Prognostic Significance of Cyclooxygenase-2 in Oropharyngeal Squamous Cell Carcinoma

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ABSTRACT

Purpose: To determine the relative prognostic significance of cyclooxygenase (COX)-2 expression in patients with oropharyngeal squamous cell carcinoma (SCC).

Experimental Design: This retrospective cohort study included 82 patients with SCC referred to the Department of Therapeutic Radiology at Yale-New Haven Hospital (Connecticut) between 1980 and 1999 who were treated with primary external beam radiotherapy or gross total surgical resection and postoperative radiotherapy. A microarray of archival tumor tissue was constructed and stained with monoclonal antibodies directed against COX-2 and scored for intensity by a pathologist blinded to the clinical outcomes of the patients. COX-2 immunoreactivity and clinicopathological data were analyzed with respect to survival endpoints using bivariate and multivariate techniques.

Results: Frequency of COX-2 overexpression was 45%. In multivariate analysis, COX-2 positivity predicted poor 3-year survival (P = 0.02; odds ratio = 0.41; 95% confidence interval, 0.20–0.84). Increasing age was significantly associated with increased 3-year survival (P = 0.03; odds ratio = 1.04; 95% confidence interval, 1.004–1.09). Positive COX-2 status trended toward predicting decreased 3-year disease-free survival.

Conclusions: COX-2 was the most important predictor of poor survival in this patient cohort. In patients with oropharyngeal SCC treated with external-beam radiation therapy, overexpression of COX-2 may affect clinical outcome, and COX-2 may therefore prove valuable both as a prognostic factor and as a therapeutic target.

INTRODUCTION

Every year in the United States, over 40,000 people will be diagnosed with squamous cell carcinoma of the head and neck (HNSCC), and over 7000 people will die from the disease (1). Despite recent advances in cancer treatment, the overall 5-year survival rate for patients with HNSCC has not improved over the last 30 years (2). The primary cause of treatment failure in patients with early-stage disease is the development of second primary tumors, whereas patients who present with locally advanced disease are at risk for local regional recurrence and metastasis, even with the use of near-tolerance doses of radiation (2).

Traditionally, clinical and pathological parameters such as Tumor-Node-Metastasis staging, surgical margin status, perineural invasion, radiation dose, and treatment schedule have been used to guide treatment decisions in the management of HNSCC. However, these factors fail to provide definitive information regarding the biological behavior of the tumor and its potential to recur. Identifying a biological marker that correlates with recurrence would provide more accurate information on prognosis and enable the treating physician to select a more aggressive course of therapy for high-risk patients. Such a marker could also potentially serve as a target for drugs designed to seek out and exploit specific molecular defects in cancerous cells.

One potential molecular marker in HNSCC that has drawn considerable interest is cyclooxygenase (COX)-2. Cyclooxygenase is well known as the enzyme that catalyzes the conversion of arachidonic acid to prostaglandin H2, the rate-limiting step in the synthesis of prostaglandins and other eicosanoids from membrane phospholipids. Two isoforms of COX are expressed in human tissue. COX-1 is constitutively expressed in most mammalian cells and generates prostaglandins necessary for normal physiological function. COX-2 is normally undetectable but is rapidly inducible in response to a variety of stimuli. Factors that have been found to up-regulate COX-2 include interleukin 1, epithelial growth factor receptor, transforming growth factor β, tumor necrosis factor α, hypoxia, the procarcinogen benzo(a)pyrene, and the oncogenes ras and src. Factors that suppress COX-2 include dexamethasone, interleukin 10, and the tumor suppressor gene p53 (3, 4). Expression of COX-2 in the cell is limited to the luminal endoplasmic reticulum and the nuclear membrane (3).

