

Expression of cyclooxygenase-2 in human esophageal squamous cell carcinomas

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Abstract

AIM: To determine whether cyclooxygenase-2 (COX-2) was expressed in human esophageal squamous cell carcinoma.

METHODS: Quantitative reverse transcription-polymerase chain reaction (RT-PCR), western blotting, immunohistochemistry and immunofluorescence were used to assess the expression level of COX-2 in esophageal tissue.

RESULTS: COX-2 mRNA levels were increased by >80-fold in esophageal squamous cell carcinoma when compared to adjacent noncancerous tissue. COX-2 protein was present in 21 of 30 cases of esophageal squamous cell carcinoma tissues, but was undetectable in noncancerous tissue. Immunohistochemistry was performed to directly show expression of COX-2 in tumor tissue.

CONCLUSION: These results suggest that COX-2 may be an important factor for esophageal cancer and inhibition of COX-2 may be helpful for prevention and possibly treatment of this cancer.

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INTRODUCTION

Esophageal cancer is the fourth most prevalent malignancy in China. Although several factors have been implicated for the recent rise in the frequency of esophageal carcinoma, including diet, activation of *c-myc* oncogenes and inactivation of tumor suppressor genes (*p53*)^[1,2], the exact pathogenic mechanisms and promoting factors of this cancer remain to be

clarified. There is no effective strategy for treatment of this disease. Recently epidemiological studies suggest intake of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin can reduce the risk of colorectal cancer^[3,4]. Based on these findings, subsequent studies have addressed the role of COX enzyme as a target of these compounds.

COX is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H₂, the precursor of several molecules including prostaglandin (PG), prostacyclin, and thromboxane. Results from recent studies have established the presence of two distinct COX enzymes, a constitutive enzyme (COX-1) and an inducible form (COX-2). They share over 60% identity at the amino acid level and have similar enzymatic activities, but both isoforms are suggested to have different biological functions^[5-9]. COX-1 is constitutively expressed in most mammalian tissues and is thought to carry out "housekeeping" functions such as cytoprotection of the gastric mucosa, regulation of renal blood flow, and control of platelet aggregation. In contrast, COX-2 mRNA and protein are undetectable in normal tissues, but can be rapidly induced by proinflammatory or mitogenic stimuli including cytokines, endotoxins, interleukins, and phorbol ester^[10-12].

Multiple lines of experimental evidence have also suggested that COX-2 is involved in carcinogenesis. For example, COX-2 is up-regulated in transformed cells^[13] and in various forms of cancer^[14-17], whereas levels of COX-1 are relatively stable. Moreover, COX-2 knockout mice develop 75% fewer chemically-induced skin papillomas than control mice. Recently, it has been shown that selective COX-2 inhibitors may significantly suppress experimentally-induced colon carcinogenesis^[18,19]. Epidemiological studies have shown that intake of aspirin was associated with up to a 90% decreased risk of developing esophageal cancer^[20,21], and in induced esophageal carcinomas, indomethacin had antitumor activity^[22,23].

Here we investigated whether COX-2 was up-regulated in esophageal squamous cell carcinomas from Chinese patients. Our data show that levels of COX-2 are markedly increased in esophageal squamous cell carcinomas, which raises the possibility that selective inhibitors of COX-2 may be useful in the prevention or treatment of this important disease.

MATERIALS AND METHODS

Samples

We examined 30 cases of esophageal squamous cell carcinoma. Both tumor tissue and surrounding normal tissue were obtained from surgical patients with esophageal squamous cell carcinoma at Tongji Hospital during operations. The age of the patients was 57±9 years (mean±SD; range, 37 to 75 years). Of the patients, 23 were men and 7 were women. A small portion from each tissue sample was immediately frozen in liquid nitrogen and stored at -80 °C. Tissue samples were fixed in 40 g/L neutral buffered formaldehyde, processed through graded ethanol solutions, and embedded in paraffin and immunostained for COX-2. A subset of paired tumor tissues and surrounding normal tissue was further examined by RT-PCR and Western blotting.

