Advances in Brief

Up-Regulation of Cyclooxygenase-2 in Squamous Carcinogenesis of the Esophagus

Awad Shamma, Hirofumi Yamamoto, Yuichiro Doki, Jiro Okami, Motoi Kondo, Yoshiyuki Fujiwara, Masahiko Yano, Masatoshi Inoue, Nariaki Matsuura, Hitoshi Shiozaki, and Morito Monden

Department of Surgery II, Osaka University Medical School [A. S., H. Y., Y. D., J. O., M. K., Y. F., M. Y., M. I., H. S., M. M.], and Department of Pathology, School of Allied Health Science, Faculty of Medicine, Osaka University [N. M.], Osaka 565-0871, Japan

Abstract

Cyclooxygenase-2 (COX-2) is overexpressed in various types of human malignancies including squamous cell carcinomas (SCCs) of the esophagus, but little is known about COX-2 expression in premalignant esophageal squamous dysplasia. To elucidate the role of COX-2 in esophageal carcinogenesis, we examined the expression of this enzyme in normal squamous epithelium (n = 42), squamous dysplasia [high-grade dysplasia (HGD, n = 41; low-grade dysplasia (LGD, n = 33)]; carcinoma in situ (n = 16), mucosal invasive carcinoma (n = 18), and advanced SCC (n = 45). Immunohistochemistry showed a significantly high COX-2 expression in HGD compared with other lesions. The COX-2 score, an index determined by intensity and positivity of COX-2 staining (maximum 3.0), was 0.29 ± 0.04 in normal esophagus, 1.75 ± 0.11 in LGD, 2.89 ± 0.05 in HGD, 2.17 ± 0.18 in CIS, 1.95 ± 0.22 in mucosal invasive carcinoma, and 1.81 ± 0.08 in advanced SCC. Results of reverse transcription-PCR assays confirmed those obtained by immunohistochemistry. COX-2 expression correlated with proliferation activity assessed by the proliferating cell nuclear antigen index in dysplastic lesions (P = 0.001) but not in SCCs. COX-2 expression in SCC did not correlate with various clinicopathological parameters including prognosis. Our results indicate that COX-2 is a sensitive marker for HGD and suggest that COX-2 may be involved in early stages of squamous carcinogenesis of the esophagus.

Introduction

Esophageal SCC is one of the most aggressive human diseases worldwide. Despite surgical treatment and adjuvant chemotherapy, the overall 5-year survival rates range from 5 to 35% (1). Squamous epithelial dysplasia of the esophagus is thought to be a precancerous lesion because it is frequently encountered in esophageal SCC (2). Long-term follow-up studies have revealed that squamous dysplasia is associated with a high risk of esophageal SCC and that approximately 70% of patients with squamous dysplasia are later diagnosed as SCC (3–5). The molecular basis of this process has been examined, and several abnormalities have been described in dysplasia, including genetic instability, DNA aneuploidy, loss of heterozygosity, mutation of the tumor suppressor gene p53, and high PCNA index (6–11). However, much remains to be clarified about the molecular pathogenesis of esophageal squamous neoplasms.

COX is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H2, the precursor of various molecules including prostaglandins, prostacyclin, and thromboxanes. Two COX genes, COX-1 and COX-2 have been identified, which share over 60% identity at the amino acid level (12). COX-1 is constitutively expressed in many tissues and responsible for various physiological functions including cytoprotection of the stomach, vasodilatation in the kidney, and production of a proaggregatory prostanooid, thromboxane, by the platelets. On the other hand, COX-2 is an inducible immediate-early gene, originally found to be induced by inflammation or ovulation or by a variety of stimuli, such as mitogens and cytokines, and various growth factors (13–15). Increased expression of COX-2 has been demonstrated in various inflammatory diseases, including rheumatoid arthritis, Crohn’s disease, ulcerative colitis, and Helicobacter pylori-infectious gastritis (16–18).