COX-2 has been shown to be selectively overexpressed in head and neck, bronchial, pancreatic, breast, gastric, and colon cancer (5–9). In a 2001 study, Chan et al. (10) demonstrated that COX-2 was overexpressed in 25 samples of HNSCC drawn from various anatomical regions of the head and neck. COX-2 was also overexpressed in a series of 23 hypopharyngeal squamous cell tumors, with significantly higher expression in N1–3 tumors than in N0 tumors (11). COX-2 overexpression in HNSCC has been found to correlate with increased tumor vas-
cularity and increased levels of prostaglandin E₂ (PGE₂), vascular endothelial growth factor, and peroxisome proliferator-activated receptor δ, with combination of high COX-2 and high PGE₂ levels being associated with decreased 5-year survival (12, 13).

In contrast to these results, a study of laryngeal squamous cell carcinoma (SCC) by Ranelliti et al. (14) showed that high levels of COX-2 were found more frequently in well differen-
tiated tumors and were associated with improved survival. Decreased 5-year survival and increased recurrence were seen with the combination of low COX-2 and elevated epithelial growth factor receptor, and in in vitro studies on an epidermal growth factor-positive COX-2-negative cell line, epidermal growth fac-
tor stimulation failed to induce COX-2 overexpression. These data suggest that COX-2 overexpression may be lost in poorly differen-
tiated laryngeal SCCs, possibly through an alteration in epithelial growth factor receptor signaling.

The goal of this study was to study the immunohistochemical expression of COX-2 in relation to 3-year survival and recurrence of disease in a large cohort of patients with oro-
pharyngeal SCC treated with radiotherapy.

MATERIALS AND METHODS

Patient Selection. Patients were included in this study if they met the following criteria: (a) presentation to the Depart-
ment of Therapeutic Radiology at Yale-New Haven Hospital (Connecticut) between 1980 and 1999 with a histologically confirmed diagnosis of SCC of the oropharynx and (b) treatment with primary external beam radiotherapy or surgical excision and postoperative external beam radiotherapy with the intent to cure. Patients were excluded from this study if they had a prior history of SCC of the head and neck, presented with metastatic disease, or failed to receive a full course of radiation therapy. A review of radiation records identified 103 patients who met the entry criteria.

After the approval of the appropriate institutional review
boards, paraffin-embedded tissue samples were obtained from hospital archives. Covariables including patient demographics, staging information, and clinical, pathological, and treatment parameters were extracted from patient charts. All patients were staged according to the American Joint Committee on Cancer Tumor-Node-Metastasis classification system (15). Clinically N₀ patients were restaged for purposes of this analysis if path-
ological examination results of the neck dissection specimens were positive for nodal metastases. Local recurrence was de-
efined as recurrence of disease at a site within the upper aerodi-
gestive tract anatomically contiguous with the primary tumor. Regional recurrence was defined as recurrence of disease within the cervical lymphatic system. Distant recurrence was consid-
ered recurrence of disease that did not meet the definitions of local or regional recurrence and was not considered to represent a second primary tumor based on its histological and/or clinical manifestations. Fifty-four patients (66%) were followed up until death. The median follow-up time of the surviving patients was 4 years, with a minimum of 0.6 years.

Patients were treated with primary radiotherapy or gross total surgical resection and postoperative external beam radio-
therapy. The mean dose of radiation was 60 Gy. Forty-seven patients (57%) received a radical or modified radical neck dissection, and 35 patients (43%) received brachytherapy. Twenty-three patients (28%) received chemotherapy, with 16 receiving the hypoxic cytotoxins porfiromycin or mitomycin C as part of an institutional protocol (16, 17).

