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Main Article

Expression of cyclooxygenase-2, vascular endothelial growth factor and matrix metalloproteinase-2 in patients with primary laryngeal carcinoma: a tissue microarray study

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Abstract

Objective: To determine the correlation between expression of cyclooxygenase-2, vascular endothelial growth factor and matrix metalloproteinase-2, in patients with laryngeal carcinoma.

Design: The study included 85 primary laryngeal squamous cell carcinoma cases. Expression was assessed using Envision immunohistochemical stains for cyclooxygenase-2, vascular endothelial growth factor and matrix metalloproteinase-2.

Subjects: A tissue microarray containing samples from the 85 primary laryngeal squamous cell carcinoma cases was assembled. Immunohistochemical testing for cyclooxygenase-2, vascular endothelial growth factor and matrix metalloproteinase-2 was performed. Using Pearson correlation, expression of these proteins was compared with the following clinicopathological variables: age, sex, clinical tumour–node–metastasis staging, and prognosis. Three-year survival curves, factored by cyclooxygenase-2, vascular endothelial growth factor and matrix metalloproteinase-2 expression, were generated for overall survival, by Kaplan–Meier analysis.

Results: The expression of cyclooxygenase-2 significantly differed between patients with different pathology, tumour–node–metastasis stage and prognosis. A marked difference in vascular endothelial growth factor expression was seen between two histological grade groups. Expression of matrix metalloproteinase-2 protein statistically significantly differed between patients with different tumour–node–metastasis stages, lymph node metastases and three-year survival rates. The expression of cyclooxygenase-2 in laryngeal carcinoma tissue was found to be associated with the expression of matrix metalloproteinase-2.

Conclusion: Cyclooxygenase-2 and matrix metalloproteinase-2 may act as clinical prognostic indicators of tumour growth and differentiation in laryngeal carcinoma.

Key words: Microarray Analysis; Head and Neck Neoplasms; Larynx Neoplasms; Immunohistochemistry; COX-2; MMP-2; Angiogenesis; Prognosis

Introduction

Cyclooxygenase-2 is an enzyme involved in the conversion of arachidonic acid to prostaglandins, induced by inflammatory and mitogenic stimuli. Recently, attention has been drawn to the role of the cyclooxygenase enzyme and its involvement in tumorigenesis. Cyclooxygenase-2 is pro-inflammatory in nature and is inducible by mitogens, cytokines, tumour promoters and growth factors. A large volume of data exists showing that cyclooxygenase-2 is over-expressed in a large number of human cancers, and correlates with tumour aggression and poor prognosis.[1–3] Cyclooxygenase-2 increases the angiogenic potential of tumour cells and upregulates vascular endothelial growth factor expression.[4–6] It is suggested that cyclooxygenase-2 plays a role in promotion of angiogenesis and increases microvascular density.

Matrix metalloproteinases play a central role in cell migration by degrading cellular and extracellular components during cancer invasion, morphogenesis, organ development and tissue damage. Some research suggests that cyclooxygenase-2 over-expression promotes the activity of matrix metalloproteinase-2 and is possibly involved in the process of invasion and metastasis. An interaction between cyclooxygenase-2

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and matrix metalloproteinases has been proposed for some cancers.[7–9]

The possibility of cyclooxygenase-2 as a factor in cancer development and progression evolved from epidemiological studies suggesting that regular use of aspirin or other non-steroidal anti-inflammatory drugs could significantly decrease the risk of cancer development in experimental animals and in humans.[10]

The use of tissue microarrays is a powerful technique which can be used to examine many clinical specimens in a single slide. It is a rapid and efficient method for testing immunohistochemical reactivity of monoclonal antibodies against multiple tissue samples simultaneously.[11] The use of tissue microarrays has been validated in breast, rectal and (recently) laryngeal neoplasms.

We studied the correlation between expression of cyclooxygenase-2, vascular endothelial growth factor and matrix metalloproteinase-2, in 85 patients with primary laryngeal carcinoma, using the tissue microarray technique. The aim was to investigate whether cyclooxygenase-2 was involved in tumour invasion and angiogenesis in laryngeal carcinoma, as well as to study the interaction between the expression of cyclooxygenase-2 and matrix metalloproteinase-2.

Materials and methods

Patients and samples

The study group consisted of 85 patients who underwent surgery for laryngeal squamous cell carcinoma at the Shanghai First People's Hospital between January 1982 and December 2000. There were 80 male and five female patients, with ages ranging from 50 to 65 years (average 61.5 years).

