

Clinicopathological significance of cyclooxygenase-2 expression in hypopharyngeal squamous cell carcinoma

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Received October 5, 2007; Accepted November 29, 2007

Abstract. The purpose of this study was to determine the expression of cyclooxygenase-2 (COX-2) in normal epithelium, dysplasia and squamous cell carcinoma of the hypopharynx and to investigate associations with clinicopathological factors and survival. Seventy-five patients with hypopharyngeal squamous cell carcinomas (HPSCC) who underwent surgical treatment at the Department of Otolaryngology, Osaka Medical Center for Cancer and Cardiovascular Diseases, were investigated. COX-2 expression was determined by immunohistochemistry and 97.3% (73/75) of samples displayed immunostaining in tumor cells. COX-2 staining was localized mainly in the cytoplasm (73/75) and was rare in stromal cells (2/75). Over half of the areas of dysplastic cells adjacent to carcinomas also showed COX-2 staining (41/70, 58.6%). There were no significant correlations between the COX-2 expression and tumor size, location and tumor growth type, T- and N-stage, tumor recurrence, lymph node metastasis and survival in this study. COX-2 expression thus does not appear to have a prognostic significance for hypopharyngeal SCC although there was a tendency for higher values in T3/T4 than T1/T2 cases. Furthermore, COX-2 was found to be more strongly expressed in poorly-differentiated than in moderately/well-differentiated carcinomas. In this study group, COX-2 was up-regulated not only in SCCs but also in the dysplastic lesions of the hypopharynx, suggesting that COX-2 inhibition may be a useful chemopreventive strategy.

Introduction

The hypopharyngeal squamous cell carcinoma is relatively aggressive among head and neck carcinomas with a very poor overall prognosis due to loco-regional recurrence and metastasis to lymph nodes despite advances in surgery, radiotherapy and chemotherapy. Therefore, new effective treatment strategies are needed.

Cyclooxygenases (COX) are rate-limiting enzymes converting arachidonic acid to prostaglandin. The two isoforms are characterized as COX-1 and -2. COX-1 is constitutively expressed in most tissues and mediates the synthesis of prostaglandins to control normal physiological functions, whereas the COX-2 expression is generally not detectable but is rapidly induced by various stimuli such as inflammatory reactions, growth factors, oncogenes and tumor promoters (1,2).

COX-2 is expressed in various tumor types and is now generally considered to promote the carcinogenesis and growth of established tumors. There is evidence that a prolonged COX-2 inhibition by aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) offers protection against colon cancer and other malignancies (3,4). COX-2 inhibitors have been shown to inhibit the proliferation of tumor cells *in vitro* and *in vivo* and to act in part by inducing tumor cell apoptosis.

Overexpression of COX-2 is found in the carcinomas of various organs, including the colon (5,6), lung (7), bladder (8), esophagus (9-14) and head and neck (15-18). Up-regulation in laryngeal (19-22), tongue (23) and oropharyngeal cancers (24) has been reported at the mRNA and protein levels. However, data are rather limited regarding hypopharyngeal cancers (15). Furthermore, the prognostic significance for COX-2 has been shown, but mainly for adenocarcinomas (26) and results for squamous cell carcinomas are equivocal (12).

We examined the COX-2 expression in hypopharyngeal squamous cell carcinomas and investigated the relationships with clinicopathological and prognostic parameters.

