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Cyclo-oxygenase-2 Over-expression Is Associated with Human Esophageal Squamous Cell Carcinoma

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Abstract

Cyclo-oxygenase (COX)-2 is not usually detectable in normal tissues but is induced in inflammation and carcinogenesis. The level of COX-2 is elevated in cancer tissues of the colon, bladder, and skin. In the esophagus, squamous cell carcinoma and adenocarcinoma are known to express COX-2. The purpose of this study was to clarify the association of COX-2 expression with clinicopathological factors of squamous cell carcinoma. The immunohistochemical expression of COX-2 was examined in 48 surgical specimens of esophageal squamous cell carcinoma. Although COX-2 over-expression was more frequently observed in tumors invading the submucosa (T1b, 76.4%), muscularis propria (T2, 57.1%), adventitia, or adjacent organs (T3～4, 83.3%), even 33.3% of mucosal cancers, such as T1a, showed COX-2 over-expression. COX-2 over-expression was present in 82.3% of lymph node-negative patients but in only 54.8% of lymph node positive patients. There was no difference in COX-2 over-expression between the earlier stages (0 and I, 60%) and more advanced stages (II～IV, 69.6%). COX-2 over-expression did not correlate with survival during 3 years of follow-up. These findings suggest that COX-2 is associated with the phenotype of the esophageal squamous cell carcinoma cells, including superficial cancer cells, and may be related to tumor growth in esophageal squamous cell carcinoma.
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Key words: COX-2, esophageal cancer, immunohistochemistry, carcinogenesis

Introduction

Cyclo-oxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins. It has two isoforms, and each isoform is encoded by separate genes and demonstrates cell-specific expression and regulation. Epidemiological data and results of clinical studies have been compared with results of studies of human tumor tissues, animal models, and cultured tumor cells[1]. Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit both the COX-1 and COX-2 proteins. COX-2[2], but not COX-1, is often expressed in human neoplasms; consequently NSAIDs are known as COX-2 inhibitors. Regular use of such NSAIDs significantly reduces the risks of tumor development and cancer spread[3,4].

The up-regulation of COX-2 has been reported in different stages of the carcinogenic sequence leading to esophageal adenocarcinoma[5,6]. Barrett's esophagus, which is a predisposing condition of esophageal

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Table 1 COX-2 and pathological factors

	Positive/Negative	%	*p* value
Sex			
Male	23/12	65.7	NS[1)]
Female	8/5	61.5	
Stage			
0-I	15/10	60.0	NS
II-IV	16/7	69.6	
Depth			
T1a	4/8	33.3	$p<0.05$ [2)]
T1b	13/4	76.5	
T2	4/3	57.1	
T3 ～ T4	10/2	83.3	
LN metastasis			
N0	14/3	82.4	NS
N1	17/14	54.8	

[1)] NS; statistically not significant
[2)] T1a compared with T1b, T2 and T3 ～ 4

adenocarcinoma (EADC), is caused by reflux of gastroduodenal juice. COX-2 is over-expressed in Barrett's esophagus and EADC and supposedly contributes to esophageal carcinogenesis[7]. It has been reported that strong expression of COX-2 is correlated with tumor progression and poor differentiation in patients with esophageal squamous cell carcinoma (ESCC)[8]. The prognosis of patients with ESCC that strongly expresses COX-2 is significantly poorer than that of patients with ESCC that weakly expresses COX-2[2]. It has also been reported that high COX-2 mRNA expression is related to treatment resistance in ESCC[9]. On the other hand, it has also been reported that COX-2 is not a prognostic marker in ESCC[10,11]. COX-2 has a non-redundant role in the regulation of cellular proliferation and tumorigenesis of esophageal epithelial cells, and COX-2 expression is more characteristic of dysplasia and carcinoma than of normal mucosa, implying a possible association with esophageal tumorigenesis[12]. It is of great importance to investigate which steps of carcinogenesis in ESCC are more closely related to COX-2 expression. Therefore, in this study we investigated the association of immunohistochemical over-expression of COX-2 with clinical and pathologic aspects in ESCC.

