

Role of Cyclooxygenase-2 in Tumor Progression and Survival of Head and Neck Squamous Cell Carcinoma

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Abstract

Inhibition of cyclooxygenase-2 (COX-2) pathways may have significant implications for the prevention and treatment of head and neck squamous cell carcinoma (HNSCC). COX-2 is overexpressed in both premalignant lesions and invasive HNSCC. We examined COX-2 expression by immunohistochemistry in normal tissues, different stages of premalignant lesions, and carcinoma *in situ* (CIS). We also evaluated the correlation between COX-2 expression and clinical characteristics of HNSCC patients. Tissue specimens were obtained from the following: premalignant lesions from 25 subjects enrolled in a biochemoprevention trial, tumor samples collected at diagnosis from 38 HNSCC patients enrolled in an induction chemotherapy trial, and normal control tissues from 10 noncancer, nonsmoking subjects. COX-2 was expressed in early and intermediate stages of premalignant lesions, increasing first in the basal and parabasal layers, then lower spinous, and upper spinous layers. This correlation was noted in normal epithelium ($P < 0.0001$), histologically normal in-field samples ($P < 0.0001$), low-grade dysplasia ($P = 0.024$), and moderate-grade dysplasia ($P = 0.009$), but was lost in the majority of high-grade dysplasia/CIS ($P = 0.896$). COX-2 expression was also noted to increase progressively through the early stages of premalignancy, and to decrease in severe/CIS stage and invasive carcinoma. COX-2 expression in tumors from patients treated with induction chemotherapy was correlated with overall survival after controlling for clinical variables. These findings elucidate the differential expression pattern of COX-2 in stages of head and neck premalignant lesions and invasive carcinoma, supporting the rationale for COX-2 inhibition as an important strategy for cancer chemoprevention. Further validation of COX-2 expression is needed in prospective ongoing chemoprevention trials.

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy that is the sixth most common neoplasm worldwide, with 47,560 new cases diagnosed and >11,260 deaths in the United States estimated in 2008 (1). HNSCC is a phenotypically and biologically heterogeneous disease with widely variable patterns of behavior that represents a paradigm for cancer chemoprevention. Advances in the molecular biology of HNSCC (1) as well as the development of novel targeted therapies have led to increased interest in the study of chemoprevention in HNSCC.

Several laboratory investigations including animal models and epidemiologic studies have provided evidence that inhibition of cyclooxygenase (COX)-2 pathways may have significant implications for cancer treatment or prevention in general (2–4), and in HNSCC in particular (5, 6). There are at least two forms of the COX isoenzyme, COX-1 and COX-2. COX-1 is constitutively expressed in most epithelial tissues and is thought to catalyze the formation of prostaglandins, which in turn promote tumorigenesis (7, 8). COX-2, in contrast, is not constitutively expressed in most tissues but is induced by a wide spectrum of growth factors and cytokines in pathophysiologic states (9). Increased expression of COX-2 is commonly found in both premalignant and malignant tissues (9–12). Overexpression of COX-2 in epithelial cells has been shown to inhibit apoptosis and increase the invasiveness of tumor cells.

Evidence suggests that COX inhibitors, including nonsteroidal anti-inflammatory drugs, protect against a variety of tumors (2, 13, 14). Selective inhibition of COX-2 avoids the side effects of nonsteroidal anti-inflammatory drugs that inhibit both COX-1 and COX-2. In patients with familial adenomatous polyposis, Celecoxib, a selective COX-2 inhibitor,

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Table 1. High COX-2 expression in patients with HNSCC in correlation with clinical variables

Characteristics	COX-2 expression above mean	P
Sex		
M	13/33	0.152
F	4/5	
Response		
CR/PR	13/31	0.677
NC/PD	4/7	
T stage		
T1/T2	2/12	0.033
T3/T4	15/26	
N stage		
N0/N1	5/10	0.727
N2/N3	12/28	
AJCC stage		
III	2/5	1.000
IV	15/33	
Site		
HP/LX	5/11	1.000
OC/OP	11/25	
Differentiation		
G1	3/6	0.913
G2	6/15	
G3	8/17	

Abbreviations: CR/PR, complete response/partial response; SD/PD, stable disease/progression of disease; AJCC, American Joint Committee on Cancer; HP/LX, hypopharynx/larynx; OC/OP, oral cavity/oropharynx.

caused a 28% reduction in the number of colorectal polyps compared with a 4.5% reduction for placebo (15).