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Key words: cyclooxygenase-2, hypopharynx, squamous cell carcinoma, dysplasia

Materials and methods

Patients. Our subjects were 86 patients with hypopharyngeal squamous cell carcinomas undergoing surgical treatment of primary tumors at the Department of Otolaryngology, Osaka Medical Center for Cancer and Cardiovascular Diseases, from January 1995 through December 2000. After receiving informed consent, medical records and surgical specimens were collected from all but nine patients, who were excluded as the samples were inadequate or of special histopathology (one adeno-squamous and one basaloid lesion). The 75 cases of HPSCC (64 men, 85.3% and 11 women, 14.7%) had a mean age at surgery of 64.0 years (range: 36-85). The classification of tumor size, lymph node status and stage grouping was performed according to the 1988 UICC criteria. The data collected for each patient included age, sex, tumor site, Brinkman index, Sake index, tumor growth type, treatment method, tumor recurrence, cause of death (where appropriate) and survival outcome. Histological grading (well-, moderately- and poorly-differentiated) was performed with H&E stained sections by a pathologist at the Osaka Medical Center for Cancer and Cardiovascular Diseases. The details of patient and tumor characteristics are given in Table I. One patient received preoperative chemo- and radiotherapy and four patients underwent surgery for recurrent lesions after radical chemo- and/or radiotherapy and three cases had radiotherapy of the hypopharynx for another disease. During the follow-up period, local recurrence occurred in 5 cases (6.7%), lymph node recurrence in 16 (21.3%) and distant metastasis in 9 (12%). An extended follow-up was possible for 66 patients and 5-year survivors numbered 20.

Pretreatment. All block samples were fixed in formalin and were cut into 7-mm slices for decalcification and fixation. Sections of 4 μ m thickness were cut from tissues embedded in paraffin wax and routinely processed. As a pretreatment for immunohistochemistry, heating for 15 min at 100°C in citrate buffer (pH 7.0) was performed in a microwave, followed by washing in PBS buffer (pH 7.5) for 5 min.

Immunohistochemistry. The Ventana NX System® (Ventana I-View DAB Universal kit®) was employed for immunohistochemistry, with anti-COX-2 Ab (160112; Cayman Chemical, 1:100) as the primary antibody. Scoring of the immunohistochemical staining results was performed by a single pathologist blinded to the patient status (clinical parameters). The intensity of staining was graded from 0 to 3: 0, negative; 1, weakly positive; 2, moderately positive and 3, strongly positive. The area of stained cells was also graded from 0 to 4: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75% and 4, 76-100%. The degree of staining (0-12) was then calculated as the product of the intensity and area of stained cells.

The pattern of tumor infiltration according to digestive organ cancer (INF- α - β - γ) and the COX-2 relative staining score (-3 to +3) in tumor invasion fronts as compared to the main tumor lesion were identified and investigated, as well as the relationship to the clinical data.

Statistical analysis. Statistical analysis was performed using StatView 5.0 (Abacus Concepts, Inc., Berkeley, CA). Clinical

Table I. Patient characteristics.

Characteristics	No.	%
Total	75	
Sex		
Male	64	85.3
Female	11	14.7
Age at surgery (years)	64.0 (36-85)	
Tumor stage		
T1	3	4.0
T2	31	41.3
T3	17	22.7
T4	24	32.0
Nodal stage		
N0	20	26.7
N1	14	18.7
N2	38	50.7
N3	3	4.0
Pathological differentiation		
Well	23	30.7
Moderate	37	49.3
Poor	10	13.3
NOS	5	6.7
Recurrence		
Local	5	6.7
Lymph node	16	1.3
Distant	9	12.0
Alive (recurrence-free)	25	33.3
5-year survival	20	26.7
Smoker	64	85.3
Brinkman index	930.2 (40-3120)	
Drinker	65	86.7
Sake index	101.0 (32-370)	

and histopathological parameters were analyzed using the Mann-Whitney U test and the Kruskal-Wallis test (non-parametric analysis of variance) and differences were considered significant at $p < 0.05$. Overall survival was estimated according to the Kaplan-Meier method (Breslow-Gehan-Wilcoxon) and differences in survival were evaluated by the log-rank test.

Results

Immunohistochemical analysis of COX-2. The samples (97.3%, 73/75) displayed COX-2 immunostaining in tumor cells of which 10 (13.3%), 35 (46.7%) and 28 (37.3%) showed a strong, moderate and weak expression, respectively. COX-2 staining was localized mainly in the cytoplasm of tumor cells (73/75) and only infrequently found in stromal cells (2/75).