RNA extraction

Protein and total RNA were extracted using TriZol Reagent (Life Technologies, GIBCO BRL). RT-PCR kit was purchased from Takara Bio Co., Ltd. COX-2 affinity-purified polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. Rabbit anti-goat IgG conjugated with horse-radish peroxidase (HRPO) was obtained from KPL, Inc., USA. ImmunoResearch Lab Enhanced chemiluminescence assay was purchased from PIERCE, Inc. SDS-PAGE standard was obtained from Bio-Rad, Inc.

Western blotting for detection of COX-2

Protein in the various samples were extracted and purified by TriZol Reagent, and the protein concentration was evaluated by Bradford method. A portion of protein (20 μ g) extracted from each tissue sample was subjected to electrophoresis in 80 g/L polyacrylamide slab gels. After transfer to PVDF membrane and blocking with fat free milk powder, blots were probed with COX-2 antibodies (1:750), followed by incubation with HRPO-conjugated secondary antibody (rabbit anti-goat 1:800). COX-2 proteins were visualized by enhanced chemiluminescence.

Semi-quantitative RT-PCR

Total RNA was isolated and purified by TriZol Reagent, and the RNA concentration was determined. Semi-quantitative analysis of the expression of COX-2 mRNA was performed using the multiplex RT-PCR technique. In this assay, we used a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal standard. A sample (1 μ g) of total RNA was reverse-transcribed using the Takara Bio RT-PCR kit according to the manufacturer's protocol. PCR was performed in a 25 μ L reaction mixture containing 5 μ L of cDNA template, 1 \times PCR buffer, 1.5 mmol/L MgCl₂, 0.8 mmol/L dNTPs, 100 nmol/L of each primer for COX-2 (sense primer, 5'-CCGAGGTGTATGTATGAGTG-3'; antisense primer, 5'-AGGAGAGGTTAGAGAAGG-3') or 100 nmol/L GAPDH (sense primer, 5'-CCTTGCCTCTCAGACAATGC-3'; antisense primer, 5'-CCACGACATACTCAGCAC-3') and 1 U of *Taq* DNA polymerase. The PCR primers used for detection of COX-2, and GAPDH yielded cDNA products of 314-bp and 340-bp, respectively. The conditions for PCR were one cycle of denaturing at 94 $^{\circ}$ C for 5 min, followed by 35 cycles of at 94 $^{\circ}$ C for 45 s, 56 $^{\circ}$ C for 45 s, 72 $^{\circ}$ C for 1 min with a final extension at 72 $^{\circ}$ C for 7 min. The electrophoresed PCR products were scanned by densitometry and the ratio of COX-2 to GAPDH was calculated in each sample.

Immunohistochemistry

Tissue sections (4 μ m thick) were deparaffinized in xylene and rehydrated. Heat antigen retrieval was performed as described previously^[24]. Slides were then processed for immunohistochemistry. In the primary antibody reaction step, slides were incubated with the COX-2 antibody (final concentration 5 μ g/mL) for 1 h at room temperature. For positive controls, sections of colon cancer expressing the COX-2 protein were included in each staining procedure. For negative controls, nonimmunized rabbit IgG or Tris-buffered saline was substituted for the primary antibody to distinguish false positive responses from nonspecific binding to IgG or the secondary antibody. In addition, preabsorbed antibody with an excess amount of immunogen abolished the staining. Staining was repeated twice to avoid possible technical errors and similar results were obtained in all cases.

Evaluation of COX-2 immunohistochemistry

All immunostained sections were coded and evaluated without

prior knowledge of the clinical or pathological parameters. In each section, five high-power fields were selected at random and a total number of at least 700 cells were evaluated. The results were expressed as the intensity of staining. The intensity of staining was judged relative to smooth muscle cells in the sample, and estimated on a scale 0-3 (0 negative; 1 weak; 2 moderate; and 3 strong). All slides were interpreted by two investigators on three different occasions. Their evaluations were similar and <10% disagreement between the investigators was noted. In case of major disagreement, the final evaluation of such sections was determined by consensus using a multihead microscope.

Immunofluorescence assay

Tissue samples were cut into 6 μ m crystal-sections, and processed with COX-2 specific antibody and fluorescein labeled secondary antibodies and all the sections were observed immediately under fluorescence microscope.

Statistical analysis

Statistical analysis was performed using SAS software. Results of immunohistochemistry were analyzed by Fisher's exact test. The correlation of RT-PCR and western blotting was analyzed by logistic regression analysis. $P < 0.05$ was considered statistically significant.

