CEO Clinical and Experimental Otorhinolaryngology Vol. 2, No. 2: 55-60, June 2009 DOI 10.3342/ceo.2009.2.2.55

Review

Animal Models of Cancer in the Head and Neck Region

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Animal models that resemble the cancers of the head and neck region are of paramount importance in studying the carcinogenesis of these diseases. Although several methods for modeling cancer in the head and neck are available, none are fully satisfactory. Subcutaneous xenograft models of cancer in nude mice are often used in preclinical studies. However, these models are problematic in several aspects as they lack the specific interactions that exist between the tumor cells and their native environment. Establishment of tumors at the orthotopic sites restore these distinct patterns of interactions between the tumor and the host organs that are lost or altered when the tumors are established in ectopic sites. With regard to the transgenic model of cancer in the head and neck region, it should be kept in mind that the transgene used to drive the malignant transformation may not be representative of the carcinogenic process found in human tumors. Low penetrance of tumor formation also translates into high cost and time commitment in performing studies with transgenic models. In this review, we will discuss some of the commonly used methods for modeling cancer in the head and neck region including squamous cell carcinoma of the head and neck as well as thyroid carcinoma.

Key Words. *Head and neck cancer, Animal model, Thyroid carcinoma, Trangenic, Orthotopic xenograft*

INTRODUCTION

The development and evaluation of novel anticancer agents require the use of an appropriate animal model, which can accurately recapitulate the disease process (1, 2). Although subcutaneous xenograft models of cancer in nude mice are often used in preclinical studies, these models are problematic in several aspects. The subcutaneous xenograft models lack the specific interactions that exist between the tumor cells and their native environment that influences the molecular, pathologic, and clinical features of the tumor (3-7). Establishment of tumors at the orthotopic sites restore these distinct patterns of interactions between the tumor and the host organs that are lost or altered when the tumors are established in ectopic sites. Furthermore, the use of orthotopic xenograft models allow for study of metastasis and the effects of agent that inhibit metastasis. However, a limitation of orthotopic xenograft models is that it does not allow modeling of the pre-neoplastic process that precedes full malignant transformation. The cancer cell lines, when injected into the test animals, already carries a fully malignant potential. Therefore, it would be wrong to conclude that there is a progression of the tumor from a premalignant to a malignant stage during the progressive growth of the xenografts. Modeling of such premalignant process requires the use of transgenic murine models. In this review, we will address the various xenograft models as well as transgenic murine models of head and neck cancer that are currently in use.

• Received June 10, 2009
Accepted after revision June 15, 2009

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SQUAMOUS CELL CARCINOMA (SCC) OF HEAD AND NECK

Carcinogen induce model

There are several methods of inducing oral cancer in animals using carcinogenic agents. The first model uses the administration of polycyclic hydrocarbon 9,10 dimethy-1,2 benzanthracene (DMBA), dissolved in benzene or acetone to the cheek pouch of hamsters. The DMBA model was first described by Salley (8) in which DMBA was painted onto the buccal surface of the cheek pouch in hamsters. The technique was later refined by Lin et al. (9) who showed that the tumor incidence can be increased up to 100% by painting the pouch with DMBA three times a week, followed by promotional painting with arecaidine six times a week for four weeks. Others have utilized promotional painting with 4-nitroquinoline 1-oxide (4NQO) or 12-O-tetradecanoylphorbol-13-acetate after initial exposure to DMBA in order to produce oral cancer with high frequency (10). The tumors produced

in this fashion have been shown to carry many molecular changes seen in human oral cancer including increased expression of epidermal growth factor receptor (EGF) and transforming growth factor receptor-α (TGF-α), increased expression of oncogenic proteins such as ras and p53, an increase in low-molecular weight keratins, and increase in proliferative markers (11-15).

