Prognostic Significance of Cyclooxygenase-2 Overexpression in Glottic Cancer

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Abstract

Purpose: Cyclooxygenase-2 (COX-2) overexpression has been associated with a poor prognosis in many cancers. However, the role of COX-2 overexpression in head and neck cancers remains undetermined. The objective of this study was to evaluate whether COX-2 is a prognostic factor in glottic cancer.

Experimental Design: This study was part of a phase III placebo-controlled randomized trial evaluating the efficacy of α-tocopherol in reducing second primary cancers (SPC) in head and neck cancer patients. Immunohistochemical analyses were conducted on pretreatment biopsies of 301 patients with early-stage glottic cancer treated by radiotherapy. The median value of 50% of positive tumor cells was the cutoff point used to define COX-2 overexpression. Outcomes considered in the statistical analysis were recurrence, SPC, and death. The Cox proportional hazards model was used to estimate the hazard ratios (HR) and their 95% confidence intervals (95% CI).

Results: The HR associated with COX-2 overexpression was 0.94 (95% CI, 0.55-1.62) for recurrence. The HR associated with SPC was 2.63 (95% CI, 1.32-5.23) for the first 3.5 years of follow-up and 0.55 (95% CI, 0.22-1.32) for the following 3.5 years. The HR associated with COX-2 overexpression was 1.57 (95% CI, 1.01-2.45) for overall mortality.

Conclusions: COX-2 overexpression in glottic cancer was associated with increased overall mortality and an increased risk of SPC during the early follow-up period. Future studies are needed to explain observed effects on SPC. COX-2 expression may prove helpful in defining an individual patient's prognosis.

Glottic cancer is the most common early-stage head and neck cancer. The 5-year overall survival rate is between 72% and 85% for stage I cancer and from 59% to 77% for stage II cancer (1–3). Local recurrence occurs in about 6% to 28% of patients within 5 years (1). Another important outcome for patients with early-stage glottic cancer is the occurrence of a second primary cancer (SPC), which is discovered in 15% to 18% of the patients within 5 years and is associated with poor survival (2, 4, 5). The most common site of SPC is the upper aerodigestive tract (2, 5). SPCs are attributed to a "field cancerization" effect induced by carcinogens such as tobacco, which can alter the respiratory, oral, and esophageal mucosa (6, 7). Although the early stage of glottic cancer is relatively well controlled by therapy, prognosis of these patients is often compromised by the occurrence of SPC.

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid into prostaglandins. It exists in two isoforms, COX-1, which is constitutional, and COX-2, which can be induced by stimuli such as smoking and radiotherapy (8–10). COX-2 overexpression increases prostaglandins, which are known to promote tumor development, invasion, and metastasis by many mechanisms, most importantly by stimulating angiogenesis through the action of vascular endothelial growth (11–13). Prostaglandins also induce cell proliferation, reduce apoptosis, suppress the immune response, and favor invasion by their action on matrix metalloproteinases (11, 14). High levels of COX-2 expression have been found in many cancers (15–21) and have been associated with poor survival and increased risk of recurrence (22–28). In head and neck cancers, COX-2 overexpression has been clearly shown (29, 30) but its effect on prognosis is still uncertain (12, 31–34).

The purpose of this study was to determine the prognostic significance of COX-2 expression in glottic cancer. It was hypothesized that tumors associated with COX-2 overexpression

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Received 8/17/07; revised 9/12/07; accepted 10/10/07.

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Note: The material contained in this article has been partially presented at the American Society for Clinical Pathology Annual Meeting, October 18-22, 2006, Las Vegas, Nevada (Resident Research Award for best presentation) and at the XXVI Congress of the International Academy of Pathology, September 16-21, 2006, Montreal, Quebec, Canada.

