

Cyclooxygenase-2 expression in squamous cell carcinoma of the oral cavity and pharynx: Association to p53 and clinical outcome

TIMO ATULA¹, JOHAN HEDSTRÖM^{1,2}, ARI RISTIMÄKI^{4,5}, PATRIK FINNE²,
ILMO LEIVO⁴, MARI MARKKANEN-LEPPÄNEN¹ and CAJ HAGLUND^{3,4}

¹Department of Otorhinolaryngology - Head and Neck Surgery, Helsinki University Central Hospital, P.O.Box 220;

²Department of Clinical Chemistry, Biomedicum Helsinki, P.O. Box 700; ³Department of Surgery, Helsinki University Central Hospital, P.O.Box 340, FIN-00029 HUS; ⁴Department of Pathology,

Helsinki University Central Hospital; ⁵Cancer Biology Research Program, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland

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Abstract. Cyclooxygenase-2 (COX-2) expression is up-regulated in transformed cells and in malignant tissues, including tumours of the head and neck, and it has prognostic significance in many types of cancer. COX-2 expression is suppressed by the wild-type but not by the mutant tumour suppressor gene *TP53*. The purpose of this study was to investigate the association between the expression of COX-2 and the clinical outcome in patients with oral and pharyngeal squamous cell carcinoma (SCC), and to examine its relationship to p53. Immunohistochemistry showed an elevated COX-2 expression in 88% (n=57; strong 38, weak 19) of the 65 tumour samples. The staining intensity was not associated with patient or tumour characteristics, nor with the immunohistochemical expression of p53. Kaplan-Meier analysis showed no significant correlation between COX-2 expression and recurrence-free or overall survival, but a strong p53 expression was associated with a poor recurrence-free (p=0.001, log-rank) and overall survival (p=0.003). We conclude that, unlike strong p53 expression, COX-2 expression does not have prognostic significance in advanced oral and pharyngeal SCCs.

Introduction

Despite the development in treatment strategies, patients with advanced oral and pharyngeal squamous cell carcinoma (SCC) have a poor prognosis. The overall 5-year survival rate in stage III and IV disease is less than 40% (1). The TNM

classification does not reliably predict the clinical outcome in individual patients, and recent research has focused on targeted therapies (1-4). Therefore, there is a need to further investigate molecular markers associated with the biological behaviour of the tumour.

Epidemiological studies suggest that the use of nonsteroid anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of cancer especially in the gastrointestinal tract (5). The best known target of NSAIDs is cyclooxygenase (COX), the rate-limiting enzyme in the conversion of arachidonic acid to prostanoids (6,7). Of the two known isoforms of COX, the expression of COX-2 is low or not detectable in most healthy tissues, but can be highly induced in response to cell activation by hormones, proinflammatory cytokines, growth factors, and tumour promoters (6,7). Through different mediators, COX-2 expression promotes angiogenesis, invasiveness, cell proliferation, immunosuppression and inhibits apoptosis (6,8). These many important processes in carcinogenesis make COX-2 an attractive therapeutic target (4,6-9).

Elevated COX-2 expression has been described in a wide variety of human premalignant and malignant conditions and in experimental animal models of carcinogenesis (6,7,10). COX-2 upregulation has been shown in head and neck squamous cell carcinomas (HNSCC) at both mRNA and protein levels (11,12), and it contributes to the growth and progression of HNSCC through multiple pathways (11,13-15).

Tumour suppressor gene *TP53* is commonly mutated in human malignancies, which leads to the accumulation of a mutant non-functional p53 protein (5,14,16). *TP53* mutations and altered p53 expression have often been associated with poor prognosis in HNSCC patients (2,16-20). p53 status is also associated with COX-2 expression (14,21-23), and the wild-type but not mutant p53 has been shown to suppress COX-2 transcription (21), and moreover, overexpression of COX-2 has been detected in HNSCCs containing mutant *TP53* (14).

Correspondence to: Dr Timo Atula, Department of Otorhinolaryngology, Head and Neck Surgery, P.O. Box 220, FIN-00029 HUS, Helsinki, Finland
E-mail: timo.atula@hus.fi

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As in various other cancer forms, the role of COX-2 as a possible target for inhibiting the growth of HNSCCs has been examined (7,9,24,25). Experimental studies show that COX-2 inhibition can suppress the growth of human squamous carcinoma cells (9,12,24,26), further supporting the role of COX-2 in HNSCC.