Another model of carcinogen induced oral cancer utilizes the chronic administration of 4NQO to rodents (16). 4NQO is water soluble and can be added to the drinking water of the rodents and have been shown to induce SCC of the palate, tongue, esophagus, and stomach. The SCC tumors produced in this fashion also displays some of the molecular changes seen in human SCC including increased expression of ras, p53, E-cadherin, Bcl-3 and Bax (17-20). Furthermore, one advantage of the 4NQO model is that the development of fully malignant SCC is clearly preceded by increasing grades of dysplastic changes that mimic the development of oral cancer in human. This feature of the 4NQO-induced oral cancer model in rodent makes it an ideal model for studying premalignant lesions and agents that can be used to reverse these changes (21). A disadvantage of the rodent 4NQO model is that the reliable development of tumors requires the administration of 4NQO for extended periods lasting over two to three months (16). Furthermore, the detection of premalignant lesions or early SCC within the oral cavity of the test animals can be difficult. Detection methods that use autofluorescence or drug-induce fluorescence has been proposed to overcome this problem and have been shown to be highly sensitive (22, 23). Lastly, carcinogen induced model does not allow for study of a specific gene in the process of oral carcinogenesis. For this purpose, utilization of xenograft model or transgenic mouse model would be necessary.

Orthotopic models

Orthotopic xenograft models of oral SCC was first described by Fitch et al. (24) in which SCC cells aspirated from subcutaneous ectopic xenografts in nude mice were injected into the tongues of nude mice. Another method, described by Dineman et al. (25), involved injection of oral SCC cell lines into the floor of mouth of nude mice transcutaneously. In this technique, the cells were injected transcutaeneously via a submandibular route into the deep tissue around mylohyoid muscles within the floor of mouth. One problematic feature of this model is that almost 40% of the mice developed pulmonary metastasis while only 5% of the mice developed cervical lymphatic metastasis. One explanation for this observation is that there was spillage of the injected tumor cells into the murine vasculature, leading to pulmonary emboli of the tumor cells. Pulmonary lesions produced in this fashion have bypassed the normal process of metastasis and contradicts the concept of orthotopic model.

Myers et al. (26) described an orthotopic model of oral cancer that was produced by submucosal injection of oral SCC cell lines into the dorsal tongue of nude mice. In this model, the resulting orthotopic xenografts produced cervical lymphatic metastasis and produced disease specific symptoms such as dysphagia and weight loss. The authors also showed that oral SCC cells injected into the tongues of nude mice had significantly higher tumorigenicity than when injected subcutaneously into the flank. This is a significant observation as it suggests that production of xenografts in orthotopic locations restores the organ-specific tumor-stromal cell interaction that is thought to be lost in subcutaneous ectopic models.

One disadvantage of above xenograft models is that the use of human cell lines necessitates the use of athymic or severe combined immunodeficiency (SCID) mice. The use of immunodeficient mice precludes the study of interactions between the tumor and the host immune system. In order to circumvent this problem, O'Malley et al. (27) proposed injecting SCC VII, a murine SCC cell line, into the floor of mouth of syngeneic C3H/HeJ mice. The xenografts produced in the floor of mouth showed local invasion into the mandible and mylohyoid muscle. Cervical lymphatic and pulmonary metastatic lesions were also identified. However, it should be noted that the SCC VII cell lines used in this model was later identified as having originated from the abdominal wall of C3H mouse and not from the oral cavity (28).

Transgenic models

Two transgenic mouse model of oral cancer has been described that utilizes the keratin 5 (K5) or keratin 14 (K14) promoter to overexpress the oncogene K-*ras*^{G12D} in oral epithelium of mice (29, 30). K5 is expressed 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 (29). As such, the K5 or K14 promoter is ideal for targeting the expression of transgenes to the oral cavity. In the first model by Caulin et al. (29), the K-*ras*^{G12D} oncogene driven by either K5 or K14 promoter was placed under control of modified Cre recombinase fused to a deletion mutant of the human progesterone receptor. In this fashion, administration of RU486 resulted in induction of the oncogene K-*ras*^{G12D} in the oral epithelium of mice. The authors found that the administration of RU486 to the transgenic mice resulted in the formation of squamous papilloma within the oral cavity. In the second model by Vitale-Cross et al. (30), the expression of K-*ras*^{G12D} oncogene driven by K5 promoter was placed under the control of *tet*-responsive elements, and the expression of K-*ras*^{G12D} was induced by the administration of doxycycline to the test animals. In contrast to the study by Caulin et al. (29), Vitale-Cross et al. (30) 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.

