

Cyclooxygenase-2 Expression in Human Gastric Tubular Adenomas and Carcinomas; Correlation with Intratumoral Microvessel Density and Apoptotic Index

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Abstract. *Background:* Cyclooxygenase (COX)-2 plays an important role in carcinogenesis in various human malignancies. This study examined the relationship among COX-2 expression, angiogenesis and apoptosis in human gastric adenoma and carcinoma. *Materials and Methods:* We examined the expression of COX-2 in 30 tubular adenomas and 111 carcinomas, comparing it with intratumoral microvessel density (IMVD) and apoptotic index (AI) by immunohistochemistry and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) procedure. *Results:* Immunohistochemistry demonstrated positive expression of COX-2 in 15 (50.0%) adenomas and in 50 (53.1%) carcinomas, respectively. The frequency of COX-2 expression was significantly higher in intestinal-type carcinomas than in diffuse-type, regardless of the tumor stage. The IMVD was significantly higher in the early and advanced carcinomas than in the adenomas and also higher in the COX-2-positive adenomas and carcinomas than in the negative ones. The AI was significantly higher in the adenomas than in the carcinomas and also in the COX-2-negative adenomas and intestinal-type early carcinomas than in their positive counterparts, respectively ($p < 0.05$). The IMVD and AI showed significant inverse correlation in both the adenomas ($p = 0.02$, $r = -0.64$) and carcinomas ($p = 0.04$, $r = -0.18$). *Conclusion:* COX-2 expression might be an early event in gastric tumorigenesis and provide a preferential advantage

for tumor cell proliferation because of its vascular-rich micro-environment and escape from tumor cell apoptosis, especially in intestinal-type gastric carcinomas.

Gastric carcinoma is one of the most frequent malignancies and remains an important cause of mortality world-wide (1). More than 90% of gastric cancers are adenocarcinomas, which are divided into two histological types, intestinal and diffuse, based on the Lauren's classification (2). Pathogenesis of the intestinal-type cancer correlates with precursory changes such as chronic atrophic gastritis, intestinal metaplasia and dysplasia, whereas the diffuse-type lacks well-recognized precursory lesions (1,3). Furthermore, these two types of gastric carcinoma possess some distinctly different and some common genetic backgrounds (1,4).

Cyclooxygenase (COX) is a key enzyme in prostaglandins synthesis and two isoforms, COX-1 and COX-2, are involved in the process (5). COX-1 is a constitutive isoform expressed in various tissues and COX-2 is an inducible enzyme (6). Various epidemiological and experimental studies have suggested that nonsteroidal anti-inflammatory drugs (NSAIDs) decrease the incidence of gastrointestinal carcinomas, especially colonic carcinomas (7). COX-2 is also known as a major target of NSAIDs (8). In fact, elevated COX-2 expression has been detected in several human malignancies in addition to colon cancer (9-12). Moreover, COX-2 expression is associated with tumor cell proliferation, escape from apoptosis and tumor angiogenesis in human esophageal squamous cell carcinomas (13). Several groups have reported COX protein expression in gastric carcinomas using immunoblotting (14-18).

This study examined the immunohistochemical expression of COX-2 in gastric adenoma and adenocarcinoma, comparing it with tumor cell apoptosis and intratumoral microvessel density to clarify the pathobiological role in human gastric tumorigenesis and proliferation.

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Key Words: COX-2, gastric carcinoma, tubular adenoma, angiogenesis, apoptosis.