Tissue Microarray Block. The tissue microarray was constructed using a dedicated arraying device (Beecher Instru-
ments). The recipient blocks were made from paraffin and measured 3.0 by 2.5 by 0.5 cm. Holes spaced 0.8 mm apart were first made in the recipient block with a coring needle. The donor blocks of formalin-fixed, paraffin-embedded archival tissue from the primary tumor were then prepared after a thorough evaluation of the H&E-stained slides. Several normal skin samples were included as negative controls. Representative tumor areas were defined and marked, and single 0.6-mm circular cores of tumor tissues were removed from each donor block and transferred to the prepared holes in the recipient block using a coring needle and stylet. The microarray block was then incu-
bated at 37°C for 15 min to adhere the cores to the walls of the array. Consecutive 5 μm-thick sections were cut from the mi-
croarray using a microtome and placed onto adhesive-coated slides (Instrumedics, Inc).

Immunohistochemistry. Immunohistochemical analysis was performed on consecutive 5 μm-thick sections from the microarray. The sections were deparaffinized in xylene followed by 100% ethanol and rehydrated with graded ethanol solutions. The sections were pretreated to promote antigen retrieval by steaming at 90°C for 20 min in DAKO Target Retrieval Solution (catalogue no. S1699; DAKO Corp., Carpinteria, CA), followed by a 20-min cool-down at room temperature in the retrieval solution. Samples were then quenched in 3% hydrogen peroxide solution for 5 min. Incubation with the primary antibody was performed at room temperature for 1 h using a mouse mono-
clonal IgG1 reactive with human COX-2 amino acids 580–599 (catalogue no. 160112; Cayman Chemical Co., Ann Arbor, MI) at a dilution of 1:100. The slides were then incubated with horse antimouse secondary antibody, labeled with avidin-biotin complex streptavidin-peroxidase (catalogue no. K0690; DAKO), incubated with the chromagen diaminobenzidine tetrahydro-
chloride, lightly counterstained with hematoxylin, and mounted. Negative controls were performed by omitting the primary antibody.

An experienced pathologist (D. P. K.) blinded to the clin-
ical outcomes examined each sample core to score the tissue sections for tumor staining intensity (0, none; +, faint; ++, moderate; +++, strong). Because the positive tumor sections stained diffusely, an estimation of percentage of tumor cells stained was not done. To maximize the power of statistical comparisons, COX-2 status was determined by dichotomizing COX-2-staining intensity with the cut point established between faint (+) and moderate (+++) staining.

Statistical Analysis. Molecular marker status and the relevant covariables were assembled into a database and ana-
yzied using the SPSS statistical software package. All tests of statistical significance were 2-sided. Follow-up time and time to recurrence were calculated from the date of initial diagnosis to the date of the relevant outcome. To enhance the power of statistical comparisons, the following categories were collapsed: T stages 1 and 2; T stages 3 and 4; and N stages 2 and 3.
Prognostic Significance of COX-2 in Oropharyngeal SCC

23 patients (28%) experienced local recurrence, whereas 12 had III, and 9 had stage II. The mean patient age was 60.1 years. Patients presented with stage IV disease, whereas 26 had stage II. The mean patient age was 60.1 years. Forty-seven (80%) in the analysis. Frequency statistics for the characteristics tissue representation in the microarray, leaving 82 patients follow-up data, and 11 were excluded because of insufficient gible for analysis, but 10 were excluded because of incomplete

RESULTS

Bivariate analyses for associations between the clinico-pathological predictor variables gender, age, race, T stage, N stage, and COX-2 status were conducted using Pearson’s correlation test. Age was considered as a continuous variable.

Bivariate analyses for the associations between the predictor variables and the main outcome variables (3-year survival, recurrence, and disease-free survival) were conducted using the Kaplan-Meier log-rank test.

For the multivariate analysis, Cox proportional hazards regression was used to determine significant predictors of 3-year survival and disease-free survival at an \( \alpha = 0.5 \) level. COX-2 status and all clinicopathological variables were included in the final model. Age was analyzed as a continuous variable.

Descriptive Statistics. A total of 103 patients were eligible for analysis, but 10 were excluded because of incomplete follow-up data, and 11 were excluded because of insufficient tissue representation in the microarray, leaving 82 patients (80%) in the analysis. Frequency statistics for the characteristics of the patient cohort are presented in Table 1. Forty-seven patients presented with stage IV disease, whereas 26 had stage III, and 9 had stage II. The mean patient age was 60.1 years.

