

Cyclooxygenase-2 Expression Is Up-Regulated in Squamous Cell Carcinoma of the Head and Neck¹

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Abstract

The purpose of this study was to determine whether cyclooxygenase-2 (COX-2) was overexpressed in squamous cell carcinoma of the head and neck (HNSCC). Quantitative reverse transcription-PCR, immunoblotting, and immunohistochemistry were used to assess the expression of COX-2 in head and neck tissue. Mean levels of COX-2 mRNA were increased by nearly 150-fold in HNSCC ($n = 24$) compared with normal oral mucosa from healthy volunteers ($n = 17$). Additionally, there was about a 50-fold increase in amounts of COX-2 mRNA in normal-appearing epithelium adjacent to HNSCC ($n = 10$) compared with normal oral mucosa from healthy volunteers. Immunoblotting demonstrated that COX-2 protein was present in six of six cases of HNSCC but was undetectable in normal oral mucosa from healthy subjects. Immunohistochemical analysis showed that COX-2 was expressed in both HNSCC and adjacent normal-appearing epithelium. Taken together, these results suggest that COX-2 may be a target for the prevention or treatment of HNSCC.

Introduction

Head and neck cancer is a major, worldwide cause of morbidity and mortality. Over 40,000 cases of HNSCC³ occur per year in the United States alone (1). Despite recent advances in radiotherapy and chemotherapy, the survival of patients with HNSCC has not improved significantly. Moreover, patients who have been cured of one cancer of the head and neck develop second primary carcinomas of the head and neck, lung, or esophagus at a rate approaching 4% per year (2). Hence, new molecular targets are needed for the prevention and treatment of HNSCC and related cancers.

COXs catalyze the synthesis of PGs from arachidonic acid. There are two isoforms of COX. One is constitutively expressed (COX-1), and the other is inducible (COX-2; Ref. 3). The *COX-2* gene is an immediate, early-response gene that is induced by growth factors, oncogenes, carcinogens, and tumor-promoting phorbol esters (3-5). The constitutive isoform, COX-1, is essentially unaffected by these factors.

A large body of evidence from a variety of experimental systems suggests that COX-2 is important in carcinogenesis. COX-2 is up-regulated in transformed cells (3, 6) and in malignant tissue (7-10). Oshima *et al.* (11) showed that knocking out the *COX-2* gene caused a marked reduction in the number and size of intestinal polyps in a

murine model of familial adenomatous polyposis, *i.e.*, APC^{Δ716} knockout mice. COX-2 knockout mice also develop about 75% fewer chemically induced skin papillomas than control mice (12). In addition to the genetic evidence implicating COX-2 in tumorigenesis, recently developed selective inhibitors of COX-2 inhibit intestinal tumor formation in experimental animals (11, 13). In this study, we investigated whether COX-2 was overexpressed in HNSCC compared with normal mucosa from healthy volunteers. Our data show that levels of COX-2 are increased in HNSCC and raise the possibility that selective inhibitors of COX-2 may be useful in the chemoprevention and/or treatment of this disease.

Materials and Methods

Materials. COX-2 primers were from Life Technologies, Inc. (Grand Island, NY). RNeasy Mini kits were from Qiagen, Inc. (Santa Clarita, CA). GeneAmp RNA PCR kits were from Perkin-Elmer (Norwalk, CT). GenElute Agarose Spin Columns were from Supelco (Bellefonte, PA). Lowry protein assay kits and secondary antibody to IgG conjugated to horseradish peroxidase were from Sigma Chemical Co. (St. Louis, MO). The COX-2 standard for immunoblotting was from Cayman Chemical Co. (Ann Arbor, MI). The COX-2 polyclonal antibody, PG-27, was from Oxford Biomedical Research, Inc. (Oxford, MI). Western blotting detection reagents (ECL) were from Amersham Pharmacia Biotech. Streck tissue fixative was from Streck Laboratories, Inc. (Omaha, NE). The tyramide signal and amplification kit was from NEN Life Science. The Vector Blocking kit was from Vector Laboratory, Inc. (Burlingame, CA).

Patient Samples. HNSCC was obtained from 24 patients who underwent resection of their tumors at Memorial Sloan-Kettering Cancer Center. Pieces (2 × 2-mm) of HNSCC were sharply excised, placed in sterile tubes, and frozen immediately in liquid nitrogen. In some cases, clinically normal contralateral or adjacent mucosa was also collected. These latter samples, which were histologically normal, are referred to as normal-appearing epithelium. Some samples were bisected before freezing; one-half of this tissue was used for immunohistochemistry, and the other half was used for quantitative RT-PCR and/or immunoblotting. Normal oral mucosa was obtained from 17 subjects; these individuals were nonsmoking, nondrinking healthy volunteers and patients undergoing ear, nose, and throat procedures for benign disease. All tissue samples for RT-PCR and Western blotting were stored at -80°C until analysis. Tissue for immunohistochemistry was placed in 5 ml of Streck tissue fixative for 12-24 h before processing. Informed consent was obtained from each patient. The study was approved by the Committees on Human Rights in Research at the participating institutions.