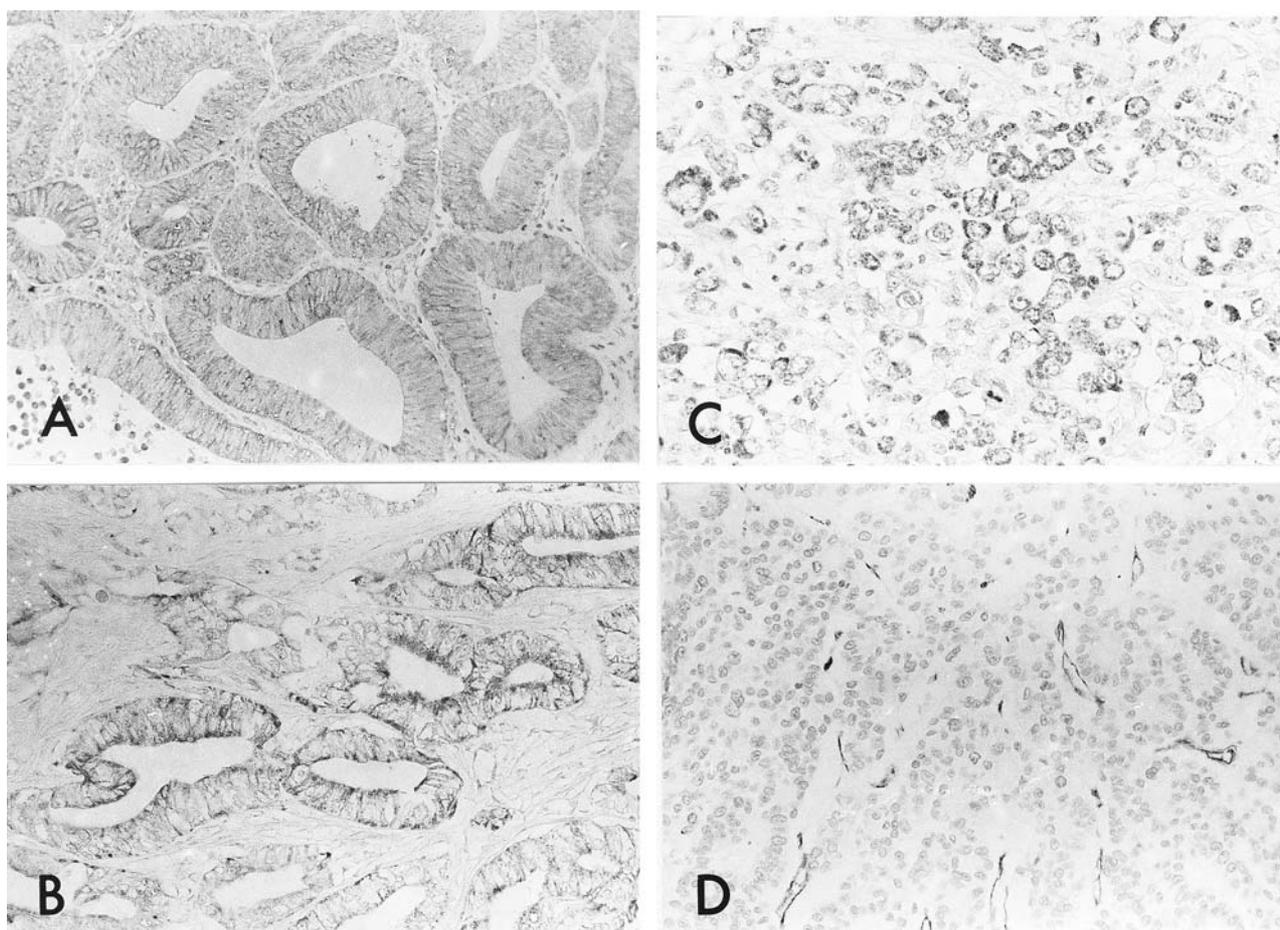


Figure 1. Immunohistochemistry of cyclooxygenase-2 (A, B, C) and CD34 (D); A-C : COX-2 immunoreactivity is shown in the cytoplasm in gastric adenoma cells (A) and gastric adenocarcinoma cells (B, C). Original magnification x50 (A, B), x100 (C). D : Endothelial cells of intratumoral microvessels are detected by CD34 immunoreactivity. Original magnification x50.