Recent studies have also highlighted the relevance of COX-2 in human carcinogenesis. Increased levels of COX-2 has been reported in carcinomas of the colon (19–21) and in carcinomas of stomach, breast, esophagus, lung, liver, and pancreas (22–30). In contrast, the levels of COX-1 are mostly similar in normal and tumor tissues (20, 25). Importantly, overexpression of COX-2 in human carcinomas seems to be of functional significance because double knockout mice for APC and COX-2 genes showed marked reduction in the size and frequency of intestinal polyps (31). There is also cumulative evidence that selective COX-2 inhibitors prevent carcinogenesis in experimental animals, and that these compounds induce apoptosis and inhibit growth in several types of cancer cells (25, 32–38).

3 The abbreviations used are: SCC, squamous cell carcinoma; COX, cyclooxygenase; PBGD, porphobilinogen deaminase; RT, reverse transcription; PAS, periodic acid Schiff; HGD, high-grade dysplasia; LGD, low-grade dysplasia; PCNA, proliferating cell nuclear antigen; CIS, carcinoma in situ; MIC, mucosal invasive carcinoma; APC, adenomatous polyposis coli.
These findings suggest that COX-2 may be associated with carcinogenesis and/or progression of certain types of human malignancies. However, only a few studies have examined the expression of COX-2 in human esophageal SCC, and, to our knowledge, COX-2 expression in premalignant lesions for esophageal SCC has not been examined thus far.

In the present study, we examined the distribution and level of COX-2 protein by immunohistochemistry in multistage esophageal squamous cell carcinogenesis. Moreover, we assessed the prognostic significance of COX-2 in patients with esophageal SCC.

Materials and Methods

Cell Lines and Tissue Samples. Three esophageal squamous carcinoma cell lines, TE2, TE3, and TE8, were obtained from the Japanese Cancer Research Resources Bank. TE2R and TE2S cell lines were subclones established from the TE2 parental cell line (39). Cells were cultured in RPMI 1640 supplemented with 10% FCS at 37°C. We also examined tissue samples obtained from 79 patients, who underwent subtotal esophagectomy because of esophageal carcinoma without preoperative irradiation or chemotherapy at the Department of Surgery II, Osaka University Medical School from 1989 to 1996. The mean follow-up period for the patients’ outcome is 24.5 ± 19.9 months. To identify dysplastic lesions, the resected esophagus was stained with Lugol’s solution (40), and unstained lesions >5 mm in diameter and at least 10 mm apart from the cancerous lesion were collected, together with the main tumors. They were fixed in 10% neutral buffered formalin, processed through graded ethanol, and embedded in paraffin. A piece of each tissue sample was immediately frozen in liquid nitrogen and stored at −80°C for RT-PCR and Western blot analysis.

Histological Diagnosis. Four-μm-thick sections were deparaffinized in xylene, rehydrated, and stained with H&E. The specimens were histologically diagnosed by two skilled pathologists from the Department of Pathology, Osaka University Medical School, according to the criteria defined by the WHO International Histological Classification of Tumors (41). Diagnosis of mucosal neoplastic lesions was based on the following criteria. Dysplastic lesions were characterized by the presence of cells with large hyperchromatic nuclei showing increased mitotic activity in intraepithelial lesions. Esophageal lesions were classified into the following types: (a) LGD: atypical proliferation zone one-half of the thickness of the epithelium; (b) HGD: atypical proliferation zone encompassing up to three-quarters of the epithelium; (c) CIS: the epithelium was either completely or almost completely composed of atypical “immature” cells without invasive growth; (d) MIC: atypical immature cells showing invasive growth that was limited to the muscularis mucosa; and (e) advanced SCC: carcinoma cells infiltrated beyond the muscularis mucosa. Among Lugol’s unstained lesions, histological examination identified 33 LGDs, 41 HGDs, 16 CISs, and 18 MICs. These minimal mucosal neoplastic lesions were examined together with 45 advanced SCCs and 42 normal squamous epithelia.