RESULTS

COX-2 mRNA expression in esophageal squamous cell carcinoma by semi-quantitative RT-PCR

To examine the level of COX-2 mRNA, RT-PCR was performed to estimate the expression of COX-2 in tumor tissues relative to that in the matched normal tissues, which was expressed as COX-2/GAPDH ratio. GAPDH was used as an internal control. Semiquantitative RT-PCR indicated that COX-2 mRNA was found in 21 of 30 esophageal squamous cell carcinomas. In surrounding normal tissue of esophageal squamous cell carcinoma, COX-2 mRNA could not be detected (Figure 1). In the 21 esophageal squamous cell carcinoma tissues, the COX-2/GAPDH ratios ranged from 0.6 to 1.0, levels of COX-2 mRNA were increased by >80-fold compared to adjacent noncancerous tissue.

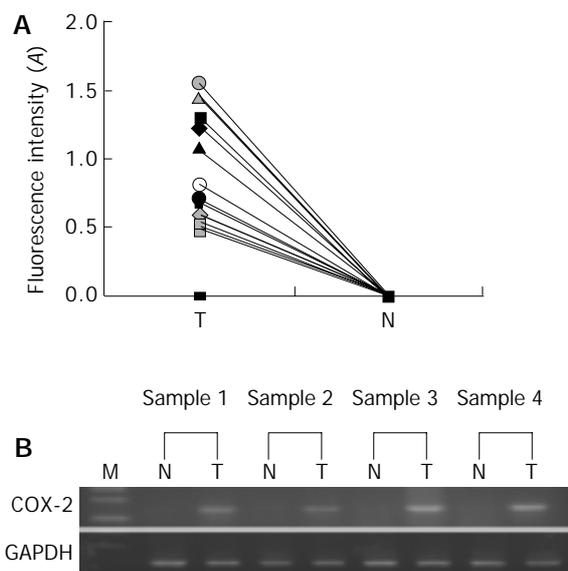


Figure 1 Semiquantitative result of RT-PCR analysis of COX-2 mRNA expression in squamous carcinoma tissues and matched normal tissues of the esophagus. A: Data are expressed as the fluorescence values of COX-2 band in tumor and nontumorous

tissue samples from 30 patients with esophageal squamous cell carcinoma. B: Representative results of semiquantitative RT-PCR, indicated COX-2 mRNA was found in esophageal squamous cell carcinoma samples (T), while in surrounding normal tissue (N), COX-2 mRNA could not be detected. The coamplified GAPDH gene served as an internal control. PCR product sizes are 314 bp for COX-2 and 340 bp for GAPDH. M indicates DNA marker. The samples in lanes 1N and 1T, 2N and 2T, 3N and 3T, 4N and 4T are paired samples from 4 patients, respectively.

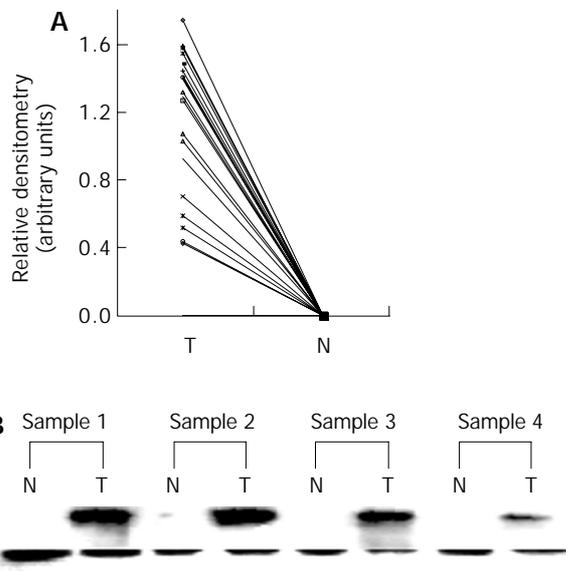


Figure 2 Western blot analysis of COX-2 in squamous carcinoma tissues and matched normal tissues of the esophagus. COX-2 expressions in representative tumor (T) and nontumorous (N) are shown. A: Data are expressed as the absorbency values of COX-2 band in tumor and nontumorous tissue samples from 30 patients with esophageal squamous cell carcinoma. B: Representative result of Western blot analysis. COX-2 protein was detected in tumor tissue but was undetectable in nontumorous tissue in the same patients. β -actin was used as an internal control. The samples in lanes 1N and 1T, 2N and 2T, 3N and 3T, 4N and 4T are paired samples from 4 patients, respectively.

