Coexpression of COX-2 and iNOS in Angiogenesis of Superficial Esophageal Squamous Cell Carcinoma

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Using immunohistochemical staining, the present study was conducted to examine whether cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) affect angiogenesis in early-stage esophageal squamous cell carcinoma (ESCC). We also analyzed the correlation between these two factors. Cyclooxygenase 2, iNOS, and angiogenesis in early-stage ESCC are unclear. Using 10 samples of normal squamous epithelium, 7 samples of low-grade intraepithelial neoplasia (LGIN), and 45 samples of superficial esophageal cancer, we observed the expression of COX-2 and iNOS. We then investigated the COX-2 and iNOS immunoreactivity scores and the correlation between COX-2 or iNOS scores and microvessel density (MVD) using CD34 or CD105. The intensity of COX-2 or iNOS expression differed significantly according to histological type ($P < 0.001$). The scores of COX-2 and iNOS were lowest for normal squamous epithelium, followed in ascending order by LGIN, carcinoma in situ and tumor invading the lamina propria mucosae (M1-M2 cancer); and tumor invading the muscularis mucosa (M3) or deeper cancer. The differences were significant ($P < 0.001$). Cancers classified M1-M2 ($P < 0.01$ and $P < 0.05$, respectively); M3; or deeper cancer ($P < 0.01$) had significantly

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higher COX-2 and iNOS scores than normal squamous epithelium. There was a significant correlation between COX-2 and iNOS scores ($P < 0.001$, $r_s = 0.51$). Correlations between COX-2 score and CD34-positive MVD or CD105-positive MVD were significant ($r_s = 0.53$, $P < 0.001$; $r_s = 0.62$, $P < 0.001$, respectively). Inducible nitric oxide synthase score was also significantly correlated with CD34 MVD and CD105 MVD ($r_s = 0.45$, $P < 0.001$; $r_s = 0.60$, $P < 0.001$, respectively). Chemoprevention of COX-2 or iNOS activity may blunt the development of ESCC from precancerous lesions.

Key words: COX-2 – iNOS – CD34 – CD105 – Esophageal cancer – Angiogenesis

The depth of invasion of superficial esophageal carcinoma is expressed in accordance with the sub-classification criteria of the Japan Esophageal Society (Guidelines for clinical and pathologic studies on carcinoma of the esophagus).¹

Angiogenesis is essential for cancer progression in order to supply nutrients and oxygen, and remove metabolic waste. Understanding the mechanism of angiogenesis, especially during the early stage of cancer progression, may uncover new targets for treatment or prevention.

We have previously reported in detail the use of microvessel density (MVD) as a feature of early-stage esophageal squamous cell carcinoma (ESCC) revealed by immunostaining using CD34 and CD105.² In that report, we mentioned that MVD based on CD34 and CD105 immunostaining increased according to the grade of atypia or depth of ESCC progression, and also that these findings were correlated with the morphological features of microvessels at the surface of superficial ESCC observed by magnifying endoscopy.

As a subsequent step, we examined the angiogenic factors that induced these newly recruited capillaries at the early stage of ESCC progression. Based on a review of the literature, we had previously reported the profiles of these angiogenic factors, and proposed a hypothesis of “multi-step angiogenesis.”^{3,4} It was found that low-grade intraepithelial neoplasia (LGIN) showed weak expression of cyclooxygenase (COX)-2, whereas carcinoma in situ showed strong expression.⁵ This pattern of COX-2 expression appears to be unique, because the majority of angiogenic factors, such as vascular endothelial growth factor⁶ and matrix metalloproteinase,⁷ show peak expression in invasive cancer. Accordingly, we considered that COX-2 and its related angiogenic factors may play an important role in promoting morphological change in the microvasculature at the early stage of cancer progression. Cyclooxygenase-2 and inducible nitric