Semiquantitative RT-PCR. RNA was extracted using Trizol Reagent in a single-step method, and cDNA was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), as described previously (42). Semiquantitative analysis of COX-2 mRNA expression was performed by multiplex RT-PCR technique, using PBGD as the internal standard (30, 43–44). To minimize inter-PCR differences, PCR was performed with COX-2 and PBGD primers in an identical tube, in an unsaturated condition. PCR was performed in a 25-μl reaction mixture containing 1 μl of cDNA template, 1× Perkin-Elmer PCR buffer, 1.5 mM MgCl₂, 0.8 mM deoxynucleotide triphosphates, 0.8 μM each primer for COX-2, 80 nM each for PBGD, and 1 unit of Taq DNA polymerase (AmpliTaQ Gold, Roche Molecular Systems, Inc., NJ). The PCR primers used for detection of COX-2 and PBGD cDNAs were synthesized as described previously, and the amplified products were 305 bp and 127 bp, respectively (44–45). The condition for multiplex PCR was set up as follows: one cycle of denaturing at 95°C for 12 min, followed by 35–40 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min, before a final extension at 72°C for 10 min. Electrophoresed PCR products were scanned by densitometry, and the relative value of COX-2 band to PBGD band was calculated in each sample.

Antibodies. Rabbit polyclonal antihuman COX-2 antibody and its blocking peptide, which was used as immunogen (17 amino acids: position 251–267), were obtained from IBL Co. (Gunma, Japan; Refs. 26, 30). Recombinant COX-2 protein was obtained from Cayman Chemical (Ann Arbor, MI) and used as a positive control in Western blot analysis. Rabbit polyclonal anti-COX-1 antibody was also obtained from IBL Co. Mouse monoclonal antihuman PCNA antibody was purchased from Novocastra Laboratories (New Castle, United Kingdom).

Immunohistochemistry and PAS Staining. After heat antigen retrieval (46), slides were processed for immunohistochemistry on the TeckMate Horizon automated staining system (DAKO, Glostrup, Denmark; Refs. 30, 47), using the Vectastain ABC-peroxidase kit (Vector Labs., Burlingame, CA; Ref. 46). In the step of primary antibody reaction, slides were incubated with the COX-2 antibody or COX-1 antibody (final concentrations, 5 μg/ml for both) for 1 h at room temperature. For positive controls, sections of colon cancer tissues expressing COX-2 protein were included in each staining procedure. For negative controls, nonimmunized rabbit IgG (Vector Labs) and preabsorbed antibody with excess amount of immunogens were used as substitute for the primary antibody. A series of staining was repeated twice to avoid possible technical errors, but similar results were obtained. Serial sections of dysplastic tissues were stained with PAS solution, which stains glycogen as well as mucin (48). Immunostaining of PCNA was performed in all of the specimens, by incubation with 2 μg/ml PCNA antibody for 1 h at room temperature, as described previously (49).
Evaluation of COX-2 Immunostaining. All immuno-
stained sections were evaluated in a coded manner without
knowledge of the clinical and pathological background of pa-
tients. In each section, five high-power fields were selected, and
a total of at least 700 cells were evaluated. The results were
expressed as percentage of cells counted that gave COX-2
positive staining. The intensity of staining was estimated on a
scale from 0 to 3 (negative, weak, moderate, and strong).
Smooth muscle cells served as internal controls within the
sample (25), and immunoreactive score was determined by
multiplication of the percentage of positive cells and staining
intensity, as reported previously (25, 30, 50), ranging from 0 to
3.0. All of the slides were interpreted by two investigators (A. S.
and H. Y.) on three different occasions. Evaluations were sim-
ilar among assessors, with <10% disagreement. A final con-
sensus was achieved between the two assessors using a multi-
head microscope. The PCNA index was calculated as a
percentage of nuclear PCNA, irrespective of intensity because
positive staining for PCNA was routinely strong.

RT-PCR
Using a RT-PCR assay, we determined COX-2 mRNA
levels in 4 esophageal squamous carcinoma cell lines, 2 normal
esophageal epithelia and 11 SCCs. TE2R and TE2S, TE3, and
TE8, 2 normal esophageal epithelia, and 11 SCCs. The relative value of COX-2 band to PBGD band was calculated for each tissue sample and noted under each lane. PCR product sizes (in bp): COX-2, 305; PBGD, 127. M, molecular marker.

RT-PCR
Using a RT-PCR assay, we determined COX-2 mRNA
levels in 4 esophageal squamous carcinoma cell lines, 2 normal
esophageal epithelia and 11 SCCs. TE2R and TE2S, TE3, and
TE8, 2 normal esophageal epithelia, and 11 SCCs. The relative value of COX-2 band to PBGD band was calculated for each tissue sample and noted under each lane. Values of the normal epithelium were relatively low (0.9 and 0.2), whereas values of SCCs varied among samples from 0.3 to 3.7. These assays were repeated at least twice, and similar results were obtained. The results of immunostaining and Western blot analysis were similar to those of RT-PCR
assay in each sample, which suggests that expression of the
COX-2 protein is regulated at a transcription level (data not
shown).

