

Chapter 2

Head and Neck Cancer

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Abstract Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer worldwide. Although advance of conventional and development of new therapeutic approaches, including fractionated radiotherapy, targeted chemotherapy and concurrent radiotherapy and chemotherapy, the improvement in overall survival in patients with HNSCC is still low. HNSCCs often metastasize to locoregional lymph nodes, and lymph node involvement represents one of the most important prognostic factors of poor clinical outcome. Experimental models of the HNSCC and its spread via lymphatic system is an essential research tool used to study all steps of HNSCC progression and evaluation of new therapeutic approaches. This chapter provides with short review of molecular events implicated in metastatic spread of the HNSCC and presents experimental approaches with emphasis on in vivo models. Importantly, methods to model and to visualize the spread of HNSCC into sentinel lymph nodes are presented.

Abbreviations

HNSCC	head and neck squamous cell carcinoma
MMPs	metalloproteinases
HPV	human papillomavirus
ECM	extracellular matrix
UADT	upper aerodigestive tract
DMBA	dimethy-1,2 benzanthraccene
4NQO	4-nitroquinoline 1-oxide
TPA	12-O-tetradecanoylphorbol-13-acetate

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2.1 Introduction

Head and neck cancer, which generally comprises the cancers of the oral cavity, pharynx and larynx, affects 49,260 Americans each year, with an average five-year survival of approximately 61 % and an estimated 11,480 deaths a year [1]. The vast majority of these cancers is of squamous epithelial cell origin and is called head and neck squamous cell carcinomas (HNSCC *or* SCCHN) [2]. The major risk factors and causative etiologies for HNSCC are exposure to tobacco, alcohol, and, increasingly, the human papillomavirus (HPV). Metastatic spread is primarily lymphatic, with spread primarily to locoregional lymph nodes, although rarely distant organs [3], and locoregional metastasis is a very poor prognostic indicator [4]. While 5-year survival for cancer overall is improving over the past 3 decades, head and neck cancers survival has been stagnant [1], due in large measure to the difficulty in obtaining regional control of the disease and preventing metastasis. Furthermore, once metastasis or recurrence occurs, few treatment options exist beyond palliation, and those that exist have not been shown to be very effective [5]. Thus, having appropriate preclinical models for HNSCC metastasis is of vital importance for developing new strategies for preventing metastasis spread and improving locoregional control. In this chapter, we explore the research into various *in vitro* and *in vivo* HNSCC models and attempt to explain the advantages and limitations of each.

2.2 Molecular Mechanisms Of Local Invasion and Metastasis

Understanding the invasion-metastatic cascade provides a basic framework of analyzing the metastatic model of cancer, including HNSCC. This model depicts a succession of cellular-biologic changes, starting with dissociation of cells from their primary site followed by local invasion of cells into the nearby lymphatic or hematogenous system which allows these cells to extravasate into the distant host parenchyma [6]. These cancer cells interact with the host tissue and acquire a distinct tumor microenvironment which leads to autonomous growth of cancer from micrometastasis to clinically evident macrometastasis. The following discussion focuses on the various putative proteins and factors responsible for the metastatic cascade in HNSCC in the various stages of metastasis.

The ability of cancer cells to dissociate from their primary host tissue is a prerequisite for metastasis. E-cadherin is a key cell-to cell adhesion molecule which facilitates the formation of adheren junctions with adjacent epithelial cells and hence, maintains the quiescence of the cells within these sheets [7, 8]. Therefore, downregulation or dysfunction of E-cadherin results in a functional loss of E-cadherin-mediated cell adhesion and enhances the ability of cells to dissociate from the primary tumor. Moreover, it is evident that as carcinomas arising from epithelial tissues progressed to higher pathological grades of malignancy, they typically developed alterations in their shape as well as in their attachment to other cells and

to the extracellular matrix (ECM). Given these information, preclinical studies on HNSCC have demonstrated a link between downregulation of E-cadherin expression and metastasis by either correlating loss of expression in the primary tumor with positive lymph node status or by revealing a significant loss of expression in metastatic lymph nodes compared with primary tumors [9–12].