Materials and Methods

Tissue specimens. Studies were conducted on 30 tubular adenomas, obtained by endoscopic mucosal resection and 111 surgically removed carcinomas (51 early and 60 advanced carcinomas). The histological type of the gastric carcinomas was classified into intestinal and diffuse-types (2). All the specimens were collected from the files of the Division of Organ Pathology, Faculty of Medicine, Tottori University, Japan, and its related teaching hospitals. We obtained approval for this study from the Institutional Review Board of Faculty of Medicine, Tottori University (Approval number; 283). Routinely processed, formalin-fixed, paraffin-embedded tissue blocks were selected from the main infiltrative portion of the tumors. Three-micrometer thick sections were examined using light microscopy, immunohistochemistry and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) procedure.

Immunohistochemistry. De-waxed paraffin sections were immunostained using the streptavidin-biotin-peroxidase complex (SAB) method (19). The following primary antibodies were used :

monoclonal antibody raised against COX-2 (dilution 1:100, 160112; Cayman) and CD34 (no dilution; NU-4A1, Nichirei, Japan). As pretreatment of COX-2 immunohistochemistry, microwave-based antigen retrieval was performed in 10 mM citrate buffer (pH 6.0). Immunoreactions were visualized with diaminobenzidine and the sections were counterstained with 3% methylgreen. To examine the specificity of immunostaining, the primary antibody was replaced with mouse normal IgG or Tris-buffered saline. In this study, the lesions were regarded as COX-2-positive when more than 20% of the tumor cells showed positive signals. The intratumoral microvessel density (IMVD) was analysed on at least five of the most highly vascularized areas and counted under a light microscope with 200-fold magnification (*i.e.*, 20 objective lens and 10 ocular lens; 0.7386 mm² per field), as described by Weidner *et al.* (20). The IMVD was recorded from the average numbers in each case through a blind method of observation by the two authors independently.

TUNEL method. To detect DNA breaks *in situ* TUNEL was performed according to the method of Gavrieli *et al.* (21), using an Apop Tag Plus *in situ* apoptosis detection kit (Intergen, N.Y., USA).

Table I. Correlation among COX-2 expression, IMVD and AI in gastric adenomas and carcinomas.

	No. of case (%)	IMVD	AI	
Adenomas (n=30)		62.8±4.04	4.55±0.62	
COX-2-positive	15 (50.0%)	71.7±6.05 *	2.17±0.23 *	* *
COX-2-negative	15 (50.0%)	53.9±4.44	6.93±0.87	
Early gastric carcinomas (n=51)		69.2±3.26	2.64±0.22	
Intestinal-type		70.6±4.32	2.80±0.31	* *
COX-2-positive	15 (50.0%) *	82.9±5.70 *	1.73±0.21 *	
COX-2-negative	15 (50.0%)	58.3±4.80	3.87±0.44	
Diffuse-type		67.2±5.02	2.41±0.30	
COX-2-positive	4 (19.0%) *	91.5±3.52 *	1.68±0.32	* *
COX-2-negative	17 (81.0%)	61.5±5.26	2.59±0.35	
Advanced gastric carcinomas (n=60)		77.1±4.10	1.73±0.15	
Intestinal-type		73.6±6.25	1.72±0.15	* *
COX-2-positive	27 (90.0%) *	76.7±6.48	1.72±0.16	
COX-2-negative	3 (10.0%)	46.3±18.25	1.70±0.61	
Diffuse-type		80.4±5.33	1.73±0.27	
COX-2-positive	13 (43.3%) *	91.4±5.50	1.27±0.23	* *
COX-2-negative	17 (56.7%)	72.0±7.95	2.09±0.42	

**p*<0.05

IMVD: intratumoral microvessel density

AI: apoptotic index

At the same time, the TUNEL procedure was also conducted with control slides of esophageal carcinoma, which were already known to show many apoptotic cells with TUNEL signals, to obtain consistent findings (13). The apoptotic index (AI; percentage of apoptotic cells) was obtained as the ratio of the number of TUNEL-positive cancer cells relative to counted tumor cells following cell counting in the well-labeled areas, as determined by scanning at low magnification. Actual counts were made at 200 magnification in ten fields until more than 1,000 different tumor cells were counted and described as apoptotic indexes (AI).