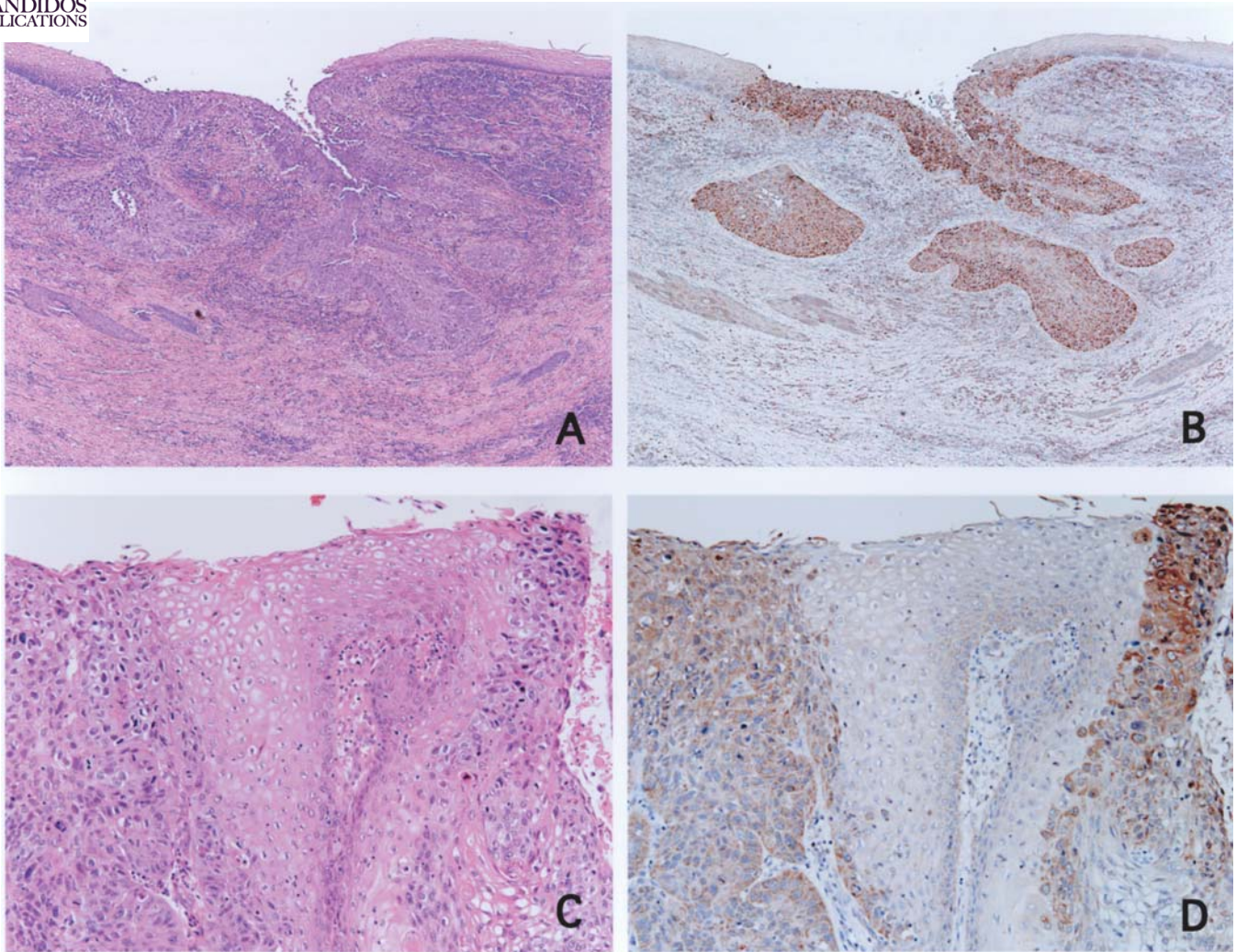


Figure 1. H&E staining (A and C) and COX-2 immunohistochemistry (B and D) of the normal epithelium, a dysplastic area and a squamous cell carcinoma of hypopharynx. Note COX-2 staining in the dysplasia and carcinoma, including an invasive front (B). Strong COX-2 staining in carcinoma. On the other hand, the normal epithelium is negative (D). Magnification: x40 (A and B) and x100 (C and D).

