An Orthotopic Model of Papillary Thyroid Carcinoma in Athymic Nude Mice

Soon-Hyun Ahn, MD, PhD; Ying Henderson, MD, PhD; Ya’an Kang, MD, PhD; Chandrani Chattopadhyay, PhD; Paula Holton, MS; Mary Wang, MS; Katrina Briggs, BS; Gary L. Clayman, DDS, MD

Objective: To develop a reproducible orthotopic model of papillary thyroid carcinoma for the BRAFV600E mutation (GenBank NM004333) and an RET/PTC rearrangement (GenBank M31213) that recapitulates the clinical picture in humans.

Design: In vitro and in vivo study.

Setting: Department of Head and Neck Surgery, M. D. Anderson Cancer Center.

Subjects: Eight- to 12-week-old athymic female nude mice.

Interventions: Either BRAF-mutated or RET/PTC1–rearranged papillary thyroid carcinoma cells were injected into the thyroid glands of athymic female nude mice. The mice were euthanized when the tumor burden exceeded 1.0 cm or when they exhibited significant morbidity.

Main Outcome Measures: Tumorigenicity, extent of tumor invasion and metastasis, cell invasion and migration, and median survival.

Results: All the BRAF-mutated cell lines and 1 selected RET/PTC1–rearranged cell line were 100% tumorigenic in mice. These mouse tumor models exhibited a wide range of biological potential, including laryngeal invasion, lymph node metastasis, and pulmonary metastasis, thus reflecting the clinical spectrum of papillary carcinoma.

Conclusions: An orthotopic model of papillary thyroid carcinoma was successfully established in nude mice using BRAF-mutated and RET/PTC1–rearranged cell lines. These models mimic the human disease and will thus be useful for evaluating the clinical potential of novel targeted therapies.


The most common thyroid carcinomas are papillary carcinomas. The prognosis of differentiated thyroid carcinoma is usually favorable, with 10-year survival exceeding 90%. Conversely, the prognosis of advanced disease is relatively poor: 10-year survival for patients with stage IV disease is approximately 50%, and for patients with metastatic disease who are older than 45 years it is approximately 32%.1 Advanced recurrent thyroid carcinomas are often refractory to conventional treatment with surgery and radioactive iodine therapy. Therefore, novel approaches to treating such tumors and tools for evaluating such approaches are needed.

Recently, the genetic alterations responsible for most papillary carcinomas have been well established. Many of these alterations involve genes encoding the kinases RET, NTRK1, ras, and BRAF. The most frequent alterations are RET/PTC rearrangements and BRAF mutations.2 The RET/PTC rearrangements occur in an estimated 20% to 40% of adult sporadic papillary carcinomas3 and in 40% to 70% of papillary carcinomas in children and young adults.4,5 In general, papillary carcinomas that exhibit an RET/PTC rearrangement are associated with younger age, a high rate of lymph node metastasis at presentation, classic (ie, typical) papillary morphologic features, and a favorable prognosis.6 The BRAF point mutation occurs in an estimated 45% of sporadic adult papillary carcinomas.7 This mutation is associated with older age, either typical or tall-cell variant papillary morphologic features, a higher rate of extrathyroidal extension, and more advanced tumor stage at presentation.8 The BRAF mutation is also typically found in poorly differentiated or anaplastic carcinomas and especially in tumors that contain some areas of well-differentiated tissue.9

To take advantage of these kinase gene alterations, several new drugs that specifically target kinases are being developed. However, testing their effects on papillary carcinomas will require appropriate animal models. Such models are lacking. Xenobiotic models can provide spontaneous tumors, but the rate of tumorigenesis in such models (eg, 1%-10% in female mice at 24 months) is inadequate for use in treat-
Xenograft models of human thyroid carcinoma cell lines have been established in severe combined immunodeficient mice. However, in many cases, the tumor cells were injected subcutaneously into the flank, and these tumors rarely developed metastases as in the human condition. In this program, an orthotopic mouse model using anaplastic thyroid carcinoma cell lines revealed lung metastases. The cell lines used for various kinds of xenograft models were, in most cases, follicular thyroid carcinoma cell lines (FTC-133, FTC-238, RTC-R2, and FRO) and anaplastic thyroid carcinoma cell lines (ARO, DRO, WRO, and KAT-4). However, only 1 mouse model using a papillary carcinoma cell line (ie, NPA187) has been described in the literature thus far. Moreover, there have been no reports of a xenograft tumor model involving cell lines exhibiting the RET/PTC rearrangement.