A transgenic mouse model that produced SCC exclusively within the oral cavity has also been described using the K-*ras*^{G12D} oncogene (31) . In this model, the mice carrying K-*ras*^{G12D} oncogene construct under the control of K14 promoter and tamoxifen-inducible Cre recombinase were crossed with p53 condition-

al knockout mice. The resulting progeny mice developed SCC exclusively in the oral cavity as early as within two weeks of tamoxifen treatment.

Another recently described transgenic model of utilized constitutive activation of Akt along with downregulation of Trp53 (32). In this model by Moral et al. (32), the K14 promoter was used to target the expression of constitutively active Akt to the oral cavity. The mice developed pre-neoplastic lesions which progressed to SCC. The oral SCC also demonstrated cervical lymphatic and pulmonary metastasis. More importantly, the authors showed that the SCC tumors showed molecular changes seen frequently in human tumors including the overexpression of epidermal growth factor receptor (EGFR) and Stat3.

Although the models described above appears to reproduce some of the major clinical characteristics of head and neck cancer, several drawbacks needs to be considered. First, the transgenic mice usually have a heterologous promoter driving transgenic expression, leading to no physiologic levels of transgene product. Second, the tumor microenvironment in the transgenic mice is different from human tumors in that the stromal cells also carry the transgene. Although the use of oral-mucosa specific promoters such as keratin 5 or 14 promoter minimizes the leakage of transgene expression, the intended tissue specificity is not absolute. Lastly, no single gene predominates the process of oral cancer carcinogenesis. Therefore, the use of one or two specific genes, such as K-*ras* or Akt, to drive the tumor formation in these transgenic mice may not necessarily reflect the carcinogenic process in human. In fact, the frequency of K-*ras* mutation in human head and neck cancer is relatively low although the presence of H-*ras* mutations in HNSCC has been previously demonstrated (33, 34).

THYROID CARCINOMA

Orthotopic models

Although the orthotopic tumor cell implantation is the xenograft model of choice in modeling human cancer, orthotopic injection of the murine thyroid gland can be technically difficult. Kim et al. (35) previously described an orthotopic model of anaplastic thyroid carcinoma (ATC) in which orthotopic injection of tumor cells into murine thyroid gland was found to be technically feasible and well tolerated by the test animals. In this technique, ATC cells were injected directly into the thyroid glands of nude mice with the use of operating microscope. The authors showed that orthotopic ATC xenografts were produced with 100% frequency at injection concentration as low as 1,000 cells per injection. The xenografts also closely replicated the clinical behavior of ATC in humans, including rapid tumor growth, tracheal and esophageal compression, laryngeal and tracheal invasion, as well as cervical and pulmonary metastasis (Figs. 1 and 2). Moreover, the ATC cells showed significantly higher tumorigenicity when injected orthotopically in the thyroid gland compared with when the cells were injected subcutaneously. Immunohistochemical analysis showed that the orthotopic xenografts had higher microvessel density as well higher expression of proangiogenic factors such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8).

The injection technique described by Kim et al. (35) was modified by Ahn et al. (36) who showed that papillary thyroid carcinoma cell lines (PTC) can also be injected orthotopically in nude mice to produce orthotopic PTC xenografts. In the study by Ahn et al. (36), PTC cell lines containing $BRAF^{V600E}$ or RET/PTC1 rearrangement were used to produce orthotopic PTC xenografts. As with the ATC xenografts, the PTC xenografts reproduced several features of human PTC including laryngeal and tracheal invasion as well as lymphatic metastasis. One limitation of this model is that the tumor is located within the deep tissues of the neck and the detection of tumor difficult in the early stages of tumor growth. Lastly, the use of athymic, nude mice precludes the examination of the interaction between the tumor and host-immune system.