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doi:10.1158/1078-0432.CCR-07-2028

Table 1. Association between COX-2 expression and standard prognostic factors in patients with glottic cancer

Factors		*n* (%)	COX-2 >50% (*n* = 139), *n* (%)	COX-2 ≤50% (*n* = 162), *n* (%)	*P*
Age (y)	<60	109 (36)	55 (40)	54 (33)	0.27
	60-69	112 (37)	45 (32)	67 (41)	
	≥70	80 (27)	39 (28)	41 (25)	
Gender	Male	264 (88)	124 (89)	140 (86)	0.46
Trial arm	Intervention	149 (49)	68 (49)	81 (50)	0.85
Smoking in the preceding year	Yes	170 (57)	73 (53)	97 (60)	0.20
Alcohol intake in the preceding year	>1 drink per day	73 (24)	31 (22)	42 (26)	0.46
Charlson comorbidity index	≥1	116 (39)	48 (35)	68 (42)	0.19
Tumor stage	I	232 (77)	104 (75)	128 (79)	0.39
Histologic grade	2 or 3	180 (60)	91 (65)	89 (55)	0.06
Radiotherapy (total dose)	>64 Gy	178 (59)	82 (59)	96 (59)	0.96

Abbreviation: *n*, number of patients.

would result in an increased risk of recurrence and overall mortality. Furthermore, it was hypothesized that COX-2 expression could be induced in the whole oral and respiratory mucosa by stimuli such as tobacco smoking, predisposing to the development of SPC. To our knowledge, the association between COX-2 and SPC has never been studied before. It is clear that an immunohistochemical marker that could predict which tumors are more at risk for death, SPC, and recurrence might prove useful, especially if this marker is a potential pharmaceutical target.

Materials and Methods

Patient selection and study design. This study was conducted as part of a phase III multicenter, randomized, double-blind, placebo-controlled chemoprevention trial, which had as primary objective to evaluate the efficacy of antioxidant vitamin supplementation in decreasing the occurrence of SPC in head and neck cancer patients (35). The vitamin supplementation consisted of DL-α-tocopherol, a synthetic form of vitamin E, given at a daily dosage of 400 IU, and β-carotene at a daily dosage of 30 mg. The supplementation was given during radiotherapy and for 3 years after the end of radiotherapy. Concerns about the adverse effects of β-carotene supplementation prompted the investigators to halt its use in January 1996 after the first 156 patients had been enrolled. The trial was then continued with α-tocopherol alone. Between October 1, 1994 and June 6, 2000, 540 patients with a first diagnosis of stage I or II head and neck squamous cell carcinoma treated by radiotherapy were recruited in five radiation oncology centers of the Province of Québec, Canada, to take part in this study. The study protocol was approved by the ethics committees of all the institutions involved. For the purpose of this immunohistochemical study, the study population was restricted to glottic larynx cancer, which is by far the most frequent early-stage head and neck cancer, to ensure homogeneity because the site of origin may influence prognosis.

Diagnostic confirmation and histologic grade assessment. One observer (M.K.S.) reviewed the histologic slides of pretreatment biopsies to confirm the diagnosis of squamous cell carcinoma and to evaluate the histologic grade of tumors in a uniform manner. Assessment of the histologic grade was based on the extent of tumor keratinization, the presence of intercellular bridges, cellular pleomorphism, and mitosis. All lesions were categorized as grade 1 (well differentiated), grade 2 (moderately differentiated), and grade 3 (poorly differentiated).

Immunohistochemical analyses. COX-2 expression was determined by immunohistochemistry on freshly cut 4-μm tissue sections of the paraffin-embedded pretreatment biopsies. Slides were deparaffinized with toluene and solutions of 100% and 95% ethanol. Microwave heat-induced epitope retrieval was done with a pH 6.0 citrate buffer solution. Endogenous peroxidase activity was blocked with a 3% solution of hydrogen peroxide. Goat serum was used to prevent nonspecific antigen binding. Slides were then incubated for 1 h with a mouse monoclonal antibody, clone COX 229 (Zymed Laboratories, Inc.), at a 1:50 dilution. A biotinylated secondary antibody (DAKO) followed by streptavidin-horseradish peroxidase (DAKO) was then incubated for 20 min each. The chromogen diaminobenzene was applied for 6 min. Slides were then counterstained with Mayer's hematoxylin, dehydrated with increasing gradients of ethanol, ranging from 50% to 100%, and treated with toluene and xylene before being mounted. A colon cancer was used as the positive control. The COX-2 antibody was omitted for the negative control.

One observer (M.K.S.), blinded to patient baseline characteristics and outcomes, assessed tumor staining. The percentage of positive tumor cell intensity was evaluated quantitatively from 0% to 100%. We elected a priori to use the median value, which corresponded to 50% of positive tumor cells, as the cutoff point to define overexpression. The intensity was also assessed using an arbitrary scale from 0% to 100% and 50% was also used as cutoff point.