All patients had a histopathological diagnosis of squamous cell carcinoma: 64 tumours were well differentiated, 19 moderately differentiated and two poorly differentiated.

Each patient's tumour–node–metastasis (TNM) stage was determined based on the clinical data and histopathological examination, according to the 2002 TNM classification system. Patients' staging was as follows: 17 T_1, 33 T_2, 21 T_3 and 13 T_4 stage; and 67 N_0, 10 N_1, six N_2 and one N_3 stage. No cases showed evidence of metastasis.

Thirty-two cases were supraglottic cancer, 52 were glottic cancer and one case was subglottic cancer.

Surgical procedures included laryngofissure, horizontal partial laryngectomy, subtotal laryngectomy, supracricoid partial laryngectomy with cricohyoidoepiglottopexy, Arslan surgery and total laryngectomy. These procedures were combined with 15 cases of unilateral modified radical neck dissection, one bilateral dissection, two thyroidectomy, one deltopectoral skin flap reconstruction and one pectoralis major myocutaneous flap reconstruction.

Epidemiological analysis showed that 17 patients were non-smokers, 30 were light smokers (i.e. less than one packet of cigarettes per day) and 38 were heavy smokers (i.e. more than one packet per day); in addition, 52 were non-drinkers, 15 were casual drinkers and 18 were alcoholics.

Following surgery, only six cases were lost over the three-year study follow-up period; the three-year follow-up rate was 92.96 per cent. The three-year survival rate was 73 per cent, while the five-year survival rate was 58.7 per cent if the lost cases were attributed to death.

Tissue microarray construction

The tissue microarrayer used (Beecher Instruments, Sun Prairie, WI, USA) was designed to produce circular sample spots 0.6 mm in diameter at a spacing of 0.7–0.8 mm. The tumour tissues were initially screened at a low magnification by a pathologist, and three tumorous sections within the one sample were marked. The surface area of each section was 0.282 mm. Cores from these sections were placed on the recipient microarray block, using the tissue microarrayer.

Immunohistochemical staining

The tissue microarray was then sectioned into 5-μm slices and placed on glass slides, using an adhesive tape transfer system (Instrumedics, Hackensack, New Jersey, USA) with ultraviolet cross-linking. Sections were incubated at 37º for 10–15 minutes, and then deparaffinated and dehydrated in xylol with graded alcohol. Murine monoclonal (anti-human) antibodies against cyclooxygenase-2, vascular endothelial growth factor and matrix metalloproteinase-2 were purchased from the Fuzhou Company (Fuzhou, Fujian, PRC), along with an immunostaining kit (streptavidin-peroxidase conjugated method). The antibodies were diluted and used as instructed by the manufacturer. The Diaminobenzidine (DAB) staining system was used. The study used both positive controls (i.e. known positive slides provided by the manufacturer) and negative controls (i.e. using phosphate-buffered saline (PBS) instead of the primary antibody).

Consecutive sections were also stained with haematoxylin and eosin for routine histopathological examination.

Determination of expression

The extent of immunostaining was observed using light microscopy with a ×40 objective lens by two investigators without knowledge of the patient's history. The percentage of tumour cells which exhibited positive cytoplasmic immunoreactivity was determined. All cancer cells in each tissue microarray spot were counted in each case. The intensity of the immunohistochemical staining for cyclooxygenase-2 was categorised as follows: negative (<5 per cent); +, mild (5–10 per cent); ++, moderate (10–20 per cent); and +++, marked (>20 per cent). Expression of matrix metalloproteinase two and vascular endothelial growth factor were graded as negative or positive, according to a 10 per cent cut-off point on the labelling index.

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Statistical analysis

Using Pearson correlation, expression was correlated with the following clinicopathological variables: age, sex, clinical TNM staging and prognosis. Overexpression of cyclooxygenase-2, vascular endothelial growth factor and matrix metalloproteinase-2 was compared with the clinical and pathological variables of gender, TNM stage, histopathological grade, tumour type, laryngeal subsite, and alcohol and tobacco consumption, using chi-square analysis. Three-year survival curves, factored by cyclooxygenase-2, vascular endothelial growth factor and matrix metalloproteinase-2 expression, were generated for overall survival, using the method proposed by Kaplan and Meier. Survival curves were compared by log-rank tests. All calculations and analyses were performed using the Statistical Package for the Social Sciences version 11.0 for Windows software (SPSS, Chicago, Illinois, USA). A p value of less than 0.05 (two-tailed test) was considered to indicate statistical significance.

