is particularly evident for adenoid cystic carcinoma of the salivary gland, which has a very high affinity for perineural spread. HNSCC also commonly exhibits perineural invasion, and this feature has been cited as a harbinger for an increased rate of locoregional recurrence and poor outcome. Brandwein-Gensler \textit{et al.} found that perineural invasion (PNI) of small and large nerves was associated with reduced overall survival, and PNI of large nerves was specifically associated with local recurrence\textsuperscript{6}. De Matos \textit{et al.} applied the same criteria\textsuperscript{6,8} to independently assess another set of OSCC cases and found a significant positive correlation between PNI and POI, as well as between POI and tumor thickness\textsuperscript{15}, or depth of invasion (DOI)\textsuperscript{3}. High levels of nerve growth factor have been found to be correlated with PNI and worse survival\textsuperscript{16}. When perineural spread is identified after surgical resection, this feature can influence the decision to administer postoperative adjuvant treatment. Clinicians have evaluated PNI as an indicator in patients with early stage (I and II) OSCC tumors to determine treatment strategies, but PNI and lymphovascular invasion (LVI) were not found to be significant risk factors for disease-free or overall survival\textsuperscript{17}.

The clinical significance of invasive properties is not limited to local disease. It has been well established that in regional lymph node metastases, invasion outside of the lymph node capsule is associated with poor outcome\textsuperscript{18,19}. Two large randomized clinical trials demonstrated that extracapsular spread of lymph node disease identified after surgical resection is an indication for maximized treatment with postoperative radiation and concurrent cisplatin\textsuperscript{20}.

The strategies that cancer cells use to achieve all of the invasive properties highlighted above likely require genetic and molecular alterations that are highly complex. The details of how these cancers acquire the ability to invade normal, organized, anatomic structures are being revealed. An improved understanding of the molecular events that lead to these invasive phenotypes may provide insight that could improve both prognostication and treatment. The
following sections summarize what is known about the mechanisms of invasion of head and neck cancer cells.

**Cell surface receptors shown to influence invasive behavior in HNSCC**

The best characterized receptor for stimulation of HNSCC invasion is the epidermal growth factor receptor (EGFR, Figure 1)\(^{21-26}\). Epidermal growth factor (EGF) is present in saliva at concentrations around 1 ng/ml\(^{27-29}\). These concentrations\(^{30,31}\) or higher\(^{32-39}\) are able to stimulate migration and invasion in oral carcinoma lines in vitro, as well as in vivo\(^{40}\). Other EGFR ligands besides EGF have been shown to stimulate invasion, including transforming growth factor alpha (TGF\(_A\)), betacellulin (BTC), heparin binding EGF-like growth factor (HBEGF) and amphiregulin (AREG)\(^{32}\). Conversely inhibition of the EGFR has been shown to block invasion in response to EGF\(^{32,37,41-43}\). Intriguingly, the EGFR also mediates invasion induced by some other receptors, notably G protein coupled receptors (GPCRs)\(^{44}\) for lysophosphatidic acid (LPA)\(^{45,46}\), bradykinin (BK), prostaglandin E2 (PGE2)\(^{47}\) and gastrin-releasing peptide (GRP)\(^{48}\). In some cases, the mechanism of cross-activation of EGFR by GPCRs has been identified as the release of EGFR ligands. EGFR ligands are initially produced as cell surface transmembrane proteins\(^{49}\) which can then be released from their transmembrane tethers by members of a disintegrin and metalloproteinase (ADAM) family of proteases\(^{50}\). ADAM17 mediates the stimulated release of TGF\(_A\) by BK and PGE2\(^{47}\). GRP stimulates the release of both AREG and TGF\(_A\)\(^{48}\). In the case of GRP, EGFR ligand release involves a pathway in which Src activates phosphatidylinositol 3-kinase (PI3K), leading to phosphoinositide dependent kinase 1 (PDK1) activation and phosphorylation of ADAM17\(^{51}\). Estrogen receptor activation can also trigger EGFR ligand release\(^{30}\). In addition, autocrine release of AREG, HBEGF, BTC and TGF\(_A\) has been shown to occur during serum starvation\(^{48,52-55}\), indicating that autocrine stimulation of EGFR can contribute to basal invasion capability.