Statistical analysis. The difference in the numerical data between the COX-2 expression and IMVD or AI was evaluated using the Mann-Whitney *U*-test. The correlation between IMVD and AI was analyzed using Spearman's rank correlation coefficient. The level of significance was set at *p*<0.05.

Results

COX-2 protein expression. COX-2 immunoreactivity was observed mainly in the cytoplasm of the gastric adenoma (Figure 1A) and carcinoma cells (Figure 1B,C). Occasionally, a few tumor cells showed COX-2 in their nuclei. On the other hand, vascular endothelial and inflammatory cells infiltrating in the gastric tumor did not express the COX-2 protein (Figure 1B,C).

Table I summarizes the frequency of COX-2-positive tumors in the gastric adenomas and carcinomas, categorizing histological type and tumor stage. COX-2 expression was noted in 15 (50%) of the 30 tubular

adenomas and 59 (53.1%) of the 111 gastric carcinomas, the frequency showing no significant difference. Among 111 carcinomas, COX-2-positive tumors were noted in 19 (37.2%) of the 51 early carcinomas and 40 (66.7%) of the 60 advanced carcinomas. The frequency of COX-2-positive tumors was significantly higher in the latter than in the former (*p*<0.05). COX-2-positive tumors were noted in 15 (50.0%) of the intestinal-type and 4 (19.0%) of the diffuse-type early carcinomas, and in the 27 (90.0%) intestinal-type and the 13 (43.3%) diffuse-type advanced carcinomas. Thus, the frequency of COX-2 expression was significantly higher in the intestinal than in the diffuse carcinomas, regardless of the tumor stage (*p*<0.05).

Correlation among COX-2 expression, IMVD and AI.

Intratumoral microvessels were clearly demonstrated with CD34 immunohistochemistry (Figure 1D). Apoptotic cells were identified by brown nuclear TUNEL signals (Figure 2). Table I also shows the correlation of COX-2 expression with IMVD or AI. The mean IMVDs were 62.8±4.04 in the 30 adenomas, 69.2±3.26 in the 51 early carcinomas and 77.1±4.10 in the 60 advanced carcinomas, the value being higher in the carcinomas than in the adenomas, although no significant difference was noted. The mean IMVDs were 71.7±6.05 in the 15 COX-2-positive adenomas and 53.9±4.44 in the 15 COX-2-negative ones, the value being significantly different (*p*<0.05). Similarly, mean IMVDs were significantly higher in the COX-2-positive carcinomas than in the COX-

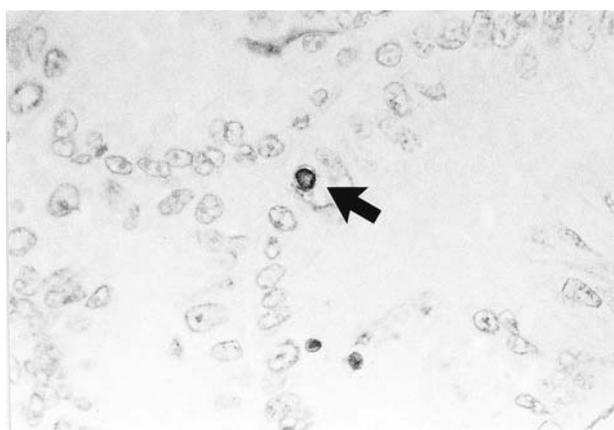


Figure 2. Intense TUNEL signals (arrows) are visible in the nuclei of apoptotic cancer cells. Original magnification x100.