Table II. The association between COX-2 staining and pathology.

Pathology	No.	COX-2 ⁺ (%)	COX-2 positive (intensity of staining)			
			0	1	2	3
Normal	75	1 (1.30)	74	1	0	0
Dysplasia	70	41 (58.6)	29	39	2	0
SCC	75	73 (97.3)	2	28	35	10

In the dysplastic areas adjacent to carcinomas, 58.6% (41/70) of samples showed COX-2 staining of which 2 (2.9%) and 39 (55.7%) showed a moderate and weak expression, respectively. There was only 1.3% (1/75) COX-2 staining in the normal squamous cells around the tumor area (Table II). Representative COX-2 immunostaining patterns in hypopharyngeal SCCs and dysplastic cells adjacent to carcinomas

are shown in Fig. 1. A clear increase in COX-2 staining was evident from normal epithelium through to dysplasia and cancers. The COX-2 expression patterns in each degree of differentiation are shown in Fig. 2.

Correlation with clinicopathological parameters (Table III). There were no significant correlations between the COX-2 expression and tumor size, location, growth type, T- and N-stage, recurrence, lymph node metastasis, or survival in this study. However, with the area of stained cells ($p=0.086$) and the degree of staining ($p=0.073$, not shown in Table), there was a tendency for higher values in T3/T4 than T1/T2. Smokers constituted 64 cases (85.3%) and the average Brinkman index (Brinkman index = numbers of cigarette smoked per day multiplied by years of smoking) was 930.2 (40-3120). The number of drinkers was 65 cases and the average Sake index (Sake index = total grams of ethanol consumed per day/22 g multiplied by years of drinking) was 101.0 (32-370). The COX-2 expression did not correlate with either of these indices.

The intensity of COX-2 staining was significantly higher in the poorly-differentiated tumors than in the well/moderately-