Data collection and patient follow-up. Baseline data collection was completed before randomization. A structured questionnaire administered to all patients by research nurses provided data on patients' socioeconomic characteristics, weight, height, medical history, alcohol and tobacco consumption, and diet. Radiation oncologists provided information on the primary tumor: site, dimensions, and clinical tumor-node-metastasis stage. Comorbidities were assessed using the Charlson comorbidity index (36). Follow-up visits with study nurses and radiation oncologist were scheduled immediately at the end of the radiation therapy, 1 month later, and then every 6 months for the first 3 years followed by once a year until the end of the study (June 30, 2003). Radiation oncologists monitored for recurrence of the primary cancer or development of a SPC. Medical notes, hospital records, and pathology reports were obtained from the medical centers. Death certificates were obtained from medical records and the "Institut de la statistique du Québec." To ensure complete ascertainment, record linkage with the Québec mortality files was done using the unique Québec health insurance identifier (37).

Clinical outcomes. The clinical outcomes considered were death, local recurrence, any recurrence (local, regional, and distant combined), and SPC. Death was subdivided into three categories according to cause: death from first (glottic) cancer, from SPC, or from other causes.

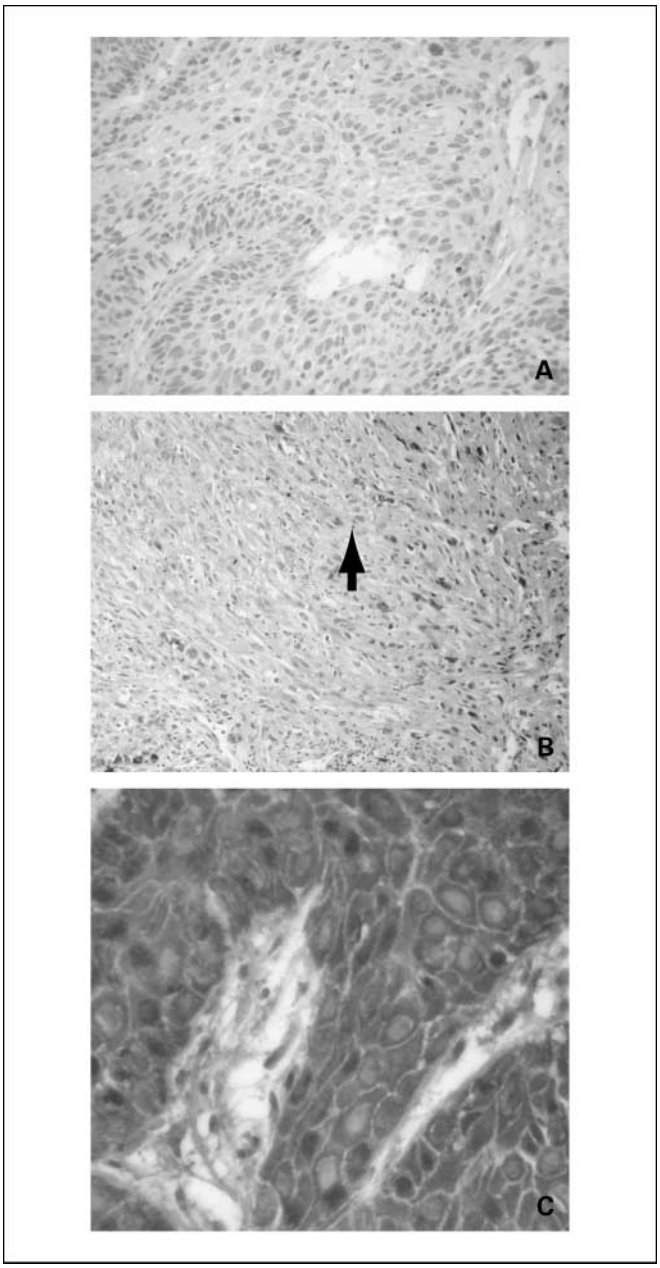

Fig. 1. Immunostaining for COX-2. *A,* no immunostaining. *B,* arrow, expression of <50% of cells. *C,* overexpression of >50% cells.