Trangenic models

In contrast to SCC of the head and neck region, the specific mol-

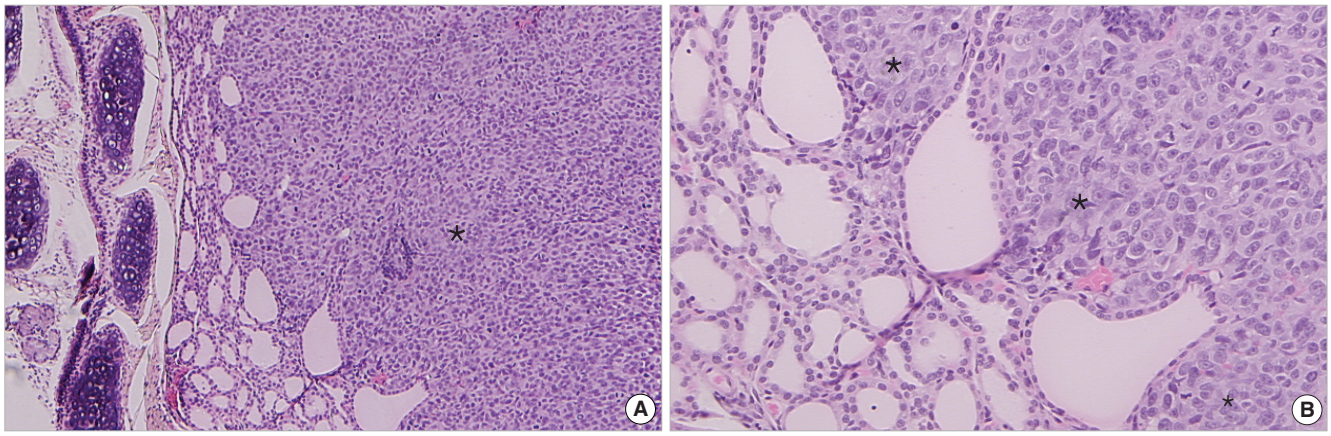

Fig. 1. Invasion of the thyroid gland by tumor (asterisk). (A) H&E stain, original magnification ×40, (B) H&E stain, original magnification ×100.