A number of other receptors that can regulate HNSCC invasion have been identified. Other GPCRs that stimulate invasion with ERK and MMP9 activation include chemokine receptors CXCR1 and CXCR\(^{56,57}\) and CXCR\(^{5,59}\). Interleukin 6 (IL6) receptors can activate the ERK and c-Jun N-terminal kinase (JNK) pathways\(^{60,61}\). Toll-like receptors (TLRs) have also been shown to stimulate invasion with activation of the NFKB pathway\(^{62,63}\). NFKB signaling can synergize with the ERK/AP1 pathways to stimulate invasion\(^{64,65}\). Wnt-5B knockdown suppresses MMP10 production and invasion indicating that Wnt signaling pathways can also contribute to invasion\(^{66,67}\). Transforming growth factor beta (TGF\(_B\)) can be produced by tumor-associated fibroblasts\(^{58}\) and induce NFKB activation\(^{69}\) and SMAD-dependent MMP production\(^{70}\) as well as EMT factors such as Snail and Slug\(^{71-76}\). Invasion is stimulated by hypoxia in a Notch-dependent fashion\(^ {77} \). Inhibition of Notch can also suppress MMP production\(^{78}\).

**Signaling pathways involved in HNSCC invasion**

Several signaling pathways have been shown to be important for invasion of HNSCC. Inhibition of ERK results in reduced invasion\(^ {37,41,79}\). An important ERK target is AP1\(^ {80}\), which can increase transcription of matrix metalloproteases (MMPs), such as MMP9\(^ {79,81,82}\).
PI3K activity is also important for invasion\(^{37,79,83,84}\). Inhibition of PI3K also results in reduced MMP9 expression\(^{37,79}\). Additional contributions of PI3K to invasion can occur through AKT to stimulate Slug and Snail expression for enhancing EMT\(^{85,86}\), or phosphorylation of ezrin\(^{87}\) and yes-associated protein (YAP)\(^{88}\). Phospholipase C (PLC) gamma-1 has been shown to be important in EGFR induced invasion in some cases\(^{79,89}\), potentially in a complex with c-Src\(^{35}\). Src family members can contribute to invasion either through enhanced release of receptor ligands as described above or through stimulation of invadopod formation, which is described in more detail below\(^{48,55,90–94}\). A number of HNSCC cell lines have been shown to have constitutively high levels of Rac1 activity, and inhibition of Rac activity results in reduced invasion capability\(^{95}\). In most cases the increased Rac1 activity correlated with increased tyrosine phosphorylation of the Rac GEF Vav2 induced by EGFR, but an alternative pathway utilized Ras activation. The role of the Janus kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) pathway in HNSCC invasion is complex. STAT3 has been shown to be important for ligand-independent invasion induced by the EGFR vIII isoform\(^{96}\), but JAK2-STAT5a signaling is important for erythropoietin (EPO) induced invasion but not EGF-induced invasion\(^{96}\). STAT3 activity may generally enhance invasion capability through suppression of PTEN\(^{97}\) and E-cadherin\(^{98}\).

### Proteases implicated in HNSCC invasion

A key distinction between migration and invasion is the ability to degrade extracellular matrix barriers in the latter activity. Thus expression of proteases, especially matrix metalloproteases (MMPs), has been shown to be important for HNSCC invasion in a number of cases\(^{99}\). The most commonly identified MMP has been MMP9. Its activity is increased by EGFR\(^{32,37,42,54,100–102}\) and integrins\(^{75}\), while its inhibition reduces invasion\(^{81,103,104}\). MMP9 can degrade type IV collagen\(^{105–107}\), a key constituent of the basement membrane, and thus has the potential to play an important role in enabling HNSCC cells to become frankly invasive. Analysis of OSCC cases by immunohistochemistry revealed that expression of MMPs corresponded to areas with loss of collagen alpha IV chain. The enzymatic activity of MMPs was assessed by zymography and was found to be enhanced in higher grade OSCC and along the advancing tumor front\(^{108}\). MMP9 is used as a marker of invasive OSCC in many studies and the extent of its expression is related to the infiltration pattern of SCC at the invasive front\(^{109}\). *In vitro* invasion assays that utilize Matrigel, which is derived from basement membranes, often show a dependence on MMP9 activity. However, MMP9 can cleave other proteins exposed to the extracellular milieu as well, activating ligands such as TGFβ and chemokines as well as cleaving cell surface receptors\(^{110}\). In addition, MMP9 may be involved in cleavage of E-cadherin, resulting in reduced cell-cell adhesion and increased invasion\(^{37}\).