Although the mechanism(s) by which nonsteroidal anti-inflammatory drugs inhibit tumor growth are not clearly understood, it could involve blockage of COXs, which suppress eicosanoid production, especially of PGs, and might affect cell proliferation, apoptosis, and immune response (5, 16). It has recently been shown that inhibition of COX pathways may also exert a negative effect on tumor angiogenesis (17, 18).

In HNSCC, COX-2 is expressed in both tumor tissue and the adjacent epithelium, with a higher expression level in invasive carcinoma compared with normal epithelium (5). Western blot analysis of tissue from subjects at different stages of carcinogenesis and of normal mucosa showed expression of COX-2 in all premalignant lesions but not in normal mucosa, suggestive of a possible role for COX-2 inhibition in chemoprevention of HNSCC (19). COX-2 inhibition has been shown to result in inhibition of cell growth in HNSCC (20–23). Based on these findings, a number of chemoprevention and therapeutic trials in HNSCC using COX-2 inhibitors are under way (24).

We sought to examine the expression of COX-2 in normal and dysplastic tissues of different stages of premalignancy, including low-grade dysplasia (LD), moderate-grade dysplasia (MD), and high-grade dysplasia/carcinoma *in situ* (CIS), to better understand the role it plays in tumorigene-

sis. We also evaluated the correlation between COX-2 expression and clinical outcomes of patients with HNSCC who were treated on an induction chemotherapy trial with TIC (25).

Materials and Methods

Tissue specimens

Specimens for this study were obtained from two separate trials, due to their availability in the University Of Texas M.D. Anderson Cancer Center. The biopsy tissues were obtained from consented patients following Institutional Review Board protocol approval.

The first group of tissue specimens included patients with premalignant lesions enrolled on the chemoprevention trial with 13-*cis*-retinoic acid, IFN- α 2a, and α -tocopherol (26–28). A total of 36 participants were enrolled on this study, and 25 tissue specimens were obtained for our analysis at the time of diagnosis. In addition, 10 tissue samples from noncancer patients who were nonsmokers were collected and used as control. These biopsy samples were classified as follows: normal control ($n = 10$), histologically normal in-field ($n = 25$), LD or mild dysplasia ($n = 14$), MD ($n = 29$), and severe dysplasia/CIS ($n = 14$). For each of the samples, basal/parabasal (B/PB), lower spinous (LS), and upper spinous/granular were available for our analysis.

The second cohort of HNSCC tissue samples were collected at the time of diagnosis from 38 patients with HNSCC who enrolled on a phase II trial of induction chemotherapy with paclitaxel, ifosfamide, and carboplatin (TIC) followed by local therapy (25). A total of 54 patients were enrolled on the trial and tissue was available for analysis from 38 patients. All eligible patients on this trial had locally advanced HNSCC and received two courses of induction chemotherapy with TIC. They subsequently underwent repeated clinical and radiologic evaluation. If they achieved complete or partial responses, they received two more courses of chemotherapy before undergoing definitive local treatment.

Clinical information for these patients was obtained from the pathology files in the University of Texas M.D. Anderson Cancer Center following the regulations of the Health Insurance Portability and Accountability Act.

Immunohistochemistry

Unstained sections from tissue blocks were prepared using appropriately coated slides and stained with anti-COX-2 specific antibody (Cayman Chemical) at a dilution of 1/100 following a standard immunohistochemistry (IHC) procedure. The slides were incubated with the primary antibody at 4°C overnight followed by incubation with a secondary antibody conjugated with horseradish peroxidase (R&D Systems) for 1 h at room temperature. Antibody staining was visualized using 3,3'-diaminobenzidine peroxidase substrate solution (Vector Laboratories). Cell nuclei were counterstained using hematoxylin QS (Vector Laboratories). Mouse IgG without the primary antibody was used as a negative control.