Results

The tissue microarray comprised a total of 255 minute, cylindrical samples within a paraffin matrix. Six samples (7.06 per cent) contained little or no neoplastic tissue. A small proportion of cases were missing afterz staining.

After cyclooxygenase-2 staining, 11 out of 85 cases were shed in the tissue microarray. In the available 68 cases, the average cyclooxygenase-2 labelling index was 13.13 per cent (range 1.00–35.00 per cent, median 10.00 per cent). Brown cytoplasmic staining was considered positive. A perinuclear staining pattern was observed, with nuclei showing no staining (cytoplasmic expression of cyclooxygenase-2 is shown in Figure 1). The tumour cells showed stronger staining near the basal membrane than in the superficial epithelium of tumour; there was generally no staining at normal mucosa adjacent to tumour tissue. Greater expression of cyclooxygenase-2 correlated with a higher T stage ($p = 0.0043$). When cases were divided into 'high expressors' and 'low expressors' by a cut-off point at 10 per cent of the labelling index, a statistically significant difference in cyclooxygenase-2 expression was found between tumours of different pathological differentiation and between those with different TNM stages, by chi-square test ($p = 0.003$, $p = 0.047$, respectively). Markedly greater expression of cyclooxygenase-2 was found in tumours of poor histological grade and advanced TNM stage. Kaplan–Meier survival curves for three-year overall survival are given in Figure 2. Patients with markedly greater cytoplasmic cyclooxygenase-2 expression demonstrated inferior overall survival ($p = 0.0053$).

In the 73 cases stained for vascular endothelial growth factor, staining of the cytoplasm, or of both the cytoplasm and the nuclei, was considered positive (cytoplasmic expression of vascular endothelial growth factor is shown in Figure 3). When the 73 cases were divided into 'high expressors' and 'low expressors' by a cut-off point at 12.2 per cent of the labelling index (i.e. the median), a statistically significant difference in vascular endothelial growth factor expression was found between tumours in the three pathological grade groups ($p = 0.0035$) and between tumours from different sites ($p = 0.04$). Much greater expression of vascular endothelial growth factor was seen in tumours of poor histological grade and in those supraglottic tumours. The data also displayed towards statistical difference between lymph node metastasis and no lymph node metastasis, and among TNM stage groups. No statistically significant difference was observed between different levels of vascular endothelial growth factor expression and three-year overall survival.

Interestingly, matrix metalloproteinase-2 was also expressed in the cytoplasm of laryngeal cancer cells, with almost no nuclear staining (cytoplasmic expression of matrix metalloproteinase-2 is shown in Figure 4). When 69 cases were divided into 'high expressors' (41/69, 59.5 per cent) and 'low expressors' (28/69, 40.5 per cent) by a cut-off point at 20

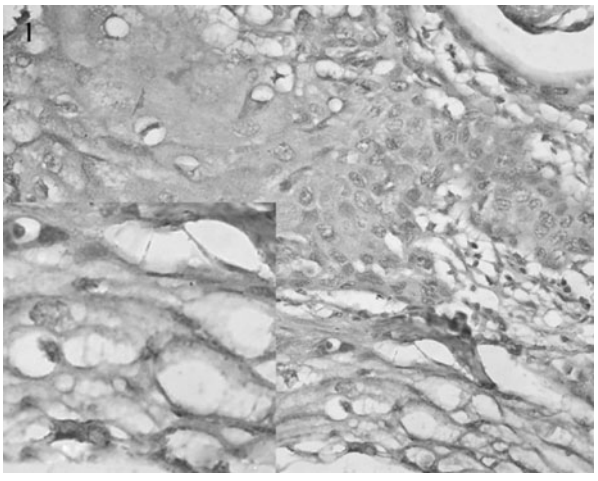

FIG. 1

Photomicrograph showing cyclooxygenase-2 expression in laryngeal cancer, seen as dark staining (DAB; ×400).

