Immunohistochemical characterization of feline oral squamous cell carcinoma

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Objective—To evaluate the expression of Ki67 and epidermal growth factor receptor (EGFR), mitotic index (MI), and microvascular density (MVD) in feline oral squamous cell carcinoma (SCC) via immunohistochemical staining on archival tumor tissues and to seek a correlation between these markers and clinical variables.

Sample—22 archived tumor samples of feline oral SCC.

Procedures—Immunohistochemical staining for Ki67, MVD, and EGFR was performed and scored. Patient survival information was obtained from the medical records. These molecular markers as well as MI were correlated with tumor locations and patient survival time.

Results—The 22 tumors had wide variation in Ki67 expression, MI, MVD, and EGFR expression. Tongue SCC had higher MVD than did mandibular and maxillary SCC. Tumor expression of EGFR was inversely proportional to survival time.

Conclusions and Clinical Relevance—Results suggested that EGFR expression might be a valuable prognostic factor for treatment outcome in feline oral SCC. It also identified higher angiogenesis in tongue SCC, compared with mandibular and maxillary SCC, which may account for a different clinical outcome. Further prospective characterization of feline oral SCC may provide a better understanding of the underlying molecular factors that drive its behavior and offer the possibility for future patient-specific treatment plans. (Am J Vet Res 2012;73:1801–1806)

Squamous cell carcinoma is the most common feline oral malignancy. It arises from the mucosal epithelial cells in the oral cavity and can invade nearby bone. Although regional lymph node involvement and distant metastasis are relatively rare, local disease progression interferes with food and water consumption and reduces patient quality of life. Historically, treatment outcomes from surgery, chemotherapy, radiation therapy, or combinations of these treatments have been discouraging enough to deter owners from pursuing treatment. Two studies that used surgical resection of feline oral SCC found poor local control and survival time. Another 2 studies of treatment of feline oral SCC with radiation therapy found no obvious improvement in either survival time or quality of life. To improve treatment outcome of this devastating disease, further understanding of the biology of this tumor is essential. To date, most of the underlying biology of this tumor still remains unknown. It is our opinion that cellular and molecular characterization involving measurable tumor variables such as MI, Ki67 expression, MVD, and EGFR expression may help elucidate aspects of feline oral SCC biological and clinical behavior.

Determination of tumor MI is a simple and quick way to estimate the proportion of proliferating cells. Mitotic index has been studied extensively in human oncology. For example, studies revealed that a higher MI value is predictive for local tumor recurrence, patient survival time, and treatment response in humans with cervical SCC. In another study, some human patients with head and neck SCC who had reduction of MI after the first fraction of radiation therapy had longer survival time than did patients who had increased MI. Due to the similarities between human

ABBREVIATIONS

CI
Confidence interval
EGFR
Epidermal growth factor receptor
IHC
Immunohistochemical
MI
Mitotic index
MVD
Microvascular density
OST
Overall survival time
SCC
Squamous cell carcinoma
EGFR is expressed in oral SCC,\textsuperscript{16} MI could be of prognostic importance in cases of oral SCC in cats.

Ki67 is a nuclear antigen with expression restricted to actively proliferating cells.\textsuperscript{17} Although the role of Ki67 in cell cycle control is unclear, its expression is widely used as a measure of the growth fraction in a cell population.\textsuperscript{17} Cats with nasal and periorcular SCC treated with electron beam radiation therapy and with fewer Ki67-positive cells had a shorter median disease-free survival time, compared with cats with the same tumors and with higher Ki67 reactivity.\textsuperscript{18} Conversely, human patients with head and neck SCC who had high expression of Ki67 protein and an absence of p53 protein expression had excellent outcome after radiation therapy even if disease was advanced.\textsuperscript{19} These studies suggest that SCC with more proliferating cells, identified by high Ki67 expression, responds better to radiation therapy. Human laryngeal and hypopharyngeal SCC, treated with surgery alone, had an inverse relationship between disease-free survival time and Ki67 expression.\textsuperscript{20,21} implying that SCCs with high Ki67 expression are more likely to recur without ancillary perioperative treatment.