COX-2 expression in the cell cytoplasm was scored by a head and neck pathologist blinded to the response or the outcome of patients using a semiquantitative scale ranging from 0 (no expression) to 3+ (highest level of expression) on a cell-by-cell basis in 10 consecutive microscopic fields ($\times 200$) in a defined area. The weighted index of COX-2 expression was calculated as the sum of the number of counted cells multiplied each by the degree of intensity of each cell (0-3+) and divided by the total number of cells counted, as follows: weighted index of COX-2 expression = (number of cells counted \times degree of intensity)/total number of cells counted $\times 100$ (31). COX-2 expression was determined in each of the epithelial layers of the different premalignant lesions and its correlation with clinical parameters was assessed.

Statistical Analysis

For the analysis of premalignant lesions, Kruskal-Wallis test was carried out to compare the median expression level of COX-2 within each premalignant lesion and between the different degrees of premalignancies.

For the analysis of the 38 patients with invasive carcinomas, COX-2 expression was dichotomized at the mean (0, COX-2 expression below the mean; 1, COX-2 expression above the mean) and the Fisher exact test for comparing two population proportions was used to compare the proportion of men and women with COX-2 expression equal to 1.

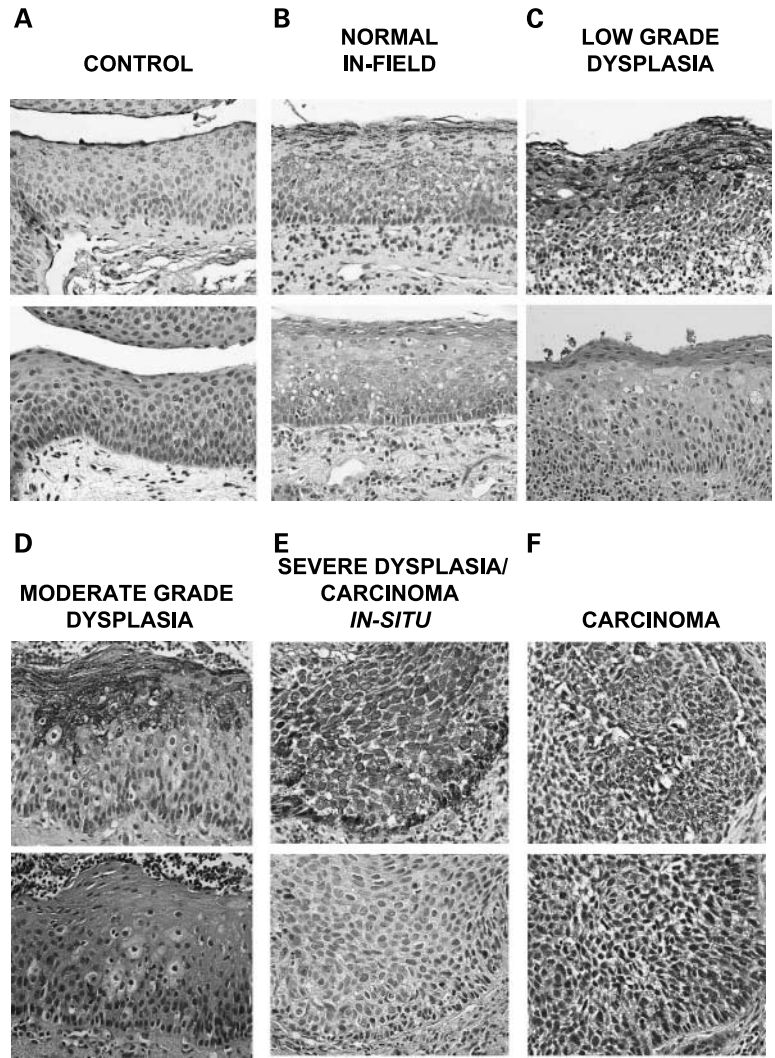
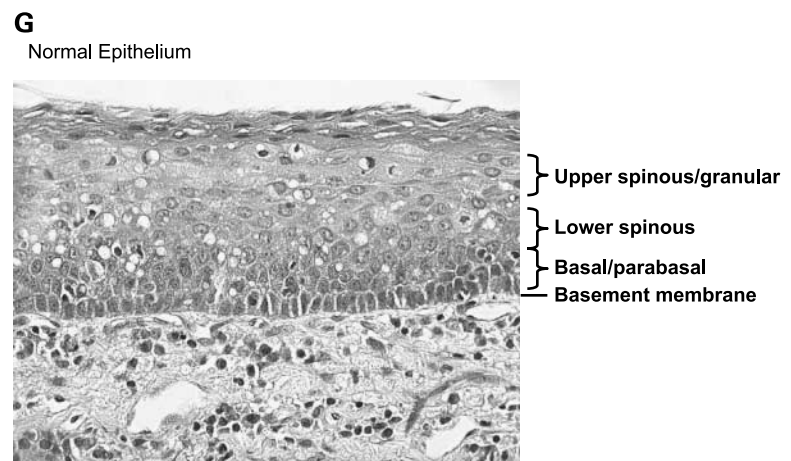