Western Blotting. Frozen tissue was thawed in ice-cold lysis buffer containing 150 mM NaCl, 100 mM Tris-buffered saline (pH 8), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Tissues were sonicated for 20 s on ice and centrifuged at 10,000 × *g* for 10 min at 4°C to remove the particulate material. The protein concentration of the supernatant was measured using the Lowry protein assay kit. Immunoblot analysis for COX-2 was performed as in previous studies (4, 5).

Construction of a COX-2 Competitor Template Containing a Nucleotide Deletion. A competitive RT-PCR deletion construct (mimic) for COX-2 was synthesized using a mutant sense primer (nucleotides 932-955 attached to

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³ The abbreviations used are: HNSCC, squamous cell carcinoma of the head and neck; COX-2, cyclooxygenase-2; PG, prostaglandin; RT-PCR, reverse transcription polymerase chain reaction; EGFR, epidermal growth factor receptor.

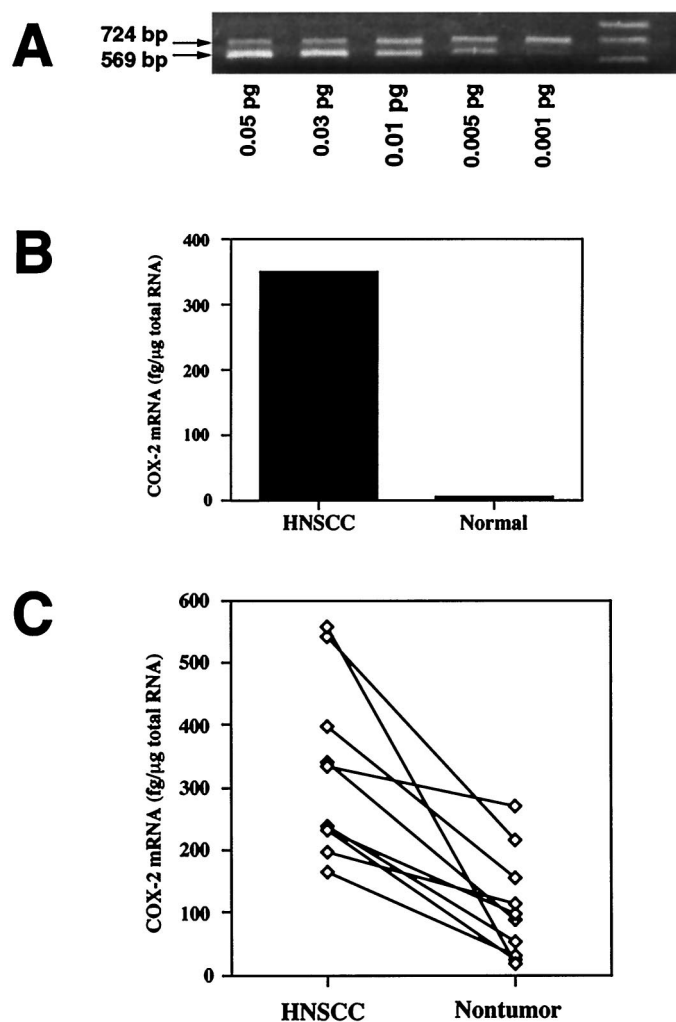


Fig. 1. Increased levels of COX-2 mRNA in HNSCC. *A*, representative quantitative RT-PCR in a case of HNSCC. Ten μ l of endogenous cDNA and known concentrations (0.001–0.05 pg) of hCOX-2 mimic are competing for a fixed amount of hCOX-2 primer in each reaction lane, giving rise to relative proportions of 569-bp mimic product and 724-bp target cDNA product. In this case, there are equal amounts of both products formed at the mimic concentration of 0.01 pg, indicating that 10 μ l of target cDNA are equivalent to 0.01 pg of COX-2 mimic. This is calculated to be 340 fg of COX-2 mRNA/ μ g of total RNA. *B*, quantitative RT-PCR was used to determine the amounts of COX-2 mRNA in 24 cases of HNSCC (mean, 350 fg/ μ g of total RNA; SD, 394 fg/ μ g of total RNA) and 17 cases of normal oral mucosa (mean, 2.4 fg/ μ g; SD, 4 fg/ μ g of total RNA). Nearly a 150-fold increase in amounts of COX-2 mRNA was detected in HNSCC versus normal oral mucosa ($P < 0.001$). *C*, quantitative RT-PCR was used to determine amounts of COX-2 mRNA in 10 pairs of HNSCC and in adjacent normal-appearing epithelium (Nontumor). Lines connect values for samples of HNSCC and adjacent normal-appearing epithelium from the same patients.