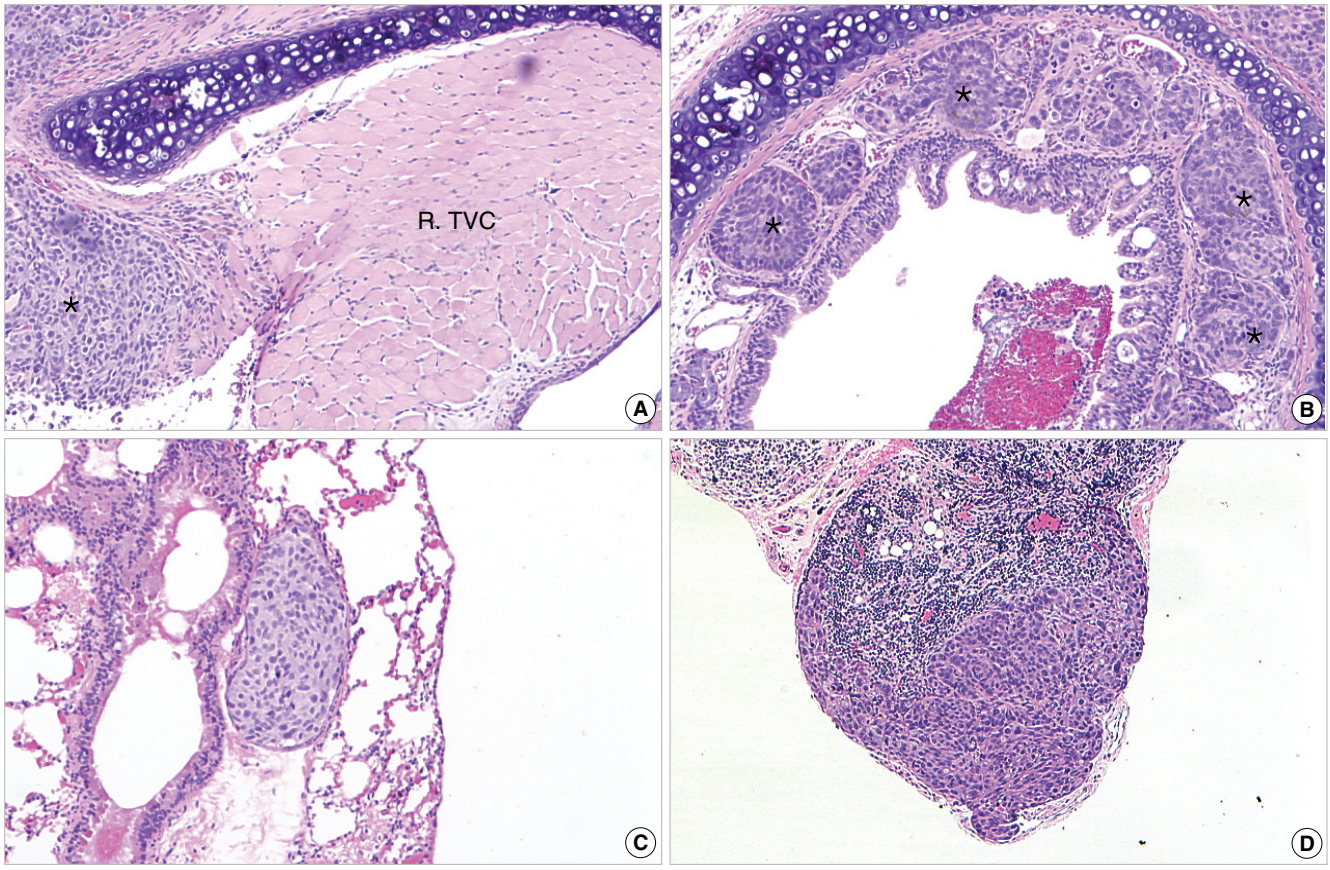

Fig. 2. (A) Axial section of the larynx showing tumor (asterisk) invasion of the paraglottic space via erosion through the inferior constrictor muscles posterior to the thyroid cartilage. (B) Tracheal invasion evident by the nest of tumor cells (asterisk) interpositioned between the tracheal mucosa and cartilage. (C) Pulmonary metastasis. (D) Cervical lymph node with subcapsular metastatic tumor. (A to D: H&E stain, original magnification ×40).

ecular changes underlying the carcinogenesis of thyroid cancer has been well-characterized. Therefore, transgenic models of thyroid cancer using these genes have significant relevancy to the human disease. The two mutations that are found with high frequency in PTC are the V600E mutation within the BRAF gene and the RET/PTC chromosomal rearrangement. $BRAF^{V600E}$ is the most common mutation found in PTC where more than 60% of PTC tumors harbor this mutation. The $BRAF^{V600E}$ mutation results in the constitutive activation of the Ras/Raf/MEK/ERK signaling pathway (37, 38). $BRAF^{V600E}$ in PTC has also been associated with poorer prognosis and higher locoregional recurrence rates (39). Knauf et al. (40) produced a transgenic murine model of PTC by targeting the expression of $BRAF^{V600E}$ to thyrocytes with the use of bovine thyroglobulin promoter. Over 90% of the mice developed goiter as well PTC, which eventually transitioned into poorly differentiated carcinomas. The tumors showed evidence of angioinvasion as well as extracapsular extension. However, none of the mice developed metastatic disease and this observation suggests that the overexpression of $BRAF^{V600E}$ alone is not adequate in full modeling of PTC.