Fig. 1. COX-2 staining in premalignant and HNSCC samples. Tissue blocks were stained with anti-COX-2 specific antibody at a dilution of 1/100 following a standard IHC procedure. Antibody staining was visualized using 3,3'-diaminobenzidine peroxidase substrate solution and cell nuclei were counterstained using hematoxylin QS. Mouse IgG without the primary antibody was used as a negative control. Representative samples of COX-2 staining in control tissue (A), normal in-field (B), LD (C), MD (D), and CIS (E) are shown. A representative image is showing the epithelial layers (basement membrane, B/PB layer, LS layer, and upper spinous layer) in a normal epithelial tissue sample, stained with hematoxylin (F).



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Table 2. COX-2 correlation with each stage of premalignancy and its corresponding epithelial layers

Epithelial layer		B/PB layer	LS layer	Upper spinous layer	Total	Correlation P
Normal control	<i>n</i>	11	11	11	33	<0.0001
	COX-2	0.0	0.0	2.0		
Normal in-field	<i>n</i>	25	25	25	75	<0.0001
	COX-2	0.0	1.0	2.0		
LD	<i>n</i>	14	14	14	42	0.024
	COX-2	1.0	0.9	1.9		
MD	<i>n</i>	29	29	29	87	0.009
	COX-2	1.3	1.0	2.0		
CIS	<i>n</i>	14	14	14	42	0.896
	COX-2	1.3	1.2	1.4		
Correlation P		<0.0001	0.002	0.1		

NOTE: *n*, total number of samples per category. COX-2, mean COX-2 value obtained for each category.

Similarly, Fisher exact test was used to compare COX-2 expression with respect to the binary variables listed in Table 1. When the variable had more than two categories, such as differentiation, the χ^2 exact test for comparing more than two population proportions was carried out. Kaplan-Meier estimator was used to estimate the survival distribution of all patients and to compare the overall survival of patients with COX-2 expression 0 relative to 1. The Cox proportional hazards model was used to compare the overall survival distributions between these two groups adjusting for relevant confounding variables.

Results

Premalignant Lesions

A total of 36 patients were enrolled on the chemoprevention trial (27, 28). Tissue samples were available from 25 subjects. Squamous mucosal samples were composed of 10 normal mucosa from healthy samples, 25 histologically normal in field of cancer, 14 with LD, 29 with MD, and 14 with severe dysplasia or CIS. For each of the samples, B/PB, LS, and upper spinous/granular were available for our analysis, as shown in Fig. 1.

COX-2 was found to be expressed in early and intermediate stages of HNSCC carcinogenesis, and increased through the different epithelial layers, its lowest expression being in the B/PB, and the highest in the US layers (Table 2; Figs. 1 and 2). This correlation was statistically significant in histologically normal, normal in-field, LD, and MD epithelium, but did not reach statistical significance in the CIS cohorts (Table 2). In addition, COX-2 expression was found to increase in premalignant stages of HNSCC carcinogenesis from normal in-field to moderate grade dysplasia and CIS. (Table 2).