MMP2 is another MMP that has been implicated in HNSCC invasion\(^{103,104}\). While MMP2 and MMP9 are secreted MMPs, the transmembrane protease MMP14 (MT1-MMP) also plays a significant role. MMP14, together with TIMP2\(^{111,112}\), is important in the activation of secreted proteases such as MMP2\(^{113}\). Suppression of MMP14 reduces overall matrix degradation activity\(^{114}\) and invasion\(^{43}\). Another protein that enhances protease activity and is important for invasion is extracellular matrix metalloprotease inducer (*EMMPRIN*)\(^{100,115–117}\). Although the mechanism is unclear, *EMMPRIN* homophilic
binding induces the expression of a number of proteases, including MMPs, cathepsin B, and urokinase plasminogen activator receptor (uPAR)\textsuperscript{100,115,117,118}. Other proteases that have been shown to contribute to HNSCC invasion include MMP1\textsuperscript{66}, MMP13\textsuperscript{80}, matriptase\textsuperscript{119}, fibroblast activation protein\textsuperscript{120}, and uPA/uPAR\textsuperscript{117,121–124}, as well as the glycosidase heparanase\textsuperscript{125}.

The roles of adhesion in HNSCC invasion

Cell-cell adhesion mediated by E-cadherin is generally found to inhibit invasion\textsuperscript{26,126}. Induction of cell-cell junctions can inhibit invasion\textsuperscript{127}. Reduction in E-cadherin expression can occur through methylation of the promoter, and demethylating agents, which reverse this, can enhance invasion\textsuperscript{128}. Inhibition of E-cadherin function also enhances invasion\textsuperscript{129}. However, the inhibitory effect of E-cadherin may be overcome by expression of other invasion-inducing proteins\textsuperscript{130,131}. Desmosomes are another cell-cell adhesion structure, which can suppress invasion\textsuperscript{132}. However, overexpression of certain adhesion proteins such as claudin\textsuperscript{113} and desmoglein\textsuperscript{313,134} can enhance invasion.

Although degradation of the extracellular matrix, especially the basement membrane, is a key property of invasive tumors, interaction with the extracellular matrix is also important for invasion and prognosis\textsuperscript{135–138}. Knockdown of the integrin beta 1 subunit (\textit{ITGB1}) using siRNAs can reduce MMP2 activity and invasive capability\textsuperscript{139}, mimicking the ability of miR-124, which has been shown to target ITGB1 and reduce invasion\textsuperscript{140}. The integrin alpha v beta 6 (\textit{AVB6}) is upregulated in wound healing, and inhibition of AVB6 also inhibits invasion\textsuperscript{141–143}. Integrin AVB6 is important in inducing protease expression\textsuperscript{142,144}. Conversely, expression of specific ECM proteins such as collagen\textsuperscript{145}, collagen XVI\textsuperscript{146,147}, or laminin\textsuperscript{148} can enhance invasion. Binding of integrins to these matrix molecules triggers a signaling cascade including integrin linked kinase (ILK)\textsuperscript{149}, talin\textsuperscript{92,150}, focal adhesion kinase (FAK)\textsuperscript{151–153}, and Src family members\textsuperscript{90,154} that then link to the MAP kinase and PI3 kinase pathways for stimulation of MMPs\textsuperscript{82,145,149,154}. It is unclear why both growth factor signaling and adhesion signaling are needed for invasion – the connection between adhesion and growth factor signaling in invadopod formation is an active area of investigation\textsuperscript{155–159}.

MicroRNAs that regulate HNSCC invasion

MicroRNAs (miRNAs) are a class of gene expression regulators that often have altered expression in various human cancers, including HNSCC\textsuperscript{160–165}. MiRNAs have emerged as having key roles in diverse cellular processes, including cancer cell proliferation, migration, invasion and metastasis\textsuperscript{160,161,166,167}. Dicer, a key enzyme involved in siRNA and miRNA function, was reduced in expression in tongue SCC lines, and reduction of Dicer led to increased cell proliferation and invasion\textsuperscript{168}. Consistent with these observations, the majority of the miRNAs that have been found to affect invasion in HNSCC are inhibitory (see Table 1).

The targets of the miRNAs that have been identified thus far can be categorized according to a number of functions involved in invasion. A large number of miRNAs target proteins
associated with signal transduction including ligands, receptors, and intracellular regulators. MiR-126 was found to downregulate protein levels of ligands epidermal growth factor-like domain 7 (EGFLD7), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (FGF2). Dickkopf 2 (DKK2), a target of miR-21, promotes cell invasion by antagonizing Wnt/beta-catenin signaling. MiR-99a was found to be a metastasis suppressor in OSCC by regulating expression of the insulin-like growth factor 1 receptor (IGF1R). The regulation of neurofibromin 1 (NF1) by miR-193b impaired cell migration and invasion via inhibiting Erk1/2 phosphorylation. MiR-155 promotes the migration and invasion of laryngeal SCC. Knockdown of miR-155 in LSCC cells suppressed invasion by negatively regulating suppressor of cytokine signaling 1 (SOCS1), which allowed increased STAT3 signaling. HNSCC cells overexpressing miR-107 had reduced cell invasion in vitro and tumor growth in vivo resulting from the targeting of protein kinase C epsilon (PRKCE).

