Overexpression of Cyclooxygenase-2 in Nasopharyngeal Carcinoma and Association With Epidermal Growth Factor Receptor Expression

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Objectives: To examine the association between cyclooxygenase-2 (COX-2) expression with epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), and latent membrane protein 1 (LMP-1) expression and with COX-2 promoter methylation status in primary nasopharyngeal cancer (NPC) tumors and to determine COX-2 promoter methylation status in NPC cell lines.

Design: Retrospective study.

Setting: Patients with NPC were referred to the Department of Otolaryngology–Head and Neck Surgery for treatment.

Patients: Formalin-fixed, paraffin-embedded NPC specimens from 42 patients were obtained.

Interventions: Immunohistochemical expression of COX-2, EGFR, VEGF, iNOS, and LMP-1 was performed in 42 NPC samples. COX-2 promoter methylation status was studied in 20 separate specimens and in 4 NPC cell lines.

Main Outcome Measures: (1) COX-2, EGFR, VEGF, iNOS, and LMP-1 expression; and (2) COX-2 promoter methylation status.

Results: COX-2 was overexpressed in 79% of NPC specimens and was associated with EGFR status (P = .03) but not with LMP-1 or iNOS. In primary NPC tissue, methylation of the COX-2 promoter was seen in 4 of 7 COX-2-negative and 1 of 13 COX-2-positive immunohistochemical cases. COX-2 promoter methylation was found in the CNE-1 cell line.

Conclusions: Nasopharyngeal cancer may be a useful target for selective COX-2 inhibition. The absence of promoter methylation may be a necessary component of COX-2 overexpression, and promoter methylation may be one of the mechanisms that regulate COX-2 expression.

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Cyclooxygenase (COX), a key enzyme in the synthesis of prostaglandins, exists in 2 isofoms: COX-1 and COX-2. COX-1 is expressed constitutively in most tissues and is responsible for the production of prostaglandins that mediate normal physiologic functions. COX-2, in contrast, is undetectable in most normal tissues and is induced by inflammatory cytokines, tumor promoters, and growth factors. COX-2 appears to have an important role in carcinogenesis. COX-2 overexpression has been detected in various malignancies, and inhibition of tumor growth, metastases, and angiogenesis by selective COX-2 inhibitors has been shown in vitro and in animal models. The mechanisms that underlie the tumorigenic effect of COX-2 include inhibition of apoptosis, increase of metastatic potential, and tumor angiogenesis via up-regulation of vascular endothelial growth factor (VEGF). Alterations in the epidermal growth factor receptor (EGFR) pathway play an important role in tumor proliferation and invasion, inhibition of apoptosis, and angiogenesis. Inducible nitric oxide synthase (iNOS) is expressed in tumors and can promote tumor growth and angiogenesis. Complex relationships among COX-2, EGFR, and iNOS expression have been observed. In a colon cancer cell line, EGFR activation induced COX-2 protein and subsequently prostaglandin release. In a murine macrophage cell line, nitric oxide derivatives induced COX-2 and enhanced the production of prostaglandins, whereas COX-2 and its eicosanoid products decreased iNOS expression in rat intestinal epithelial cells.

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World Health Organization type III or undifferentiated type nasopharyngeal cancer (NPC) is endemic in many areas of Asia. Undifferentiated NPC is associated with Epstein-Barr virus infection and is characterized by the expression of Epstein-Barr virus oncoproteins: latent membrane proteins (LMPs) 1 and 2 and Epstein-Barr nuclear antigen gene 1.

A previous study found expression of COX-2 by Western blot in 8 of 14 fresh NPC specimens and correlation between LMP-1 and COX-2 expression. Cell line work in the same study showed induction of the COX-2 promoter by LMP-1 through nuclear factor κB (NF-κB) and increased VEGF production induced by LMP-1 in a COX-2–dependent manner. Thus, a complex interaction exists between COX-2 and VEGF, iNOS, EGFR, and LMP-1 in tumorigenesis, apoptosis, and angiogenesis. Aberrant promoter methylation is associated with the transcriptional silencing of various genes in cancer and aging. There is evidence to suggest that COX-2 expression is regulated partly by the methylation status of its promoter region. Methylation of the COX-2 promoter region was associated with the loss of COX-2 expression in colorectal tumors and gastric cancer cell lines. This study aimed to assess whether COX-2 overexpression was associated with EGFR, iNOS, LMP-1, and VEGF by immunohistochemical analysis, to evaluate COX-2 overexpression in more than 50% of cells. A score of 2 or higher was considered positive for or overexpressing COX-2 and EGFR.