oxide synthase (iNOS) are two critical inducible enzymes showing increased expression in many human cancers, including ESCC.^{8,9} Their metabolites, prostaglandin E2 (PGE2) and nitric oxide (NO), can affect cell proliferation, differentiation, and angiogenesis.¹⁰ Expression of the genes for COX-2 and iNOS is correlated in human lung, colon, prostate, pancreas, and gastric cancers.^{11–15} In an animal model of ESCC, Chen *et al*^{16,17} also reported a correlation between COX-2 and iNOS. However, to our knowledge, there have been no detailed reports mentioning the correlation between COX-2 and iNOS in early-stage human ESCC. In the present study, using a series of samples of normal squamous epithelium, LGIN, and superficial ESCC that we² used previously to investigate MVD using CD34 and CD105 immunostaining, we analyzed the immunohistochemical correlation between COX-2 and iNOS expression, and the correlation between these two angiogenic factors and MVD.

Materials and Methods

The study was performed under a protocol approved by our hospital ethics committee.

Tissue samples

We employed 62 samples from 47 patients who underwent histological examination at Saitama Medical Center, Saitama Medical University, between 2006 and 2012. The tissue samples comprised 10 specimens of normal squamous epithelium, 7 specimens of LGIN, and 45 specimens of esophageal cancer (M1: 12 lesions; M2: 7 lesions; M3: 7 lesions; tumor invading the upper third of the submucosal layer [SM1]: 5 lesions; tumor invading the middle third of the submucosal layer [SM2]: 3 lesions; tumor invading the lower third of the submucosal layer [SM3]: 11 lesions).

Fig. 1 (a) Immunohistochemical detection of COX-2 ($\times 400$). Cyclooxygenase 2 expression was detected in the cytoplasm and around the nuclei of cancer cells. The percentage of positive cells in this picture was 84.6%. (b) Immunohistochemical detection of iNOS ($\times 400$). Inducible nitric oxide synthase expression was also detected in the cytoplasm and around the nuclei of cancer cells. The percentage of positive cells in this picture was 52.8%.

Tissue samples were obtained by esophageal biopsy ($n = 4$), endoscopic resection ($n = 14$), or esophagectomy ($n = 44$). We excluded specimens from patients who had undergone radiotherapy and/or chemotherapy before lesion resection. In order to evaluate MVD, we also excluded cases where it was not possible to observe the lamina propria mucosae or submucosa in biopsy samples. We selected 10 samples of normal squamous epithelium that were located distantly from the cancer lesion in esophagectomy specimens.

Pathological diagnosis was made according to the Guidelines for Clinical and Pathologic Studies on Carcinoma of the Esophagus (10th edition).¹⁸

Sections were cut from 3-mm-wide step-sectioned blocks of endoscopic resection specimens, or from 5-mm-wide blocks obtained from surgically resected esophagi, and stained with hematoxylin and eosin (H&E).

We divided the specimens into four different histological types (*i.e.*, normal squamous epithelium, LGIN, M1-M2 cancer, and M3 or deeper cancer).

Immunohistochemical staining

Tissue samples fixed in 10% formalin and embedded in paraffin were cut into sections 4- μ m thick and mounted on slides.

Details of the protocols used for CD34 and CD105 immunostaining and quantification of MVD were described in our previous report.²

For cyclooxygenase 2 and iNOS immunostaining, after dewaxing and dehydration, the sections were pretreated using an autoclave in citrate buffer (pH 6.0) at 121°C for 15 minutes for immunostaining with anti-COX-2 antibody (clone CX229, diluted 1:100, overnight, Cayman Chemical Co, Ann Arbor,

Michigan) and anti-iNOS antibody [rabbit polyclonal antibody (ab3523), diluted 1:200, 30 minutes, Abcam Co, Cambridge, Massachusetts], which was performed using a highly sensitive indirect immunoperoxidase technique (Histofine Simple stain MAX-PO, Nichirei, Tokyo, Japan) with diaminobenzidine as the chromogen, followed by hematoxylin counterstaining.