2-negative carcinomas, regardless of stage and histological type. This difference was significant in the early carcinomas ($p < 0.05$), but not in the advanced carcinomas.

The mean AIs in the 30 adenomas were highest in significance, with the value being 4.55 ± 0.62 , followed by the 51 early carcinomas (2.64 ± 0.22) and the 60 advanced carcinomas (1.73 ± 0.15). The mean AIs were 2.17 ± 0.23 in the 15 COX-2-positive adenomas and 6.93 ± 0.87 in the COX-2-negative ones ($p < 0.05$). Among intestinal-type early gastric carcinomas, the mean AIs were 1.73 ± 0.21 in the 15 COX-2-positive cases and 3.87 ± 0.44 in the 15 COX-2-negative ones, the value being significantly higher in the latter than in the former ($p < 0.05$). On the other hand, no significant difference of AIs was noted in either the 21 diffuse-type early carcinomas or the 60 advanced carcinomas, regardless of histological type, although the AIs tended to be lower in the COX-2-positive tumors than in the negative ones. The AIs in advanced carcinomas were lower than in early ones, but no significant difference was found among histological types.

Regression analysis of the Spearman rank correlation coefficient, on plots of AI versus IMVD on a per case basis, showed a significant inverse correlation between AI and IMVD in both the adenomas and carcinomas ($p = 0.02$, $r = -0.64$; $p = 0.04$, $r = -0.18$; for adenomas and adenocarcinomas, respectively; Figure 3).

Discussion

Recently, we demonstrated AI and IMVD, as well as expression of thymidine phosphorylase in intramucosal intestinal-type gastric adenocarcinomas and tubular gastric adenomas to clarify the pathobiological properties of gastric

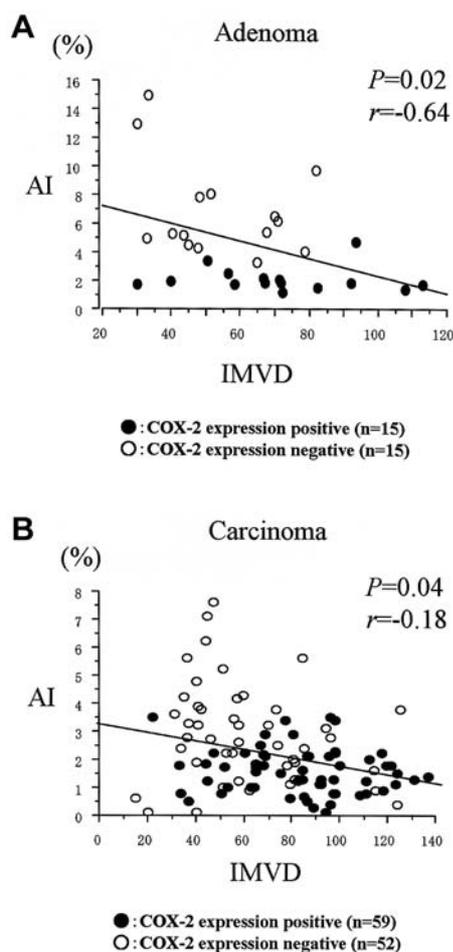


Figure 3. Spearman's rank correlation coefficient test for the correlation between apoptotic index (AI) and intratumoral microvessel density (IMVD) for all cases. A: Tubular adenoma; $r = -0.64$, $p = 0.02$. B: Carcinoma; $r = -0.18$, $p = 0.04$.

intraepithelial tumors (22). Recent studies have suggested that COX-2 expression is associated with a variety of pathobiological factors in human malignancies (13,23,24). These reports prompted us to analyse COX-2 expression, comparing AI and IMVD in gastric carcinomas and tubular adenomas. Moreover, we have already confirmed that COX-2 expression correlated with a high IMVD and low AI in human esophageal carcinomas (13). Esophageal carcinoma cell lines also expressed COX-2 in a varying degree, demonstrated by Western blot, which confirmed the specificity of the antibody used.