Materials and Methods

Patients and Tissues

Surgical specimens were collected from 48 patients with primary ESCC who had undergone radical esophagectomy without preoperative treatments, such as chemotherapy or radiotherapy, from 1999 through 2003 at Nippon Medical School Hospital (Tokyo). As shown in **Table 1**, the patients were 35 men and 13 women, with a median age of 63 years (age range, 45 to 81 years). Twenty-five patients (52.1%) had early-stage disease (Stage 0 and I), and 23 patients (47.9%) had more advanced disease (Stage II～IV). The median follow-up period was 21.5 months. The clinicopathologic stage was determined according to the TNM classification system of the International Union Against Cancer. This study was carried out in accordance with the principles embodied in the Declaration of Helsinki, 1975, and informed consent for using esophageal tissues was obtained from each patient.

Immunohistochemistry

Paraffin-embedded sections (4 μm thick) were immunostained using a Histofine Simple Stain PO (G) Max kit (Nichirei, Tokyo). After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol

Table 2 COX-2 and pathological factors

	Positive/Negative	%	*p* value
Lymphatic invasion			
Positive	18/6	75.0	NS[1)]
Negative	13/11	54.2	
Vascular invasion			
Positive	8/4	66.7	NS
Negative	23/13	63.9	
Intraepithelial spread			
Positive	3/2	60.0	NS
Negative	28/20	58.3	
Differentiation			
Well	12/8	60.0	NS
Mod	10/2	83.3	
Por	9/7	56.3	

[1)] NS; statistically not significant

for 30 min. For immunostaining, the sections were pretreated in an autoclave at 121℃ for 10 min in 10 mM/L citrate buffer (pH 6.0). The tissue sections were incubated with primary antibodies (Immuno-Biological Laboratory Co., Ltd., Tokyo; 1：100 diluted for the anti-antibody) in phosphate-buffered saline containing 1% bovine serum albumin for 16 hr at 4℃. Bound antibodies were detected using diaminobenzidine-tetrahydrochloride as the substrate, and the sections were counterstained with Mayer's hematoxylin. To confirm the specificity of the anti-antibody, the antibody was preincubated with its blocking peptide overnight at 4℃ and then applied to the sections. Negative controls were prepared by omitting the primary antibodies.

Evaluation of Immunohistochemical Analysis

The immunoreactivity of COX-2 was located in the cytoplasm of esophageal cancer cells. The expression of COX-2 protein was classified with a method described in a previous report with minor modifications[13]. The specimens with distinct cytoplasmic staining in more than 10% of the cancer cells were considered positive for the COX-2 protein, whereas specimens not fulfilling this criterion were considered negative. Two investigators separately evaluated all the specimens without knowledge of the patients' clinical background.

Statistical Analysis

Either the chi-square test or Fisher's exact test was used to analyze the correlation between COX-2 expression and clinicopathologic findings. Cumulative survival rates were calculated with the Kaplan-Meier method, and the differences in survival rates were analyzed with the log-rank test; $p<0.05$ indicated significant differences in all analyses. Calculations were performed with the StatView J version 4.5 software package (SAS Institute, Inc., Cary, NC, USA).

Results

Relationship between COX-2 Over-expression and Clinicopahological Findings

COX-2 immunoreactivity was observed mainly in the cytoplasm of the esophageal cancer cells **(Fig. 1a, b)**. COX-2 immunoreactivity was not detected in normal epithelial cells **(Fig. 1c)**. When the anti-COX-2 antibody pre-incubated with its blocking peptide was applied to the sections, no positive immunoreactivity was seen in the cancer cells (data not shown). A total of 31 of 48 patients (64.6%) were positive for COX-2. Although COX-2 over-expression was more frequently observed in tumors invading the submucosa (T1b, 76.5%), muscularis propria (T2, 57.1%), adventitia, or adjacent organs (T3～4, 83.3%), even 33.3% of mucosal cancers, such as T1a, showed COX-2 over-expression ($p<0.05$, **Table 1**). COX-2 over-expression was observed in 82.4% of lymph node-negative patients but in only 54.8% of the lymph node-positive patients, but the difference was