Strict criteria were applied for the diagnosis of SPC to distinguish them from first cancer recurrence (35).

Statistical analyses. The clinical prognostic factors evaluated as potential confounders in the statistical analysis were tumor-node-metastasis stage, histologic grade, age, sex, intervention arm of the trial, smoking, alcohol consumption, Charlson comorbidity index, and radiotherapy variables (2).

Standard statistical tests were used to assess the association between COX-2 expression and other prognostic factors. For overall mortality, Kaplan-Meier survival curves were used to describe time until the occurrence of the outcome depending on the level of COX-2 expression. For the survival analyses, follow-up time was calculated from the time of randomization until the time of the last visit or the date of the outcome of interest (recurrence, SPC, or death). For death, follow-up was calculated from the time of randomization until death or December 31, 2004.

The Cox proportional hazards model was used to estimate the associations between COX-2 overexpression and recurrence of the first primary cancer, occurrence of a SPC, and death (overall and from specific causes). The proportionality assumption of the Cox models was evaluated using models with a time-dependent coefficient. The effect of supplementation on the occurrence of a SPC varied according to time ($P = 0.007$). We then conducted multivariate analyses evaluating the risk of SPC associated with COX-2 overexpression according to two time periods: (*a*) from entry until 3.5 years and (*b*) beyond 3.5 years after randomization. We also examined whether the associations between COX-2 expression and outcomes were modified by trial arm using the Wald test. There was no statistically significant interaction between expression of COX-2 and trial arm for recurrence (P value for interaction test = 0.91), SPC (P value for interaction test ≥ 0.37), or for overall and specific death (P value for interaction test ≥ 0.34). These results indicated that the effect of COX-2 on the selected outcomes could be evaluated among all patients without stratifying for trial arm. For each outcome, the Cox model was also used to take into account potential confounders, such as trial arm, standard prognostic variables, as well as variables strongly associated with each outcome in the data. For overall mortality, the factors included in the model were age (continuous), tumor-node-metastasis stage (I versus II), histologic grade (1 versus 2 or 3), trial arm (supplementation arm versus placebo arm), and COX-2 expression (≤50% versus >50%). For SPC, the factors included in the model were age (continuous), Charlson comorbidity criteria (<1 versus ≥1), smoking status in the year preceding the randomization (smoker versus nonsmoker), trial arm (supplementation arm versus placebo arm), and COX-2 expression (≤50% versus >50%). To evaluate whether COX-2 added prognostic information to standard prognostic factors, we also generated the likelihood ratio test, which is the difference between the maximized log likelihood statistics for the models including standard prognostic factors with and without the COX-2 variable. All analyses were done using Statistical Analysis System, version 8.2 (SAS Institute). A two-sided α was fixed at 0.05 for all statistical tests.

Table 2. Crude HRs of recurrence, SPC, and mortality associated with COX-2 expression

Outcomes	COX-2 >50% (*n* = 139), no. events (%)	COX-2 ≤50% (*n* = 162), no. events (%)	Crude HR (95% CI)
Any recurrence	23 (17)	30 (19)	0.94 (0.55-1.62)
Local recurrence	21 (15)	26 (16)	0.99 (0.56-1.62)
SPC			
During the first 3.5 y of follow-up	25 (18)	12 (7)	2.63 (1.32-5.23)
Beyond 3.5 y of follow-up	7 (8), *n* = 84	16 (14), *n* = 113	0.55 (0.22-1.32)
Death			
From all causes	44 (32)	35 (22)	1.57 (1.01-2.45)
From first cancer	10 (7)	12 (7)	1.04 (0.45-2.40)
From SPC	16 (12)	10 (6)	2.04 (0.93-4.50)
From other causes	18 (13)	13 (8)	1.71 (0.84-3.49)

Results

Patient and tumor characteristics. Of the 348 patients with glottic cancer in the clinical trial, 301 (86.5%) had material available for immunohistochemistry. The main baseline patient characteristics are presented in Table 1. The mean age was 63 years (SD, 10). Two hundred thirty-two (77%) patients had stage I cancer. The majority of patients were males and were smokers. One hundred forty-nine (49%) patients with glottic cancer were in the trial arm. All patients were treated for their glottic cancer by first intention radiation therapy. The mean dosage was 61 Gy (SD, 7.4). COX-2 staining was cytoplasmic in all cases. There was also some staining in the adjacent normal or dysplastic mucosa. Both COX-2 overexpression and staining intensity were correlated with prognostic factors and outcome. However, the intensity of the staining did not provide any significant information and overexpression only was further investigated.