Immunohistochemical Analysis of COX-2
Because dysplasia and early carcinoma are minimal path-
ological lesions, we examined COX-2 expression by immuno-
histochemistry rather than by Western blotting or RT-PCR
assays in these specimens. Normal epithelium and advanced
SCCs were also examined, for comparison.
Specificity of COX-2 Antibody in Immunohistochemistry. Positive control sections of colon carcinoma expressing COX-2 protein displayed strong staining for COX-2, whereas no staining was observed when the primary antibody was substituted by nonimmunized rabbit IgG (data not shown). Preabsorbed antibody with excess amount of the immunogen abolished staining on the sections (data not shown), which indicated that the COX-2 antibody used in this study was highly specific to COX-2 protein in the examined sections.

COX-2 Expression in Normal Squamous Epithelium. A total of 42 normal epithelial specimens that did not include significant inflammation or dysplastic changes were evaluated. COX-2 was weakly expressed in the cytoplasm and around the nuclei of cells that were mainly located in the parabasal and spinous cell layers of the normal esophageal epithelium (Fig. 3A). In general, the percentage of COX-2 positive cells was approximately 25%. In the lamina propria, infiltrating mononuclear cells, fibroblasts, and vascular endothelial cells displayed a moderate-to-strong staining for COX-2.

COX-2 Expression in Squamous Dysplasia. COX-2 expression was detected in the cytoplasm and around nuclei of LGD cells, mostly with a moderate staining intensity (Fig. 3B), whereas strong immunoreactivity for COX-2 was usually noted in the cytoplasm of HGD cells (Fig. 3C). Because esophageal dysplasia grows underneath nonneoplastic epithelial cells, the positivity of COX-2 in dysplasia was determined as a percentage of COX-2-positive cells among cells in the neoplastic layer. The positivity for COX-2 in LGDs varied from 40 to 100%, whereas 37 (90%) of 41 HGDs showed 100%.

COX-2 Expression in Esophageal SCC. Moderate-to-strong COX-2 expression was noted in CIS, whereas the intensity of COX-2 staining varied from weak to strong among cases of MIC and advanced SCC (Fig. 3D). CISs and MICs usually showed a homogeneous COX-2 expression, whereas advanced SCCs often displayed a heterogeneous COX-2 expression. The intensity and percentage of COX-2 staining in the normal and neoplastic lesions are summarized in Table 1, A and B.

Immunoreactive Score of COX-2 Expression

COX-2 immunoreactive scores were determined in all of the specimens (Fig. 4). The mean value of COX-2 score in different tissues was as follows: (a) normal esophagus: 0.29 ± 0.04; (b) LGD: 1.75 ± 0.11; (c) HGD: 2.89 ± 0.05; (d) CIS: 2.17 ± 0.18; (e) MIC: 1.95 ± 0.22; and (f) advanced SCC: 1.81 ± 0.08. COX-2 expression was significantly high in each lesion compared with the normal epithelium (P < 0.0001 for
Furthermore, COX-2 expression in HGD was significantly higher than in LGD, CIS, MIC, and advanced SCC ($P < 0.0001$ for each).

### Relationship between COX-2 Expression and Clinicopathological Parameters in Advanced SCCs

To evaluate the role of COX-2 in tumor progression, patients with advanced SCC were classified into two groups; high COX-2 expressors ($n = 25$) and low COX-2 expressors ($n = 20$), using a cutoff score value of 1.81, representing the mean value of COX-2 score of advanced SCCs. We then analyzed COX-2 expression in relation to various clinicopathological features, including age, gender, site of tumor, histological grade, depth of invasion, nodal involvement, and tumor-node-metastasis stage. There was no correlation between COX-2 expression and the selected clinicopathological parameters (Table 2). In addition, no significant association was found between COX-2 expression and the overall survival rate of patients (Fig. 5).