The importance of tumor angiogenesis has been extensively studied in human oncology. Microvascular density is an indicator of angiogenesis in the tissue microenvironment and can be assessed with the endothelial marker von Willebrand factor.\textsuperscript{22–24} In human SCC, MVD correlates with histopathologic grade, nodal metastasis, and patient outcome.\textsuperscript{22–27} Microvascular density has not been studied in feline oral SCC.

Epidermal growth factor receptor is a transmembrane receptor tyrosine kinase that controls downstream pathways such as cell cycle regulation, apoptosis, and differentiation.\textsuperscript{28} In human head and neck carcinoma, EGFR expression is a strong independent prognostic indicator for overall and disease-free survival time and is a good predictor for locoregional relapse.\textsuperscript{29} As a result, EGFR is a good therapeutic target in human oncology and some anti-EGFR drugs have been approved as anticancer drugs.\textsuperscript{28} In cats, EGFR expression is a prognostic indicator in cutaneous SCC and EGFR is expressed in oral SCC;\textsuperscript{30,31} however, its clinical importance is not well studied and only a few reports\textsuperscript{30,32} suggest that clinically obtained feline oral SCC and cultured feline oral SCC cell lines express EGFR. The goal of the study reported here was to deepen our understanding about feline oral SCC by retrospectively evaluating some cellular and molecular characteristics in archival paraffin-embedded feline oral SCC tissues and by correlating the obtained data with the clinical outcome as well as the tumor location.

**Materials and Methods**

**Patient selection**—Archived paraffin embedded feline oral SCC tissues from 42 cats collected over an 11-year period (1997 to 2008) were obtained from the Diagnostic Laboratory at Colorado State University. All tumors were confirmed as feline oral SCC by a board-certified pathologist (EJE). Twenty-two of 42 blocks were not decalcified, and 20 of 42 blocks were considered decalcified on the basis of the presence or absence of bony tissue in the H&E-stained slides. These 20 blocks were excluded due to the impact of the decalcification process on IHC staining.\textsuperscript{30,31} The remaining 22 blocks consisted of tissues from 13 neutered males, 1 sexually intact male, and 8 spayed female cats. Breed distribution was 10 domestic shorthair, 9 domestic longhair, 1 Siamese, 1 Ragdoll, and 1 Persian. The median age of the cats was 10.3 years (range, 5 to 19 years). Tumor locations included 6 maxillary, 3 mandibular, 2 buccal mucosa, 2 laryngeal, and 9 tongue or sublingual locations. Corresponding patient records were reviewed. If needed, additional information was obtained through a questionnaire distributed to the referring veterinarians to verify information regarding date of diagnosis and death as well as cause of death or reason for euthanasia.

**Western blot analysis**—Briefly, the SCCF1 cell line\textsuperscript{36} was maintained as described.\textsuperscript{16} The cells were lysed with a commercial lysis buffer.\textsuperscript{37} Lysate supernatant was stored at –80°C until analyzed. Protein concentration in the lysate was measured with a commercially available kit.\textsuperscript{38} The cell lysate was electrophoresed with a protein molecular weight ladder\textsuperscript{36} and horizontally blotted onto a polyvinylidene difluoride membrane.\textsuperscript{38} The membrane was incubated with blocking buffer\textsuperscript{39} for 1 hour, then the membrane was incubated with mouse anti-human monoclonal EGFR antibody\textsuperscript{39} (diluted in the blocking buffer 1:50) overnight at 4°C followed by incubation with a goat anti-mouse secondary antibody conjugated with horseradish peroxidase\textsuperscript{39} for 2 hours at room temperature (approx 22°C). The band was visualized with a chemiluminescent enzyme substrate.\textsuperscript{39} A charge-couple device camera equipped with a chemiluminescent imager\textsuperscript{39} was used to capture images.