The prognostic significance of COX-2 has been shown especially in certain types of adenocarcinoma (10). Its prognostic significance in SCC is less investigated. In esophageal SCCs, it does not seem to have prognostic value (27). The prognostic significance of COX-2 in HNSCC has been examined by three groups reporting controversial results (13,15,28). For this reason, we investigated the association between the expression of COX-2 and the clinical outcome in patients with SCC of the oral cavity and pharynx, and in addition, the association between p53 and COX-2 expression.

Patients and methods

Patients and tumours. Formalin-fixed and paraffin-embedded tissue specimens from 65 patients with previously untreated invasive SCC of the oral cavity and pharynx were obtained from the files of the Department of Pathology, Helsinki University Central Hospital. The samples were originally drawn from a consecutive series of patients undergoing primary radical surgery including microvascular free-flap reconstruction at Helsinki University Central Hospital between 1989 and 1998. All samples were re-evaluated by the pathologist experienced in head and neck pathology (I.L.) to confirm the diagnosis. The data collected for each patient included age, sex, primary tumour site, tumour size, cervical nodal status, overall TNM classification and tumour stage, histological grade, treatment, tumour recurrence, and cause of death. The mean age at the time of diagnosis was 58 years (range 31-80). Twenty-one of the patients were women and 44 men. Of the 65 patients, 32 (49%) had a carcinoma in the oral cavity, 19 (29%) in the oropharynx, and 14 (22%) in the hypopharynx. According to the UICC (Unité internationale contre le cancer) TNM classification, none had stage I, 10 (15%) had stage II, 23 (35%) had stage III, and 32 (49%) had stage IV cancer. Thirty-eight patients (58%) had metastases in the neck and none had distant metastases at the time of diagnosis. Four patients (6%) received radiotherapy preoperatively, 55 patients (85%) postoperatively, whereas 6 patients (9%) underwent surgical treatment only. The median follow-up time was 1.49 years (range 0.14-9.9) for all the patients, and 2.1 years (range 0.64-9.9) for those who survived until the end of the follow-up.

Immunohistochemistry. The specimens were sectioned (4 μ m), deparaffinized, and microwaved for 4x5 min at 700 W in 0.01 mol/l Na-citrate buffer (pH 6.0) for antigen retrieval. The slides were immersed in 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity and then in blocking solution [1.5:100 normal horse serum in phosphate-buffered saline (PBS)] for 15 min to block non-specific binding sites. Immunostaining was performed with a COX-2-specific anti-human monoclonal antibody (160112; Cayman Chemical Co., Ann Arbor, MI) in a dilution of 1:200 (2.5 μ g/ml) in PBS containing 0.1%

sodium azide and 0.5% bovine serum albumin at room temperature overnight. Then the sections were treated with biotinylated horse anti-mouse immunoglobulin (1:200; Vector Laboratories Inc., Burlingame, CA) and avidin-biotin peroxidase complex (Vectastain ABCComplex, Vector Laboratories). The peroxidase staining was visualized with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO). Counterstaining was performed with Mayer's hematoxylin. For the control, the primary antibody was pre-adsorbed with human COX-2 control peptide (1-10 μ g/ml, Cayman Chemical) for 1 h at room temperature prior to the staining procedure. α -smooth muscle cell actin peptide (50 μ g/ml; Dako, Glostrup, Denmark) was used as a non-COX-2 peptide. The intensity of staining was scored into four groups; 0, no staining; 1, weak diffuse cytoplasmic staining (may contain stronger intensity in <10% of the cancer cells); 2, moderate/strong granular cytoplasmic staining in >10% of the cancer cells; 3, intense granular cytoplasmic staining in >50% of the cancer cells. The slides were scored by two pathologists (I.L. and A.R.) unaware of the clinical data.

The principle of immunohistochemistry for p53 (clone DO7, Dako), which recognizes both the mutant and wild-types of p53 was similar to that described for COX-2. The primary antibody was diluted 1:300. For antigen retrieval of p53, the sections were processed in a 700 W microwave oven for 4x5 min in a 0.3% citrate buffer (pH 6.0). Formalin-fixed paraffin-embedded sections of a known breast cancer specimen positive for the antigen served as the positive control in every staining batch. Sections treated with PBS instead of the primary antibody served as the negative controls. Staining for p53 was considered weak when <20% and strong when >20% of cancer cell nuclei showed staining (29).