Quantification of COX-2 and iNOS

In each section, three high-power fields ($\times 400$) were selected. In all cases, including submucosal cancer, the invasive front of cancer infiltration was studied. The results were expressed as the percentage of cells counted that showed positive immunostaining for COX-2 or iNOS (Fig. 1). The intensity of staining was estimated on a scale from 0 to 3 (0: negative; 1: weak; 2: moderate; 3: strong). The immunoreactivity score was determined by multiplying the percentage of positive cells by the staining intensity score. All assessments were performed by 2 investigators (YK, MH) who were blinded to the clinical data. A final consensus was achieved between the 2 investigators using a multiheaded microscope.

Statistical analysis

Data are expressed as median and range. Spearman's rank correlation test was applied for examining correlations between variables. Differences between groups were analyzed by Kruskal-Wallis test followed by Dunn's test. Differences at $P < 0.05$ were considered significant. Computations were performed using a statistical software package (StatFlex, version 6.0, Artech Co, Osaka, Japan).

Fig. 2 Immunohistochemical detection of COX-2 (a–d) and iNOS (e–h). Normal esophageal mucosa ([a, e]: negative for both COX-2 and iNOS, $\times 200$); Low-grade intraepithelial neoplasia ([b, f]: weak staining for both COX-2 and iNOS, $\times 200$); M2 cancer ([c, g]: moderate staining for both COX-2 and iNOS, $\times 200$); and submucosal cancer ([d, h]: strong staining for both COX-2 and iNOS, $\times 200$). Expression of both COX-2 and iNOS was detected in the cytoplasm and around nuclei of epithelial cells or cancer cells.

Results

MVD after immunostaining for CD34 and CD105²

The median MVD (range) for CD34 staining in the normal esophageal mucosa, LGIN, M1-M2 cancer, and M3 or deeper cancer was 24.8 (12.7–69.7); 36.0 (20.0–55.3); 47.3 (24.3–80.0); and 55.3 (23.0–115.7), respectively. Microvessel density assessed on the basis of CD34 positivity was lowest for normal squamous epithelium, followed in ascending order by LGIN, M1-M2 cancer, and M3 or deeper cancer,

the correlation being significant but weak ($P < 0.001$, $r_s = 0.51$).

The median MVD (range) for CD105 immunostaining in normal esophageal mucosa, LGIN, M1-M2 cancer, and M3 or deeper cancer was 0.5 (0–2.5), 7.0 (0–17.5), 13.0 (5.0–19.5), and 22.0 (4.0–65.0), respectively. Microvessel density assessed on the basis of CD105 positivity was also lowest for normal squamous epithelium, followed in ascending order by LGIN, M1-M2 cancer, and M3 or deeper cancer, the correlation being significant and strong ($P < 0.001$, $r_s = 0.76$).

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

	COX-2, intensity of positive cells				COX-2, percentage of positive cells			
	Negative	Weak	Moderate	Strong	0-25	26-50	51-75	76-100
Normal squamous epithelium (n = 30)	29	1	—	—	—	—	—	—
LGIN (n = 21)	8	8	5	—	—	—	—	—
M1-M2 cancer (n = 57)	16	26	15	—	—	—	—	—
M3 or deeper cancer (n = 78)	13	38	21	6	—	—	—	—
Normal squamous epithelium (n = 10)	—	—	—	—	10	—	—	—
LGIN (n = 7)	—	—	—	—	5	1	1	—
M1-M2 cancer (n = 19)	—	—	—	—	9	4	3	3
M3 or deeper cancer (n = 26)	—	—	—	—	10	3	6	7

χ^2 test: $P < 0.001$.

χ^2 test: $P = 0.09$.

COX-2 and iNOS expression in normal and neoplastic squamous tissue of the esophagus

The intensity and percentage of COX-2 staining for each histological type and depth of cancer invasion are summarized in Table 1. Cyclooxygenase 2 expression was detected in the cytoplasm and around nuclei of epithelial cells or cancer cells. In normal squamous epithelium, 30 regions of 10 cases were analyzed. Expression of COX-2 was observed in one region with weak expression in normal squamous epithelium. Thirteen of 21 regions (61.9%) of LGIN, 41 of 57 regions (71.9%) of M1-M2 cancer, and 65 of 78 regions (83.3%) of M3 or deeper cancer revealed positive COX-2 expression. Strong expression was observed in 6 regions (7.7%) of M3 or deeper cancer. The differences in intensity of COX-2 expression among the histological types were significant ($P < 0.001$), whereas no such differences in the percentage of positive cells was observed among the histological types (Fig. 2a-2d).