**IHC analysis**—Tumor samples were sectioned to a thickness of 5 µm and mounted on positively charged glass slides. The IHC protocols used have been described in detail.\textsuperscript{38} Briefly, the slides were deparaffinized and rehydrated through graded xylene and alcohol. Antigen retrieval was conducted with citrate buffer\textsuperscript{40} for Ki67 and von Willebrand factor (the latter used as an endothelial marker for MVD analysis) and with protease\textsuperscript{40} for EGFR. This was followed by incubation with a blocking reagent\textsuperscript{40} for 10 minutes. The primary antibody (mouse anti-human Ki67 monoclonal, 1:50 dilution; rabbit anti-human polyclonal von Willebrand factor, 1:300 dilution; EGFR, 1:50 dilution)\textsuperscript{40} was applied and incubated overnight at 4°C. Slides were then incubated with 3% hydrogen peroxide for 10 minutes, followed by incubation with a universal secondary antibody\textsuperscript{40} for Ki67 and von Willebrand factor and with another universal secondary antibody\textsuperscript{40} for EGFR for 20 minutes at room temperature. A diaminobenzidine substrate kit\textsuperscript{40} was used to detect immunoreactive complexes. The

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slides were counterstained with Mayer hematoxylin and permanently mounted. Appropriate positive control slides were used for each batch and for all antibodies (feline lymph nodes for Ki67 and feline urinary bladder for von Willebrand factor and EGFR). Negative controls were stained exactly the same as tumor slides except for omission of the primary antibody.

**Grading of IHC stains**—Evaluation of Ki67 and EGFR was performed by 2 readers (HY and JBC) who graded all slides independently. Discrepancies were reviewed together at a multihead microscope and consensus was reached. Grading was confirmed by a board-certified pathologist (EJE). If no consensus was obtained, the reading was repeated.

**Scoring of the protein expression of Ki67**—Ki67 staining was evaluated with a scoring system based on the percentage of positively stained tumor cells (0, 0%; 1, 1% to 5%; 2, 6% to 20%; 3, 21% to 50%; and 4, ≥ 51%) and the mean intensity of positively stained tumor cells (0, negative; 1, weak; 2, moderate; 3, strong; and 4, very strong). The percentage of positive cells and intensity were multiplied to obtain a total score of 0 to 16 (termed total Ki67).

**Scoring of the protein expression of EGFR**—Epidermal growth factor receptor staining was evaluated on the basis of a reported method. The percentage of positively stained tumor cells was graded as 0, 0%; 1, < 10%; 2, 11% to 30%; 3, 31% to 60%; and 4, ≥ 61%. The intensity of positively stained tumor cells was graded as 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of positive cells and intensity were multiplied to obtain a total score of 0 to 12 (total EGFR).

**MVD analysis**—Microvascular density analysis was done by evaluating blood vessels positive for von Willebrand factor. Image analysis was performed with a microscope equipped with a charge-couple device camera and image analysis software. Briefly, the entire tumor section was scanned under low power (40X magnification) to determine the highest MVD area close to tumor cells. Then, at 200X magnification, 2 areas with the highest MVDs were picked from each slide and captured. Microvascular density was determined as the ratio of positively stained pixels of representative endothelium over the total amount of image pixels (percentage MVD). The actual numbers of pixels of positively stained endothelial cells was also determined.

**Tumor locations and molecular markers**—Analysis was performed to evaluate whether there was any relationship between tumor location and expression of these molecular markers. Locations included mandible, maxilla, and tongue or sublingual. Mandible and maxilla were combined into a single group to increase statistical power. Because of the smaller sample population, laryngeal and buccal mucosa were excluded from this analysis. A 2-sample t test was used to evaluate differences in molecular marker expression between tumor locations. The normality assumption was verified with normal-probability plots.