The aim of the present study was to help fill this research gap. Therefore, we describe the establishment of a reproducible orthotopic xenograft mouse model that might be used to evaluate novel molecular therapies targeting 2 of the most common molecular alterations underlying papillary carcinoma tumorigenesis: the RET/PTC1 rearrangement and the \( \text{BRAF}^{\text{V600E}} \) point mutation.

**METHODS**

### CELL LINES, CULTURE, AND PREPARATION

The following human papillary thyroid carcinoma cell lines were used: BHP2-7, BHP7-13, BHP18-21, BHP10-3, BHP14-9, and BHP5-16 (gifts from Jerome Hershman, MD, PhD, University of California at Los Angeles); NPA187 (a gift from Jeffrey Meyers, MD, M. D. Anderson Cancer Center); and TPC1 (a gift from Robert Gagel, M. D. Anderson). The cell lines exhibiting the RET/PTC1 rearrangement were BHP2-7, BHP7-13, BHP10-3, BHP18-21, and TPC1. Those exhibiting the \( \text{BRAF}^{\text{V600E}} \) point mutation were BHP5-16, BHP14-9, and NPA187. All the cells were cultured to confluence in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 1 nonessential amino acids, and antibiotics. For injection, the cells were diluted to a final concentration of \( \times 10^7 \)cells/mL with PBS.

### CELL GROWTH ASSAYS

Cultured cells of each line were assayed for growth as follows. First, cells were plated in triplicate at concentrations of 10 000 cells per well in a 24-well. After incubation for 0, 1, 2, or 3 days, each well was supplemented with the reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dissolved in PBS to a final concentration of 1 mg/mL. After 3 hours of incubation, the precipitate that had formed was dissolved in dimethyl sulfoxide and evaluated for color intensity at 570 nm using a multidetection microplate reader (Synergy HT; BioTek Instruments, Winooski, Vermont).

### SELECTION OF TUMORIGENIC CLONES

Because the cell lines exhibiting the RET/PTC1 rearrangement were not initially tumorigenic, more tumorigenic clones were selected in vitro by means of culture in soft agar. Briefly, \( 1 \times 10^5 \)cells of the applicable lines were plated in 6-well plates containing a top layer of 0.3% agar and a bottom layer of 0.6% agar in RPMI media and incubated for 21 days. The resulting clones were selected, cultured in routine media, and injected. Orthotopic tumors established in vivo in this way were then recultured, expanded in vitro, and reinjected within the orthotopic model.

### ORTHOTOPIC MOUSE MODEL OF THYROID CANCER

This orthotopic model of thyroid cancer was developed in 8- to 12-week-old female nude mice. In brief, each mouse was anesthetized by means of intraperitoneal injection of a cocktail of ketamine, 10 mg/mL, and xylazine, 1 mg/mL, through a 25-gauge needle at a dose of 0.1 mL/10 g of body weight. Then, the anterior neck and cervical areas were scrubbed with 70% ethanol, and the mouse was placed under a dissecting microscope. A 1-cm-long midline incision was made on the anterior neck. The underlying submandibular glands were dissected by means ofovic electrocautery and reflected superiorly to reveal the underlying laryngotracea and strap muscles. The strap muscles were grasped using a fine forceps, and the laryngotracea was rotated slightly to the right. This maneuver exposed the left lobe of the thyroid gland (Figure 1), which was readily visible through the semitransparent strap muscles. Then, \( 1 \times 10^6 \) cultured cells in PBS were drawn into a 25-μL Hamilton syringe tipped with a 30-gauge needle and injected through the exposed strap muscles into the thyroid gland. (Ten mice were used for each cell line.) The retracted submandibular glands were returned to their normal position, and the skin incision was stapled closed. The mice were monitored for tumor development for 6 months. Mice were humanely killed when they exhibited any signs of morbidity or distress, experienced a weight loss of more than 20% of their preinjection body weight, bore tumors that had grown to more than 1 cm in diameter, or reached 6 months of follow-up observation. In all cases, mice were euthanized via carbon dioxide inhalation. Median survival was calculated for each group of treated mice. All experimental studies involving the mice were performed in accordance with a protocol approved by M. D. Anderson’s Institutional Animal Care and Use Committee.