Association between COX-2 and prognostic factors. Figure 1 shows examples of immunostaining for COX-2. There was no statistically significant association between any of the prognostic factors studied and COX-2 overexpression (Table 1). Although not statistically significant, there was a trend for higher-grade tumors to overexpress COX-2. Of patients whose tumor overexpressed COX 2, 65% had grade 2 or 3 tumors, whereas of those whose tumor underexpressed COX 2, 55% had grade 2 or 3 tumors ($P = 0.06$).

COX-2 overexpression and outcomes. After a median follow-up of 4.3 years, a total of 53 patients had a recurrence of their first glottic cancer (including 47 local recurrences), and 60 patients had a SPC. The main site of SPC was the lung and trachea ($n = 25$). After a median follow-up of 6.5 years, there were 79 deaths: 22 from the first glottic cancer, 26 from SPC, and 31 from other causes.

Crude associations between COX-2 expression and outcomes are reported in Table 2. There was no association between COX-2 and recurrence of the first glottic cancer [hazard ratio (HR), 0.94; 95% confidence interval (95% CI), 0.55-1.62]. Similarly, there was no association between mortality from the first glottic cancer and COX-2 overexpression (HR, 1.04; 95% CI, 0.45-2.40). Figure 2A shows that patients who overexpressed COX-2 had an increased risk of overall mortality compared with those with <50% of COX-2 expression ($P = 0.04$). Table 3 shows that, even after taking into account other prognostic factors, the association between COX-2 and overall mortality remained similar and statistically significant (HR, 1.62; 95% CI, 1.04-2.53). Survival curves showed that an increased risk of SPC was observed in patients with COX-2 overexpression; however, this effect was only observed during the first 3.5 years of the trial (Fig. 2B). After adjustment for other prognostic factors of SPC, COX-2 overexpression remained strongly associated with an increased risk of SPC during the first 3.5 years of the trial (HR, 3.05; 95% CI, 1.52-6.13), whereas there was an inverse nonsignificant association beyond 3.5 years (Table 4).

Discussion

In our study, COX-2 tumor overexpression was associated with a significantly increased risk of overall mortality. In addition, patients who had COX-2 overexpression in their first glottic tumor presented an increased risk of having a SPC during the first 3.5 years of the clinical trial.

Current literature reveals conflicting results for the risk of overall mortality associated with COX-2 overexpression. Our results agree with those of Gallo et al. (12) who found that COX-2 overexpression was associated with poorer overall survival. They studied in multivariate analysis 52 patients with stage I to IV head and neck cancer treated by surgery. In addition, Chang et al. (34) reported an association between COX-2 overexpression and decreased 3-year survival (odds