**OST and correlation analysis between OST and tumor locations and markers**—Overall survival time was defined as the time from diagnosis to either death or last follow-up evaluation. An additional survival analysis including only patients that lived > 7 days was also performed. Data from patients with unknown date of death were censored.

**Statistical analysis**—All statistical tests were 2 sided, and values of P < 0.05 were considered significant. For each case, mean and maximum MI were correlated.
with Ki67 scores. The association between Ki67 scores and MI scores was evaluated by performing a nonparametric Spearman rank correlation analysis. The Kaplan-Meier method was used to estimate the median OST. The comparison of OST between tumor location groups was performed with the log-rank test. Multivariate Cox proportional hazard analysis was performed to determine the prognostic value of the molecular markers for predicting OST. The proportional hazard assumption was verified via plots of the log (–log) survival curves and Schoenfeld residuals. Statistical data analyses were performed with commercially available software.

**Results**

**Western blot analysis**—Cross-reactivity of the EGFR antibody was confirmed by performing western blot analysis even though this antibody was reported to cross-react with feline tissue. Human EGFR is reported to have a molecular weight of 170 kDa, and feline EGFR protein has a similar molecular weight. Only a single intense band was observed at approximately 170 kDa (Figure 1), suggesting that this antibody cross-reacted and was specific for feline EGFR protein.

**IHC staining**—Ki67 had nuclear localization, von Willebrand factor had cytoplasmic localization, and EGFR had membranous and cytoplasmic localization (Figure 2). The median and range of scores for each marker in all patients (22 cats) were summarized (Table 1).

**MI and Ki67 grades**—There were significant correlations between maximum MI and Ki67 intensity (P = 0.05; 95% CI, 0.01 to 0.73), maximum MI and total Ki67 (P = 0.004; 95% CI, 0.23 to 0.82), and mean MI and total Ki67 (P = 0.04; 95% CI, 0.05 to 0.75).

**Tumor location and molecular markers**—Values of P of the t test were summarized (Table 1). Although not significant, the tongue tumor group had higher percentage MVD, compared with the mandible and maxilla group (P = 0.088). There was no difference detected between other markers on the basis of tumor location.

**OST and correlation analysis between OST and tumor location and marker**—Among 22 cats, there were 14 death events in this cohort. Median survival time of the 14 cats was 10.5 days. Although not significant (P = 0.097), Cox proportional hazard analysis revealed an inverse relationship between total EGFR and

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Table 1—Mean (range) values of MI, Ki67, MVD, and EGFR variables in feline oral SCC.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean of total MI in up to 10 hpf</th>
<th>Maximum</th>
<th>Positively stained tumor cells (%)</th>
<th>Intensity</th>
<th>Total*</th>
<th>Positively stained pixels (%)</th>
<th>No.</th>
<th>Positively stained tumor cells (%)</th>
<th>Intensity</th>
<th>Total*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (22)</td>
<td>2.3 (0.1–6.4)</td>
<td>6 (1–10)</td>
<td>4 (2–4)</td>
<td>3 (2–4)</td>
<td>12 (6–16)</td>
<td>2.8 (0.9–4.4)</td>
<td>208 (107–532)</td>
<td>2 (0–4)</td>
<td>2 (0–3)</td>
<td>2 (0–12)*</td>
</tr>
<tr>
<td>Mandible or maxilla (9)</td>
<td>1.4 (0.1–6.4)</td>
<td>3 (1–9)</td>
<td>4 (2–4)</td>
<td>3 (2–4)</td>
<td>12 (6–16)</td>
<td>1.4 (0.9–4.1)</td>
<td>181 (128–325)</td>
<td>1 (0–4)</td>
<td>1 (0–3)</td>
<td>2 (0–8)</td>
</tr>
<tr>
<td>Tongue or sublingual (9)</td>
<td>3.6 (0.2–5.5)</td>
<td>6 (1–10)</td>
<td>3 (2–4)</td>
<td>3 (3–4)</td>
<td>12 (6–16)</td>
<td>3.01 (1.1–4.4)</td>
<td>212 (107–532)</td>
<td>2 (1–4)</td>
<td>2 (1–3)</td>
<td>4 (1–12)</td>
</tr>
<tr>
<td>P value</td>
<td>0.380</td>
<td>0.420</td>
<td>0.600</td>
<td>0.350</td>
<td>0.890</td>
<td>0.088</td>
<td>0.410</td>
<td>0.280</td>
<td>0.520</td>
<td>0.250</td>
</tr>
</tbody>
</table>