Immunostaining for VEGF and iNOS was semiquantitatively scored as follows: 0, negative; 1, weak (<10% expression of cells); 2, moderate (10%-50% expression of cells); and 3, strong (expression in >50% of cells). A score of 2 or higher was considered positive. Scoring for each LMP-1 specimen was as follows: no staining was considered a negative result and any staining was considered a positive result. The pathologist, who was unaware of the patient’s clinical details, performed the scoring.

**METHYLATION-SPECIFIC POLYMERASE CHAIN REACTION FOR COX-2 PROMOTER**

COX-2 promoter methylation status was determined by methylation-specific polymerase chain reaction (PCR) as described previously. Genomic DNA was extracted from the tumor with TRI Reagent (Molecular Research Center Inc, Cincinnati, Ohio) and then subjected to bisulfite treatment. We analyzed DNA from 4 NPC cell lines (C666-1, HK-1, CNE-1, and CNE-3) and 20 primary NPC specimens. A different set of NPC samples was used for methylation because there was an adequate amount of tumor left from the original 42 specimens to adequately perform methylation. The sequences of the COX-2–specific primers are as follows: methylated reaction, 5′-AAA AGT TTT GTT ATC GGG TTT AC-3′ (forward primer) and 5′-ACA ACA ACA AAA CGC GAA CG-3′ (reverse primer), 310–base pair (bp) product; and unmethylated reaction, 5′-AAA ATT TGT TTT TTA GGT GGT TTA T-3′ (forward primer) and 5′-ACA CAA CAA CAA AAC ACA AAC A-3′ (reverse primer), 314-bp product. These primers have been tested for no amplification for bisulfited DNA. The PCR was performed in a 12.5-μL reaction mixture that consisted of 0.6 mM primers, 0.2 mM dNTP, 2 mM magnesium chloride, ×1 PCR Buffer II (Applied Biosystems Inc, Foster City, Calif), 0.94 U of AmpliTaq Gold (Applied Biosystems), and 0.5 μL (25 ng) of template DNA. The PCR was conducted at 95°C for 10 minutes, then 40 cycles (94°C, 30 seconds; 55°C, 30 seconds; and 72°C, 30 seconds), followed by 72°C for 5 minutes. Normal nasopharyngeal mucosa samples were used as controls. The PCR products were analyzed on 1.8% agarose gels.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Blocks were cut to 4- to 5-μm thickness and mounted on precoated slides. One tissue sample underwent histologic review, and the remaining sections underwent immunohistochemical staining. After deparaffinization and rehydration, sections were microwaved and immersed in hydrogen peroxide and then in normal horse serum in phosphate-buffered saline. Slides were then incubated overnight with anti–COX-2 (Cayman Chemical Company, Ann Arbor, Mich), anti–EGFR (Merck KGaA, Darmstadt, Germany), anti–VEGF (Santa Cruz Biotechnology Inc, Santa Cruz, Calif), and anti-iNOS (Santa Cruz Biotechnology) monoclonal antibodies at dilutions of 1:500, 1:30, 1:200, and 1:50, respectively. Immunoreactive complexes were detected using an immunohistochemical visualization system (Dako Envision system; Dako, Carpinteria, Calif) and visualized with diaminobenzidine. Sections were counterstained with hematoxylin-eosin. For LMP-1, sections were digested with trypsin and incubated with anti-LMP-1 antibody (Novocastra Laboratories Ltd, Newcastle upon Tyne, England) at 1:200 dilution. Omission of the primary antibodies and substitution with normal serum constituted the negative control. Known tumor cases positive for COX-2, EGFR, VEGF, iNOS, and LMP-1 were used as positive controls, and 5 histologically normal nasopharyngeal biopsy specimens were used as controls for the immunohistochemical studies.

**METHODS**

**PATIENTS AND SPECIMENS**

Formalin-fixed, paraffin-embedded NPC specimens from 42 patients (median age, 45 years; age range, 22-72 years; 34 men and 8 women) were obtained. Clinical data were obtained by medical record review, and staging was recorded according to the American Joint Committee on Cancer criteria of 1997.