Dysadherin, a recently characterized cancer related glycoprotein, has been shown to down-regulate E-cadherin protein and thus, function as an “anti-adhesion” molecule [13]. This downregulation appears to occur in a post-transcriptional level without affecting the mRNA levels of the protein [6, 13, 14]. In HNSCC, dysadherin immunostaining is seen in the membranes of the cancerous cells and is predominantly localized in the intercellular borders of cancer cells and in poorly differentiated tumors [14, 15]. Muramatso and colleagues established an association between dysadherin and prognosis of HNSCC patients treated with radiation therapy. They found that patients with tumors that expressed higher level of dysadherin achieved a statistically higher complete response rate compared to those with lower expression levels of dysadherin. This study also found that patients who showed high level of dysadherin and low levels of E-cadherin had higher risks of having cervical metastasis at the time of diagnosis.

The movement of cancer cells from the site of origin into lymphatic or vascular channels require the breakdown of ECM that normally acts to providing an anchoring mechanism. This process has been found to be dependent on metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that degrade components of the basement membrane and ECM. All MMPs share the pro-domain and the catalytic domains, and can act on broad spectrum substrates within the ECM [7]. They are classified into secreted (soluble) and membrane-anchored MMPs; both types are initially synthesized as pro-enzymes, which then require activation either extracellularly or intracellularly. Among the 20 plus MMPs that have been characterized, MMP2 and MMP9 overexpression in HNSCC tumor samples has been correlated with invasion, metastasis, and poor prognosis [10, 16]. These studies also found that the expression of MMPs correlated inversely with the amount of ECM found around the tumor cells. Similarly, high protein expression levels of MT1-MMP, a membrane-bound metalloproteinase have also been linked to HNSCC metastasis. MT1-MMP itself has proteolytic activity against ECM components but is also important in the activation of MMP2 [12].

Although the mechanisms responsible for MMP upregulation and activation in HNSCC are not fully elucidated, interaction with tissue inhibitors of metalloproteinases (TIMPs) has been shown to be partly responsible in regulating the functional activity of MMP. The anti-invasive effects of TIMPs have been studied in HNSCC and levels of TIMP-1 have been found to be higher in HNSCC tumor specimens matched with non-metastatic cases than in metastatic cases [17]. More recently, MMP-10 has been shown to be functionally upregulated in metastatic HNSCC in an *in vitro* model [18]. In a study that utilized a combination of microarray and immunohistochemistry analysis, the overexpression of MMP-10 was found to promote the invasion of HNSCC cells *in vitro*. Conversely, the downregulation of MMP-10 resulted in the inhibition of invasion of HNSCC cells.

Vascular endothelial growth factor (VEGF) is classically thought of as a proangiogenic protein that is produced in response to hypoxia. Tumor-produced VEGF is responsible for promoting ingrowth of tumor vessels. Overexpression of VEGF has been reported in many solid tumors, including HNSCC, and is generally associated with increased tumor progression, increased resistance to chemotherapy, and poor prognosis. Although the VEGF family of cytokines modulate metastasis via the promotion of angiogenesis, recently studies have shown that VEGF may also have autocrine effects on tumor cells. Several tumor types, including HNSCC, have been found to express VEGFRs and the activation of tumoral VEGF/VEGFR axis has been shown to promote migration and invasion of the tumor cells [19]. Conversely, several studies have shown that the downregulation of VEGF leads to a decrease in invasion and migration, suggesting that VEGF acts to promote invasion and migration [7, 20, 21]. The promotion of migration and invasion of cancer cells by VEGF appears to be due to the ability of VEGF to promote epithelial-mesenchymal transition (EMT) [22]. A study by Bock et al showed that the overexpression of VEGF-C in HNSCC cell line SCC116 resulted in a 30% higher baseline rate of invasion compared with that in control cells, and that downregulation of VEGF-C expression by short hairpin RNA led to decreased invasion [23]. Given that hypoxia a stress condition for tumors cells, it not surprising that VEGF has an autocrine effect on tumor cells with the end-effect being the movement of the tumors cells away from areas of hypoxic stress.