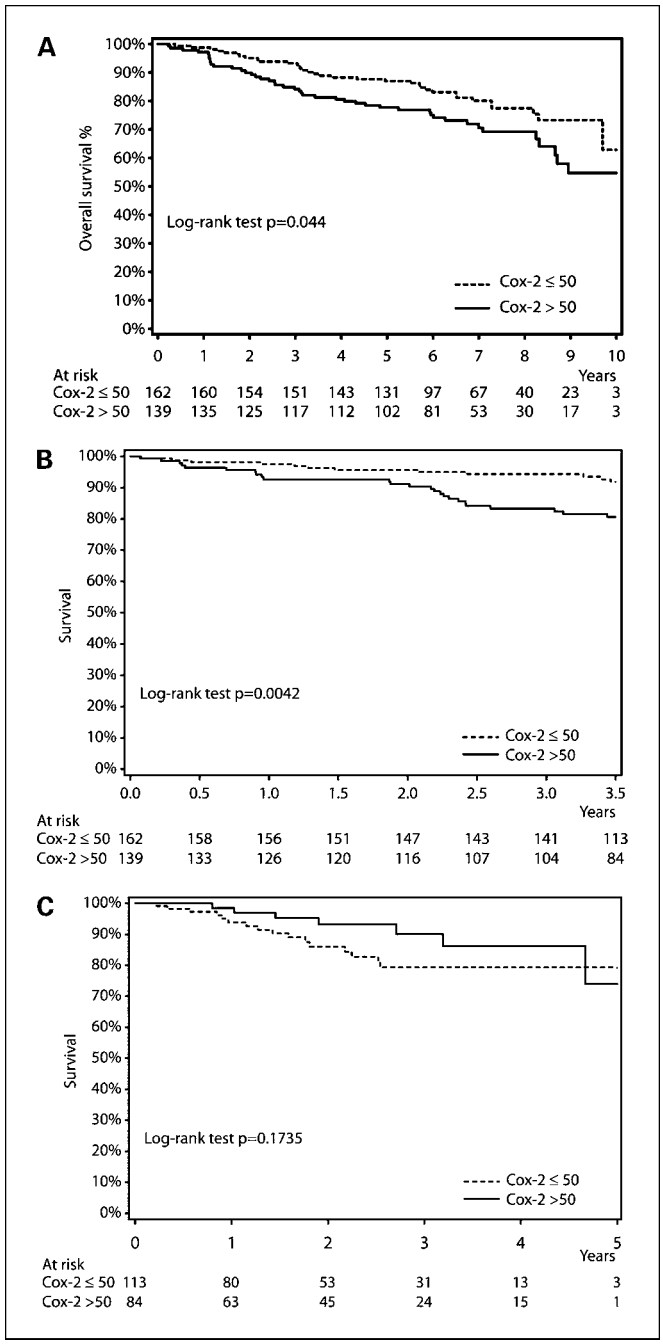

Fig. 2. *A,* overall survival according to COX-2 expression. Kaplan-Meier survival curves until occurrence of a SPC according to COX-2 expression during the first 3.5 y after randomization (*B*) and beyond 3.5 y after randomization (*C*).

Table 3. Adjusted HRs of overall mortality associated with COX-2 expression and other prognostic factors

Factors	Adjusted HR (95% CI)
Age (continuous)	1.06 (1.03-1.08)
Tumor-node-metastasis stage	
Stage I	1.00
Stage II	1.56 (0.95-2.58)
Histologic grade	
Grade 1	1.00
Grade 2 or 3*	1.12 (0.70-1.78)
Trial intervention arm	
Placebo arm	1.00
Supplementation arm	1.54 (0.98-2.58)
COX-2 expression	
≤50%	1.00
>50%	1.62 (1.04-2.53)
Likelihood ratio test statistic for COX-2 expression = 4.51; ddl = 1; $P = 0.03$	

*There were 16 cases with grade 3.

ratio, 0.41; 95% CI, 0.20-0.84) in 82 patients with stage I to III oropharyngeal cancer receiving several treatment modalities. However, results were adjusted only for age. On the other hand, Ranelletti et al. (33) found that low levels of COX-2 expression were associated with a decrease in overall survival. This study was conducted among 61 patients with stage I to IV larynx cancer having received different treatment modalities. Bayazit et al. (31) found no correlation between COX-2 expression and survival, but this study was restricted to a small group of 39 patients with stage I to IV larynx cancer treated by surgery. All these studies were carried out on patients with different tumor-node-metastasis stages and/or exposed to a variety of therapies. It is well recognized that the prognosis differs between tumors from different head and neck sites as well as between early-stage (I and II) and late-stage (III and IV) cancer (38). Furthermore, the literature suggests that COX-2 overexpression is induced by radiotherapy (10, 11) but not by surgery. The difference in these results might also be explained in part by the lack of a uniform measurement method for COX-2 expression and definition for COX-2 overexpression. One of the strengths of our study is that it has the largest and most uniform group of patients in terms of tumor location, cancer staging, and treatment modalities.