### NECROPSY AND TISSUE PREPARATION

After euthanization, all experimental mice were subjected to necropsy as follows. Briefly, the whole soft tissue of the neck, from the tongue to the upper mediastinum and including the thyroid, larynx, and trachea in continuity, was dissected and placed in 10% formalin solution. In addition, the lungs and spinal column were collected together and also placed in 10% for-

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**Figure 1.** Injection procedure. Using forceps to grasp the semitransparent strap muscles and rotating the laryngotracea, the physician revealed the thyroid gland (*) beneath the strap muscles.
and extensive visceral invasion.

bars represent SD. B, Survival curves for mice injected with cell lines exhibiting the RET/PTC1 rearrangement. Growth curves of tumor cell lines measured by means of 3-(4,5-

Figure 2. Cell growth curve in vitro and survival curves in vivo. A, Cell growth curves of tumor cell lines measured by means of 3-(4,5-

-.../H11003

105 cells were placed in serum-free medium in the top wells of each Boyden chamber, and normal medium–containing fetal bovine serum was placed as a chemoattractant in the bottom well. After incubation for 22 hours, cells that had invaded or migrated to the other (ie, bottom) side of the filter were fixed and stained (Harleco Hemacolor staining kit; EMD Chemicals, Gibbstown, New Jersey). Three different fields on each fixed and stained filter were examined under a microscope (magnification ×200), and the cells in each field were counted. All the assays were performed in duplicate, and the results of at least 3 independent experiments were averaged. The results for all treatment groups were compared using unpaired t tests.

IMMUNOHISTOCHEMICAL ANALYSIS

Tumor tissue sections were analyzed immunohistochemically as follows. First, the sections were deparaffinized and rehydrated by successive washing with xylene and decreasing concentrations of ethanol in water. Then the sections were heated to retrieve antigens and treated to block endogenous peroxidase and proteins. The sections were incubated overnight at 4°C in medium containing a 1:200 dilution of anti–p53 antibody (SC-6243; Santa Cruz Biotechnology, Santa Cruz, California), anti–Ki67 antibody (RM-9106-S1; Laboratory Vision Corp, Fremont, California), or control rabbit immunoglobulin G. Then the sections were washed and incubated in medium containing horse-radish peroxidase–linked secondary antibody and streptavidin. Next the sections were stained with 3,3’-diaminobenzidine (Vectorstain ABC staining system; Vector Laboratories, Burlingame, California) and counterstained with hematoxylin. Finally, the sections were placed under a microscope (magnification ×400), and stained cells were counted. The Ki67 index was calculated using the NIH Image J program.15

STATISTICAL ANALYSIS

Survival in the various treatment groups was analyzed using the Kaplan-Meier method and Cox regression analysis. The degree of invasiveness and the incidence of metastasis were analyzed using the χ2 test. All the statistical analyses were conducted using software programs (SPSS 12.0; SPSS Inc, Chicago, Illinois, and GraphPad Prism 4.0; GraphPad Software Inc, San Diego, California).

RESULTS

TUMORGENICITY

In vitro, cells exhibiting the BRAF V600E mutation grew more slowly than cells exhibiting the RET/PTC1 rearrangement (Figure 2A). In vivo, however, all 3 BRAF-mutated cell lines were extremely tumorigenic, producing tumors in all injected mice except 1 (98%, 59/60) (Table 1). That lone exception was a mouse that died during follow-up and could not be evaluated for ultimate tumorogenicity. Median survival for these mice was 41 to 47 days (Table 1 and Figure 2B). Moreover, median survival was not significantly altered by injecting mice with recultured in vivo xenografted cells instead of original cell lines (data not shown). In contrast, cell lines exhibiting the RET/PTC1 rearrangement were rarely tumorogenic, producing tumors in only 20% (2 of 10) of...
mice injected with BHP10-3 cells and 10% (1 of 10) of those injected with TPC1 cells (Table 1).