Invasive Carcinoma

A total of 38 of the 54 patients with invasive HNSCC had COX-2 expression analyzed in tissue samples. Their clinical characteristics are shown in Table 1. The male to female ratio was 7:1. Response to systemic therapy was reported as 81% (31 of 38), including complete and partial remissions. The majority of patients (87%) had stage IV disease (33 of 38). We used a cutoff point of COX-2 expression of 0.856 to differentiate between low versus high expressers in our survival analysis. Four patients had no expression of COX-2. Seventeen

patients had COX-2 expression below the mean (<0.856), whereas 21 had expression above the mean (>0.856). Of note is the lack of association between COX-2 expression and gender, response to therapy, nodal stage, American Joint Committee on Cancer stage, site, and degree of differentiation. There was, however, a correlation noted with primary tumor T stage ($P = 0.033$).

The 5-year overall survival rate of the 38 patients was 63% (Fig. 3), which is comparable with that in more recent trials using induction chemotherapy in treatment of HNSCC (32). Expression of COX-2 seemed to correlate with overall survival. Patients whose tumor had COX-2 expression of <0.856 (below the mean) had a 5-year survival rate of 78%, significantly greater than that in patients whose COX-2 level was >0.856 ($P = 0.026$), who had a 5-year survival rate of 49% (hazard ratios, 3.19; Fig. 4). A multivariable analysis adjusting for clinical

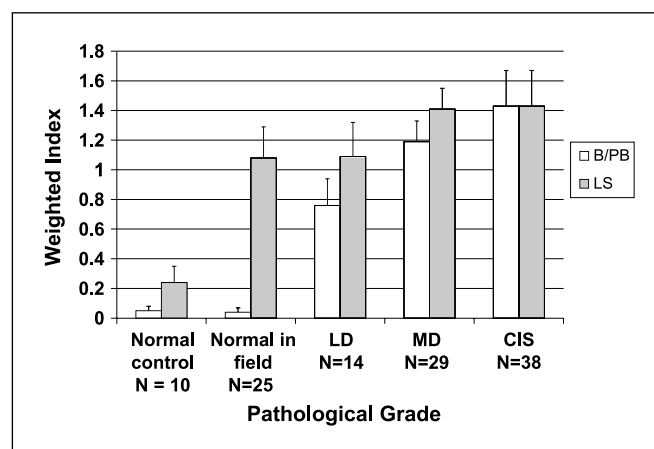


Fig. 2. COX-2 expression level by IHC in different premalignant tissues. COX-2 expression in the cell cytoplasm was scored using a semiquantitative scale ranging from 0 (no expression) to 3+ (highest level of expression) on a cell-by-cell basis in 10 consecutive microscopic fields ($\times 200$) in a defined area. The weighted index of COX-2 expression was calculated as follows: weighted index of COX-2 expression = (number of cells counted \times degree of intensity)/total number of cells counted $\times 100$. COX-2 expression is shown for the B/PB and LS layers of the different premalignant lesions. *N*, total number of samples for each category.

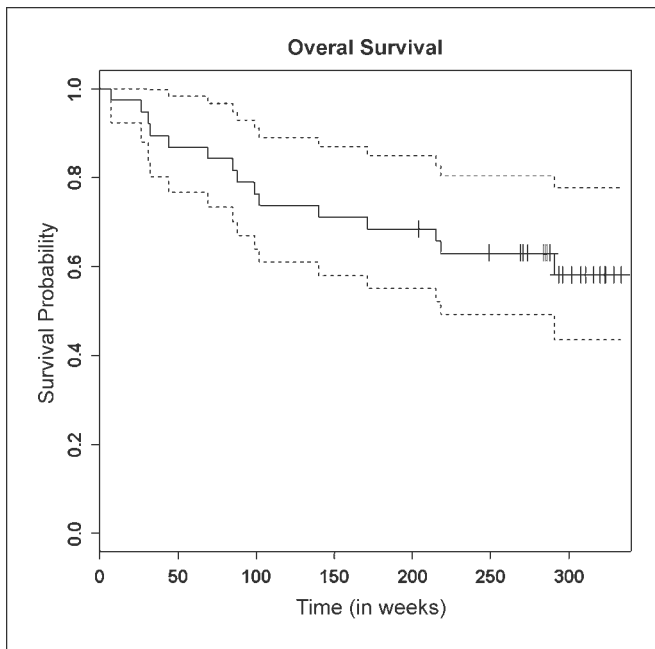


Fig. 3. TIC induction trial: reported overall survival. The 5-y overall survival rate of the 38 patients treated on the TIC induction trial.