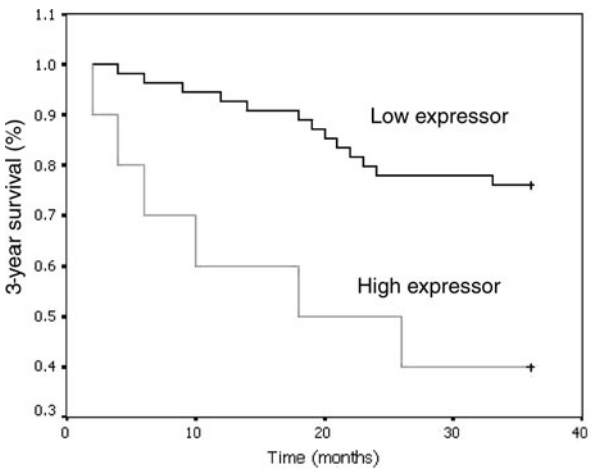

FIG. 2

Kaplain–Meier estimate of 3-year overall survival, for different levels of cyclooxygenase-2 cytoplasmic expression.

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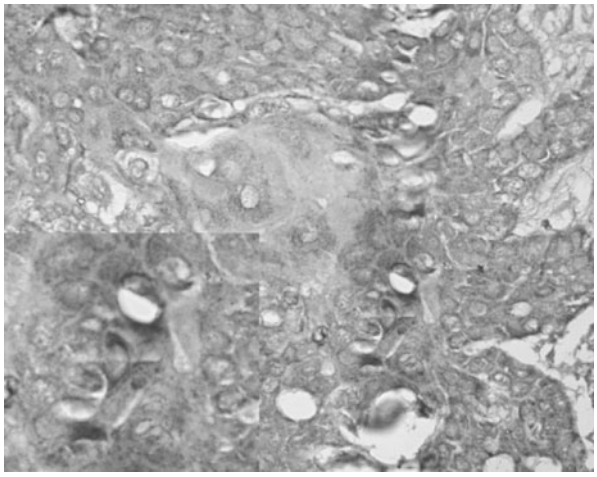

FIG. 3
Photomicrograph showing vascular endothelial growth factor expression in laryngeal cancer, seen as dark staining (DAB; ×400).

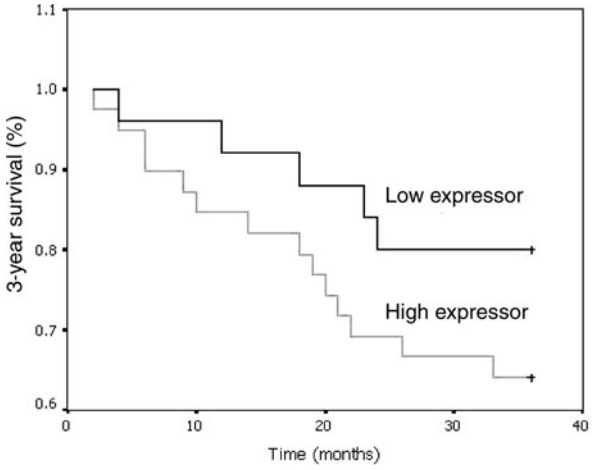

FIG. 5
Kaplain–Meier estimate of 3-year overall survival, for different levels of matrix metalloproteinase-2 cytoplasmic expression.

per cent of the labelling index (i.e. the median), a statistically significant difference in matrix metalloproteinase-2 expression was found between tumours of different TNM stages and between tumours of varying lymph node metastasis stages ($p = 0.012$, $p = 0.049$, respectively). Kaplan–Meier survival curves for three-year overall survival are shown in Figure 5. Patients with greater cytoplasmic matrix metalloproteinase-2 expression demonstrated inferior overall survival ($p = 0.017$). No correlation was found between matrix metalloproteinase-2 expression and smoking, drinking, gender, age or histopathology ($p > 0.05$).

Data on the expression of cyclooxygenase-2, vascular endothelial growth factor and matrix metalloproteinase-2 are listed in Table I.

From analysis of immunostained cells, a significant correlation was found between the expression of cyclooxygenase-2 and matrix metalloproteinase-2 ($r = 0.28$; $p = 0.009$), while no correlation was found between the expression of cyclooxygenase-2 and vascular endothelial growth factor, or between the expression of matrix metalloproteinase-2 and vascular endothelial growth factor.