Statistical analysis. The relationship between the staining intensity and pattern of COX-2 and p53, sex, age (>60 years), tumour site, grade, size (T-status), metastases (N-status), and stage were assessed with the Chi-square test. Fisher's exact test was used when necessary because of low expected values. Overall survival time was defined as the interval between primary surgical treatment and death or the end of follow-up (event: death, censored at the end of follow-up). Correspondingly, the end-point for recurrence-free survival time was the detection of recurrent disease or the end of follow-up (event: recurrence of disease, censored at the end of follow-up). Probabilities of overall and recurrence-free survival were calculated according to the Kaplan-Meier method. Differences in survival probability between the groups were assessed using the log-rank test. The Cox proportional hazards regression was used to estimate relative risks adjusted for other prognostic factors. All p-values were two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Expression of COX-2 and p53. Staining for COX-2 was detected in 88% (57 of 65) of the SCC samples (Fig. 1). The staining was negative (scored 0) in 11% (7/65), weak (score 1) in 58% (38/65), strong (score 2) in 28% (18/65), and intense

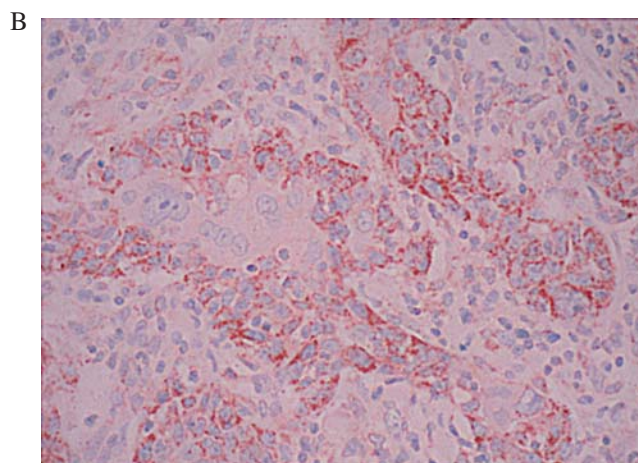
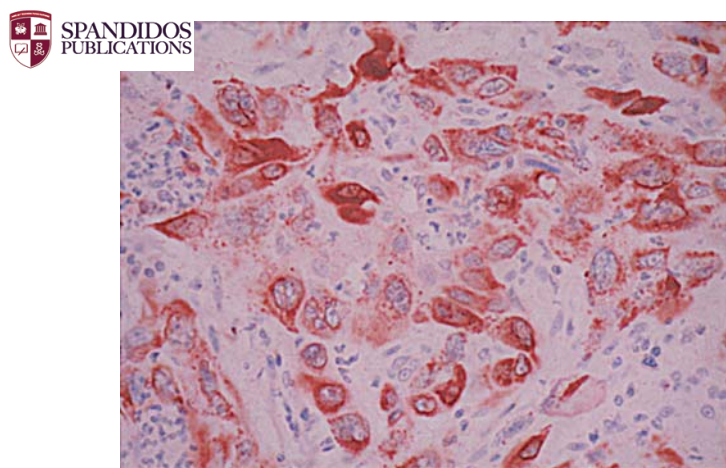


Figure 1. Immunohistochemistry for cyclooxygenase-2 (COX-2) in oral squamous cell carcinoma. (A) Strong cytoplasmic immunostaining is visible in moderately differentiated carcinoma cells. (B) Weak cytoplasmic immunostaining is seen in well-differentiated squamous cell carcinoma.

(score 3) in 1.5% (1/65) of the samples. For statistical analysis, COX-2 scores 0 and 1 (no staining or weak staining) and likewise scores 2 and 3 (strong and intense staining) were combined. The staining intensity for COX-2 showed no significant correlation with patients' sex or age, tumour site, grade, size (T-status), metastases (N status) or stage.

p53 expression showed no staining in 30% (19/63), it was weak in 29% (18/63) and strong in 41% (26/63) of the samples. For statistical analysis of p53, samples with no staining or weak staining were combined and compared with those with strong staining. p53 expression did not correlate with patients' sex or age, tumour site, grade, size (T-status), metastases (N status) or stage, and nor with COX-2 staining ($p=0.64$).