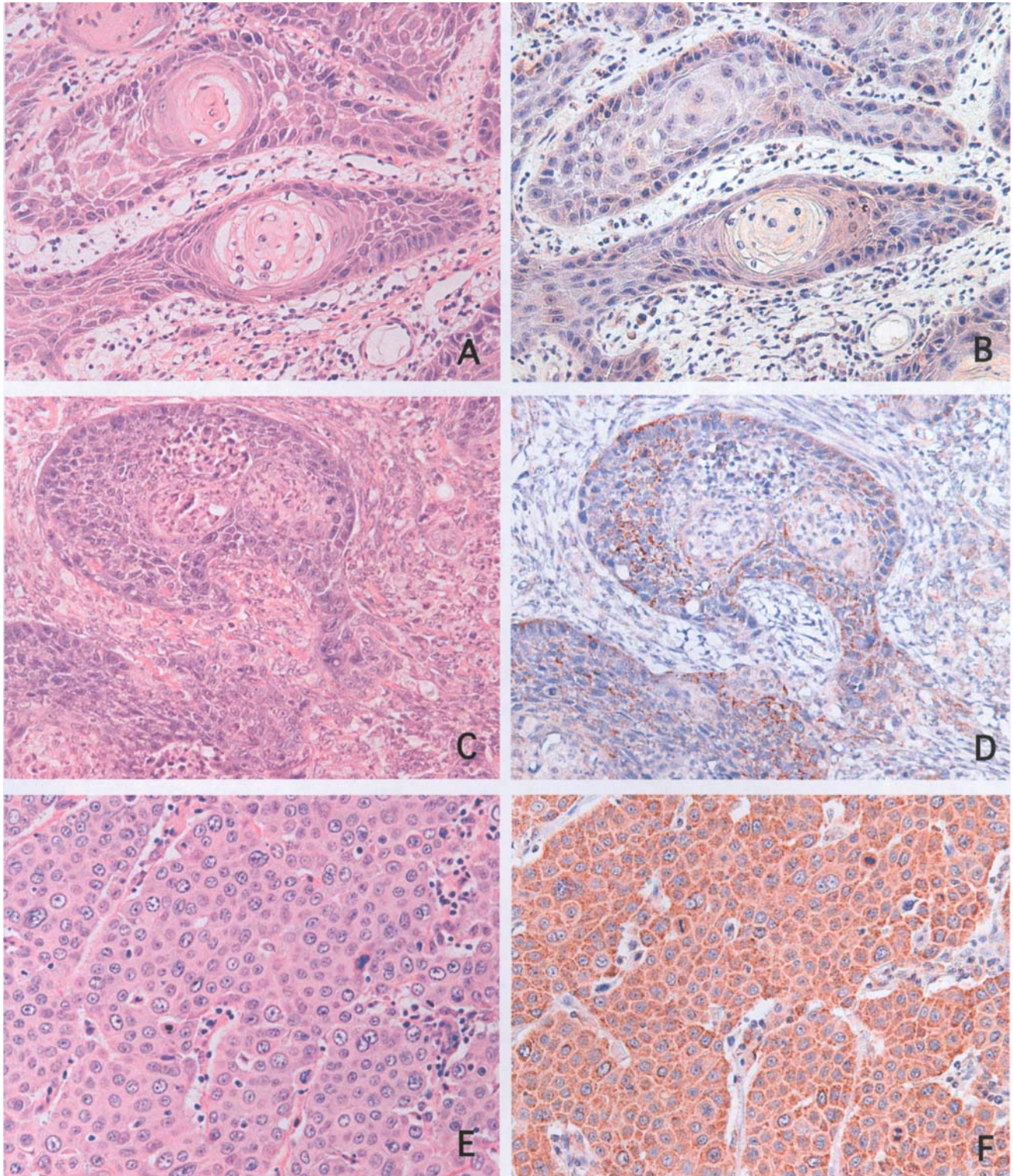


Figure 2. H&E staining (A, C and E) and COX-2 immunohistochemistry (B, D and F) in hypopharyngeal carcinoma. Well (A and B), moderate (C and D) and poorly (E and F) differentiated carcinomas are shown, respectively. The example of various intensities of COX-2 staining in cytoplasm. Weakly positive in well-differentiated carcinomas (B). Moderately positive in moderately-differentiated carcinomas (D). Strong COX-2 staining in almost all areas of the tumor cells with poor differentiation (F). Magnification: x200.

differentiated lesions ($p=0.039$). However, no significant difference was found between poor- and well-differentiated carcinomas ($p=0.087$) and there was no relationship with the tumor infiltration pattern (INF- α - β - γ).

Discussion

In this study, the COX-2 expression was found to gradually increase with progressive tumorigenesis in the hypopharynx.

Clinicopathological variables	No.	COX2 ⁺	COX-2 intensity of staining					p-value	COX-2 area of stained cells					p-value
			0	1	2	3	0		1	2	3	4		
Tumor stage														
T1/T2	34	33 (97.1)	1	16	13	4	0.163	1	2	6	7	18	0.086	
T3/T4	41	40 (97.6)	1	12	22	6		1	2	3	5	30		
Lymph nodes														
Negative	20	20 (100)	0	7	10	3	0.540	0	1	3	3	13	0.872	
Positive	55	53 (96.4)	2	21	25	7		2	3	6	9	35		
Differentiation														
Well/moderate	60	58 (96.7)	2	23	29	6	0.039	2	2	6	10	40	0.201	
Poor	10	10 (100)	0	2	4	4		0	2	2	1	5		