and demographic variables including smoking status showed that COX-2 expression was still a statistically significant predictor of survival ($P = 0.033$) with an adjusted hazards ratio of 3.64. When we stratify by smoking status, the test of hypothesis that COX-2 expression is the same in all strata had a P value of 0.1694.

Discussion

Our findings support those of other published reports in showing that COX-2 is involved in early and intermediate stages of carcinogenesis in HNSCC, although we found its expression to decrease in invasive carcinoma.

Studies looking at COX-2 expression in various stages of carcinogenesis are scarce. COX-2 expression has been investigated in other premalignant and malignant lesions besides HNSCC, such as gastric lesions from *Helicobacter Pylori*-infected patients with chronic active gastritis, gastric atrophy, intestinal metaplasia, and frank carcinoma (31). Other studies have suggested a close relationship between hepatitis B infection and COX-2 expression in chronic hepatitis B and hepatocellular carcinoma (32). In squamous cell carcinoma of the lung, there seems to be a clear correlation between the stage of the preneoplastic lesion and the degree of COX-2 expression, with severe dysplasia being a cutoff point and seemingly a key step in early lung carcinogenesis. In this and other similar studies, COX-2 expression seems to be weak in metaplasia and only increases in dysplasia and CIS, which is consistent with our findings in premalignant lesions of the head and neck (33).

Our findings indicate that COX-2 is involved in early and intermediate stages of carcinogenesis in HNSCC. As carcinogenesis progresses, COX-2 levels increase from the B/PB layer to the LS and upper spinous layers in all stages of carcinogenesis except in CIS. Although we cannot explain the observed pattern of COX-2 expression in different epithelial layers from

a biological standpoint, it may reflect the role COX-2 plays in HNSCC carcinogenesis. It is worth noting that other markers such as epidermal growth factor receptor and proliferating cell nuclear antigen expression have been noted to be over-expressed in the basal and parabasal layers compared with the superficial layers in premalignant head and neck tissues (34–36). This, in addition to our findings, suggests that these distinct epithelial layers may play different roles in cellular differentiation and carcinogenesis. These patterns although only descriptive at the present, may have a bearing on future understanding of the role COX-2 plays in the different stages of cell differentiation and malignant transformation.

COX-2 expression in premalignant oral lesions has been studied by several groups. COX-2 has been shown to be extensively expressed in oral leukoplakia and premalignant lesions. Its expression seems to increase in high-grade premalignant lesions (37). In a study looking at COX-2 gene expression in healthy persons, persons with oral dysplasia, and carcinomas, COX-2 was up-regulated from healthy (3%) to premalignant lesions (41%) to oral carcinomas (88%; ref. 38). In contrast to our findings, there was no correlation found in this study between histologic grading of oral premalignant lesions and the level of COX-2 expression. In another study of similar design, the level of COX-2 expression in oral premalignancies including oral leukoplakia and leukoplakia with and without dysplasia was significantly higher than those in normal mucosa ($P < 0.05$), but lower than those in oral invasive carcinoma ($P < 0.01$). There was also no statistically significant difference in the expression of COX-2 among the oral premalignancy groups ($P > 0.05$; ref. 39). Other studies reported a correlation between COX-2 level and grade of dysplasia, with the highest expression found in severe dysplasia (40). Our findings are therefore consistent with others, showing that the level of COX-2 expression increases throughout the process of carcinogenesis. Possible biases in our study include the fact that not all tissues were available for IHC for both premalignant as well as carcinoma samples from both premalignant and carcinoma cohorts. Although this could have potentially biased the