The frequency of COX-2 expression did not differ between gastric adenomas and early intestinal carcinomas, indicating that COX-2 expression might act as one of the factors related to early tumorigenesis in the stomach. Of interest is that the frequencies of COX-2 expression were significantly higher in

the advanced carcinomas than in the early carcinomas and higher in intestinal-type carcinomas than in diffuse-type carcinomas. COX-2 expression may be more significantly involved in the progression of intestinal-type carcinomas than in diffuse-type ones. Lim *et al.* (25) reported that there was no correlation between COX-2 expression and histological type in 104 gastric adenocarcinomas. This discrepancy might be partly due to the different criteria used in the results of immunohistochemistry and/or antibodies used for COX-2 detection.

Angiogenesis is essential for the development and proliferation of various human tumors (26). Prevascular tumors less than 3 mm in diameter might remain dormant *in situ* for months to years (27). Consequently, a phenotypically transformed subclone of prevascular cells possessing angiogenic activity can show rapid growth, progression and even metastasis (26,27). The process of intratumoral angiogenesis is regulated by the local balance of various growth factors or cytokines released by both tumor and normal mesenchymal cells (27). This study confirmed that COX-2 expression was correlated with an increase in IMVD in both the adenomas and the early gastric carcinomas. A similar tendency was noted in the advanced carcinomas, regardless of histological type, although no significant difference was found. These results suggested that COX-2 expression might be involved in angiogenesis in human gastric adenomas and carcinomas.

Apoptosis is regulated by the balance between pro- and anti-apoptotic factors and also depends on cell type. In fact, apoptosis is more easily induced in cultured hematopoietic cells than in carcinoma cells (28). Our data showed that the mean AIs were lower in COX-2-positive tumors than in negative tumors, except for intestinal-type advanced gastric carcinomas. A few experimental studies have demonstrated a positive correlation between COX-2 expression and resistance to apoptosis in human cells (29). Tsujii *et al.* (30) found that transfection of the COX-2 gene resulted in the overexpression of antiapoptotic Bcl-2 protein and a reduction in susceptibility to apoptosis in rat intestinal epithelial cells. Recently Uefuji *et al.* (31) showed that a selective COX-2 inhibitor, JTE-522, induced apoptosis in human gastric carcinoma cell lines. Li *et al.* (32) showed that a selective COX-2 inhibitor, NS398, induced apoptosis and this may occur *via* the cytochrome c pathway in esophageal carcinoma cells. Thus, COX-2 expression plays an important role in the escape from apoptosis in human gastric carcinoma cells both *in vivo* and *in vitro*, although the precise role has not been sufficiently elucidated.

Most tumor cells retain the susceptibility to undergo apoptosis in response to hypoxic stress (33). Therefore, it is conceivable that there exists a close correlation between tumor angiogenesis and apoptosis. Little attention, however, has been paid to the relation between angiogenesis and

apoptosis of tumor cells. The relationship between AIs and IMVD has been reported in human esophageal (13) and renal cell carcinomas (34). Our study also demonstrated a significant correlation between AI and IMVD in 30 gastric adenomas and 111 gastric carcinomas. Our results are consistent with previous reports (13,34) in which apoptosis was significantly affected by the extent of neovascularization, suggesting that tumor angiogenesis contributes to a reduction in apoptosis in tumor cells. Recently, it has been demonstrated that hypoxia-inducible factor-1 α (HIF-1 α) regulates neovascularization *via* induction of vascular endothelial growth factor (35), and suppresses the apoptosis induction in human pancreatic cancer cells (36). Moreover, Liu XH *et al.* confirmed the relationship between HIF-1 α and COX-2 expressions in human prostate cancer *in vitro* (37). Further study is needed to clarify the precise roles of COX-2 expression and angiogenesis in gastric tumors.

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