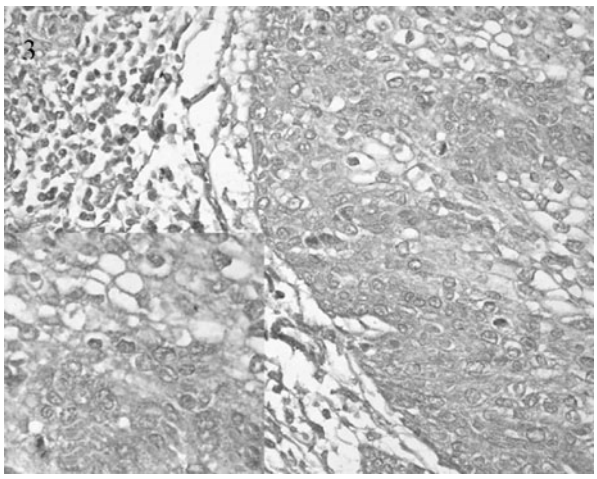

FIG. 4
Photomicrograph showing matrix metalloproteinase-2 expression in laryngeal cancer, seen as dark staining (DAB; ×400).

Discussion

Squamous cell carcinoma of the head and neck comprises about 4 per cent of all malignancies. Tumours of the larynx are quite common, representing 22 per cent of all head and neck cancers. In the USA, about 11 300 new cases of laryngeal cancer are anticipated in 2007.[12] Current pathological diagnostic procedures are unable to differentiate between head and neck squamous cell carcinomas of varying sites of origin. All head and neck squamous cell carcinomas have a similar histological appearance, when using traditional tissue-staining techniques (such as haematoxylin and eosin) or immunohistochemical analysis for epithelial markers such as cytokeratin. Thus, currently, a tumour's site of origin can only be determined by clear anatomical visualisation; traditional histological techniques are of no assistance. Similarly, imaging studies (including computed tomography, magnetic resonance imaging and positron emission tomography) are usually unhelpful in differentiating the site of origin when clinical examination is inconclusive.

One alternative is to characterise tumours at the molecular level. Using immunohistochemical techniques, protein expression can be semi-quantitatively evaluated. One of the drawbacks of immunohistochemistry performed on individual slides is the potential for staining differences between cases due to varying staining conditions. This is virtually eliminated using the tissue microarray technique. This new technique is not to be confused with deoxyribonucleic acid microarrays, in which each spot represents a unique, cloned complementary DNA

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TABLE I

CORRELATION BETWEEN EXPRESSION OF COX-2, MMP-2 AND VEGF AND CLINICAL FEATURES OF LARYNGEAL SCC

SCC feature	COX-2 expression (%)						MMP-2 expression (%)				VEGF expression (%)			
	<5 (‘−’)	5–10 (‘+’)	10–20 (‘++’)	>20 (‘+++’)	Total	*p*	≤10 (‘−’)	>10 (‘+’)	Total	*p*	≤10 (‘−’)	>10 (‘+’)	Total	*p*
Differentiation														
Well	5	17	17	13	52		20	33	53		25	30	55	
Moderate	7	6	2	1	16	0.0073	8	8	16	0.3104	14	4	18	0.0254
T stage														
T_1–T_2	5	13	7	11	36		15	24	39		19	22	41	
T_3–T_4	7	10	12	3	32	0.0937	13	17	30	0.3589	20	12	32	0.1488
N stage														
N_0	7	16	14	14	51		17	36	53		27	30	57	
N_x	5	7	5	0	17	0.3442	11	5	16	0.0168	12	4	16	0.0688
Pt survival status														
Alive	4	18	11	12	45		14	32	46		28	21	49	
Dead	8	5	8	2	23	0.0165	14	9	23	0.0234	11	13	24	0.2626

COX-2 = cyclooxygenase-2; MMP-2 = matrix metalloproteinase-2; VEGF = vascular endothelial growth factor; SCC = squamous cell carcinoma; T = tumour; N = node; Pt = patient

(cDNA) or oligonucleotide. In tissue microarrays, the spots are larger and contain small histological tissue sections from unique patients or tumours. The arrays are assembled by taking needle core ‘biopsies’ of pre-existing, paraffin-embedded tissues and then re-embedding them in an arrayed master block, using the established technique of Wan and Furmanski together with apparatus recently developed by Konenen *et al.*[11,13] Appropriate positive and negative controls can also be included in this block. Using this technique, up to 600 cases can be analysed together, under identical conditions. Tissue microarray use has been validated in breast, rectal and (recently) laryngeal neoplasms.[14–16] The combination of tissue microarray and immunohistochemistry is a powerful approach, enabling the detection of protein expression at a molecular level, in large series of head and neck carcinoma specimens.[16–18]