### COX-2 Expression and Tissue Proliferation

To investigate a possible effect of COX-2 on cell proliferation, we determined the PCNA index for all of the specimens. There was a progressive rise in the mean PCNA index from normal esophagus to advanced SCC (Fig. 6A). This trend was statistically significant ($P < 0.0001$). We also examined the relationship between COX-2 expression (COX-2 score) and proliferation activity (PCNA index) at each stage of carcinogenesis, i.e., normal epithelium ($n = 42$), dysplasia (LGD and

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**Table 1** COX-2 expression in normal and neoplastic squamous tissues of the esophagus

<table>
<thead>
<tr>
<th></th>
<th>0 (negative)</th>
<th>1 (weak)</th>
<th>2 (moderate)</th>
<th>3 (strong)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal squamous epithelium ($n = 42$)</td>
<td>2</td>
<td>22</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
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<td>3</td>
<td>24</td>
<td>6</td>
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<td>HGD ($n = 41$)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>40</td>
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<td>0</td>
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<td>7</td>
<td>8</td>
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<tr>
<td>MIC ($n = 18$)</td>
<td>0</td>
<td>5</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Advanced SCC ($n = 45$)</td>
<td>0</td>
<td>5</td>
<td>35</td>
<td>5</td>
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<table>
<thead>
<tr>
<th></th>
<th>0–25%</th>
<th>26–50%</th>
<th>51–75%</th>
<th>76–100%</th>
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<tr>
<td>Normal squamous epithelium ($n = 42$)</td>
<td>35</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LGD ($n = 33$)</td>
<td>0</td>
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<td>10</td>
<td>22</td>
</tr>
<tr>
<td>HGD ($n = 41$)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>CIS ($n = 16$)</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>MIC ($n = 18$)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Advanced SCC ($n = 45$)</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>32</td>
</tr>
</tbody>
</table>

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* $p<0.0001$
Table 2

<table>
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<th>Markers</th>
<th>COX-2 immunostaining</th>
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<tr>
<td></td>
<td>High n (%)</td>
</tr>
<tr>
<td>No. of patients</td>
<td>25 (56%)</td>
</tr>
<tr>
<td>Age</td>
<td>9 (50%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>21 (57%)</td>
</tr>
<tr>
<td>Women</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
</tr>
<tr>
<td>Cervical</td>
<td>1 (33%)</td>
</tr>
<tr>
<td>Upper thoracic</td>
<td>11 (50%)</td>
</tr>
<tr>
<td>Lower thoracic</td>
<td>13 (65%)</td>
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<tr>
<td>Histological grade</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>6 (50%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>11 (65%)</td>
</tr>
<tr>
<td>Poor</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>T2</td>
<td>8 (67%)</td>
</tr>
<tr>
<td>T3</td>
<td>9 (34%)</td>
</tr>
<tr>
<td>T4</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12 (50%)</td>
</tr>
<tr>
<td>No</td>
<td>13 (62%)</td>
</tr>
<tr>
<td>TNM clinical stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4 (57%)</td>
</tr>
<tr>
<td>II</td>
<td>14 (67%)</td>
</tr>
<tr>
<td>III</td>
<td>7 (70%)</td>
</tr>
</tbody>
</table>

*a* NS, not significant; TNM, tumor-node-metastasis.

**Table 2** Clinicopathological parameters of patients with esophageal SCC in relation to COX-2 expression

HGD, n = 74), early cancer (CIS and MIC, n = 34), and advanced SCC (n = 45), Fig. 6B shows a significant correlation between the two parameters in dysplastic lesions (P = 0.001) but not in other types of tissues.

**Diagnostic Value of COX-2 and PAS Staining for Detection of HGD**

Negative PAS staining is one of the hallmarks of esophageal carcinoma and dysplasia (49). To evaluate the usefulness of COX-2 staining in the differential detection of HGD from dysplasia, COX-2 staining—together with PAS staining—was performed in a total of 74 dysplasias (33 LGDs and 41 HGDs). PAS staining and COX-2 staining were often negatively correlated in dysplasia (Fig. 7A). For statistical analyses, dysplasias were divided into two groups according to COX-2 score, using a cutoff score value of 2.38, representing the mean score of the entire group of dysplastic lesions. A complete loss of PAS staining was found in 6 (18%) of 33 LGDs, and in 35 (85%) of 41 HGDs, and high COX-2 expression was found in 5 (15%) of 33 LGDs, and in 38 (93%) of 41 HGDs. When PAS negativity was combined with high COX-2 expression, this category (PAS−/high COX-2) showed a high sensitivity for detection of HGD (97% or 32 of 33 HGDs) compared with the other three combinations, i.e., PAS−/low COX-2, PAS+/low COX-2, and PAS+/high COX-2 (Fig. 7B).