The COX-2 expression was detectable in almost all hypopharyngeal SCCs and was higher in the adjacent dysplastic squamous epithelium than in normal tissue. The overexpression of COX-2 has been documented in various carcinomas, as well as in precancerous and dysplastic lesions (7,14,27,28) and increases with the grade of dysplasia and progression (16,23,29). It has been reported that mild-to-moderate COX-2 immunoreactivity was also present in the epithelial cells of normal-appearing oral mucosal epithelium adjacent to the head and neck SCCs and linked to the up-regulation of COX-2 mRNA (15). Thus this may be an early event in tumorigenesis. With hypopharyngeal carcinomas, COX-2 expression may also be detectable in tissues adjacent to cancer nests and the possibility was suggested that a chronic inflammatory reaction leads to the induction of COX-2 expression and the promotion of the development of carcinoma (25).

The clinicopathological significance of COX-2 expression in SCCs is controversial, despite a number of investigations of the esophageal (11,12) and the head and neck (21,22,24) lesions. In the current study, there was no clear relationship with clinicopathological parameters. There was a tendency for COX-2 expression to be higher in T3/T4 than T1/T2 lesions but there was no prognostic significance, in line with earlier findings (6,17,22,27). Again the available data are conflicting. In various cancers, COX-2 overexpression is associated with a more malignant phenotype and worse prognosis (9,24,29) following local relapse, lymph node (20,26) and distant metastasis (18). In the single investigation of 23 hypopharyngeal carcinomas by RT-PCR analysis (25), the frequency of COX-2 overexpression was significantly higher in the N1-N3 group than in the N0 group. However, Raneletti *et al* (21) described COX-2 overexpression in less aggressive laryngeal SCCs. In the esophagus, a high COX-2 expression only appears to impact on the prognosis in adenocarcinomas (12), with no relation to the N stage, loco-regional and distant metastasis and survival with SCCs.

The links with the degree of differentiation for SCCs of the esophagus (9,11,30), cervix (31) and head and neck region (17,23,29,32) are also unclear. In an earlier laryngeal study COX-2 was found to be strongly expressed in well-

differentiated areas and apparently absent in very poorly-differentiated tissue, the more node positive cases all being COX-2 negative (21). The authors concluded that the COX-2 expression is lost when laryngeal SCCs progress to a more aggressive phenotype. Another study concerning laryngeal carcinomas (19) also documented a higher expression in well- than in poorly/moderately-differentiated SCCs and in N0 rather than N+ cancer cases.

Regarding the hypopharyngeal SCC, the only previous study (25) showed there was no correlation with the degree of differentiation and COX-2 expression. In this study, COX-2 was strongly expressed in the poorly-differentiated tumor. The intensity of COX-2 staining was significantly higher in the poorly-differentiated tumors than in the well/moderately-differentiated tumors ($p=0.039$). Therefore, COX-2 may be related to tumor growth and differentiation as well as the early tumor phenotype.

The prognosis of hypopharyngeal carcinoma is worse than other head and neck cancers in spite of advances in surgery, radiotherapy and chemotherapy. Furthermore, the QOL of the patients frequently decreases after operations. Therefore, effective non-invasive therapy is a high priority. If COX-2 up-regulation occurs in the early stages of tumorigenesis inhibitors may be useful for prevention (16). It has been reported that selective COX-2 inhibitors inhibit cancer cell proliferation and induce apoptosis in colon, bladder and esophageal adenocarcinomas (10,13,28,33). An investigation of a head and neck squamous carcinoma cell line (34) showed a similar suppression of tumor growth caused by anti-angiogenesis, cell cycle arrest and inhibition of the telomerase activity of the tumor cells. Moreover, it has been shown that COX-2 inhibitors enhance the radiosensitivity of the surviving cells (18,33).