The intensities and percentages of iNOS staining for each of the histological types and depths of cancer invasion are summarized in Table 2. Inducible nitric oxide synthase expression was also detected in the

cytoplasm and around nuclei of epithelial cells or cancer cells. Expression of iNOS was observed in 4 out of 30 regions with weak expression in normal squamous epithelium. Thirteen of 21 regions (61.9%) of LGIN, 46 of 57 regions (80.7%) of M1-M2 cancer, and 77 of 78 regions (98.7%) of M3 or deeper cancer showed positive iNOS expression. Strong expression was observed in 3 regions (3.8%) of M3 or deeper cancer. The differences in intensity of iNOS expression among the various histological types were significant ($P < 0.001$) (Fig. 2e-2h). There were significant differences in the percentages of positive cells among the various histological types ($P < 0.001$).

COX-2 and iNOS immunoreactivity scores

The COX-2 and iNOS scores for the various histological types are shown in Fig. 3. The median COX-2 score (range) for normal esophageal mucosa, LGIN, M1-M2 cancer, and M3 or deeper cancer was 0.0 (0.0-0.0); 0.04 (0.0-1.20); 0.41 (0.0-1.86); and 0.50 (0.0-2.97), respectively (Fig. 3a). The cyclooxygenase 2 score was lowest for normal squamous epithelium, followed in ascending order by LGIN, M1-M2 cancer, and M3 or deeper cancer, and the difference

Table 2 Expression of iNOS in normal and neoplastic squamous tissues of the esophagus

	iNOS, intensity of positive cells				iNOS, percentage of positive cells			
	Negative	Weak	Moderate	Strong	0-25	26-50	51-75	76-100
Normal squamous epithelium (n = 30)	26	4	—	—	—	—	—	—
LGIN (n = 21)	8	13	—	—	—	—	—	—
M1-M2 cancer (n = 57)	11	40	6	—	—	—	—	—
M3 or deeper cancer (n = 78)	1	55	19	3	—	—	—	—
Normal squamous epithelium (n = 10)	—	—	—	—	10	—	—	—
LGIN (n = 7)	—	—	—	—	5	—	1	1
M1-M2 cancer (n = 19)	—	—	—	—	9	1	1	8
M3 or deeper cancer (n = 26)	—	—	—	—	—	3	4	19

χ^2 test: $P < 0.001$.

Fig. 3 (a) Correlation between COX-2 score and each histological type. Kruskal-Wallis test; $P < 0.001$, Post hoc Dunn's test. * $P < 0.01$. (b) Correlation between iNOS score and each histological type. Kruskal-Wallis test. $P < 0.001$, Post hoc Dunn's test. * $P < 0.01$. ** $P < 0.05$. (c) Correlation between COX-2 score and iNOS score ($P < 0.001$, $r_s = 0.51$).

Fig. 4 Correlation between COX-2 score and (a) CD34 or (b) CD105 MVD ($r_s = 0.53$, $P < 0.001$ and $r_s = 0.62$, $P < 0.001$, respectively).

was significant by Kruskal-Wallis test ($P < 0.001$). Cancers classified as M1-M2 ($P < 0.01$) and M3 or deeper ($P < 0.01$) both showed a significantly higher score than that for normal squamous epithelium by post-hoc Dunn's test.

The median iNOS score (range) for normal esophageal mucosa, LGIN, M1-M2 cancer, and M3 or deeper cancer was 0.0 (0.0–0.5); 0.01 (0.0–0.98); 0.33 (0.0–1.58); and 1.0 (0.30–3.0), respectively (Fig. 3b). Inducible nitric oxide synthase score was also lowest for normal squamous epithelium, followed in ascending order by LGIN, M1-M2 cancer, and M3 or deeper cancer, and the difference was significant by Kruskal-Wallis test ($P < 0.001$). Cancers M1-M2 ($P < 0.05$) and M3 or deeper cancer ($P < 0.01$) also showed significantly higher scores than normal squamous epithelium by post-hoc Dunn's test.