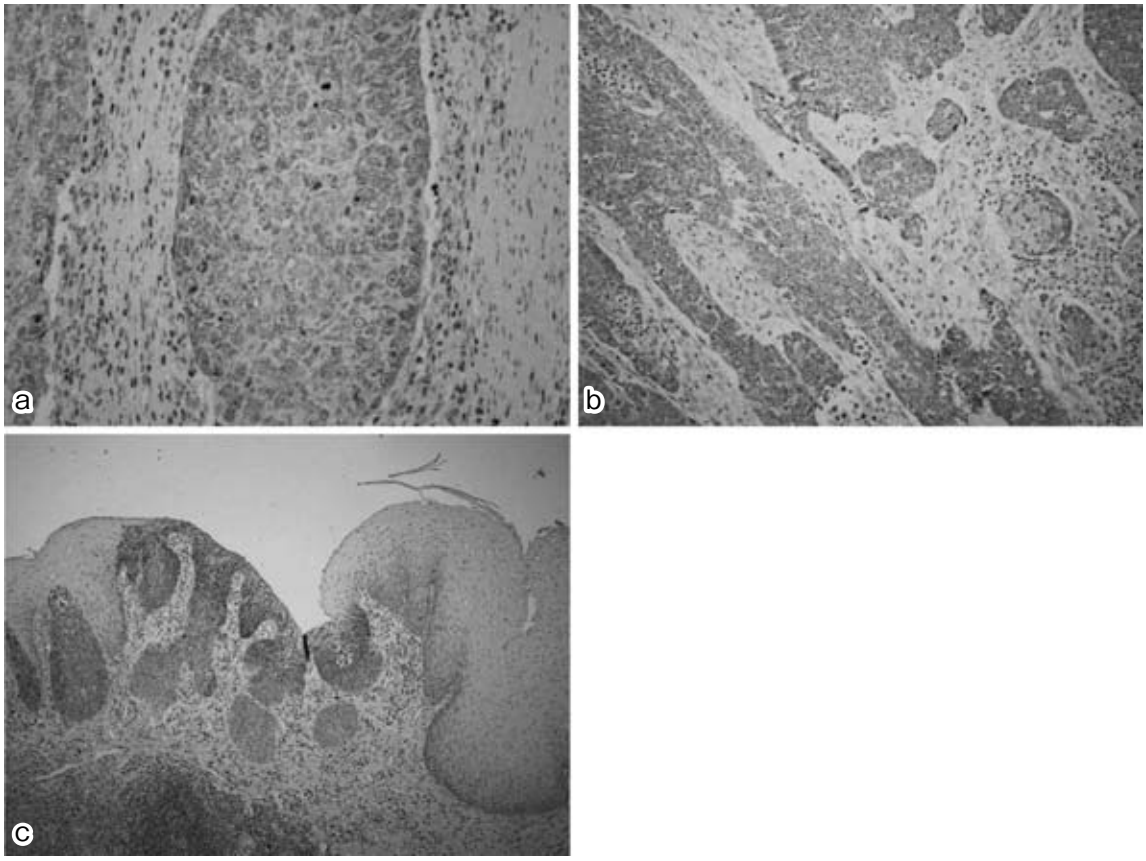

Fig. 1 Immunohistochemical staining of ESCC tissues. **a**: The immunostaining of COX-2 was localized in the cytoplasm of ESCC cells. **b**: Immunoreactivity was present in invasive tumors. **c**: Immunoreactivity of COX-2 was found in cancer cells in the mucosa and submucosa but was not present in the normal mucosa around the tumor.

not statistically significant. Therefore, there was no significant difference in COX-2 over-expression between earlier stages (0 and I, 60%) and more advanced stages (II～IV, 69.6%). As shown in **Table 2**, there was no correlation between COX-2 over-expression and histological findings, such as lymphatic invasion, vascular invasion, and intraepithelial spread. The COX-2 over-expression was not correlated with the histological differentiation of ESCC.

Cumulative Kaplan-Meier survival curve

Kaplan-Meier analysis showed that COX-2 over-expression was not correlated with survival during the 3-year follow-up **(Fig. 2)**.

Discussion

In this study, we have demonstrated COX-2 mucosal over-expression in ESCC, although over-expression was detected more frequently in invading tumors. There was no correlation between COX-2 over-expression and any histological factors, such as invasion of lymph and blood vessels. No significant correlation was found between COX-2 over-expression and lymph node metastasis. As a consequence, COX-2-over-expression had no effects on stages or prognosis. These findings indicate that COX-2 is more closely associated with the phenotype of ESCC cells, including superficial cancer cells, and that COX-2 over-expression may be an early biomarker of ESCC. These possibilities are supported by other studies demonstrating that COX-2 is upregulated in the majority of squamous dysplasia and ESCC[12,14,15]. Accumulating evidence also suggests that COX-2 plays a significant role in carcinogenesis in various tumors. Although most reports are derived from studies of colorectal cancer,