Patient outcome data are summarized in Table 2. A total of 23 patients (28%) experienced local recurrence, whereas 12 had regional recurrence (15%), and 9 (11%) had distant recurrence. Overall, 33 patients (40%) had \( \geq 1 \) recurrences of disease within 3 years after diagnosis. Fifty-four patients (66%) died during the follow-up period. The 3-year survival for this cohort was 33%.

The results of the COX-2 immunohistochemistry were as follows: 6 patients (7%) had strong staining, 31 patients (38%) had moderate staining, 31 patients (38%) had faint staining, and 14 patients (17%) had no staining. The negative controls exhibited no staining. The pattern of staining observed in the tumor samples was predominantly granular and cytoplasmic. To increase the statistical power of this study, the COX-2 staining variable was dichotomized, with the cut point set between faint and moderate staining. Therefore, the 37 patients (45%) with moderate and strong staining were defined as positive for overexpression of COX-2, whereas the 45 patients (55%) with faint or no staining were defined as negative for overexpression of COX-2.

Bivariate Analysis. The results of the Pearson’s correlation tests for associations between the clinicopathological predictor variables and COX-2 status are listed in Table 3. There was no association between the predictor variables or between the predictor variables and COX-2 status at \( \alpha = 0.10 \).

Results from the Kaplan-Meier log-rank tests for associations between the predictor variables and 3-year survival and disease-free survival are presented in Table 4. COX-2 positivity

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients</th>
<th>% of patients</th>
<th>No. COX-2* positive</th>
<th>% of patients</th>
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<tr>
<td>T1</td>
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<td>16</td>
<td>37%</td>
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<td>38%</td>
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<td>46%</td>
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<td>Porphiromycin</td>
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<td>11%</td>
<td>4</td>
<td>44%</td>
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<td>9%</td>
<td>2</td>
<td>29%</td>
</tr>
<tr>
<td>Brachytherapy</td>
<td>35</td>
<td>43%</td>
<td>15</td>
<td>43%</td>
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</table>

* COX, cyclooxygenase; TNM, Tumor-Node-Metastasis; OP, oropharynx.

Table 1. Clinicopathologic variables and patient characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total no. patients</th>
<th>3-year survival</th>
<th>Local relapse-free</th>
<th>Relapse-free</th>
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<td>72%</td>
<td>60%</td>
</tr>
<tr>
<td>Positive</td>
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<td>22%</td>
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<td>51%</td>
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<tr>
<td>Negative</td>
<td>45</td>
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<td>67%</td>
</tr>
<tr>
<td>TNM stage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>9</td>
<td>33%</td>
<td>67%</td>
<td>56%</td>
</tr>
<tr>
<td>III</td>
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<td>46%</td>
<td>69%</td>
<td>58%</td>
</tr>
<tr>
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</tr>
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<td>T1–2</td>
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<td>81%</td>
<td>72%</td>
</tr>
<tr>
<td>TNM stage</td>
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<td></td>
</tr>
<tr>
<td>N0</td>
<td>23</td>
<td>35%</td>
<td>70%</td>
<td>65%</td>
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<td>N1</td>
<td>24</td>
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<td>Soft Palate</td>
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<td>32%</td>
<td>73%</td>
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<td>20%</td>
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<td>66%</td>
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* COX, cyclooxygenase; TNM, Tumor-Node-Metastasis; BOT, base of tongue; OP, oropharynx.
was associated with decreased 3-year survival ($P < 0.01$; log-rank statistic $7.1$). Dichotomized T stage was also significant in the bivariate analysis, with $T_3$ or $T_4$ stage being associated with decreased 3-year survival ($P = 0.02$; log rank $5.5$). A Kaplan-Meier survival curve illustrating 3-year survival with respect to COX-2 status is presented in Fig. 1.