Association with clinical outcome. High tumour stage correlated with poor overall survival ($p=0.044$) while the T-status alone did not predict survival. Nodal status of N2 or N3 was associated with a shorter overall survival than the nodal status of N1 or NO ($p=0.006$). Tumour grade showed a weak trend with overall survival ($p=0.063$). Tumours in the oropharynx were associated with more favorable recurrence-free ($P=0.018$) and overall survivals ($p=0.043$) than oral or hypopharyngeal tumours, but until a follow-up time of two years these showed only small differences in survival rates.

For all the patients, the probability of surviving two years was 0.53 (95% CI 0.40-0.65), while the probability of recurrence-free survival was 0.48 (95% CI 0.35-0.62). COX-2 staining intensity did not correlate significantly with recurrence-free ($p=0.11$, log-rank test) or overall ($p=0.75$) survival (Fig. 2). The 2-year recurrence-free survival rates were 0.64 for the strong staining group and 0.43 for patients with weak or no staining. The corresponding rates for overall survival were 0.56 and 0.52.

The staining of p53 correlated with recurrence-free survival ($p=0.001$, log-rank test) and overall survival ($p=0.003$) (Fig. 3). When combining COX-2 staining intensity and p53 in a Cox regression analysis, p53 remained significant (relative risk 2.4, 95% CI 1.3-4.4, $p=0.004$), while COX-2 (strong vs weak or no staining) did not ($p=0.996$).

Discussion

We examined the association between COX-2 expression and patient outcome in oral and pharyngeal SCC, but found no correlation with recurrence-free or overall survival. In contrast to our results, elevated COX-2 expression is associated with unfavourable outcome in various cancers, such as gastrointestinal, breast and ovarian cancer (10,22). Three previous reports dealing with the prognostic significance of COX-2 in HNSCC show contradictory results (13,15,28). In a study by Ranelletti *et al* (28), including an immunohistochemical analysis of 61 laryngeal SCCs, stronger COX-2 staining was associated with a more favourable overall survival and reduced risk of tumour recurrence. In another study by Gallo *et al* (13), immunohistochemical analysis of 52 SCCs of the oral cavity, oropharynx, and larynx showed that strong COX-2 staining was associated with a reduced disease-free and overall survival. In a recent study of 70 patients including oral, laryngeal and a large number of lip carcinomas, COX-2 expression alone did not correlate with survival, but it was a sign for poor prognosis when combined with VEGF-C (15).

Differences in results between the studies may partly be explained by different kinds of patient materials. In addition to different distributions of tumour sites, our material consisted of more advanced tumours. In other studies, the proportion of stage I or II diseases varied from 23% (13) to 66% (15), while we had no stage I tumours and only 15% stage II tumours. There were also methodological differences between the studies. i) The antibodies were different. ii) A computer-based analysis of sample staining intensity was used in one study, and different cut-off values were tested in the survival analysis (28). In other studies, including ours, staining was analyzed by pathologists unaware of the clinical data. In our series, COX-2 was expressed in the majority (88%) of SCC samples as detected by immunohistochemistry. This is in line with other studies showing COX-2 overexpression from 44 to 96% of HNSCCs (15,30). COX-2 staining was localized to the cytoplasm of neoplastic cells, whereas the tumour stroma remained unstained. Normal squamous epithelium neighbouring the tumour showed weak