Many adhesion proteins are also targets of miRNAs. Laminin beta 3 (LAMB3), a component of laminin-332 in the extracellular matrix, is targeted by miR-218. MiR-29s (miR-29a, miR-29b and miR-29c) target another component of laminin-322, laminin gamma 2 (LAMC2). MiR-29a also target adhesion molecule integrin alpha 6 (ITGA6) and miR-29a was found to upregulate the ECM protease MMP2. ADAM10, another protease, is a target of miR-140–5p. There is also a significant group of miRNAs whose targets are associated with cell protrusions and the cytoskeleton, including transgelin 2 (TAGLN2), targeted by miR-1, moesin (MSN) and actin-related protein 2/3 complex subunit 5 (ARPC5), targeted by miR-133a, and podoplanin (PDPN) targeted by miR-363. The cytoskeleton proteins, Ras homology family member C (RHOC) and Rho-associated coiled-coil containing protein kinase (ROCK2) are targeted by miR-138, which also targets the EMT-associated protein vimentin (VIM). Other miRNA targets associated with EMT in terms of general gene expression are twist basic helix-loop-helix transcription factor 1 (TWIST1) and forkhead box C1 (FOXC1), targeted by miR-181a and miR-639.

Transcription factors that may regulate genes associated with motility and invasion are also targets of miRNAs. MiR-29b suppresses Sp1 expression, which impaired OSCC invasion via reduced Akt activation. MiR-375 targets metadherin (MTDH) and miR-504 targets forkhead box 1 (FOXP1), also important for invasion. Finally, there is a small group of targets, whose direct function in invasion are unclear, such as programmed cell death 4 (PDCD4) targeted by miR-21, BCL2 binding component 3 (BBC3) and manganese superoxide dismutase 2 (SOD2) targeted by miR-222.

### Invadopodia as mediators of HNSCC invasion

Invadopodia (Figure 2) are specialized actin-rich structures that mediate extracellular matrix (ECM) proteolysis and are thought to play a key role in cell invasion. An important component and marker of invadopodia is cortactin. HNSCC cells that have chromosome 11q13 amplification and subsequently overexpress cortactin have elevated binding and activity of Arp2/3 complex together with increased HNSCC cell motility and invasion. The EGFR inhibitor, gefitinib, impairs motility in HNSCC cells with the degree of inhibition positively correlated with the level of cortactin in the cells. High levels of cortactin

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reduce EGFR down-regulation and extend ERK activation\textsuperscript{203}. Cortactin is important for both invadopodium assembly and ECM degradation\textsuperscript{204}. Cortactin was shown to promote MMP2 and MMP9 secretion, as well as the cell surface levels of MT1-MMP in invadopodia of HNSCC cells\textsuperscript{204}. Figure 3 shows an example of an \textit{in vitro} assay to assess invadopodial matrix degradation, with cortactin and Tks5 as markers for invadopodia.

Src is an important regulator of invadopodium formation and activity. Saracatinib, a Src inhibitor, inhibited Src activation and phosphorylation of FAK, p130 CAS and cortactin in HNSCC cells along with reduction of invadopodia formation, ECM degradation, and MMP9 secretion\textsuperscript{91}. Intriguingly, although constitutively active Src induces invadopodium formation, wild-type Src expression is also needed in HNSCC cells for invadopodium-associated matrix degradation\textsuperscript{205}. Abelson (Abl) kinase activity diminishes invadopodial ECM degradation in HNSCC cells: the inhibition of Abl and Arg by imatinib, an Abl family inhibitor, led to EGFR activation through increased HB-EGF production and shedding, and as a result of increased EGFR signaling, Src and ERK activity induced tyrosine and serine phosphorylation of cortactin\textsuperscript{55}. These findings differ from a proposed model of EGFR-invadopodia signaling in breast cancer where elevated EGFR signaling induces Src activation, which subsequently leads to activation of Abl/Arg and tyrosine phosphorylation of cortactin by Abl/Arg\textsuperscript{206}. In addition to the EGFR\textsuperscript{207}, transforming growth factor beta receptor 1 (TGFBR1) can induce invadopodium formation through stimulation of insulin-like growth factor II mRNA binding protein 3 (IMP3) and podoplanin (PDPN), both of which are important for invadopodium formation, ECM degradation and MMP9 activity\textsuperscript{73,208}.