Spearman's rank correlation test demonstrated a statistically significant positive relationship between COX-2 and iNOS scores ($P < 0.001$, $r_s = 0.51$; Fig. 3c).

Correlation between COX-2 score or iNOS score and MVD based on CD34 or CD105 immunostaining

Correlations between COX-2 score and CD34-positive MVD or CD105-positive MVD are shown in Fig.

4. Spearman's rank correlation test demonstrated a significant correlation between COX-2 score and both CD34 MVD and CD105 MVD ($r_s = 0.53$, $P < 0.001$ and $r_s = 0.62$, $P < 0.001$, respectively).

Similarly, iNOS score revealed a significant correlation in terms of both CD34 MVD and CD105 MVD ($r_s = 0.45$, $P < 0.001$ and $r_s = 0.60$, $P < 0.001$, respectively; Fig. 5).

Discussion

The esophagus is the only organ where morphological changes in the superficial microvasculature from normal squamous epithelium to invasive cancer can be observed using magnifying endoscopy in vivo.^{19–22} Inoue *et al* reported the terminal capillary inside the epithelial papillae of the normal squamous epithelium named intra-papillary capillary loop (IPCL).¹⁹ In cancers classified M1 or M2, the IPCLs at the tumor surface retain the shape of normal squamous epithelium and shows dilation and elongation. At the surface of M3 or deeper cancer, the newly developed tumor vessels appear dilated and irregularly branched, with a shape that obviously differs in comparison with the IPCL-like

Fig. 5 Correlation between iNOS score and (a) CD34 or (b) CD105 MVD ($r_s = 0.45$, $P < 0.001$ and $r_s = 0.60$, $P < 0.001$, respectively).

capillaries of M1 or M2 cancer. Japanese endoscopists currently apply these features for diagnosing the depth of tumor invasion, and in this context, high accuracy of preoperative magnifying endoscopy has been reported.^{20–22} Considering these observations, we compared the four different categories of normal squamous epithelium, LGIN (borderline malignancy), M1 and M2 cancer, and M3 or deeper cancer.

Using cancerous and precancerous lesion of the esophagus, Shamma *et al*⁵ reported that the COX-2 immunoreactivity score was highest in high-grade dysplasia, and then gradually decreased according to cancer progression (at the present time, “high-grade dysplasia” is considered to be “high-grade intraepithelial neoplasia” in the Japanese classification,¹ which is included in “M1 and M2 cancer” in the present report). Our present study revealed positive staining for 71.9% of COX-2 and 80.7% of iNOS among M1, M2 regions. Furthermore, the intensity of the positive cells and immunoreactivity scores for both COX-2 and iNOS increased in ascending order from normal squamous epithelium to invasive cancer. In addition, M1 and M2 cancer or M3 or deeper cancer showed significantly higher immunoreactivity scores for COX-2 and iNOS than

normal squamous epithelium. These results suggest that switching of both COX-2 and iNOS expression is activated at the early stage of cancer progression.

In order to assess MVD, we employed 2 different antibodies: anti-CD34 antibody and anti-endoglin/CD105 antibody. Anti-CD34 antibody is a panendothelial marker that stains whole microcapillaries including newly formed and pre-existing vasculature.^{23,24} Endoglin (CD105), a member of the transforming growth factor 1 receptor complex, is well acknowledged to be the most reliable marker of endothelial cell proliferation, and is overexpressed on tumor vessels.²⁵ Here we observed a significant and marginally strong correlation between the immunoreactivity scores for COX-2 or iNOS and both CD34 or CD105 MVD. This result suggests that COX-2 and iNOS from cancer cells induce angiogenesis from the early stage of ESCC progression.