Western blotting

To determine whether levels of COX-2 protein were also increased in esophageal squamous cell carcinomas, western blot analysis of paired tumorous and nontumorous tissue was performed. COX-2 protein was detected in tumor tissue from 21 of 30 patients but was undetectable in nontumorous tissue in the same patients. β -actin was used as an internal control

(Figure 2). Furthermore, the results of western blot analysis paralleled those of mRNA RT-PCR analysis. There was a significant positive correlation between western blot analysis and RT-PCR analysis results ($r=0.708$, $P<0.01$) (Figure 3).

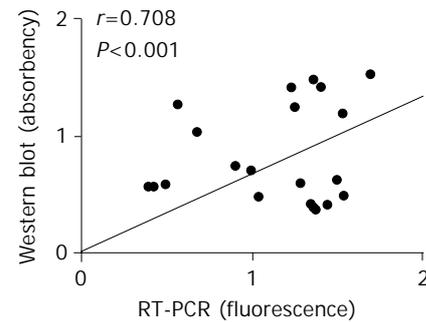


Figure 3 Correlation between western blot analysis and RT-PCR analysis results. The results showed that the expression of COX-2 in cancer tissues (western blot analysis) was highly correlated with by RT-PCR analysis (r value=0.708, $P<0.001$).

Table 1 Expression of COX-2 in esophageal squamous cell carcinomas detected by immunohistochemistry

	Intensity				
	0	1	2	3	
Tumor tissue	10	2	12	6	$P<0.05$
Normal tissue	27	3	0	0	

The intensity of staining was estimated on a scale 0–3. (0, negative; 1, weak; 2, moderate; and 3, strong).

COX-2 expression in esophageal squamous cell carcinoma by immunohistochemistry

Immunohistochemistry was performed to directly indicate cell specific COX-2 protein expression. In nontumorous tissue specimens, a weak COX-2 staining or no COX-2 staining was detected. In contrast, a moderate to strong expression of COX-2 was noted in many esophageal squamous cell carcinomas of the same patients. Specifically, a strong expression of COX-2 protein was present in 6 of 30 (20%) esophageal squamous cell carcinomas, moderate expression was present in 12 of 30 (40%), and weak expression was present in 2 of 30 (15%). The level of COX-2 expression in esophageal squamous cell carcinomas was significantly higher than that in surrounding normal tissue (Table 1). Expression of COX-2 was localized to tumor cells, not to surrounding stromal cells or infiltrating inflammatory cells (Figure 4).

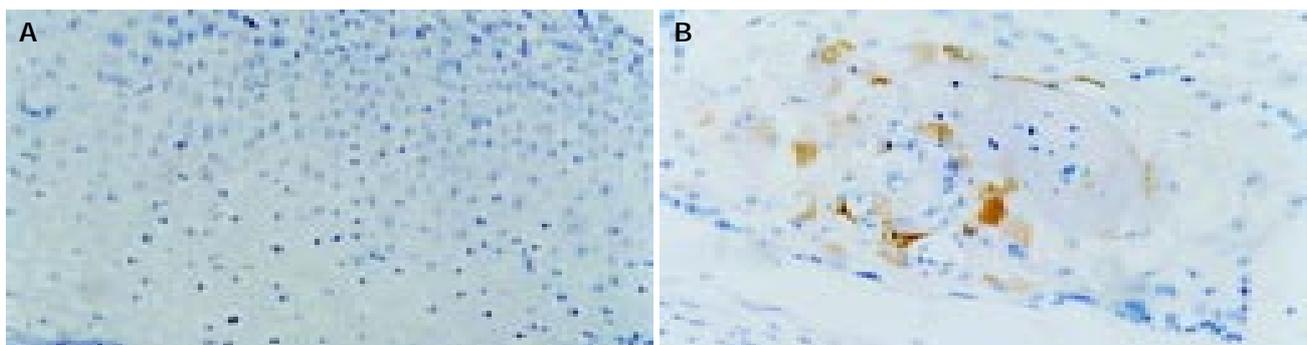


Figure 4 Representative results of immunohistochemical analysis of COX-2 in squamous carcinoma tissues and matched normal tissues of the esophagus from the same patient (Magnification $\times 200$). In normal esophageal tissue there is no positive staining for COX-2 (A) but in esophageal squamous cell carcinoma tissue, carcinoma cells display a strong staining for COX-2, which indicate COX-2 expression specifically in cancer tissues (B).