PI3K activity also regulates the formation and activity of invadopodia\textsuperscript{209,210}. It has been shown in breast cancer cell lines that PI3K signaling induced formation of invadopodia, and specifically the p100 alpha catalytic subunit of PI3K was responsible for the effect\textsuperscript{210}. In HNSCC cells, the mechanism of PI3K signaling differs from the breast cancer model\textsuperscript{209}. LY294002, a PI3K inhibitor, suppressed invadopodia numbers and ECM degradation, as well as focal adhesion numbers and size. Stimulation of invadopodia by PI3K may be in part due to production of P(3,4)P\textsubscript{2} from PIP\textsubscript{3} through the action of SH2-containing inositol 5'-phosphatase (SHIP2)\textsuperscript{209}. Expression levels of formin homology domain protein 1 (FHOD1), an actin nucleating protein, are dependent upon PI3K\textsuperscript{211}. Silencing FHOD1 reduced cell migration, invasion, invadopodium formation and invadopodium-mediated ECM degradation\textsuperscript{211}. Protein kinase C alpha (PKCA) can provide feedback negative regulation of cells with mutant PI3K, although in cells with wild-type PI3K, PKCA appears to stimulate invadopodium formation\textsuperscript{209}.

Adhesion signaling has also been shown to be important for invadopodium formation and maturation. Adhesions rings form around invadopodia and are recruited after invadopodium formation\textsuperscript{114}. Integrin activity and ILK are needed for adhesion ring formation, MT1-MMP accumulation in invadopodia and invadopodia-associated ECM degradation\textsuperscript{114}. MT1-MMP is necessary for ECM degradation, but not for the adhesion ring formation. Invadopodia are key docking sites for multivesicular endosomes (MVEs) containing smaller secreted vesicles termed exosomes, and are secretion sites for exosomes\textsuperscript{212}. Exosomes were also shown to
promote invadopodia formation, enable the exocytosis of MT1-MMP at invadopodia and stimulate invadopodia-associated matrix degradation\textsuperscript{212}.

**Epithelial to mesenchymal transition, cancer stem cells, and invasion**

Epithelial to mesenchymal transition (EMT) is a highly regulated process in which epithelial cells acquire a mesenchymal morphology through coordinated changes in gene and protein expression that lead to decreased cell adhesion and cell polarity, resulting in a more invasive phenotype\textsuperscript{26,213–219}. Common protein indicators of EMT are decreased expression of E-cadherin, and increased expression of N-cadherin and vimentin. The expression of these genes is controlled by transcription factors including Snail, ZEB, and Twist. There are also changes in cellular proteins such as integrins and alpha-smooth muscle actin, as well as ECM proteins, such as collagen, laminin, and fibronectin\textsuperscript{149,213–216,220–223}. HNSCC cells that undergo EMT have been shown to be more invasive\textsuperscript{57,77,152,215,217,221–224}. The loss of E-cadherin associated with EMT has shown to be an indicator of poor prognosis in HNSCC\textsuperscript{26}.

EMT can induce the generation of a sub-population of cells that have the potential for tumor initiation, called cancer stem cells (CSCs)\textsuperscript{225,226}. CSCs exhibit the properties of self-renewal, potential to behave as tumor progenitor cells, and the ability to differentiate \textsuperscript{223,225–227}. CSCs are slow-dividing, and therefore often more resistant to chemotherapy\textsuperscript{225–227}. CSCs are found in invasive fronts, and in many tumors are near the blood vessels\textsuperscript{217,223,225–227}. EMT can generate cells within tumors that have stem-like properties\textsuperscript{216,219,223,228}. In head and neck cancer, Twist, a regulator of EMT, was found to induce expression of Bmi-1, a regulator of stemness\textsuperscript{219,228}. Twist and Bmi-1 can act together to repress expression of E-cadherin and p16\textsuperscript{INK4a}. Higher expression of Twist and Bmi-1 has been correlated with lower E-cadherin and p16\textsuperscript{INK4a}, and was associated with poor prognosis\textsuperscript{219}.