nucleotides 1111–1130; 5'-GGTCTGGTGCCTGGTCTGATGATGGAGTGGCTATCACT TCAAAC-3') and an antisense primer (nucleotides 1634–1655; 5'-GTCCTTTCAAGGAGAA TGGTGC-3'), producing a 569-bp PCR product. The mutant sense primer contains the primer-binding sequence of endogenous target (from nucleotide 932 to 955) attached to the end of an intervening DNA sequence (a 156-bp deletion from nucleotides 956 to 1110). Thus, the mimic DNA has primer binding sequences identical to the target cDNA. The 569-bp mimic was further amplified using the sense primer (5'-GGTCTGGTGCCTGGTCTGATGATG-3') and the antisense primer (5'-GTCCTTTCAAGGAGAA TGGTGC-3') in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate, 2.5 units AmpliTaq DNA polymerase, and 400 nM primers for 35 cycles consisting of denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s in a Perkin-Elmer 2400 thermal cycler. The PCR products were electrophoresed on 1% agarose gels and gel-purified using GenElute Agarose Spin Columns according to the manufacturer's protocol.

RNA Isolation and Reverse Transcription. Total RNA was isolated from head and neck tissue (~50 mg) using RNeasy Mini kits from Qiagen. Total RNA (0.6 μ g) was reverse transcribed using the GeneAmp RNA PCR kit according to the manufacturer's protocol.

Quantitative PCR for COX-2 in Human Head and Neck Tissue. Each PCR was carried out in 25 μ l of a reaction mix, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate, 2.5 units AmpliTaq DNA polymerase, and 400 nM primers (sense primer, 5'-GGTCTGGTGCCTGGTCTGATGATG-3'; antisense primer, 5'-GTCCTTTCAAGGAGAA TGGTGC-3'). Five- μ l aliquots of the reverse-transcribed cDNA samples and various known amounts of COX-2 mimic (between 0.001 and 0.05 pg), adjusted to the abundance of the target cDNA, were added to the reaction mix and coamplified for 35 cycles: denaturation at 94°C for 20 s, annealing at 65°C for 20 s, extension at 72°C for 90 s, and final extension at 72°C for 10 min. Ten μ l of PCR products, 724-bp fragments from endogenous target cDNA, and 569-bp fragments from mimic COX-2 were then separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. A computer densitometer (Eagle Eye II; Stratagene, La Jolla, CA) was used to determine the density of the bands. A comparison of the band densities yielded the quantity of COX-2 mRNA in the reaction.

Immunohistochemistry. Tissues from 10 patients with HNSCC were fixed in Streck's solution, embedded in paraffin, cut into 4- μ m sections, and mounted onto polylysine-coated slides. Sections were dewaxed in xylene, rehydrated in descending alcohols, and blocked for endogenous peroxidase (3% H₂O₂ in methanol) and avidin/biotin (Vector Blocking kit). The sections were permeabilized in TNB-BB [0.1 M Tris (pH 7.5), 0.15 M NaCl, 0.5% blocking agent, 0.3% Triton-X, and 0.2% saponin] and incubated in primary antibody overnight at 4°C. The polyclonal antiserum to COX-2 (PG-27; Oxford Biomedical Research, Inc.) was used at a 1:500 dilution in TNB-BB. Control sections were incubated with antisera in the presence of a 100-fold excess of human recombinant COX-2 protein or with isotype-matched IgG normal rabbit serum. Immunoreactive complexes were detected using tyramide signal amplification (TSA-indirect) and visualized with the peroxidase substrate, AEC. Slides were then counterstained in aqueous hematoxylin, mounted in crystal mount, and coverslipped in 50:50 xylene/Permount.

Statistics. Comparisons between groups were made by the Student's *t* test. A difference between groups of $P < 0.05$ was considered significant.

Table 1 Levels of COX-2 mRNA are increased in HNSCC originating in different sites of the head and neck

Quantitative RT-PCR was used to determine the amounts of COX-2 mRNA in 24 cases of HNSCC from different sites in the head and neck.