Since the COX-2 expression was found to gradually increase from normal epithelium, through dysplasia to poorly differentiated SCC, but without any clear link to prognosis, its importance may be related to tumor growth. COX-2 inhibitors may therefore be beneficial, particularly for patients with dysplastic lesions in the hypopharynx and/or persistent chronic inflammation due to heavy drinking or smoking.

Acknowledgements

This work was supported by a Grant-in-Aid for Young Scientists (B))(18791211) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Vane JR, Bakhle YS and Botting RM: Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* 38: 97-120, 1998.
2. Smith WL and Song I: The enzymology of prostaglandin endoperoxide H synthases-1 and -2. *Prostaglandins Other Lipid Mediat* 68-69: 115-128, 2002.
3. Giovannucci E, Egan KM, Hunter DJ, Stampfer MJ, Colditz GA, Willett WC and Speizer FE: Aspirin and the risk of colorectal cancer in women. *N Engl J Med* 333: 609-614, 1995.
4. Schreinemachers DM and Everson RB: Aspirin use and lung, colon and breast cancer incidence in a prospective study. *Epidemiology* 30: 138-146, 1994.
5. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S and DuBois RN: Up-regulation of cyclooxygenase-2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 107: 1183-1188, 1994.
6. Fux R, Schwab M, Thon KP, Gleiter CH and Fritz P: Cyclooxygenase-2 expression in human colorectal cancer is unrelated to overall patient survival. *Clin Cancer Res* 11: 4754-4760, 2005.
7. Hida T, Yatabe Y, Achiwa H, *et al*: Increased expression of cyclooxygenase-2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res* 58: 3761-3764, 1998.
8. Mohammed SI, Knapp DW, Bostwick DG, *et al*: Expression of cyclooxygenase-2 (COX-2) in human invasive transitional cell carcinoma (TCC) of the urinary bladder. *Cancer Res* 59: 5647-5650, 1999.
9. Nozoe T, Ezaki T, Kabashima A, Baba H and Maehara Y: Significance of immunohistochemical expression of cyclooxygenase-2 in squamous cell carcinoma of the esophagus. *Am J Surg* 189: 110-115, 2005.
10. Zhi H, Zhang J, Zhou C, *et al*: Significance of COX-2 expression in human esophageal squamous cell carcinoma. *Carcinogenesis* 27: 1214-1221, 2006.
11. Yang GZ, Ding HY and Zhou JS: Cyclooxygenase-2 is over-expressed in Chinese esophageal squamous cell carcinoma and correlated with NF- κ B: An immunohistochemical study. *Exp Mol Pathol* 79: 214-218, 2005.
12. Heeren P, Plukker J, Dullemmer HV, Nap R and Hollema H: Prognostic role of cyclooxygenase-2 expression in esophageal carcinoma. *Cancer Lett* 225: 283-289, 2005.
13. Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE and Schror K: Cyclooxygenase-2 expression in human esophageal carcinoma. *Cancer Res* 59: 198-204, 1999.
14. Maaser K, Daubler P, Barthel B, *et al*: Oesophageal squamous cell neoplasia in head and neck cancer patients: Up-regulation of COX-2 during carcinogenesis. *Br J Cancer* 88: 1217-1222, 2003.
15. Chan G, Boyle JO, Yang EK, *et al*: Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. *Cancer Res* 59: 991-994, 1999.
16. Nathan CAO, Leskov IL, Lin M, Abreo FW, Shi R, Hartman GH and Glass J: COX-2 expression in dysplasia of the head and neck. *Cancer* 92: 1888-1895, 2001.
17. Erovic BM, Pelzmann M, Turhani D, *et al*: Differential expression pattern of cyclooxygenase-1 and -2 in head and neck squamous cell carcinoma. *Acta Otolaryngol* 123: 950-953, 2003.