In the bivariate analysis of disease-free survival, COX-2 was significantly associated with any recurrence of disease ($P = 0.05$; log rank $4.0$). There was a trend toward dichotomized T stage as a predictor of any recurrence of disease ($P = 0.07$; log rank $3.3$). When local recurrence, regional, distant, loco-regional, and any site recurrence were tested individually against COX-2 status, there was no significant association at $P < 0.1$ nor was there any association between the clinicopathological predictor variables and any of the aforementioned morbidity outcomes.

**Multivariate Analysis.** The Cox proportional hazards models included all 82 patients. COX-2 status and the clinicopathological predictors were entered into the models. The results of the multivariate analysis are listed in Table 5. At a final $P < 0.05$, COX-2 positivity was the most significant predictor of decreased 3-year survival ($P = 0.02$; odds ratio $0.41$; 95% confidence interval, 0.20–0.84). Increasing age was also found to be significantly associated with increased 3-year survival ($P = 0.03$; odds ratio $1.04$; 95% confidence interval, 1.004–1.09).

In the analysis of disease-free survival, none of the variables was significant at a level of $P = 0.05$. There was a trend toward positive COX-2 status and increased recurrence at any site ($P = 0.10$; odds ratio $0.51$; 95% confidence interval, 0.23–1.13). Increasing age also trended toward an association with decreased frequency of recurrence at any site ($P = 0.06$; odds ratio $1.04$; 95% confidence interval, 1.00–1.09).

**DISCUSSION**

In this study, a uniform cohort of patients with SCC of the oropharynx treated with external beam radiation was selected to assess the prognostic value of COX-2 immunohistochemical staining. The resulting analysis shows that patients whose tumors overexpressed COX-2 had decreased 3-year survival. Increasing age was also found to be a significant predictor of...
increased 3-year survival. When compared with standard clinicopathological predictors of outcome such as Tumor-Node-Metastasis staging, gender, age, and race, in a multivariate analysis, COX-2-positive status was the most significant predictor of poor survival outcome.

The patient cohort used in this study was relatively homogeneous, consisting only of oropharyngeal tumors, in contrast to some past studies of HNSCC, which have included tumors from diverse sites along the aerodigestive tract. This study also benefits from the use of a monoclonal antibody, which may have enhanced specificity for COX-2 compared with polyclonal products.

Despite the effort to confine the patient sample to oropharyngeal tumors, this cohort still contains tumors from sites as diverse as base of tongue, tonsils, tonsillar fossa, and soft palate. Larger studies of tumors from these particular areas will be needed to validate the conclusions drawn in this study. Furthermore, this study relied on qualitative immunohistochemistry to assess levels of COX-2 protein. Although fast, relatively inexpensive, and readily available to most pathology departments, this type of test can be subject to interobserver variability. The particular scoring system used in this study will also need to be further evaluated in subsequent studies.

The results from this study are in line with those of Chan et al. (10) who found COX-2 protein to be overexpressed in HNSCC in comparison with normal tissue and with a recent study showing that high levels of COX-2 were associated with decreased 5-year survival (18). These data stand in contrast to a study that demonstrated a correlation between higher levels of COX-2 expression and shorter survival and disease-free survival (14) in a series of laryngeal tumors, indicating that perhaps COX-2 behaves differently in carcinoma of the oropharynx.

It is notable that increasing age was significantly associated with increased 3-year survival and trended toward predicting decreased recurrence at any site. These findings suggest that older patients in this cohort may have developed less aggressive tumors.

The trend toward an association between COX-2 positivity and increased recurrence at any site in this cohort of patients treated with radiotherapy suggests that COX-2 overexpression may decrease tumor radioresponse, rendering the patient vulnerable to disease recurrence. Prostaglandins are known to protect normal tissues from radiation damage (19), and Milas (20) has recently published data showing that selective COX-2 inhibition with SC-236 [4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzene sulfonamide] enhanced the response of human glioma xenograft tumors in mice to irradiation, suggesting that the COX-2 enzyme may protect tumor cells from the cytotoxic effects of radiation. Larger studies will be needed to confirm whether overexpression of COX-2 is indeed associated with increased recurrence in HNSCC.