Site	n	COX-2 mRNA ^a
Tongue	11	323
Oral cavity	8	252
Tonsil	1	1515
Larynx	1	397
Hypopharynx	1	541
Maxillary sinus	2	187
All sites	24	350 ^b

^a Mean levels of COX-2 mRNA in fg/ μ g of total RNA for tumors in each site.

^b Mean level for all tumors in fg/ μ g of total RNA (SD, 394 fg/ μ g of total RNA).

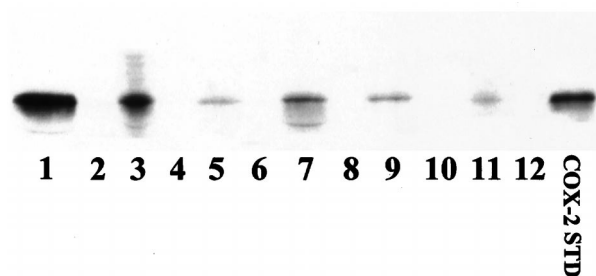


Fig. 2. Levels of COX-2 protein are increased in HNSCC. Immunoblotting was performed on HNSCC from six patients (odd lanes) and normal oral mucosa from six healthy volunteers (even lanes). Equal amounts of protein (100 μ g/lane) were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibody specific for COX-2. Purified ovine COX-2 was used as a standard.

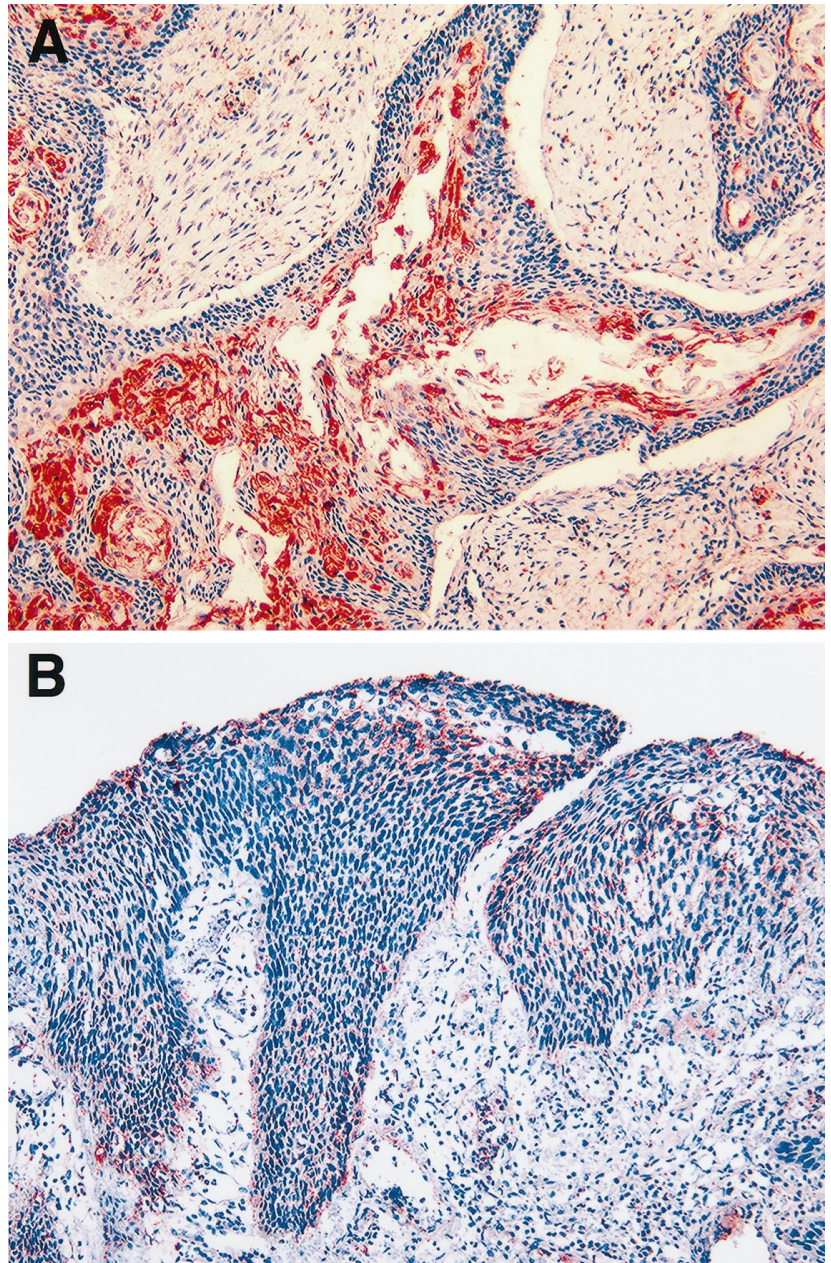


Fig. 3. COX-2 is expressed in malignant epithelial cells in HNSCC. *A*, this invasive, moderately differentiated squamous cell carcinoma shows diffuse and intense granular cytoplasmic immunoreactivity with anti-COX-2 antibody ($\times 100$). *B*, severe dysplasia shows moderately intense cytoplasmic COX-2 expression that is most apparent in the superficial strata of this squamous epithelium ($\times 100$).