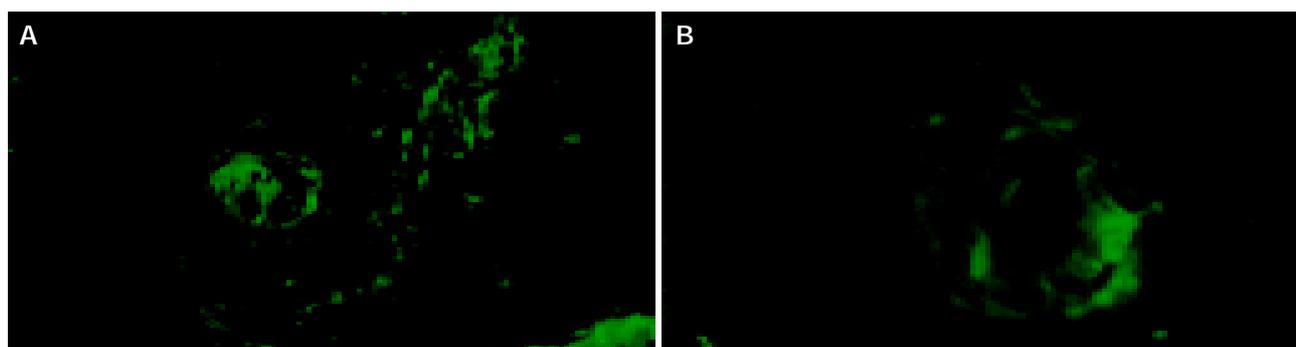


Figure 5 Immunofluorescence analysis of COX-2 in squamous carcinoma tissues (Magnification, x400). A and B are from 2 different patients with esophageal squamous cell carcinoma, Both display a strong green fluorescence staining for COX-2 in cancer-nests.

COX-2 expression in esophageal squamous cell carcinoma by immunofluorescence

Immunofluorescence was performed to directly indicate whether COX-2 protein was expressed in esophageal cancer. COX-2 expression was detected in esophageal squamous cell carcinomas. Immunofluorescence in esophageal squamous cell carcinomas displayed a cancer-nest structure (Figure 5).

DISCUSSION

Epidemiological studies indicate that the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) results in a marked reduction in adenoma size and number and, not infrequently, leads to a complete regression of colonic polyps in patients with familial adenomatous polyposis^[25-27]. NSAIDs also reduce the risk of sporadic colorectal, breast, and lung cancers^[28,29]. These effects of NSAIDs on the inhibition of carcinogenesis have also been demonstrated in animal experiments, including pancreatic tumor model induced by N-nitrosobis (2-oxopropyl) amine in hamsters^[30]. Several lines of evidence indicate that the antitumor effects of NSAIDs may be due in part to the inhibition of COX-2 activity^[19,31-33]. Indeed, in carcinomas of the colon and lung and also in gastric and esophageal carcinomas, the level of COX-2 expression is significantly increased. These findings suggest that up-regulation of this enzyme might be a common mechanism for carcinogenesis of cells of an epithelial origin. Indeed, in the present large sampled study we found that COX-2 expression was increased at both the RNA and protein levels in a large proportion of esophageal squamous cell carcinoma compared with paired normal surrounding esophageal tissues by RT-PCR and Western blot analyses. RT-PCR analyses indicated that 21 of 30 (70%) esophageal squamous cell carcinoma cases displayed increased COX-2 mRNA. Furthermore, the results of mRNA analysis paralleled those of western blot and immunohistochemistry. Thus, approximately 67% of the carcinoma samples exhibited positive staining of COX-2 protein, whereas paired normal tissues showed little or no COX-2 staining. Up to 60% of carcinoma cases showed moderate to strong COX-2 immunostaining (intensity 2 or 3). Also, immunofluorescence demonstrated the expression of COX-2 in esophageal squamous cell cancer-nests. These findings suggest that COX-2 may also be a marker for the malignant potential of esophageal squamous cell carcinoma, although this function remains to be clarified. Taken into consideration of the limited number of specimens, a conservative interpretation of the increased COX-2 mRNA and protein expression is necessary. Examination of a larger number of paired samples of tumor tissue and nonmalignant mucosa from esophageal cancer patients, as well as normal mucosa from persons without esophageal cancer in future studies will be necessary to more clearly determine the status of COX-2 in normal esophageal mucosa and in