More tumorigenic clones of the cell lines exhibiting the RET-PTC1 rearrangement were selected by growth in soft agar. Although TPC1 cell lines did not colonize in soft agar, the 4 other lines (BHP2-7, BHP7-13, BHP18-21, and BHP10-3) did. Two colonies from each of these 4 cell lines were selected and injected into mice (n=10 for each cell line), but only 1 of these colonies produced tumors (ie, a colony of BHP10-3 cells). This colony, which we designated BHP10-3 SC, produced tumors in 60% (6 of 10) of injected mice and resulted in median survival of 88.5 days. When tumor cells from one of these mice were harvested and recultured to create a new clone, which we designated BHP10-3 SC<sub>mouse</sub>, the new clone turned out to be extremely tumorigenic, producing tumors in 100% (11 of 11) of injected mice and reducing median survival to 30 days (Table 1 and Figure 2C). Together, these findings demonstrated that we had successfully established an orthotopic animal model that could produce papillary carcinomas exhibiting either the BRAF<sup>V600E</sup> mutation or the RET/PTC1 rearrangement.

**PATHOLOGIC FINDINGS**

In this orthotopic model, tumors growing between the trachea and the strap muscle displaced the trachea to the right. This was apparently due to tumors invading and compressing the normal mouse thyroid (Figure 3B and C), which happens to be the case in human thyroid carcinoma. Tumors also seemed to invade the laryngeal airway (Figure 3D) and, in some cases, to metastasize to the lymph nodes (10.5% of cases) (Figure 3E) and lung (23.7%) (Figure 3F). Tumors exhibiting the RET/PTC1 rearrangement seemed to be more aggressive and to metastasize more frequently than tumors exhibiting the BRAF<sup>V600E</sup> mutation (Table 2).

**CELL INVASION AND MIGRATION ASSAY FINDINGS**

Cell migration assays revealed no significant difference between tumors derived from the BHP10-3 and BHP10-3 SC cell lines (Figure 4). On the other hand, cell invasion assays showed striking differences (Figure 4). First, the BHP10-3 SC line that generated tumors in 60% of injected mice was more invasive than its parent line BHP10-3, although not significantly so. Second, the BHP10-3 SC<sub>mouse</sub> line derived from the BHP10-3 SC line that generated tumors in 60% of mice was significantly more invasive than either of its parent cell lines (BHP10-3 [P = .003] and BHP10-3 SC [P = .009]).

**HISTOLOGIC COMPARISON OF TUMORS EXHIBITING THE RET/PTC1 REARRANGEMENT VS THE BRAF<sup>V600E</sup> MUTATION**

Tumors arising from the extremely tumorigenic BHP10-3 SC<sub>mouse</sub> line were much more differentiated than tumors arising from BRAF-mutated (BHP5-16) cells (Figure 5A and D). In fact, they demonstrated many characteristics of papillary thyroid carcinoma. For example, tumors induced by BHP10-3 SC<sub>mouse</sub> cells exhibited large, round-to-ovoid or elongated nuclei characterized by marked clearing and occasional grooving as well as wispy cyttoplasmic clearing. They grew in small solid nests surrounded by a vascular network and occasionally by scant colloid areas and trabeculation, and occasional follicle/lumen formation was also observed.

In comparison, tumors arising from BRAF-mutated cell lines (BHP5-16, BHP14-9, and NPA187) exhibited no features characteristic of well-differentiated papillary thyroid carcinoma. Instead, they seemed to be poorly differentiated tumors whose nuclear morphologic features were reminiscent of Hurthle or oncocytic cell tumors. For example, these BRAF-mutated tumors exhibited large, round-to-ovoid, and markedly uniform nuclei; very prominent and large nucleoli; and variable amounts of cytoplasm (although the nuclear to cytoplasmic ratio was usually high). Histologically, they appeared as predominantly solid sheets containing relatively few vessels and demonstrating no follicular or colloid areas but occasional areas of cellular necrosis.