*Total was calculated as product of percentage of positively stained tumor cells and intensity of each marker.

OST (hazard ratio, 1.91; 95% CI, 0.97 to 1.47; Figure 3). A cutoff value of 2 for total EGFR was determined on the basis of the median of the patient population. All other markers had no significant correlations. There was no significant difference in OST between the maxilla or mandible group and the tongue or sublingual group. In the additional survival analysis that included only patients that lived > 7 days, total EGFR was not prognostic for patient survival time (P = 0.14).

**Discussion**

Feline oral SCC is resistant to conventional treatments, but the mechanism of resistance is still unknown. The main purposes of this retrospective study were to analyze expression of Ki67 and EGFR, MI, and MVD in a larger patient population than in previous studies to understand and characterize the biology of feline oral SCC better as well as to seek a correlation between these markers and clinical outcome.

Significant positive correlations were detected between some Ki67 scores and MI scores. To our knowledge, the present study is the first to reveal a significant correlation between MI and Ki67 scores in feline oral SCC. Ki67 is expressed in all cell cycle phases of actively proliferating cells but not in quiescent cells (gap

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In contrast, MI takes into account cells in mitosis but not cells in other cell cycle phases or G0. Melzer et al. reported that there was a correlation between Ki67 reactivity and disease-free intervals in feline nasal and periorbital SCC. In dogs with cutaneous mast cell tumors, Ki67 is a prognostic factor. Both Ki67 and MI are known to be related to patient outcomes in human breast cancer and lung SCC.17-30 Thus, observing correlations between the 2 indices in this study was not surprising. However, the correlations found may imply that many feline oral SCC cells positive for Ki67 are able to pass cell cycle checkpoints and continue proliferating rather than undergoing senescence or apoptosis, which are both Ki67-negative processes.17 Given the role of Ki67 and MI in human and veterinary oncology as a prognostic factor in multiple tumor types, we expected to find significant correlations between these 2 markers and patient OST. The present study did not find a significant correlation with patient OST, possibly due to the small sample size.

Epidermal growth factor receptor is a transmembrane growth hormone receptor, and activation of its receptor-coupled tyrosine kinase leads to activation of signaling cascades such as cell cycle regulation and cytoskeleton reorganization.28 In humans, EGFR is a prognostic indicator in many malignancies, including head and neck SCC.30 Although not significant, the present study found a negative correlation between total EGFR and OST. Considering that EGFR activation leads to cell cycle progression, invasion, and inhibition of apoptosis and that accumulating data suggest similarities between human head and neck SCC and feline oral SCC, EGFR may be useful as a prognostic indicator for OST in cats with oral SCC.30 Because of the retrospective nature of the present study, treatments that patients received varied widely from no treatment to aggressive treatments such as multiple radical surgical excisions or radiation therapy. There was an initial decrease in patient survival time in both low and high EGFR groups (Figure 3). This decrease was attributed to euthanasia chosen by owners immediately following diagnosis. The additional survival analysis revealed that total EGFR had a similar, albeit nonsignificant (P = 0.14), relationship as in the original analysis (P = 0.097). This might support the negative, nonsignificant correlation found in the primary analysis. Despite some limitations (eg, small patient population and wide treatment variety), the negative relationship revealed in this study was an interesting finding. If a well-controlled prospective study can verify this correlation, drugs targeting the EGFR pathway may be beneficial to cats with oral SCC.