Tumor angiogenesis is a critical aspect of tumor progression and various inhibitors of the VEGF pathway have been developed and have been approved for anticancer therapy. However, it should be pointed out that several preclinical studies have demonstrated increased metastasis of tumors after short-term administration of VEGF inhibitors [24, 25]. The mechanism behind this observation remains unclear but it is thought that the short-term administration of VEGF inhibitors leads to the production of cytokines in the host animal that ultimately results in a prometastatic condition [24, 25].

Cancer stem cells have attracted significant interest among researchers in recent years as evidence have suggested that a small tumor cell subpopulation among the primary tumor mass might be responsible for tumor initiation, growth, maintenance and spreading. These cells are therefore able to self-renew and have immense capability to self-propagate and seed once they are released from the primary host tumor [26]. While the cellular origin of CSC/CIC and the associated molecular pathways are still matter of discussion, there are evidence for the existence of primitive cancer stem cells (pCSC) which are responsible in the development of tumour neo-vasculogenesis [7, 27]. This is due to the fact that vascular endothelial derived growth factor receptor 2 (VEGFR-2) is considered to be the molecular marker of pCSCs [27, 28]. In breast cancer and lymphoma, the process of angiogenesis from bone marrow derived circulating endothelial progenitors have been implicated for sustaining tumor growth and metastasis. These pCSCs and CSCs have the potential of transforming into cells with endothelial characteristics. These observations have resulted increasingly research to extrapolate these observations in HNSCC. There have been some success in propagating and isolating CSCs in HNSCC and the

coming years will likely see a paradigm change in understanding how these CSCs acquire the metastatic features through the molecular signalling pathways.

2.3 In Vitro Models

In vitro models are extensively being used to study HNSCC. Several hundred HNSCC cell lines have been established by various investigators and used to study a broad spectrum of questions related to head and neck cancer. Moreover, several techniques exist for the culture of normal epithelial cells from the upper aerodigestive tract (UADT) and to model the multistep process of malignant transformation using HPV infection, oncogenes over-expression of and/or exposing to various carcinogens. This approach also allows for the comparison of cancerogenic effects of various irritants like tobacco smokes. In order to approximate experimental conditions to real tissue and to simulate histological complexity, three dimensional model systems are being applied.

Culture techniques for growing dissociated primary tumor cells for short term experimental analysis are being used as well. However, most experimental studies are being performed with established cell culture. Unlike many other cancer types, a wide variety of primary and metastatic HNSCC cell lines are available. An extended guide with classification of available cancer cell lines and discussion of important aspects of culturing is review of Dr. Charles J. Lin in “Head & Neck” 2007. All conventional cell culture techniques and assays, like colorimetric cell proliferation, wound-healing, trans-well, zymography assays as well as non-adherent culturing and cell sphere formation presented in other chapters of this issue are applicable for HNSCC research.

2.4 In Vivo Models

The use of an appropriate animal model which can accurately recapitulate the disease process is an essential aspect of anticancer drug discovery, as it allows easy, reproducible testing of agents or mechanistic hypotheses in a wholly intact biological system [29, 30]. One commonly used preclinical model is nude mice with subcutaneous cancer xenografts. However, such models lack the specific interactions that exist between the tumor cells and their native environment—interactions that influence the molecular, pathologic, and clinical features of the tumor [31–35]. Because these distinct interactions are lost or altered when the tumors are established in ectopic sites, it is preferable to establish tumors at orthotopic sites. Furthermore, since orthotopic models recreate the specific subsite and thus the pattern of spread distinct to the cancer of interest, they allow for study of metastasis and the effects of agent that inhibit metastasis. However, there are inherent drawbacks to orthotopic models as well. The cancer cell lines in orthotopic xenograft models already possess

a fully malignant potential when injected into the test animals—thus, orthotopic xenograft models do not allow modeling of the pre-neoplastic processes preceding full malignant transformation. Modeling of such premalignant process requires the use of transgenic (e.g. murine) models.

Additionally, in each of these models, there are a variety of potential methods for assessing both primary tumor burden and metastasis. Identification of the sentinel lymph node and non-invasive imaging of tumor cells which have metastasized to the lymphatics are two methods which are of increasing interest in animal models. These techniques allow the study of the process of metastasis in earlier stages than traditional histology, which may rely on metastatic tumors becoming grossly apparent, by which time the process of metastasis is very far along.