**REVERSE-TRANSCRIPTASE PCR FOR COX-2 EXPRESSION IN NPC CELL LINES**

Total RNA was extracted from cell pellets by TRI Reagent. Reverse transcriptase (RT)–PCR primers used were COX-2F (5′-CAA AAG CTG GGA AGC CTT CT-3′) and COX-2R (5′-AAC TGA TGC GTG AAG TGC TG-3′). The RT-PCR was performed using AmpliTaq Gold with 5% dimethyl sulfoxide in the following conditions: (1) initial denaturation at 95°C for 10 minutes; (2) amplification for 37 cycles (94°C for 30 seconds, 55°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds), followed by 72°C for 5 minutes. Normal nasopharyngeal mucosa samples were used as controls. The PCR products were analyzed on 1.8% agarose gels.
STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS statistical software version 11.5 (SPSS Inc, Chicago, Ill). Association between COX-2 expression and other molecular markers was determined by the \( \chi^2 \) test or Fisher exact test as appropriate. Associations were considered statistically significant if the 2-tailed \( P \leq .05 \).

RESULTS

Two of the specimens were squamous cell carcinoma, and 40 were undifferentiated carcinoma. Specimens included 13 patients with stages I and II, 17 with stage III, and 12 with stage IV NPC.

IMMUNOHISTOCHEMICAL ANALYSIS

Overexpression of COX-2, VEGF, EGFR, and iNOS was detected in 33 (79%), 42 (100%), 33 (83%), and 29 (69%) of 42 tumor samples, respectively, and LMP-1 was positive in 19 cases (45%). The staining pattern for COX-2 was cytoplasmic, granular, and uniform within only the tumor cells. Inflammatory cells showed focal COX-2 staining; the normal nasopharyngeal epithelium, vascular endothelium, and stromal cells were COX-2 negative (Figure 1A-C). Except for EGFR, which was a membran stain (Figure 2), other antigens showed a cytoplasmic staining pattern. COX-2 overexpression was associated with EGFR overexpression (\( P = .03 \)) but not with iNOS or LMP-1 (Table). The association between COX-2 and VEGF overexpression was not tested because VEGF was overexpressed in all samples.

METHYLATION STATUS OF THE COX-2 PROMOTER

The NPC cell lines C666-1, HK-1, CNE-1, and CNE-2 were characterized in terms of methylation of the COX-2 promoter. The COX-2 promoter methylation was found only in CNE-1. In the 20 NPC samples, the methylation status of the promoter region of the COX-2 gene was as-
sessed, and results were compared with COX-2 expression determined by immunohistochemical analysis. The COX-2 promoter was methylated in 4 of 7 COX-2-negative samples and weakly methylated in 1 of 13 COX-2-positive samples (Figure 3A).

**EXPRESSION OF COX-2 IN NPC CELL LINES**

The level of expression of the COX-2 gene at the transcript level was assessed with RT-PCR in 4 NPC cell lines: C666-1, HK-1, CNE-1, and CNE-2. All 4 NPC cells lines expressed COX-2 transcripts (Figure 3B). However, a relatively lower expression level was observed in the CNE-1 cell line, where both methylated and unmethylated alleles were detected.

**COMMENT**

In this study, we report the relationship between COX-2 and EGFR, VEGF, iNOS, and LMP-1 by immunohistochemical analysis in primary NPC tissue. COX-2 was overexpressed in 79% of NPC specimens and was associated with EGFR status. We also looked for an association between COX-2 overexpression and COX-2 promoter methylation status in primary NPC tissues. This report confirms a previous study on COX-2 expression in NPC. COX-2 was detected by Western blot in 8 (57%) of 14 primary NPC tissues. Differences in the frequency of COX-2 may partly be explained by differences in sample size and the methods used to detect COX-2. Using immunohistochemical analysis, the pattern of COX-2 in tumor tissue was consistent with the cellular distribution of COX-2 in other malignancies, where COX-2 staining was localized within the cytoplasm of tumor cells.

Previous studies, as well as the present study, confirm that EGFR was frequently overexpressed in NPC. More important, this is the first study that shows a significant association between COX-2 and EGFR in primary tumor tissue. In contrast, the association between COX-2 and EGFR by immunohistochemical analysis was not found in ovarian cancer or cervical cancer. It is possible that the relationship between COX-2 and EGFR might be tissue specific, thus explaining the lack of association in other tumors.

Results from this study support the extensive in vitro data that suggest a link between EGFR and COX-2. In colon cancer cell lines, EGFR activation resulted in the induction of the COX-2 protein and the subsequent release of prostaglandins. In the same study, EGFR inhibition re-

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**Table. Relationship Between COX-2 and EGFR, INOS, and LMP-1**

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Abbreviations: COX-2, cyclooxygenase-2; EGFR, epidermal growth factor receptor; INOS, inducible nitric oxide synthase; LMP-1, latent membrane protein 1.