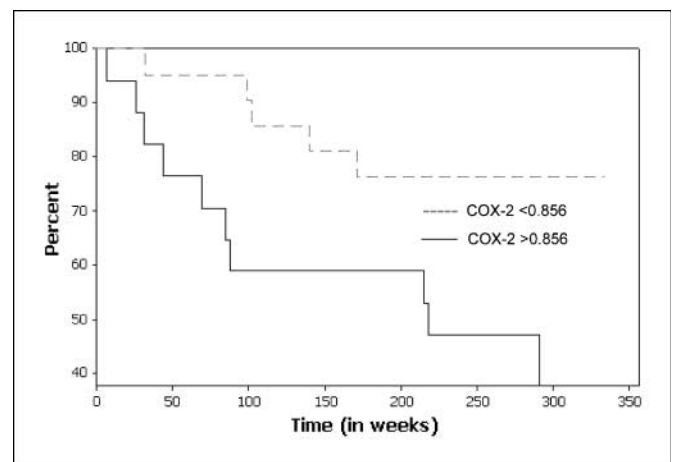


Fig. 4. COX-2 expression: impact on survival in induction trial. Kaplan-Meier estimator was used to estimate the survival distribution of all patients and to compare the overall survival of patients with high versus low COX-2 expression. A cutoff point of COX-2 expression of 0.856 was used to differentiate between low versus high expressers in the survival analysis.

results, no specific characteristics could be singled out that would predict the lack of tissue availability on either studies. Further investigation will be required to determine the exact role of COX-2 in the different premalignancy stages of head and neck carcinogenesis.

For HNSCC, our results suggest a correlation between COX-2 expression and HNSCC tumor size, which duplicates findings from other studies (41), showing a correlation between COX-2 mRNA level in peripheral blood and both tumor size and prognosis, although no correlation was found with nodal status, response to therapy, site, overall stage, and grade of disease in our study. These and similar reported findings suggest the potential use of COX-2 expression as a reliable tumor marker. In contrast to other reports, however, we found a lower level of COX-2 expression in HNSCC compared with that in premalignant lesions. These findings however, do not necessarily reflect the actual pattern of COX-2 expression between premalignant and malignant lesions. The lack of progressive increase in COX-2 may be related to the heterogeneity as well as a possible modulation of expression when progressing through the different stages of premalignancies and ultimately to carcinoma. This may partially explain the decrease in COX-2 expression we observed in carcinoma compared with premalignant samples. It is also not inconceivable that a genetic alteration occurring at a later stage in the carcinogenic process may interact with COX-2 and affect its expression in malignant tissues.

In looking at COX2 expression in malignant HNSCC samples, there was no specific rationale for choosing patients treated with TIC chemotherapy beyond the fact that they were treated on the same study that promoted homogeneity. We felt

that this would provide more reliable information compared with a retrospective analysis of patients outside of a clinical study. It is also worth noting that all analyzed tissues were from biopsies and were obtained before any cancer therapy was administered. Validation of COX-2 expression and its prognostic significance in invasive carcinomas of the head and neck needs further confirmation in large sample-size clinical trials.

In conclusion, our findings elucidate the expression level of COX-2 in different stages of head and neck premalignant lesions, and correlate COX-2 expression with clinical outcome in patients with invasive HNSCC. It indicates that COX-2 seems to play a role in the process of transformation from lower to higher grades of premalignancies, and continues to play a role in the behavior of invasive carcinoma where its higher expression seems to correlate with worse clinical outcome. An expanding body of evidence indicates that down-regulation of the COXs will be an important strategy for preventing cancer. Whether this down-regulation will translate into benefits to patients with invasive carcinomas as well is the current topic of investigation on clinical studies.

Evaluation of COX-2 inhibition seems to be justified in trials focusing on both primary chemoprevention, second primary tumor prevention for HNSCC, as well as possible therapy for invasive HNSCC. Evaluation of the role of COX-2 inhibitors as targeted therapies in combination with other potentially chemopreventive agents, such as epidermal growth factor receptor inhibitors, in premalignant head and neck lesions as well as invasive carcinomas is the subject of currently ongoing studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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