18. Chen WC, McBride WH, Che SM, *et al*: Prediction of poor survival by cyclooxygenase-2 in patients with T4 nasopharyngeal cancer treated by radiation therapy: clinical and *in vitro* studies. *Head Neck* 27: 503-512, 2005.
19. Kawata R, Hyo S, Maeda T, Urade Y and Takenaka H: Simultaneous expression of cyclooxygenase-2 and microsomal prostaglandin E synthase in squamous cell carcinoma of the larynx. *Acta Otolaryngol* 126: 627-632, 2006.
20. Cho EI, Kowalski DP, Sasaki CT and Haffty BG: Tissue microarray analysis reveals prognostic significance of COX-2 expression for local relapse in T1-T2N0 larynx cancer treated with primary radiation therapy. *Laryngoscope* 114: 2001-2008, 2004.
21. Ranelletti FO, Almadori G, Rocca B, *et al*: Prognostic significance of cyclooxygenase-2 in laryngeal squamous cell carcinoma. *Int J Cancer* 95: 343-349, 2001.
22. Bayazit YA, Buyukberber S, Sari I, *et al*: Cyclooxygenase-2 expression in laryngeal squamous cell carcinoma and its clinical correlates. *ORL* 66: 65-69, 2004.
23. Renkonen J, Wolff H and Paavonen T: Expression of cyclooxygenase-2 in human tongue carcinoma and its precursor lesions. *Virchows Arch* 440: 594-597, 2002.
24. Chang BW, Kim DH, Kowalski DP, Burleson JA, Son YH, Wilson LD and Haffty BG: Prognostic significance of cyclooxygenase-2 in oropharyngeal squamous cell carcinoma. *Clin Cancer Res* 10: 1678-1684, 2004.
25. Peng JP, Su CY, Chang HC, Chai CY and Hung WC: Overexpression of cyclo-oxygenase-2 in squamous cell carcinoma of the hypopharynx. *Hum Pathol* 33: 100-104, 2002.
26. France M, Drew PA, Dodd T and Watson DI: Cyclooxygenase-2 expression in esophageal adenocarcinoma as a determinant of clinical outcome following esophagectomy. *Dis Esophagus* 17: 136-140, 2004.
27. Shamma A, Yamamoto H, Doki Y, *et al*: Up-regulation of cyclooxygenase-2 in squamous carcinogenesis of the esophagus. *Clin Cancer Res* 6: 1229-1238, 2000.
28. Yu HP, Shi LY, Lu WH, Su YH, Li YY and Xu SQ: Expression of cyclooxygenase-2 (COX-2) in human esophageal cancer and *in vitro* inhibition by a specific COX-2 inhibitor, NS-398. *J Gastroenterol Hepatol* 19: 638-642, 2004.
29. Gallo O, Masini E, Bianchi B, Bruschini L, Paglierani M and Franchi A: Prognostic significance of cyclooxygenase-2 pathway and angiogenesis in head and neck squamous cell carcinoma. *Hum Pathol* 33: 708-714, 2002.
30. Ratnasinghe D, Tangrea J, Roth MJ, *et al*: Expression of cyclooxygenase-2 in human squamous cell carcinoma of the esophagus; an immunohistochemical survey. *Anticancer Res* 19: 171-174, 1999.
31. Chen J, Wang LS, Wang PH, *et al*: High cyclooxygenase-2 expression in cervical adenocarcinomas. *Gynecol Oncol* 88: 379-385, 2003.
32. Kyzas PA, Stefanou D and Agnantis NJ: COX-2 expression correlates with VEGF-C and lymph node metastases in patients with head and neck squamous cell carcinoma. *Mod Pathol* 18: 153-160, 2005.
33. Ruegg C, Zaric J and Stupp R: Non-steroidal anti-inflammatory drugs and COX-2 inhibitors as anti-cancer therapeutics: hopes and reality. *J Natl Med* 35: 476-487, 2003.
34. Nishimaru G, Yanoma S, Mizuno H, Kawakami K and Tsukuda M: A selective cyclooxygenase-2 inhibitor suppresses tumor growth in nude mouse xenografted with human head and neck squamous carcinoma cells. *Jpn J Cancer Res* 90: 1152-1162, 1999.