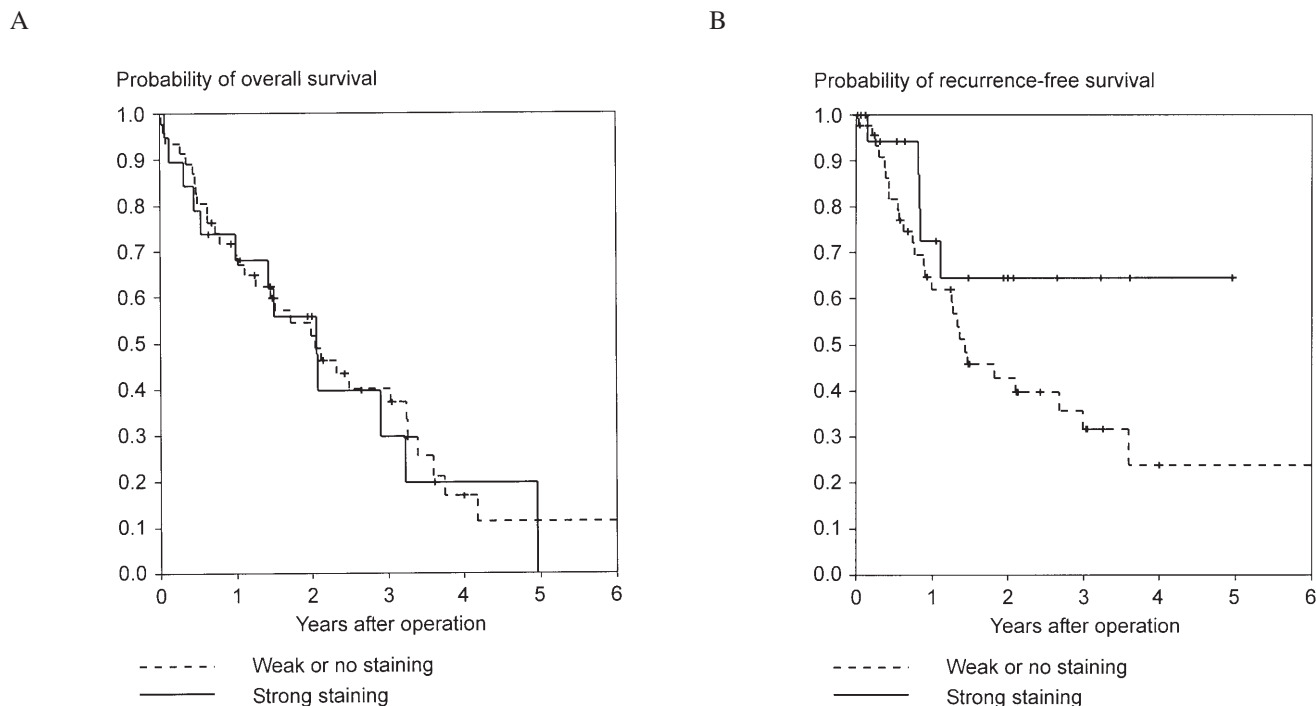


Figure 2. Staining intensity of COX-2 in tumour cells. (A) Kaplan-Meier curves for the corresponding overall survival (log-rank test, $p=0.75$). (B) Kaplan-Meier curves for the corresponding recurrence-free survival (log-rank test, $p=0.11$).