2.4.1 Carcinogen Induction Models

Carcinogenic agents offer a way to induce cancer in an animal model that is similar to the development of cancer in humans, and there are several models in existence. For example, polycyclic hydrocarbon 9,10 dimethy-1,2 benzanthracene (DMBA) may be dissolved in benzene or acetone and administered to the cheek pouch of hamsters. This model, in which DMBA is painted onto the buccal surface of the cheek pouch in hamsters, was first described by Salley [36]. It was later refined by Lin et al. [37] who showed that tumor incidence can be increased (up to 100%) by painting the pouch three times a week for eight weeks followed by painting with arecaidine six times a week for four weeks in order to promote the initial carcinogenesis. Other promotional agents that have been used after initial exposure to DMBA include 4-nitroquinoline 1-oxide (4NQO) and 12-O-tetradecanoylphorbol-13-acetate (TPA), which have been used to produce oral cancer with high frequency [38]. These tumors have been shown to possess many molecular changes seen in human oral cancer. They show increased expression of epidermal growth factor receptor (EGF), transforming growth factor receptor- α (TGF- α), and oncogenic proteins such as ras and p53, as well as an increase in low-molecular weight keratins and proliferative markers [39–43].

Chronic administration of 4NQO to rodents may also be used to produce oral cancer models [44]. 4NQO is a water soluble agent, and thus, it can easily be added to the drinking water of the rodents (requiring less effort than the painting technique above). This method has been shown to induce SCC of the palate, tongue, esophagus, and stomach. These tumors also display several of the molecular changes seen in human SCC, including increased expression of ras, p53, E-cadherin, Bcl-2 and Bax [45–48]. An advantage of this model is that it mimics the development of human oral cancer, with fully malignant SCC being clearly preceded by increasing grades of dysplastic changes. It thus becomes an ideal model for studying premalignant lesions and potential agents that can be used to reverse malignant transformation [49]. However, the reliable development of tumors requires the administration of 4NQO for extended periods lasting over two to three months [44].

One additional disadvantage to all the models above is that the carcinogen induction model does not allow for study of specific genes or protein expression in the process of oral carcinogenesis. For this purpose, xenograft or transgenic mouse models are necessary.

2.4.2 *Orthotopic Models*

The majority of orthotopic models of HNSCC are oral squamous cell carcinoma models in rodents. Fitch et al. first described orthotopic xenograft models of oral SCC in which SCC cells, aspirated from subcutaneous ectopic xenografts in nude mice, were subsequently injected into the tongues of nude mice [50]. Oral SCC cell lines have since been implanted by other means; for example, they may also be injected into the floor of mouth of nude mice transcutaneously [51]. In this technique, which was first reported by Dinesman et al., cells are injected via a submandibular route into the deep tissue surrounding the mylohyoid muscles within the floor of mouth. However, the authors found that almost 40 % of the mice developed pulmonary metastasis while only 5 % of the mice developed cervical lymphatic metastasis [51], numbers that do not replicate the more locoregional pattern of metastasis seen in human HNSCC [3]. One possible explanation for this observation is spillage of the injected tumor cells into the murine vasculature during injection, leading to pulmonary emboli of the tumor cells. Unfortunately, pulmonary lesions produced in this fashion have bypassed the normal process of metastasis in HNSCC and, thus, this model contradicts the concept of orthotopic model.

Myers et al. [52] described another orthotopic model of oral SCC that was produced by injection into the tongues of nude mice, similar to the model by Fitch et al. [50]. However, this model employed submucosal injection of oral cell lines directly into the dorsal tongue of nude mice. The resulting xenografts reproduced several features of human HNSCC (e.g. cervical lymphatic metastasis and disease specific symptoms such as dysphagia and weight loss). More importantly, oral cells injected into the tongues of nude mice had significantly higher tumorigenicity than oral SCC cells injected subcutaneously into the flank. This observation is significant for validating orthotopic xenograft models since the organ-specific tumor-stromal cell interaction that is thought to be lost in subcutaneous ectopic models was able to be reproduced. Similarly, Cabanillas et al. have produced a highly metastatic intraoral and submucosal model using the human glottis cancer line SCC 38 in nude mice, with a 100 % lymphatic and perineural invasion, and 22 % bone destruction and vascular invasion, and, similar to most HNSCC in humans, showed no hematogenous spread [53].