COX-2 most likely plays a complex role in the initiation and growth of HNSCC. The importance of COX-2 in tumorigenesis was initially demonstrated by a study showing that in rodent models of familial adenomatous polyposis, a genetic disease leading to gastrointestinal cancer in humans, loss of COX-2 via genetic deletion, or selective inhibition suppressed the formation of polyps (21). Celecoxib, a selective COX-2 inhibitor is now approved for use against this condition in humans.

A variety of mechanisms, both prostaglandin-mediated and nonprostaglandin-mediated, have been proposed implicating COX-2 in carcinogenesis. PGE2, a COX-derived eicosanoid, has been shown to induce the antiapoptotic protein Bcl-2 and reduce the levels of Bax, a proapoptotic protein (22). Forced overexpression of COX-2 in endothelial cells leads to decreased apoptosis (23), whereas inhibition of COX-2 by NSAIDs induces apoptosis in a number of in vitro systems (23, 24). Chronic inflammation, a known risk factor for epithelial cancer (25), may lead to carcinogenesis via the overexpression of COX-2 in the inflamed tissues. Immunosuppression has also been correlated with the occurrence and growth of tumor (26). PGE2 inhibits the proliferation of T and B-cells and the cytotoxic activity of natural killer cells (27–29), as well as inhibiting production of tumor necrosis factor α and increasing levels of the anti-inflammatory cytokine interleukin 10 (30). Angiogenesis is a recognized requisite for tumor growth (31), and vascular endothelial growth factor has been shown to be a negative prognostic factor in HNSCC (32). A recent study linked COX-2 levels to levels of PGE2 and vascular endothelial growth factor and to degree of tumor vascularization in HNSCC (18), whereas in another study, decreased tumor growth and vascularity were observed in COX-2 knockout mice in comparison with normal and COX-1 knockout mice (33). Finally, COX-2 may be related to increased incidence of tumor metastasis. In one study, forced overexpression of COX-2 was associated with increased expression of CD44, the cellular receptor for hyaluronate, whereas blockade of CD44 decreased tumor invasiveness (34). Human colon cancer cells engineered to overexpress COX-2 show increased prostaglandin production and more invasive behavior (35), whereas selective blockade of COX-2 has also been seen to suppress metastasis in animal studies (36).

Among nonprostaglandin-mediated models for COX-2 carcinogenesis, one theory holds that COX-2, which is induced by
benzo(a)pyrene in tobacco smoke (37), catalyzes the conversion of this procarcinogen from benzo(a)pyrene P-7,8 diol to benzo(a)pyrene P-7,8 diol-9,10-epoxide, a powerful mutagen (38). In this way, benzo(a)pyrene P-mediated overexpression of COX-2 may amplify the carcinogenic effects of tobacco smoke on the epithelium of the aerodigestive tract.

Additional studies to specifically examine the biochemistry of COX-2 in epithelial carcinogenesis will be required to elucidate the role of this protein in SCC. If results from these investigations are promising, human clinical trials of COX-2 inhibition in the treatment of HNSSC would be warranted. The existence of relatively safe, well-tolerated, and commercially available COX-2-inhibiting drugs raises the possibility that the selective blockade of COX-2 could be easily incorporated as an adjunct in existing therapeutic regimens or as a chemopreventive agent.

In conclusion, the results of this study show that patients whose tumors express high levels of COX-2 protein may be at risk for decreased 3-year survival. If these studies are confirmed, COX-2 could become recognized as a key factor in establishing prognosis and selecting treatment modalities for oropharyngeal SCC and as a therapeutic target.

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