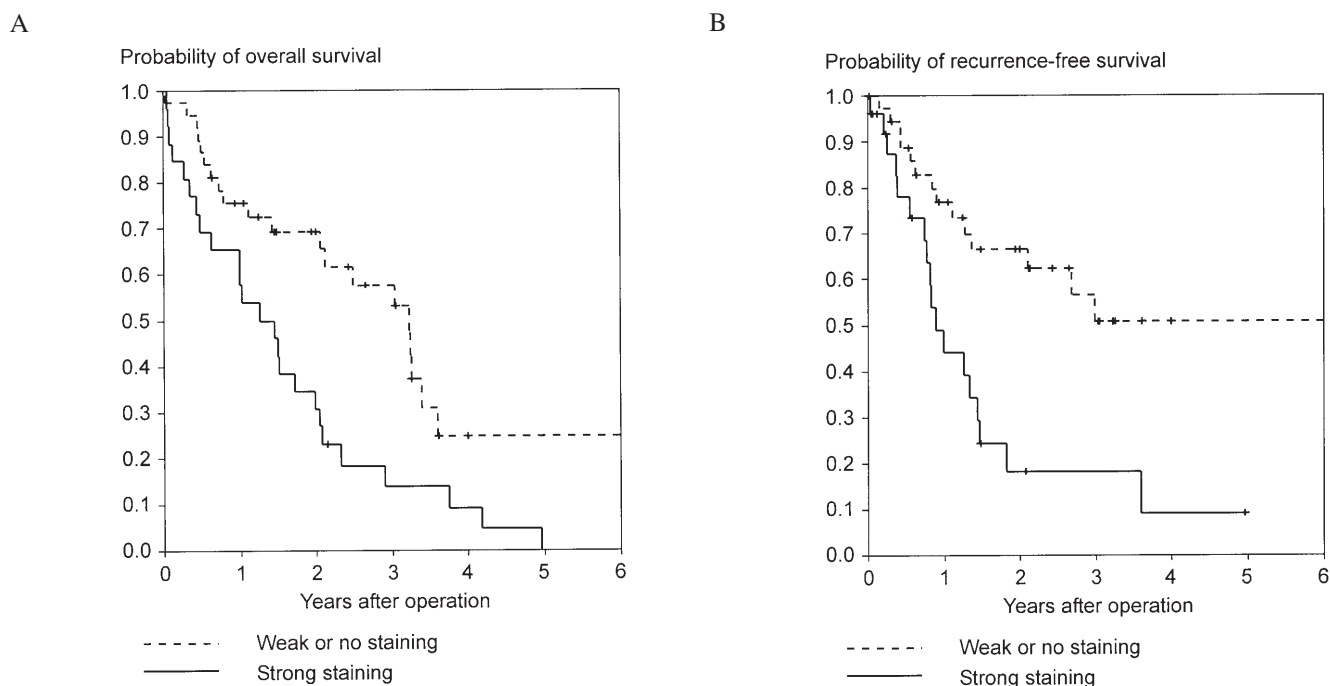



Figure 3. Staining intensity of p53 in tumour cells. (A) Kaplan-Meier curves for the corresponding overall survival (log-rank test, $p=0.003$). (B) Kaplan-Meier curves for the corresponding recurrence-free survival (log-rank test, $p=0.001$).

or no expression of COX-2. This is also in accordance with earlier studies of tumour specimens from HNSCC (12,13,30), nasopharyngeal carcinomas (31), as well as cancers from other anatomical and histological sites (10). However, the semiquantitative nature and limitations of immunohistochemistry may contribute to the discrepancies. Taken together, COX-2

expression does not have evident prognostic value in HNSCC, but obviously the number of patients included in studies on HNSCC is too limited to make definite conclusions. Furthermore, our study does not show whether COX-2 expression has prognostic significance at the early stage tumours. Reports on gastric and pulmonary

 SPANDIDOS PUBLICATIONS: inomas indicate that COX-2 overexpression appears a marker for poor survival at early, but not at advanced stages (10,32).

Lymph node metastasis is the most significant prognostic sign in HNSCCs, and not surprisingly, our material showed a correlation between metastases and poor survival. Association between COX-2 expression and the development of metastases in HNSCC has been reported previously (15,30), but our study failed to support these findings.

Our study showed no significant association between COX-2 and p53 expression. To our knowledge, both p53 and COX-2 expressions have not been evaluated previously from the same HNSCC tumour specimens. Overexpression of p53 was detected in 70% of the tumours, which is consistent with other reports on HNSCC (3,19,20). As carcinomas containing mutant *TP53* have been demonstrated to overexpress COX-2 (14,22,23), one could assume a correlation between the overexpression of p53 and COX-2. One explanation for the failure to demonstrate such a correlation may be the fact that p53 staining is not a direct marker for gene mutation (17,33). In contrast to COX-2, strong p53 immunostaining predicted a poor recurrence-free and overall survival. Previous studies on p53 expression and survival in HNSCC are inconclusive (16,18-20), and the study settings variable, e.g. some reports focus on the expression of p53 in terms of response to radiation therapy (16,19) or chemotherapy (20). However, *TP53* mutations are associated with poor survival (16-18).

The series of the samples for this study were collected from a period before chemoradiation protocols (given either alone followed by salvage surgery when needed, or as a concomitant postoperative treatment) became part of the standard treatment strategy for these patients at our institution. Thus, the treatment of patients in our material is quite uniform. There is an ongoing search for molecular markers which could predict response to chemotherapy (1,3,4). A recent study on esophageal SCCs showed an association of low COX-2 expression and poor prognosis in patients who had received neoadjuvant chemotherapy (cisplatin and etoposide) (27). This finding indicates that COX-2 may serve as a marker for favourable response to chemotherapy regimens.

To conclude, in our material of advanced oral and pharyngeal SCCs, the staining intensity of COX-2 did not correlate with prognosis. No correlation between COX-2 and p53 expression was found, but strong p53 expression was associated with tumour recurrence and poor overall survival. These findings do not rule out the possibility that COX-2 has a prognostic value at the earlier stages of the disease or in certain subgroups of patients, such as those with a favourable response to chemotherapy.

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