**Discussion**

Various clinical and investigative studies have strongly suggested that squamous epithelial dysplasia of the esophagus is a precancerous lesion (2–5, 24). In our previous study, we identified frequent loss of heterozygosity in 3p and 17p loci of the chromosome even in squamous dysplasia (6). There are two lines of sequence in carcinogenesis of the esophagus. Dysplastic lesions in Barrett’s esophagus are considered precancerous for adenocarcinoma, whereas dysplasias in squamous esophagus are premalignant lesions for SCC. The former line is frequently found in the United States and western countries whereas the latter line is common in Asia, especially in China and Japan. In
either type of carcinoma, prognosis after surgery is extremely poor compared with other types of human malignancies. Therefore, interruption of the dysplasia-carcinoma sequence may be an effective strategy against esophageal carcinoma. Although other investigators have shown overexpression of COX-2 in Barrett’s esophagus, adenocarcinoma, and SCC of the esophagus (24, 25), to our knowledge, the present study is the first that has examined COX-2 expression in premalignant squamous dysplasia.

Our immunohistochemical studies showed frequent high expression of COX-2 in premalignant lesions and associated SCC of the esophagus. Importantly, COX-2 score progressively increased from normal esophagus to LGD, and the highest expression was noted in HGD; then it gradually decreased during progression from early cancer to advanced SCC (Fig. 4). These findings suggest that COX-2 may be involved in the early stages of carcinogenesis of squamous SCC. The hypothesis that COX-2 is involved in human neoplastic transformation is supported by several lines of evidence. Overexpression of COX-2 in precancerous lesions is found in lesions in other organs. For example, COX-2 is induced even in colonic polyps, in atypical adenomatous hyperplasia and atypical alveolar epithelium of the lung, and in Barrett’s esophagus (19, 24, 26–27). Furthermore, selective COX-2 inhibitors have been shown to inhibit polyp

- **Fig. 6** A. PCNA index in different histological stages of esophageal lesions. There is a progressive increase in PCNA index from normal esophageal tissue to advanced SCC ($P < 0.0001$). B. relationship between PCNA index and COX-2 score in different stages; normal epithelium ($n = 42$), dysplasia (LGD and HGD, $n = 74$), early cancer (CIS and MIC, $n = 34$), and advanced SCC ($n = 45$). A significant correlation between the two parameters was found in dysplastic lesions ($P = 0.001$) but not in other categories.

**A**

<table>
<thead>
<tr>
<th>PCNA index(%)</th>
<th>Normal</th>
<th>LGD</th>
<th>HGD</th>
<th>CIS</th>
<th>MIC</th>
<th>Advanced SCC</th>
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<tbody>
<tr>
<td>100</td>
<td></td>
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<td>80</td>
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<td>60</td>
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<td>40</td>
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<td>20</td>
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<td>0</td>
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</table>

**B**

- **Normal epithelium (n=42)**
  - $r = 0.146$
  - N.S.
- **Dysplasia (n=74)**
  - $r = 0.450$
  - $P = 0.001$
- **Early cancer (n=34)**
  - $r = 0.282$
  - N.S.
- **Advanced SCC (n=45)**
  - $r = 0.231$
  - N.S.
formation in Min mice and carcinogenesis of colon and lung cancers in various animal models (32–34). Experimentally, mice that are null for both COX-2 and APC genes show a marked reduction of polyp formation relative to APC-null mice alone (31). These findings strongly suggest that COX-2 may be involved in the carcinogenesis of these organs.

The present study showed that COX-2 was a marker for proliferative activity in esophageal dysplastic lesions (Fig. 6B). From normal esophagus to LGD to HGD, there was a stepwise increase in both the PCNA index and the COX-2 score. However, in early and advanced SCCs, the COX-2 score tended to slightly decrease although the PCNA index further increased progressively (Fig. 4 and 6A). These findings suggest that COX-2 may be involved in the carcinogenesis of these organs.