The results of the present study show that overexpression of cyclooxygenase-2 correlated with cancer progression and poor prognosis. This finding adds to the increasing evidence for the contribution of this enzyme to the growth and prognosis of head and neck squamous cell carcinoma. Previous studies revealed the role of cyclooxygenase-2 in cancer progression, via various pathways. One of these pathways involves the mediation, by vascular endothelial growth factor and matrix metalloproteinase, of tumour angiogenesis induction.[19,20] Tumour metastasis and angiogenesis play important roles in the development of malignant tumours. Angiogenesis is critical for tumour growth and metastasis, and occurs when the endothelial cells proliferate and migrate through the matrix. Both events require the degradation of basement membranes and matrix. Plasminogen activators and matrix metalloproteinases are major enzymes which degrade proteins in tissue basement membranes and the extracellular matrix, and so facilitate endothelial cell and tumour cell migration, angiogenesis and metastasis.[21–23]

We also evaluated the expression of vascular endothelial growth factor and matrix metalloproteinase-2. Vascular endothelial growth factor expression was found to correlate with tumour differentiation, in the same manner as cyclooxygenase-2. Many studies have suggested that cyclooxygenase-2 can stimulate nitric oxide synthase, platelet-dirived growth factor (PDGF) and vascular endothelial growth factor, thereby promoting inflammatory and tumour neovascularisation and raising tissue microvessel density, and thus nourishing tumours and facilitating their metastasis. Cyclooxygenase-2 up-regulation can activate free radicals and thus cause mutation, which might increase the risk of malignancy and inhibit cell apoptosis.[24–26]

The first obstacle to invasive epithelial cancer is the basilar membrane, which is composed mainly of collagen I, IV, V, X/XI. Collagenase type IV includes matrix metalloproteinases 2 and 9, and primarily decomposes collagen I, geltin and fibronectin. Matrix metalloproteinases 2 and 9 play a crucial role in tumour invasion and metastasis. Elevated levels of

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cyclooxygenase-2 activate matrix metalloproteinase-2 and membrane metalloproteinase; thus, the former enzyme may facilitate adhesion and penetration.[27] In the study of Young-Ae *et al.*, prostaglandin E2 PGE2 increased matrix metalloproteinase-2 activity, which in turn caused an increase in type one collagen degradation.[19] Prostaglandin E2 has been implicated in the potention of matrix metalloproteinase production in some cell cultures.[28,29] In these cases, matrix metalloproteinase-2 expression correlated with cyclooxygenase-2 expression, confirming the aforementioned biological hypothesis of a synergistic interaction between the two enzymes.

- **Cyclooxygenase-2 is an enzyme involved in the conversion of arachidonic acid to prostaglandins, and is induced by inflammatory and mitogenic stimuli**
- **Recently, attention has been drawn to the role of this enzyme and its involvement in tumorigenesis**
- **Tissue microarray is a powerful technique which can examine many clinical specimens in a single slide; it is a rapid and efficient method for testing the immunohistochemical reactivity of monoclonal antibodies against multiple tissue samples simultaneously. This technique was used to examine 85 cases of laryngeal squamous cell carcinoma**
- **This study raises the possibility that cyclooxgenase inhibitors might have anti-angiogenic and antitumour effects on head and neck cancers, similar to their effect in colon cancer**

Various epidemiological studies have investigated the association between use of cyclooxygenase-2 inhibitors (e.g. nonsteroidal anti-inflammatory drugs) and reduction in colon cancer risk.[30,31] *In vitro* and *in vivo* head and neck tumour studies also suggest that aspirin and indomethacin might have an analogous effect in retarding tumour growth.[32–34] Our study raises the possibility that cyclooxygenase inhibitors may, in a similar fashion, have anti-angiogenic and antitumour effects on head and neck cancers, thus suggesting new therapeutic strategies for the treatment and prevention of laryngeal cancer.

Conclusion

These findings suggest that cyclooxygenase-2, matrix metalloproteinase-2 and vascular endothelial growth factor are important prognostic indicators in patients with laryngeal cancer. These proteins increase the malignant potential of laryngeal tumours. Further investigation will be required in order to determine the exact role of cyclooxygenase-2 in laryngeal carcinoma, as well as the mechanism by which this enzyme affects laryngeal carcinoma vascularisation and progression.

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