In general, Ki67 expression occurred more frequently in BRAF-mutated tumors. However, this difference in frequency seemed to vary depending on which section of a tumor the compared samples came from. For example, when Ki67 expression was compared in BHP10-3 SC<sub>mouse</sub> tumors (RET/PTC1 rearrangement) vs BHP5-16 tumors (BRAF mutation), the Ki67 index was markedly different between samples obtained from the center of the tumor (16.0% vs 32.8%) (Figure 5B and E) but markedly similar in samples obtained from the periphery (58.6% vs 52.6%). P53 expression was negligible in tumors exhibiting the RET/PTC1 rearrangement but strongly positive in tumors exhibiting the BRAF mutation (Figure 5C and F).

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**COMMENT**

Papillary thyroid carcinoma is the most common cancer of the endocrine organs. It is also one of the few cancers whose incidence is increasing. Poorly differentiated papillary thyroid carcinomas tend to be more aggressive, less frequently radioiodine avid, and more metastatic than their...
Table 2. Comparison of Tumors Exhibiting the \textit{BRAF}^{V600E} Mutation vs the RET/PTC1 Rearrangement

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>\textit{BRAF}^{V600E} (n=58)</th>
<th>RET/PTC1 (n=18)</th>
<th>Total (N=76)</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>Tumor invasion$^a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modest/mild</td>
<td>24 (41)</td>
<td>2 (11)</td>
<td>26 (34)</td>
<td>.02</td>
</tr>
<tr>
<td>Moderate/severe</td>
<td>34 (59)</td>
<td>16 (89)</td>
<td>50 (66)</td>
<td></td>
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<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>55 (95)</td>
<td>13 (72)</td>
<td>68 (90)</td>
<td>.02</td>
</tr>
<tr>
<td>Yes</td>
<td>3 (5)</td>
<td>5 (28)</td>
<td>8 (10)</td>
<td></td>
</tr>
<tr>
<td>Lung metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>54 (93)</td>
<td>12 (67)</td>
<td>66 (87)</td>
<td>.009</td>
</tr>
<tr>
<td>Yes</td>
<td>4 (7)</td>
<td>6 (33)</td>
<td>10 (13)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable.

$^a$ Modest/mild is defined as 1+ and 2+; moderate/severe is defined as 3+ and 4+. This scale is described in the "Necropsy and Tissue Preparation" subsection of the "Methods" section.
more differentiated counterparts. New therapeutic approaches and the means for testing them are needed.

Some recent phase 1 and 2 clinical trials have attempted to treat aggressive and progressive thyroid papillary carcinomas with RAS/RAF/MEK pathway inhibitors. However, as in vitro studies in cell lines have shown, the effect of the kinase inhibitors depends on the underlying genetic mutations in the targeted cells. For example, several raf kinase inhibitors (AAL881, LBT613, and BAY43-9006) turned out to be more sensitive and approximately 10 times more potent in tumor cells exhibiting an RET/PTC rearrangement than in those exhibiting a BRAF mutation. Subsequently, with this in mind, investigators performing in vivo laboratory studies of papillary thyroid carcinoma have used only BRAF-mutated tumor cell lines. For example, one research group used nude mice that had been subcutaneously injected with BRAF-mutated (NPA) tumor cells. Other groups have frequently used anaplastic cell carcinoma cell lines (eg, ARO)

Figure 4. Migration and invasion assays of tumors derived from the parent tumor cell line exhibiting the RET/PTC1 rearrangement (BHP10-3) and more tumorigenic clones (BHP10-3 SC and BHP10-3 SC mice). A, Migration and invasion assay chamber (Harleco Hemacolor staining kit [EMD Chemicals, Gibbstown, New Jersey], original magnification ×200). B, Cell count analysis of samples in part A. Error bars represent SD.
as a surrogate for BRAF-mutated cell lines and, there being no papillary carcinoma cell lines exhibiting an RET/PTC rearrangement that are tumorigenic in mice, TT cell lines made from medullary carcinoma harboring RET/C634W as a surrogate for RET/PTC–rearranged cell lines. The clinical study was performed in patients with iodine-refractory thyroid cancers irrespective of underlying genetic mutations. Consequently, the literature contains no published methods to support the in vivo selection of appropriate novel drug therapies for patients with aggressive papillary thyroid carcinomas.