The present study showed that COX-2 was a marker for proliferative activity in esophageal dysplastic lesions (Fig. 6B). From normal esophagus to LGD to HGD, there was a stepwise increase in both the PCNA index and the COX-2 score. However, in early and advanced SCCs, the COX-2 score tended to slightly decrease although the PCNA index further increased progressively (Fig. 4 and 6A). These findings suggest that COX-2 may be involved in the regulation of cell proliferation from normal esophagus to HGD. In malignant tissues, classes of factors other than COX-2 may regulate the growth of carcinoma cells. One such candidate is cyclin D1, a cell cycle-positive regulator that is known to progressively increase in multistage carcinogenesis, together with Ki-67 (46). The tumor suppressor gene p53 may be also involved. Although this gene has various biological functions, it is known to regulate progression of cell cycle at G1-S phase transition through activation of the cyclin-dependent kinase inhibitor p21\(^{WAF1/CIP1}\) (52). Indeed the rate of mutation of the p53 gene was reportedly shown in 35% of dysplasia and increased up to 55% in SCC (7).

In advanced SCC, COX-2 protein was detected in the majority of cases, and the COX-2 score varied among samples (Fig. 4). Western blot analyses and RT-PCR assays provided similar results, which indicated that a subset of SCCs displayed a high level of COX-2 protein or mRNA (Figs. 1 and 2). A recent study (21) that examined COX-2 in colon cancer showed gradual up-regulation of COX-2 mRNA in tumors with larger size or deeper invasion, which suggested that COX-2 may play a role in tumor progression. In contrast, the present clinicopathological survey and our previous study (30) on pancreatic carcinoma tissues showed no correlation between COX-2 expression and several clinicopathological parameters including prognosis. Other investigators also reported a lack of significant association between COX-2 level and tumor progression (25, 28). Therefore, it is suggested that COX-2 may play distinct roles among different types of carcinoma.

Although the role of COX-2 in esophageal SCC is not clear...
at present, it is possible that COX-2 is involved in the regulation of cell survival and maintenance of growth because COX-2 inhibitors are known to induce apoptosis and inhibit growth in esophageal carcinoma cells (Ref. 25 and our unpublished observation). Several mechanistic studies suggest that carcinoma cells that overexpress COX-2, and not cells that lack COX-2, are sensitive to COX-2 inhibitors (25, 35, 53). These findings are potentially important from a therapeutic point of view because COX-2 was overexpressed in a subset of esophageal SCC.

During endoscopic examination of high-risk patients for esophageal SCC, we often encounter epithelium that is unstained with Lugol’s solution. These minimal lesions represent areas of esophagitis and early SCC, and these lesions show loss of PAS staining (48, 54–57). We found that the combination of high COX-2 score and negativity for PAS was a useful biomarker for the detection of squamous HGD. This finding is clinically useful because the combination of COX-2 and PAS staining would help in determining the timing of endoscopic mucosal resection in patients who have lesions unstained by Lugol’s solution and who undergo periodic endoscopic examinations.

The main finding of the present study was overexpression of COX-2 among HGDs. This finding is of great importance from the point of view of chemoprevention against esophageal SCC. As mentioned in the “Introduction” section, there is evidence that squamous dysplasia is associated with high risk of esophageal SCC, and that approximately 70% of patients with squamous dysplasia later develop SCC (2–5). On the other hand, it is known that patients who have surgery for head and neck tumor are often diagnosed later to have SCC of the esophagus (57). If our hypothesis that COX-2 may be associated with squamous carcinogenesis of the esophagus is correct, pharmacological antagonism using specific COX-2 inhibitors may be a novel chemopreventive strategy for squamous dysplasia in the future.

In conclusion, we report here that the level of COX-2 protein was up-regulated from normal esophagus to HGD and that COX-2 was overexpressed in a subset of esophageal SCCs. The present study provides important clinical implications with regard to chemoprevention and therapy of esophageal SCC.

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References


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Awad Shamma, Hirofumi Yamamoto, Yuichiro Doki, et al.


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