Results

To analyze the expression of COX-2, we developed a sensitive competitive RT-PCR assay in which the amount of COX-2 mRNA could be measured from small quantities of RNA. This method relies on the coamplification in the same tube of known amounts of competitor DNA with COX-2 cDNA obtained after reverse transcription from total tissue RNA. The competitor and target use the same PCR primers but yield amplicons with a different size (Fig. 1A), allowing their separation on a gel at the end of the reaction. There was nearly a 150-fold increase in amounts of COX-2 mRNA in HNSCC (mean, 350 fg/ μ g total RNA) *versus* normal mucosa (mean, 2.4 fg/ μ g total RNA; Fig. 1B). Interestingly, levels of COX-2 mRNA were also increased, although to a lesser degree, in normal-appearing epithelium adjacent to HNSCC (mean, 106 fg/ μ g total RNA) compared with normal mucosa. We also compared levels of COX-2 mRNA in paired samples of HNSCC and the adjacent normal-appearing epithelium. As shown in Fig. 1C,

levels of COX-2 mRNA were consistently higher in HNSCC. Increased expression of COX-2 was detected in HNSCC from all sites in the head and neck (Table 1).

To determine whether amounts of COX-2 protein were also increased in HNSCC, Western blot analysis was performed. An immunoblot comparing six samples of HNSCC *versus* six samples of normal oral mucosa from healthy volunteers is shown in Fig. 2. COX-2 protein was detected in six of six cases of HNSCC but was undetectable in normal mucosa. Immunohistochemical analysis of 10 cases of HNSCC revealed that COX-2 expression was multifocal and moderate to strong in intensity in all cases. Expression of COX-2 was localized to tumor cells (Fig. 3A). The staining pattern was granular and localized to the cytoplasm. Mild-to-moderate COX-2 immunoreactivity was also present in the epithelial cells of normal-appearing epithelium adjacent to HNSCC and in two cases of leukoplakia and one case of carcinoma-associated severe dysplasia (Fig. 3B). This staining was specific for COX-2 because immunoreactivity was lost

when the antiserum to COX-2 was preincubated with human recombinant COX-2.

Discussion

This study demonstrates that COX-2 is markedly up-regulated in HNSCC, which is likely to cause the increased levels of PGs observed in HNSCC (14, 15). Several different mechanisms could provide an important link between COX-2 and HNSCC. Enhanced synthesis of PGs, a consequence of up-regulation of COX-2, can increase cell proliferation (16), promote angiogenesis (17), and inhibit immune surveillance (18). All of these effects favor the growth of malignant cells. Additionally, overexpression of COX-2 inhibits apoptosis (19) and enhances invasiveness (20). In extrahepatic tissues, like the head and neck, which have low mixed function oxidase activity (21), COX-2 may also be important for activating xenobiotics to reactive electrophiles that are carcinogenic. For example, COX catalyzes the oxidation of the tobacco procarcinogen benzo[*a*]pyrene-7,8-dihydrodiol to benzo[*a*]pyrene-diol epoxide, which is a highly reactive and strongly mutagenic carcinogen (22). Additional studies are needed to determine which of these mechanisms are important in HNSCC.

Deregulated signaling through the EGFR pathway is recognized to be an early event in the development of head and neck cancers (23). Previously, we reported that EGF, a ligand of EGFR, induced COX-2 and PG synthesis in oral epithelial cells (24). It is reasonable to postulate, therefore, that activation of the EGFR/Ras pathway contributes to the up-regulation of COX-2 in HNSCC. Additional studies are needed to confirm this mechanism.

Nonselective inhibitors of COX-1 and COX-2, such as piroxicam and indomethacin, prevent HNSCC in experimental animals (25). Recently, selective inhibitors of COX-2 have been developed. These compounds possess anticancer properties (11, 13) and appear to be safer than traditional nonsteroidal anti-inflammatory drugs. Based on the results of this study, it will be important to establish whether selective inhibitors of COX-2 are useful in preventing or treating HNSCC.

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