Helping to fill this gap gave us the rationale for devising the orthotopic animal model described herein. This model can use cell lines exhibiting 2 of the most common genetic mutations found in aggressive papillary thyroid carcinomas (ie, the BRAF mutation and the RET/PTC1 rearrangement). Thus, we addressed the previous lack of an animal model of human tumors exhibiting the RET/PTC rearrangement. This model also allows for the serial selection of biologically more tumorigenic clones from established lines, which in the future may prove useful for identifying and comparing molecular regulators of the development and spread of papillary thyroid carcinomas.

The tumors generated in this model seemed to differ depending on the underlying mutation. The BRAF-mutated tumors were much more poorly differentiated and in some cases totally undifferentiated. In comparison, tumors exhibiting the RET/PTC1 rearrangement seemed to be more differentiated, displaying features characteristic of papillary carcinoma (eg, ground glass nuclei and nuclear grooving). In some cases, these tumors also showed features characteristic of a variant of papillary carcinoma (ie, solid/trabecular) found predominantly in children exposed to radiation from the Chernobyl nuclear accident in the late 1980s. This is not surprising, since an RET/PTC rearrangement is known to occur more frequently in patients with a history of radiation exposure and to produce typically aggressive cancers in younger patients.
In our model, tumors derived from RET/PTC–rearranged BHP10-3 SCmice cell lines grew faster in vitro and were seen to invade adjacent tissue and metastasize to the lymph nodes and lungs more frequently in vivo than did BRAF-mutated cell lines. As shown immunohistochemically, however, the BRAF-mutated tumors clearly expressed more Ki67 and p53. This is an important finding because Ki67 and p53 levels are known to be high in poorly or undifferentiated carcinomas and very low in well-differentiated carcinomas. To summarize, tumors derived from cells exhibiting the RET/PTC1 rearrangement (BHP10-3 SCmice) grew faster and metastasized earlier than BRAF-mutated tumors but exhibited more favorable histopathologic features, whereas tumors derived from BRAF-mutated cell lines (NPA187, BHP5-16, and BHP14-9) grew more slowly and metastasized less frequently but exhibited histopathologic features characteristic of more aggressive undifferentiated papillary carcinomas.

The orthotopic animal model has several obvious advantages compared with the available subcutaneous models. First, the former models tumor invasion into adjacent organs. Second, it models metastasis to the cervical lymph nodes and lung, thus mimicking its human counterpart. No subcutaneous cancer models do either. Third, the present model seems to be capable of providing reproducible survival curves. Because the tumor in the present model is located deep in the neck, its detection in the early stages would be more difficult than that of subcutaneous tumors. Thus, median survival may be an important outcome measure in evaluating investigational agents. Fourth, the procedure for generating this model is technically feasible and well tolerated by mice, as previously reported.

However, this model has at least 1 important limitation, namely, its reliance on athymic nude mice. Because these mice lack normal immune function, they cannot be used to evaluate the immune components of tumor progression. Thus, this model does not completely reflect the human disease. Nevertheless, this model can still presumably provide important information in the run-up to clinical trials in humans.

In conclusion, we successfully established an orthotopic papillary thyroid carcinoma model in nude mice. This model represents the first successful attempt to model papillary thyroid carcinoma using tumor cell lines that exhibit the RET/PTC1 rearrangement. Moreover, it allows the creation of 2 distinctly different phenotypes based on 2 of the most common mutations seen in papillary thyroid carcinomas, namely, the BRAFV600E mutation and the RET/PTC1 rearrangement. This animal model will provide a useful tool for evaluating novel targeted therapies for papillary thyroid carcinoma.

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Correspondence: Gary L. Clayman, DDS, MD, Department of Head and Neck Surgery, Unit 4+1, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030 (gclayman@mdanderson.org).

Author Contributions: Dr Clayman had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Drs Ahn and Henderson contributed equally to this work.


Financial Disclosure: None reported.

Additional Contributions: Michelle D. Williams, MD (Department of Pathology, M. D. Anderson), assisted in the histopathologic examination of tumors and Clifton Stephens, DVM, PhD (Section of Veterinary Pathology, M. D. Anderson), assisted in grading primary tumor invasion.

REFERENCES


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