Nitric oxide (NO) is synthesized from the amino acid L-arginine by a family of enzymes known as nitric oxide synthases (NOS). There are at least 3 NOS isoenzymes responsible for NO production in cells, each being the product of a distinct gene. Neuronal NOS²⁶ and endothelial NOS²⁷ are constitutively expressed in neurons and endothelial cells, whereas inducible NOS (iNOS) is produced by

neutrophils, macrophages, epithelial cells, and other cell types.²⁸ Increased NO progression by tumor cells plays a critical role in cancer development. The iNOS enzyme has been implicated in carcinogenesis,²⁹ tumor angiogenesis and cancer progression³⁰ and metastasis.³¹

Prostaglandin endoperoxide synthase, synonymous with COX, catalyzes the formation of prostanoids including prostaglandins A₂, D₂, E₂, F_{2 α} , I₂, J₂, and thromboxane A₂.³² Two cyclooxygenase genes, COX-1 and COX-2, have been identified. Increased levels of COX-2 have been reported in carcinomas of the colon, stomach, breast, esophagus lung liver, and pancreas.^{8,10,11–15} Cyclooxygenase 2 is important for tumorigenesis because prostaglandins, especially prostaglandin E₂, affect cell proliferation, differentiation, apoptosis, angiogenesis, and metastasis.¹⁰

Nitric oxide produced by iNOS has been reported to enhance the activity of COX-2.³³ Several possible mechanisms to explain the interaction of COX-2 and iNOS have been proposed. Expression of iNOS and COX-2 is controlled by transcription factors including the activator protein (AP)-1 complex. Activator protein 1 is composed of the Jun family (c-Jun, Jun-B, and Jun-D) and the Fos family (c-Fos, Fos-B, Fra-1, and Fra-2), which regulate the expression of iNOS and COX-2 by binding to their promoter sequences.³⁴ In addition, NO (the product of iNOS) influences its intracellular targets through stimulation of guanylyl cyclase by directly binding to iron in heme at the active site of guanylyl cyclase,³⁵ or S-nitrosylation of protein targets on appropriate cysteines.³⁶ Because COX-2 has heme at its active site³⁷ and contains 13 cysteines,³⁸ these are the possible targets. Furthermore, it has been shown that iNOS binds specifically to COX-2 and that S-nitrosylates it, thus enhancing COX-2 catalytic activity.³⁹

Our data also revealed a significant correlation between the immunoreactivity scores for iNOS and COX-2. To our knowledge, this is the first report to indicate a significant correlation between COX-2 and iNOS at the early stage ESCC progression, suggesting that inhibition of COX-2 and iNOS might be a possible target for treatment or prevention.

One example of such an approach is COX-2 inhibition, which has previously been investigated for chemoprevention of several other cancers including colon cancer.^{40,41} Inhibition of COX-2 results in suppression of neovascularization and regression of solid tumors, especially those in the early stages. Several reports have suggested that COX-2 expression is upregulated in ESCC and that inhibition of

this enzyme results in a significant reduction of tumor growth.⁴²

More recently, cancer chemoprevention using a “food-based” approach has been emerging as an alternative to the use of single compounds. Black raspberries down-regulate COX-2, iNOS, c-Jun, and vascular endothelial growth factor with reduced levels of PGE₂ in the esophagus, and this is correlated with reduced levels of MVD.^{16,17}

Endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) is unequivocally indicated for the treatment of early-stage ESCC.⁴³ However, it is important to pay special attention to metachronous, multiple esophageal cancers in patients after EMR or ESD, because the entire esophagus could be a source of new lesions including metachronous cancers.^{44,45} As described above, our present series revealed a high frequency of positive staining for both COX-2 and iNOS in M1, M2 lesions. In addition, M1 and M2 lesions of our series showed significantly high immunoreactivity scores for both COX-2 and iNOS.

Chemoprevention of COX-2 or iNOS may blunt the development of precancerous lesions to cancerous lesions. Thus, a chemopreventive approach after endoscopic treatment may reduce the frequency of occurrence of metachronous cancer.

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