Although the above models all use mice to model oral SCC, other HNSCC models do exist. For example, Bao et al. produced a rat model of human HNSCC through subcutaneous injection of the human HNSCC cell line SCC-4 in athymic nude rats at the level of the scapulae, thus producing a non-oral HNSCC orthotopic model [54]. However, although the size of the animals in this model enabled the

easy use of ^{18}F -FDG PET imaging, no metastases, either cervical or distant, were reported, although this could be due to the particular cell line used.

This last example demonstrates point that it is helpful to use cell lines for in vivo models that have been associated with metastasis. Thus, the HNSCC cell lines used may be of particular interest for metastasis research, and may be from a variety of sources. Human cell lines have been developed from metastases from multiple head and neck squamous cell carcinoma subsites. For example, UM-22B cells were derived from lymph node metastases from hypopharyngeal cancer [55], PCI-37B cells were derived from laryngeal cancer lymph node metastases [56]. Detroit 562 cells were derived from a pharyngeal cancer lung metastasis and are still in use many years after their establishment [57, 58]. UMSCC2 and UMSCC17B lines have been shown to be highly metastatic to locoregional lymph nodes after injection into the tongue in a nude mouse model [59].

A disadvantage of the aforementioned xenograft models is that although the use of human cell lines is an attempt to more faithfully replicate human HNSCC, the use of such lines requires immunosuppression of the animal, typically using either athymic or severe combined immunodeficiency (SCID) mice. Indeed, the use of immunodeficient mice precludes the study of interactions between the host immune system and the tumor. O'Malley et al. [60] proposed bypassing this problem by injecting SCC VII, a murine SCC cell line, into the floor of mouth of syngeneic C3H/HeJ mice. In this way, xenografts have been produced in the floor of mouth that show local invasion into the mandible and mylohyoid muscle, with cervical lymphatic and pulmonary metastasis. However, SCC VII cell lines were later found to have originated not from the oral cavity, but from the abdominal wall of the C3H mouse [61]. Still, these cells are frequently used in head and neck cancer experiments because of their utility in replicating metastatic behavior in the head and neck. Behren et al. recently effectively used GFP transfected SCC VII cells in the floor-of-mouth of BALB/c-NU mice resulting in the formation of invasive tumors that metastasize to regional lymph nodes [62]. Similarly, Yu et al. successfully developed several highly metastatic cell lines through injection of SCC VII cells into mouse auricles and subsequent excision and serial passaging in culture [63]. Matsumoto et al. have taken a similar murine squamous cell line, NR-S1, which is usually poorly metastatic, and through in vivo selection, have developed the highly metastatic line NR-S1M which metastasizes rapidly to locoregional lymph nodes [64]. Judd et al. have since been able to combine both the carcinogen induction approach with the orthotopic xenograft approach and avoid the use of immunosuppression while still having an orthotopic model [65]. The authors used DMBA to produce murine OSCC lines in C57BL/6 mice that were then injected into the either the floor of mouth/buccal region or into the flank in syngeneic mice, and they were able to demonstrated cervical metastasis from orthotopic transplants at the same rate as metastasis to lymph nodes draining the flank [65]. Such combination models may hold promise as they lack some of the drawbacks to the individual models above.

2.4.3 Transgenic Models

Transgenic models hold the advantage of a stable introduction of cancer using specifically targeted oncogenic pathways within the animal of interest, allowing for an intact, relatively untampered biological system. These models allow to study initial steps of cancer development and local spreading, to consider host-tumor interaction and to estimate role of immune system.

There are multiple models that have been produced. The first model type involves the use of the keratin promoter, and there are two such models of oral cancer that have been described. These models utilize the keratin 5 (K5) or keratin 14 (K14) promoter to overexpress the oncogene *K-ras*^{G12D} in the oral epithelium of mice [66, 67]. These two promoters allow for specific orthotopic models due to differences in distribution: K5 is normally expressed both within the basal epithelium of the tongue and the forestomach, whereas K14 is mainly expressed in the basal layer of the oral mucosa and tongue [66]. Subsequently, either promoter is ideal for targeting transgenic expression within the oral cavity.