Tumor vasculization is an important factor for tumor oxygen and nutrient supply as well as for tumor metastasis.31 Tumors often create less organized and less dense vascular networks, compared with normal vasculature.29 This structural difference might result in ineffective oxygen delivery to tumor cells, creating hypoxic areas. von Willebrand factor has been used to identify endothelial cells to evaluate MVD.23 A so-called hot spot technique has been widely used to quantitate MVD and correlations between MVD and outcomes in several human tumors have been reported.31 In the present study, neither percentage MVD nor the actual numbers of pixels of positively stained endothelial cells correlated with patient survival time. However, percentage MVD in some tongue SCCs was higher than percentage MVD in mandibular and maxillary SCC. This result may indicate higher vascularity in feline SCC of the tongue, although the tongue may normally have higher MVD; thus, this comparison is of uncertain importance. We quantified MVD within the tumor tissue (hot spot technique), not within the normal tongue, but it is unclear whether the vascularity of the normal tissue had an influence on the MVD in the tumor. On the basis of our clinical experience, we feel that feline SCC in the tongue is more difficult to treat, but this may be due to its location and greater impact on a patient’s ability to eat. At this point, we cannot fully explain this contradiction between our clinical impression about the treatment response of the tongue SCC and higher percentage MVD in the tongue. However, we believe that this might be more clearly evaluated by evaluating more patients and prospectively measuring tissue oxygen tension directly.

The present study revealed large variations in Ki67, MI, MVD, and EGFR among feline oral SCCs. The negative, albeit nonsignificant, relationship between total EGFR and OST might suggest a similarity between feline oral SCC and human head and neck SCC. The present study suggested the role of EGFR as a potential prognostic indicator for patient survival time and suggested that therapeutic interference of the EGFR pathway might be a novel means to control this aggressive cancer. A prospective characterization study may help identify further prognostic factors and enhance understanding of the biology of this devastating tumor.

References

a. Cell line supplied by Dr. Thomas Rosol, Department of Veterinary Biosciences, The Ohio State University, Columbus, Ohio.
b. M-Per mammalian protein extraction reagent, Thermo Fisher Scientific, Fremont, Calif.
c. SDS, Fisher Scientific, Fair Lawn, NJ.
d. Complete Mini, Roche, Indianapolis, Ind.
e. Sodium orthovanadate, Sigma-Aldrich, St Louis, Mo.
f. Phenylmethylsulfonyl fluoride, Sigma-Aldrich, St Louis, Mo.
g. BCA protein assay reagent, Thermo Fisher Scientific, Fremont, Calif.
h. Precision plus protein kaleidoscope standards, Bio-Rad Laboratories, Hercules, Calif.
i. PVDF membrane, Bio-Rad Laboratories, Hercules, Calif.
k. ab-10, Thermo Fisher Scientific, Fremont, Calif.
l. 1:10,000 diluted in TBST, Millipore, Billerica, Mass.
m. SuperSignal West Pico, Thermo Fisher Scientific, Fremont, Calif.

n. ChemiDoc XRS system, Bio-Rad Laboratories, Hercules, Calif.
o. Dako target retrieval solution, Dako, Carpinteria, Calif.
p. Dako cytomation proteolytic enzyme, Dako, Carpinteria, Calif.
q. Background sniper, Biocare Medical, Concord, Calif.
r. MIB-1, Dako, Carpinteria, Calif.
s. A0082, Dako, Carpinteria, Calif.
t. Dako Envision+ Dual link, Dako, Carpinteria, Calif.
u. DAB substrate kit for peroxidase, Vector Laboratories, Burlingame, Calif.
w. Carl Zeiss Axioplan 2 imaging scope, Carl Zeiss, Thornwood, NY.
x. AxioCam HRc Carl Zeiss camera, Carl Zeiss, Thornwood, NY.
References