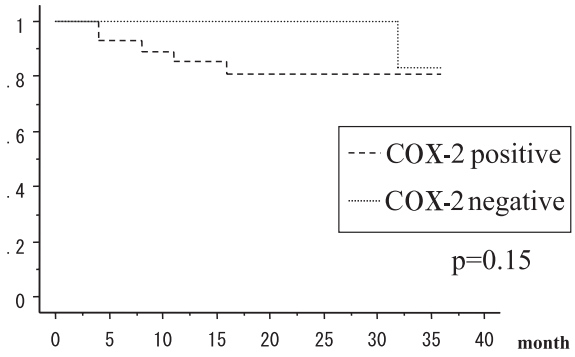

Fig. 2 Kaplan-Meier analysis. There was no significant difference in 3-year survival rates between patients positive and negative for the over-expression of COX-2.

data obtained from recent studies are similar to those of our study suggesting that COX-2 plays an important role in neoplastic transformation[1,16]. Furthermore, other reports indicate that COX-2 expression is extremely high in well-differentiated parts of squamous cell carcinoma and is not related to a poor outcome[10]. It has also been reported that elevated COX-2 expression is a prognostic factor for reduced survival in patients with EADC but is not a prognostic marker in ESCC[10,23]. Similarly, low COX-2 expression is associated with a poor prognosis in patients receiving neoadjuvant therapy for ESCC, and examining COX-2 mRNA expression is useful for predicting the effect of chemoradiotherapy in patients[11]. Therefore, it may be important for treatment to identify COX-2 over-expression in ESCC. In contrast, Yang et al. have reported that COX-2 expression becomes increasingly significant as the clinical stage advances in ESCC[2]. Nozoe et al. have also indicated that strong expression of COX-2 is correlated with tumor progression and poor differentiation in ESCC[8]. Although the difference did not reach the level of statistical significance, our results also indicated that the deeply invading tumors, such as T3～4, tended to have COX-2 over-expression. Therefore, COX-2 may be related to tumor growth or invasion as well as the early tumor phenotype.

COX-2 gene is over-expressed in inflammatory diseases such as reflux esophagitis and Barrett's esophagus[7]. It has been reported that cancer cells with high COX-2 over-expression are found significantly more often in the middle and lower esophagus than in the cervical and upper esophagus[17]. This finding suggests that carcinogenesis in ESCC may also be related to the inflammation caused by the reflux of gastroduodenal juice. In this regard, the over-expression of COX-2 is thought to be associated with carcinogenesis through inflammation. COX-2 is induced by various factors, including hypoxia and cytokines[18]. Our previous study found that hypoxia inducible factor (HIF)-α is also over-expressed in superficial ESCC[19], and there is an important association between vascular endothelial growth factor (VEGF) and COX-2[20,21]. Cytokines such as interleukin-1, tumor necrosis factor alpha, epidermal growth factor, and transforming growth factor beta, also induce COX-2[22]. As tumors grow, there may be more inflammatory reactions that generate these cytokines inducing COX-2. In the tumor environment, VEGF, which is known to be induced by HIF-α, possibly stimulates endothelial cells to form blood vessels. However, our study did not show a correlation between COX-2 over-expression and vessel invasion. For more precise correlations, the microvessel density should be evaluated.

The effects of specific COX-2 inhibitors have been investigated experimentally and clinically. The use of NSAIDs is associated with a reduction in the risk of developing EADC[4,24]. A marked reduction in the number of polyps results from COX-2 gene knockout as well as from selective COX-2 inhibition in a mouse model of human familial adenomatous polyposis[25]. Nonselective NSAIDs, such as aspirin, and selective COX-2 inhibitors, such as celecoxib (SC-58635) and NS-398, suppress azoxymethane-induced colon carcinogenesis in rats[26]. Aspirin, indomethacin, and ibuprofen decrease cultured lung cancer cell proliferation. Selective inhibition of COX-2 is preferable to nonselective inhibition because it reduces cancer cell proliferation, induces cancer cell apoptosis, and spares COX-1-induced cytoprotection of the gastrointestinal tract. Because COX-2 over-expression was also observed in mucosal cancer in this study, chemoprevention with COX-2 inhibitors may be promising strategy for ESCC in predisposed

populations, such as smokers and drinkers. Furthermore, inhibiting COX-2 over-expression in advanced ESCC may be of great benefit to reduce inflammation-associated cancer invasion.

In conclusion, COX-2 is associated with the phenotype of ESCC cells, including superficial cancer cells, and may be related to tumor growth and invasion in ESCC. Investigating the COX-2 status in ESCC and its relevance to the clinical and pathologic features may provide a rationale for possible chemopreventive and therapeutic strategies for ESCC.

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