esophageal cancer. Because COX-2 was up-regulated at the transcriptional level, which can increase COX-2 protein expression, subsequent determination of COX-2 protein synthesis and bioactivity at the protein level is important. It is also important to study the possible link between the known genetic alterations and COX-2 in esophageal cancer. However, the present results indicate COX-2 is elevated in esophageal cancer in a fashion similar to that in intestinal tumors.

COX-2 could potentially cause carcinogenesis via multiple mechanisms, and the role of COX-2 in tumor development and progression *in vivo* is not known. The most obvious possibility is that overexpression of COX-2 leads to high levels of prostaglandins (PGs) in tumor tissue. This hypothesis is supported by the finding of elevated levels of PGs in cancer tissues, compared with corresponding normal tissues^[34,35]. PGs produced by COX-2 may subsequently facilitate tumor progression by acting as differentiation and growth factors, immunosuppressors and angiogenic agents^[36-38]. Additionally, it has been shown that elevated PGE₂ levels in COX-2 overexpression cancer cells correlate with the metastatic potential of the cancer cells, and can be reduced in a dose-dependent manner by COX inhibitors^[39]. On the other hand, there is also evidence that NSAIDs might exert their antineoplastic effect by a PG-independent pathway, and the COX-2 enzyme itself may promote cancer development and progression^[40]. Thus, NSAIDs suppressed proliferative activity in colon cancer cells devoid of cyclooxygenase and PGs^[31]. Moreover, in extrahepatic tissues in which cytochrome P450 content is low, COX may be important for generation of carcinogens. For example, several classes of chemical carcinogens, e.g., dihydrodiol derivatives of polycyclic aromatic hydrocarbons, aromatic amines and heterocyclic amines, are activated to mutagenic derivatives by COX^[41]. The generation of carcinogens by COX-2 may be important, therefore, for understanding the link between cigarette smoking or consumption of grilled or fried meat and esophageal cancer^[42]. Additional studies are needed to determine which of these mechanisms are more important in esophageal squamous cell carcinoma.

Selective inhibition of COX-2 in esophageal cancer cells induces apoptotic cell death and reduces proliferative activity and these effects correlate with the inhibition of PG synthesis. Similar results have been reported^[43-45]. The significance of COX-2 likely varies between different classes of tumors. We cannot conclude from this and other published data whether the antiproliferative and proapoptotic effects of COX-2 inhibition on esophageal cancer cells are exclusively mediated through the inhibition of PG synthesis or if other mechanisms are also involved. However, our data provide a theoretical basis for evaluation of long-term intake of COX inhibitors on reduction of the incidence of esophageal cancer. Defining actual response of individual tumors to COX-2 inhibitors *in vivo*, however,

awaits further evaluation, especially since the level of COX-2 expression varies substantially between different tumors. Based on our data, a more thorough study on COX-2 expression is needed in patients with esophageal squamous cell carcinoma. Patients with a high COX-2 expression may benefit from specific or nonspecific COX-2 inhibitor medication, such as aspirin, for potential carcinogenic prevention of these cancers. Long-term intake of COX-2 inhibitors may be recommended for family members, especially with a positive family history of esophageal squamous cell carcinoma.

Taken together, our study suggests that COX-2 may play a role in esophageal squamous cell carcinoma development and/or progression and COX-2 inhibitors may be potential agents for the prevention or treatment of human invasive esophageal squamous cell carcinoma. Therefore, long-term intake of NSAIDs, such as aspirin, might reduce the risk of esophageal squamous cell carcinoma. To verify this hypothesis studies to evaluate the efficacy of COX-2 inhibitors in relevant animal models of esophageal squamous cell carcinoma are needed. In addition, studies to define the possible role of COX-2 in esophageal squamous cell carcinoma and the mechanisms by which COX-2 inhibitors exert antitumor activity are essential.

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