The relationship found between COX-2 overexpression in the first glottic tumor and the risk of SPC is intriguing. Several hypotheses can be proposed to explain this finding. First, we cannot exclude the possibility that this significant association might have been obtained by chance because we conducted several comparisons to assess the effect of COX-2 overexpression on several outcomes. However, this association is plausible in light of our knowledge about COX-2 expression and the biology of SPC. COX-2 is an inducible enzyme that has been reported to be overexpressed in the oral mucosa of active smokers as well as in premalignant and malignant tissue (39, 40). The etiology of SPC, especially those of the aerodigestive tract, may be in part explained by the clinical phenomena of field cancerization (6, 41). Mucosa may have undergone changes due to carcinogen exposure, such as tobacco and alcohol, and is therefore susceptible to the development of many foci transformation (42, 43). In addition, epidemiologic studies supported the role of tobacco exposure on the occurrence of SPC. Dikshit et al. (44) reported an increased risk of SPC of the lung in heavy cigarette smokers among a cohort of 876 patients with laryngeal or hypopharyngeal carcinoma. Day et al. (45) showed that smoking status collected at the time of detection of the first primary oral/pharyngeal cancer was a predictor of a second aerodigestive tract cancer. Current smokers have a 4-fold increased risk of SPC compared with nonsmokers and former smokers. Altogether, these studies support the hypothesis that COX-2 overexpression of the first primary cancer, induced by smoking, might be an indicator of the presence of a latent SPC. The fact that the association between COX-2 overexpression of the first tumor and the occurrence of SPC was only observed during the early years of follow-up may support this hypothesis.

Although our study found no relationship between COX-2 and recurrence, some authors found that COX-2 overexpression increased the risk of recurrence. Cho et al. (32) studied 123 patients with early-stage laryngeal cancer treated by radiotherapy. In a univariate analysis, there was no significant

Table 4. Adjusted HRs of SPC associated with COX-2 expression and other prognostic factors according to two periods

Factors	During the intervention period (first 3.5 y of the trial), HR (95% CI)	During the follow-up period (follow-up beyond 3.5 y), HR (95% CI)
Age (continuous)	1.03 (1.00-1.07)	1.03 (0.98-1.09)
Charlson comorbidity index		
<1	1.00	1.00
≥1	1.64 (0.84-3.35)	1.06 (0.44-2.57)
Smoking status in the preceding year		
Nonsmokers	1.00	1.00
Smokers	1.67 (0.84-3.35)	1.40 (0.56-3.48)
Trial intervention arm		
Placebo arm	1.00	1.00
Supplementation arm	2.21 (1.11-4.43)	0.29 (0.10-0.79)
COX-2 expression		
≤50%	1.00	1.00
>50%	3.05 (1.52-6.13)	0.54 (0.22-1.38)
	Likelihood ratio test for COX-2 = 10.70; ddl = 1; $P = 0.001$	Likelihood ratio test for COX-2 = 1.72; ddl = 1; $P = 0.19$

association between COX-2 overexpression and local recurrence (HR, 1.49; 95% CI, 0.73-3.02), but adjusted analyses showed an association with a relative risk of local recurrence of 2.57 (P = 0.01). Gallo et al. (12) found that COX-2 overexpression was associated with a decreased risk of disease-free survival. Surprisingly, Ranelletti et al. (33) found that disease-free survival at 5 years was 84% in patients with COX-2–positive tumors compared with 30% in those with COX-2–negative tumors (P = 0.0012). These contradictory data are difficult to explain but may be related in part to heterogeneity in tumor sites, stage, and treatment modalities among different studies.

The significant association between COX-2 overexpression with SPC and mortality and lack of association with local recurrence is also intriguing. We do not have a good explanation for this finding. Although we cannot exclude the possibility that this significant association might have been obtained by chance, we think that it might relate to the biology of COX-2. Indeed, our analyses showed that the standard prognostic factors were different, at least in part, for the overall mortality, SPC, or local recurrence. This suggests that COX-2 may act on a variety of biological variables and that overexpression may affect the mortality from other causes than cancer.

In conclusion, COX-2 overexpression in glottic cancer was shown to be significantly associated with an increased risk of overall mortality and an increased risk of SPC during the first 3.5 years of the follow-up period. The potential clinical implications are that the level of COX-2 expression may prove helpful in defining more precisely an individual patient's prognosis and assist in tailoring therapy. The use of COX-2 inhibitors in larynx cancer is a promising therapeutic avenue that will need to be explored (11, 46).

Acknowledgments

We thank Gilles Hamel who did the immunohistochemistry techniques, Dr. Raynald Morency for his expert insight on head and neck cancer, and Dr. Claude Lavigne for his advice.

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