There are a number of common molecules and pathways involved in invasion, EMT, and stemness. TGF beta regulates transcription of EMT transcription factors via SMADs\textsuperscript{229}. CD44, a cell surface glycoprotein that binds hyaluronic acid, is involved in cell-cell crosstalk, cell adhesion, and migration, and interacts with c-Met and EGFR\textsuperscript{227,230}. EGF can induce EMT in HNSCC cell lines through PI3K/Akt signaling\textsuperscript{223}. Expression of Bmi-1, ALDH, and CD44 also increases with EGF treatment, indicating an acquisition of stem-like properties. Notch signaling can induce EMT under hypoxic conditions, with increased expression of Notch pathway molecules and Snail, decreased expression of E-cadherin, and an increase in invasiveness\textsuperscript{77}. In addition to inducing EMT and increased invasiveness, Snail has also been shown to induce stem-like properties in OSCC\textsuperscript{224}. Adhesion-related signaling molecules that are important for EMT and invasion include ILK\textsuperscript{149} and FAK\textsuperscript{152}. In terms of proteases, membrane type 1 matrix metalloproteinase (MT1-MMP/MMP14) has been shown to induce EMT and stem-like properties in OSCC as well as invasion, in parallel with increased levels of Twist and ZEB\textsuperscript{221}. However, there may be subtypes of cancer stem cells, which differ in their invasive ability\textsuperscript{231}.
One aspect of invasion that needs more examination in HNSCC is collective invasion, in which cell-cell contacts may be maintained during invasion. Three-dimensional reconstructions of the invasive front of 14 oral tongue SCCs reveal that most invasive tumor cells are in multicellular structures of variable size, suggesting the presence of cell-cell contacts. There is an ongoing debate regarding the relative importance of single cell and collective invasion for patient prognosis. One possibility is that a partial EMT could maintain cell-cell contacts but stimulate invasive properties sufficient to generate a more aggressive tumor that results in a worse prognosis.

Tumor microenvironment and invasion

Cancer associated fibroblasts (CAFs) are the major stromal cells present in the microenvironment of HNSCC, and these tend to be myofibroblasts showing increased expression of alpha-smooth muscle actin. Myofibroblasts and CAFs have been shown to enhance HNSCC invasion in vitro in a variety of assays. Treatments that can enhance the ability of fibroblasts to stimulate tumor cell invasion include irradiation and reactive oxygen species (ROS), as well as lifestyle-correlated factors such as cigarette smoke and areca nut extract. Fibroblasts have been shown to stimulate tumor cell invasion through secretion of a number of factors, including chemokine (C-C motif) ligand 2 (CCL2), CCL7, stromal cell-derived factor-1 (SDF1), TGFB, IL33, MMP2, MMP3, MMP9, and hepatocyte growth factor (HGF). CAFs can also produce extracellular matrix molecules that enhance invasion, such as thrombospondin-1 and fibronectin. The mechanism of stimulation of tumor cell invasion by CAFs can include induction of EMT.

In a number of studies, a paracrine interaction between tumor cells and fibroblasts has been shown to stimulate invasion. For example, CCL2 from fibroblasts can stimulate invasion and ROS production by tumor cells, which in turn stimulates fibroblast senescence and CCL2 production from the fibroblasts. Galectin production by tumor cells can also stimulate CCL2 production from fibroblasts. IL1 from OSCC stimulates CCL7, HGF and TGFB production by fibroblasts, while endothelin stimulates ADAM17 mediated release of EGFR ligands. Interestingly, there is a report of TGFB from tumor cells inducing HGF production by fibroblasts.

Other features of the tumor microenvironment have also been identified as stimulating HNSCC invasion, although not as thoroughly as CAFs. The perivascular niche has been associated with cancer stem cells and invasion. Endothelial cells can secrete EGF to induce EMT and invasion. Tumor associated endothelial cells have been shown to stimulate invasion of HNSCC cells in vitro through the secretion of IL8 and CXCL1. Macrophages have been shown to stimulate Axl-mediated invasion and perineural invasion through production of GDNF. However, macrophages did not enhance invasion in response to EGF in an in vivo invasion assay. Hypoxia in the tumor microenvironment can induce Notch and EMT factors, such as Slug and Snail, to increase invasion. MMP9 can be induced by hypoxia to enhance invasion in an NHE1-dependent fashion. Hypoxia-inducible factor (HIF) 2 alpha leads to EGFR activation.
while HIF 1 alpha increases integrin alpha 5 and fibronectin\(^{277}\) in invasion. Of concern for treatments utilizing erythropoietin (EPO) to mitigate chemotherapy side effects, hypoxia can induce the EPO receptor in tumor cells, enabling EPO to stimulate tumor cell invasion\(^{278}\).

**Genomic changes in invasion-associated genes**

DNA changes such as mutations and copy number variations (CNVs) at the genomic level have been implicated in cancer progression\(^{279}\). Higher numbers of CNVs in the genome are associated with the development of cancer, and more CNVs are accumulated with tumor progression\(^{279}\). It has been shown in HNSCC that disease-specific survival and recurrence can be predicted by CNVs\(^{280}\). We used HNSCC TCGA data downloaded from the UCSC Cancer Browser to evaluate the genes discussed in this review\(^{281}\). The 25 genes with the highest copy number variation or number of mutations in HNSCC discussed here are presented in Table 2. PIK3CA is top in copy number increase and second in mutations, with \(EGFR\) and \(RAC1\) also being high in both mutations and copy number increases, emphasizing the potential importance of these genes in HNSCC.