2.4.3.1 *K-ras*^{G12D}

Vitale-Cross et al. produced an animal model in which the expression of *K-ras*^{G12D} oncogene, driven by K5 promoter, was placed under the control of *tet*-responsive elements, and they were able to induce *K-ras*^{G12D} expression by the administration of doxycycline [67]. Furthermore, they found premalignant lesions of varying dysplasia as well as malignant SCC in the skin, oral mucosa, tongue, esophagus, forestomach, or uterine cervix of the mice [67]. In another model by Caulin et al. [66], the *K-ras*^{G12D} oncogene, this time driven by either K5 or K14 promoter, was placed under the control of a modified Cre recombinase fused to a deletion mutant of the human progesterone receptor. Administration of RU486 in this model resulted in *K-ras*^{G12D} oncogene induction in the oral epithelium of mice. In contrast to the previous model, only squamous papilloma formation was found within the oral cavity [66].

The *K-ras*^{G12D} oncogene has also been used to produce SCC exclusively within the oral cavity [68]. Mice carrying the *K-ras*^{G12D} oncogene construct, under the control of both K14 promoter and tamoxifen-inducible Cre recombinase, were crossed with p53 conditional knockout mice. As early as two weeks after the beginning of tamoxifen treatment, the resulting progeny mice had developed SCC exclusively in the oral cavity [68].

2.4.3.2 *Trp53*^{F/F}; K14Cre myrAkt

There are other oncogenes that may be manipulated as well to produce transgenic models. Constitutive activation of Akt along with downregulation of Trp53 has been

used to Moral et al. to produce an oral cancer models [69]. In this model, the K14 promoter is again used to target the expression specifically to the oral cavity, this time producing constitutively active Akt. The authors found that mice developed pre-neoplastic lesions which progressed to oral SCC which also demonstrated cervical lymphatic and pulmonary metastasis. Perhaps more importantly, the authors showed that the SCC tumors produced this way possessed many of the molecular changes frequently seen in human HNSCC, including the overexpression of epidermal growth factor receptor (EGFR) and Stat3.

It is important to keep in mind that there are several drawbacks to the use of transgenic models, despite the reproduction of some of the major clinical characteristics of head and neck cancer. First, the transgene expression is usually driven through the use of a heterologous promoter, meaning that there are non-physiologic levels of transgene product. Second, the tumor microenvironment in the transgenic mice is significantly different from typical carcinogenesis in that the stromal cells also carry the transgene, as opposed to the mutation only existing in tumor cells. True, the use of oral-mucosa specific promoters (e.g. K5, K14) minimizes the leakage of transgene expression, the intended tissue specificity is unfortunately not absolute. Finally, and most importantly, except in very rare cases, no single gene predominates the process of oral cancer carcinogenesis., and thus this is a flawed system that may have limited applicability. The use of one or two specific genes (e.g. *K-ras* or Akt) to drive the tumor formation will not necessarily reflect the carcinogenic process in humans. For instance, *K-ras* mutations in human head and neck cancer are relatively infrequent, although the presence of *H-ras* mutations in HNSCC has been previously demonstrated [70, 71].

2.5 Sentinel Lymph Node

One important aspect of metastasis modeling is the identification of the sentinel lymph node, whether in the search for early metastasis that is not apparent grossly or by the imaging methods below, or whether the process of lymphatic spread is being studied. Sentinel lymph node mapping for HNSCC is an investigational tool that is not routine in the clinical setting and currently is not the standard of care due to the lack of large randomized clinical trials [72]. However, several recent studies have shown that it has promise and may eventually become standard of care [73–76].

Lymph node identification in mice can be difficult due to their small size and lack of distinct features from surrounding tissue. However, two distinct groups of lymph nodes in the neck can be identified in mice: a superficial group and a deep group of cervical lymph nodes. The superficial group of cervical lymph nodes is located at the lateral border of the submandibular gland. There are two lymph nodes on each side. (Fig. 2.1a). Solitary lymph nodes can be also found under submandibular gland. (Fig. 2.1b). These lymph nodes are the ones most frequently involved in metastasis from tumor xenografts in the oral cavity such as the tongue, floor of

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