RET/PTC rearrangement is found in up to 85% of the sporadic and radiation induced PTC (41-43). RET is a receptor tyrosine kinase that binds glial-derived neurotropic factor (GDNF). The wild type RET is not normally expressed in thyroid follicular cells. In the RET/PTC rearrangement, however, the RET protein looses the ligand binding domain and the chimeric protein is expressed in thyrocytes under the control of newly acquired promoters. Ligand-independent tyrosine phosphorylation of RET/PTC protein is then is induced by constitutive dimerization of the fusion protein (44). Jhiang et al. (45) produced a transgenic mouse model of PTC by overexpressing the RET/PTC1 chimeric oncogene under the control of bovine thyroglobulin promoter. The mice developed marked hypothyroidism as well as thyroid tumors with cytological features suggestive of papillary thyroid carcinoma. However, these mice did not develop invasive features until they were crossed with p53-/- mice (46). The resulting progeny mice that overexpressed RET/PTC1 in addition to the loss of p53 exhibited tumors that were more anaplastic and showed higher rate of local invasion. Neverthless, none of the mice developed metastatic disease. Taken together, these observations suggest that RET/PTC rearrangement is an early event in the pathogenesis of papillary thyroid cancer and that other subsequent

mutations are necessary for emergence of a fully malignant phenotype.

Although $BRAF^{V600E}$ mutation and RET/PTC rearrangement are a frequent finding in PTC, these mutations are rarely found in follicular thyroid cancer (FTC). Rather, FTC tumors are characterized often by mutation in Ras which can be found in over 50% of the cases (44, 47). Vitagliano et al. (48) has reported a transgenic murine model of follicular thyroid cancer that was produced by targeting the expression of N-ras oncogene to thyroid follicular cells using bovine thyroglobulin promoter. Over 40% of the mice developed invasive follicular FTC, in some cases with mixed papillary/follicular morphology. More significantly, about 25% of the mice also developed metastatic lesions in a pattern similar to human FTC by developing metastatic disease within the lungs, bone, or liver. These finding suggest that overexpression of N-ras oncogene is able to drive the formation of thyroid tumors that can progress to metastatic disease.

CONCLUSION

The availability of a proper animal model is critical in studying the carcinogenesis of head and neck cancer. However, none of models discussed in this review are without shortcomings and the investigator needs to choose the model that suites the stage of disease they are interested in. The orthotopic model of head and neck cancer is invaluable in reproducing the clinical features of the human disease. It is particularly useful in studying the metastasis of the primary disease or therapeutic agents that inhibit metastasis. Furthermore, orthotopic models can accurately reproduce organ-specific morbidity of tumor growth. Therefore, the effect of tumor growth or therapeutic agents on disease-specific survival can be studied. Lastly, orthotopic models allow for proper reconstitution of tumor-stromal or tumor-endothelial interaction that can be lost with ectopic, subcutaneous xenograft models. Nonetheless, most orthotopic models utilized human cell lines, and the subsequent need for immunodeficient mice can hamper the study of tumor-host immune interaction.

With regard to the transgenic model of cancer in the head and neck region, it should be kept in mind that the transgene used to drive the malignant transformation may not be representative of the carcinogenic process found in human tumors. Low penetrance of tumor formation also translates into high cost and time commitment in performing studies with transgenic models. There still remains a need for development of animal models in which the early stage of carcinogenesis can be replicated. Such model will allow for identification of predictive and correlative biomarkers in studying a particular therapeutic approach. Perhaps an ideal model would be a combination of carcinogen induced model and a transgenic model where the application of carcinogenic agents to transgenic mice leads to early formation of tumors. This would be analogous to chronic exposure of a patient to tobacco and alcohol in an individual with genetic predispositions for developing cancers in the head and neck region. Until a disease model with greatest resemblance to human cancer can be created, the advantages and disadvantages of each model should be considered carefully and utilized judiciously.

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