**Conclusions**

Studies of invasion have identified a number of possible applications in the treatment of HNSCC. The most straightforward approach is to develop specific inhibitors targeted to molecules that are identified as being important in invasion. In particular, proteases such as MMP2, MMP9 and MMP14 are commonly identified as being important for invasion, and thus could be targeted for therapeutic development. However, there has been extensive development of broad spectrum MMP inhibitors with very limited success\(^{282}\). Inhibition of the beneficial roles of MMPs resulted in unacceptable side effects\(^{283}\), and thus there is now caution in considering therapeutics targeting MMPs, with the assumption that such treatments likely need to be brief in order to avoid side effects caused by longer term treatments\(^{284}\). A possible use for brief treatments could be in counteracting the effects of radiation. Radiation has been found to induce migration and invasion\(^{285}\), and treatment with invasion inhibitors during radiation treatment could then potentially enhance its efficacy\(^{286,287}\). Inhibitors targeting \(EGFR\)\(^{288}\), \(Na^+/H^+\) exchanger 1 (NHE1)\(^{275}\), Src\(^{91}\) and microtubules\(^{289}\), can inhibit invasion at concentrations which are not toxic to cells and could be useful in counteracting radiation-induced spread.

For longer term treatments, potentially lower toxicity compounds for inhibiting invasion have been identified whose targets are unknown. These include extracts from herbs, green tea and other natural sources\(^{41,101,290–300}\). Such compounds could constrain further tumor spread and extend patient survival without directly killing tumor cells. Limiting tumor spread could develop additional benefits due to the consequences of keeping tumor cells clustered together. As noted above, there is an intimate relationship between invasion, EMT, and stem cell properties. It is possible that by inhibiting invasion of HNSCC cells, there is partial reversion of EMT and loss of stemness, which could result in greater sensitivity to cytotoxic treatments. Compounds have been identified which on their own inhibit invasion and EMT without affecting viability\(^{292}\) but then sensitize HNSCC cells to cisplatin\(^{292,301}\).

Identification of invasion inhibitors which can enhance sensitivity to chemoradiation while
suppressing the spread of tumor cells would provide a valuable addition to the therapy options of head and neck cancer patients.

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Figure 1. Signaling pathways in HNSCC invasion involving the EGFR. Epidermal growth factor receptor (EGFR), activated by EGFR ligands, can activate ERK/AP1, PI3K/Akt, or Src activity, which all lead to increased invasion in head and neck cancer. EGFR can be cross-activated by GPCRs, whose downstream signaling leads to the release of EGFR ligands from their transmembrane precursors through activity by the ADAM family of proteases.
Figure 2. Proteins that contribute to invadopodium structure and function.
Invadopodia are actin-rich structures that specialize in mediating the degradation of the ECM. Their formation can be induced by EGFR signaling. The key components of the core structure are cortactin, Tks5, N-WASP, Arp2/3, Nck-1 and −2, and cofilin. Adhesion rings, made up of adhesion proteins such as integrins and ILKs, are important for the formation and stabilization of invadopodia. Proteases, such as MMP2 and MMP9, are secreted at invadopodia. MT1-MMP can be transported by exosomes, which are secreted from multivesicular endosomes.
Figure 3. Invadopodial matrix degradation assay. UMSCC1 cells, a human oral cavity squamous cell carcinoma cell line, were plated onto an Alexa Fluor-405 labeled gelatin matrix. After 4 hours the cells were fixed and stained for cortactin and Tks5, two invadopodia markers. Representative images of the degraded fluorescent matrix (A) Tks5 (B) and cortactin (C) and the merged (D) (cortactin: red, Tks5: green, matrix: blue) staining at 60X magnification. The merged image shows colocalization of Tks5- and cortactin-rich structures associated with degradation holes in the Alexa Fluor-405 matrix.
Table 1:
MicroRNAs (miRNAs) That Affect Invasion in Head and Neck Squamous Cell Carcinoma