*Data are presented as number of samples unless otherwise indicated.*
sulted in decreased COX-2 immunoreactivity and reduced levels of prostaglandins and mitogenesis. In a murine model and in cell line studies, COX-2-derived prostaglandin E2 transactivated EGFR, leading to cell proliferation, migration, and invasion. Thus, preclinical evidence for a molecular link between COX-2 and EGFR is now supported by evidence from primary tumor tissue. The association between VEGF expression and angiogenesis in NPC has been reported. In our study, VEGF was overexpressed in 100% of samples studied, further supporting the importance of angiogenesis in this tumor. In vitro studies have suggested a role for COX-2-mediated regulation of VEGF. However, multiple mediators of VEGF production are known, and the high frequency of VEGF overexpression in NPC tissue suggests that COX-2 overexpression is only one of the mechanisms by which VEGF expression is mediated.

Sixty-nine percent of NPC overexpressed iNOS, whereas in a recent report, iNOS was positive in all NPC tumor samples by immunohistochemical analysis. Differences in the scoring system and definition of iNOS overexpression may account for the differences in results. The cellular distribution of iNOS was in the cytoplasm and nucleus of the tumor cells, which is consistent with the findings by Jayasurya et al in which immunohistochemical analysis, immunoelectron microscopy, and in situ hybridization were used. Previous studies of non-NPC tumors that used immunohistochemical analysis have reported similar findings. COX-2 and iNOS are co-overexpressed in a variety of malignancies, and cross-talk between iNOS and COX-2 enzymes has been reported. In contrast to other malignancies, no correlation between COX-2 and iNOS expression was found in this study. This difference might be due to differences in the methods used to detect iNOS and COX-2.

LMP-1, an Epstein-Barr virus–encoding membrane protein that is expressed in NPC tumor cells, transforms 3T3 cells and increases tumor frequency in a transgenic mouse model. Murono et al found that COX-2 was induced by LMP-1 and that COX-2 was positive in 7 of 10 LMP-1–positive cases and in 1 of 4 LMP-1–negative tumors. Regulation of the COX-2 promoter by LMP-1 was also demonstrated. Our study in clinical specimens does not support the association. However, different methods used in the assessment of the presence of LMP-1 and COX-2 and sample size might explain the differences in results.

Our study may be limited by the validity of immunohistochemical staining for COX-2, EGFR, VEGF, iNOS, and LMP-1. The optimal method to assess expression of these biological markers remains to be defined. Currently, there is no consensus on the optimal method to assess COX-2 overexpression. Western blot, RT-PCR, and immunohistochemical staining each have their advantages and disadvantages. The cytoplasmic localization of COX-2 by immunohistochemical staining was observed in all COX-2–positive NPC cells, and its staining pattern was consistent with previous reports in other malignancies, suggesting its validity. Staining patterns for EGFR, VEGF, iNOS, and LMP-1 in NPC were consistent with previous reports.

Promoter methylation plays a major role in the regulation of expression of many genes. Together with histone acetylation modification, promoter methylation acts as a major mechanism of silencing genes such as tumor suppressor genes. In our study of primary NPC tissue, methylation of the COX-2 promoter region was detected in 2 of 7 COX-2–negative samples and only weakly in 1 of 13 COX-2–positive samples. Similarly, methylation of the COX-2 promoter was seen in only 1 of 4 NPC cell lines that showed relatively lower expression of COX-2 by RT-PCR. Methylation of the COX-2 promoter is rare in NPC tissue that overexpresses COX-2, suggesting that the absence of promoter methylation is a necessary but not sufficient factor in allowing COX-2 overexpression. Histone acetylation may account for the silencing of COX-2 in COX-2–negative tumors without COX-2 promoter methylation, or alternatively, the methylation of another unknown critical regulatory region of COX-2 was missed by our methylation-specific PCR approach. Further work is required to elucidate the involvement of other mechanisms in regulating COX-2 expression.

COX-2 overexpression has been shown to correlate with worse clinical outcome in a variety of cancers but not as yet in NPC. Further research is needed to evaluate COX-2 overexpression as an adverse prognostic factor in NPC.
Clinical studies of a variety of tumors are ongoing to evaluate the usefulness of COX-2 inhibitors in the chemopreventive and therapeutic setting. With in vitro evidence of interaction between EGFR and COX-2 and in view of the correlation between COX-2 and EGFR in clinical NPC specimens, simultaneous targeting of EGFR and COX-2 for therapeutic intervention may become attractive in the near future. A previous study reported that the combination of a selective COX-2 inhibitor with an EGFR tyrosine kinase inhibitor was effective in the prevention of colonic polyps in a murine model of intestinal polyposis. Further studies are warranted to determine whether selective COX-2 inhibitors alone or in combination with EGFR inhibitors may be useful in patients with NPC.

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REFERENCES