| miRNA   | Effect on invasion | Target                          | Function                        | Reference |
|---------|--------------------|---------------------------------|---------------------------------|-----------|
| hsa-miR-1 | Reduction          | TAGLN2                          | Cytoskeleton                    | 178       |
| hsa-miR-107 | Reduction        | PRKCE                           | Signaling                       | 174       |
| hsa-miR-29a,b,c | Reduction      | LAMC2, ITGA6, MMP2, SP1         | Adhesion, Proteolysis, Gene expression | 148,176,188 |
| hsa-miR-99a | Reduction        | IGF1R                           | Signaling                       | 171       |
| hsa-miR-126 | Reduction        | EGFL7, VEGF, FGF2               | Signaling                       | 169       |
| hsa-miR-133a | Reduction       | CAV1, MSN, ARPC5                | Cytoskeleton                    | 179,180,302 |
| hsa-miR-138 | Reduction        | RHOC, ROCK2, VIM, ZEB2, EZH2    | Cytoskeleton, EMT                | 212,213,184,185 |
| hsa-miR-140–5p | Reduction      | ADAM10                          | Proteolysis                     | 177       |
| hsa-miR-181a | Reduction       | TWIST1                          | EMT                             | 187       |
| hsa-miR-218 | Reduction        | LAMB3                           | Adhesion                        | 175       |
| hsa-miR-363 | Reduction        | PDPN                            | Cytoskeleton                    | 181       |
| hsa-miR-375 | Reduction        | MTDH                            | Gene expression                 | 189,193   |
| hsa-miR-491–5p | Reduction     | GIT1                            | Adhesion                        | 303       |
| hsa-miR-639 | Reduction        | FOXC1                           | Gene expression, EMT            | 194       |
| hsa-miR-21  | Increase         | DKK2, PDCD4                     | Signaling, Protein Synthesis    | 97,170,195 |
| hsa-miR-134 | Increase         | WWOX                            | Cell death                      | 304       |
| hsa-miR-155 | Increase         | SOCS1                           | Signaling                       | 173       |
| hsa-miR-193b | Increase         | NF1                             | Signaling                       | 172       |
| hsa-miR-504 | Increase         | FOXP1                           | Gene expression                 | 194       |
| hsa-miR-222 | Mixed            | BBC3, SOD2                      | Cell death/ROS                  | 196,197   |

Abbreviations: EMT – epithelial to mesenchymal transition, ROS – reactive oxygen species
Table 2:
Ranking of the 25 Genes With the Greatest Number of Mutations or Copy Number Variation (CNV) in Head and Neck Squamous Cell Carcinoma

| Gene     | CNV Rank | Mut Number | Copy Number Variation Rank | CNV Rank | Mut Number |
|----------|----------|------------|---------------------------|----------|------------|
| CDKN2A   | −0.77299 | 66         | PIK3CA                    | 0.919765 | 64         |
| PIK3CA   | 0.919765 | 64         | CLDN1                     | 0.896282 | 0          |
| NOTCH1   | 0.166341 | 59         | PTK2                      | 0.827789 | 4          |
| EGFR     | 0.485323 | 14         | MTDH                      | 0.704501 | 2          |
| NOTCH3   | −0.04892 | 14         | PIK3CB                    | 0.696673 | 5          |
| NOTCH2   | −0.03327 | 13         | SNAI2                     | 0.614481 | 1          |
| FN1      | −0.20744 | 13         | CTTN                      | 0.571429 | 4          |
| TLN1     | 0.009785 | 12         | EGFR                      | 0.485323 | 14         |
| ITGB1    | −0.18395 | 10         | GDNF                      | 0.401174 | 1          |
| RAC1     | 0.373777 | 9          | RAC1                      | 0.373777 | 9          |
| NF1      | 0.060665 | 9          | SRC                       | 0.362035 | 1          |
| ZEB2     | 0.050881 | 9          | TWIST1                    | 0.358121 | 1          |
| PIK3CG   | 0.23092  | 8          | IGFBP3                    | 0.34638  | 1          |
| HGF      | 0.203523 | 8          | SNAI1                     | 0.320939 | 0          |
| ZEB1     | −0.18591 | 8          | MMP9                      | 0.318982 | 0          |
| TLR4     | 0.242661 | 7          | PLCG1                     | 0.313112 | 5          |
| ABL2     | 0.195695 | 7          | EPO                       | 0.293542 | 2          |
| IGF1R    | 0.023483 | 7          | WNT5B                     | 0.25636  | 1          |
| COL1A1   | 0.107632 | 6          | LPAR5                     | 0.25636  | 0          |
| ROCK2    | 0.099804 | 6          | TLR4                      | 0.242661 | 7          |
| COL16A1  | −0.03523 | 6          | ABL1                      | 0.238748 | 0          |
| AXL      | −0.04501 | 6          | LPAR1                     | 0.236791 | 2          |
| THBS1    | −0.08023 | 6          | FOSL1                     | 0.236791 | 0          |
| EGF      | −0.20548 | 6          | PIK3CG                    | 0.23092  | 8          |
| PIK3R1   | −0.34442 | 6          | VAV2                      | 0.23092  | 1          |