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Expression of Cyclooxygenase-2 and Its Correlation with Clinicopathologic Factors of Ampulla of Vater Cancer

There has been no report for the expression of cyclooxygenase-2 (COX-2) and its clinicopathologic and biologic significance in ampulla of Vater cancer. This study was aimed for the clarification of COX-2 expression and its biologic roles in ampulla of Vater cancer. Fourty-six patients with ampulla of Vater cancer were enrolled and their COX-2 expression and clinicopathologic features were analyzed. The median age of patients was 60 yr and the mean duration of follow-up was 35 months (range: 14-82 months). Immunohistochemical stainings for COX-2, Ki-67, CD34 and TUNEL staining were performed. The immunoreactive COX-2 expression was present in 24 (52.2%) patients of ampulla of Vater cancer and mainly localized in cytosolic and perinuclear region. There was no significant difference in the length of survival between COX-2 postive and negative group (p=0.9420 by Log Rank test). Also, there were no significant differences of proliferation index (p=0.326), apoptotic index (p=0.764) and microvessel density (p=0.135) between COX-2 positive and negative group. Initial pTNM stage (p= 0.0028 by Log Rank test) and blood transfusion over 4 pints during operation (p=0.0254 by Log Rank test) were independent prognostic factor in patients with ampulla of Vater cancer. It is suggested that immunoreactivity of COX-2 is not correlated with clinicopathologic and biologic features of ampulla of Vater cancer.

Key Words : *Vater's Ampulla; Duodenal Neoplasms; Prostaglandin-Endoperoxide Synthase; Ki-67 antigen; Apoptosis; Antigen CD34*

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INTRODUCTION

Ampulla of Vater cancer accounts for approximately 6% of the tumors occurring in the region of the head of the pancreas (1). The majority of tumors in this region are pancreatic adenocarcinomas and the remainders are divided among bile duct carcinomas, duodenal carcinomas, endocrine tumors and metastatic lesions. Patients with cancer of the ampulla of Vater account for up to 36% of those undergoing surgery for pancreato-duodenal malignacies and are the only patients among those affected by cancers of biliopancreatic origin who have upto a 50% chance of being cured by surgery alone (2-9). In addition, these tumors grow more slowly than cholangiocarcinoma and pancreatic adenocarcinoma.

Ampulla of Vater cancer appears as a heterogenous disease from the pathophysiological and molecular point of view. They can arise as a feature of a familial cancer syndrome or as a sporadic and quite uncommon event among gastrointestinal malignancies. In the latter case, they can rise from a preexisting adenoma, closely resembling the colon adenoma-carcinoma sequence. This group of ampullary neoplasm has the histologic appearance of a tubular colon adenocarcinoma, shows an expansive pattern of growth and is characterized by a K-*ras* mutational rate similar to that of colon cancer (10). The existence of these types of ampullary tumors, which share some biological characteristics with colon carcinomas, may partially explain their striking difference from pancreatic and biliary tract cancers in terms of growth behavior and patient survival.

Cyclooxygenase (COX), also referred to as prostaglandin endoperoxide synthase, is the rate-limiting enzyme for the metabolic conversion of arachidonic acid to prostaglandins (PGs) and related eicosanoids. There are at least two isoforms of cyclooxygenase, COX-1 and COX-2. COX-1 is constitutively expressed in many tissues and cell types (11), but in some cases is increased during differentiation (12). By contrast, the expression of COX-2 is frequently up-regulated by mitogens, cytokines and tumor promoters.

Greater than 80% of colon cancers in humans have increased COX-2 levels when compared to adjacent normal tissue (11). The antitumor effect of COX-inhibitor (NSAIDs) in colorectal tumorigenesis is evidenced by abundant data from epidemiologic studies (12, 13), studies in patients with familial adenomatous polyposis (FAP) (14, 15), and multiple experiments in rodent models of colon cancer (16-19). One potential mechanism for the antitumor effect of NSAIDs

could involve inhibition of prostaglandin (PG) synthesis. An important role for PGs in the pathogenesis of colon cancer has been suggested by several lines of evidence: (a) the level of PGE_2 (20) and the mRNA for COX-2 (21) are elevated in human colon cancers; (b) PGs stimulate the proliferation of colon cancer cells (22); (c) PGE_2 may interfere with host antitumor immunologic functions (23). Tsujii et al. (24) have shown that NSAIDs can inhibit angiogenesis by inhibiting COX-2 activity in colon carcinoma cells and downregulating the production of angiogenic factors. Another study has shown that NSAIDs could reduce the proliferation of HT-29 colon cancer cells in vitro, and they caused cell cycle quiescence and apoptosis, both of which could account for their anti-proliferative effect (25).

To our knowledge, there has been no report for the expression of COX-2 in ampulla of Vater cancer. For the intestinal type of ampulla of Vater cancer, which is the most common among the ampullary cancers, an adenoma-carcinoma sequence, which is similar to colorectal cancer, has been postulated, and we hypothesized that biological study for COX-2 expression in ampulla of Vater cancer will demonstrate the similar clinicopathological significance with that of colorectal cancers. Hence, we have collected the surgical specimens of ampulla of Vater cancer and executed the immunohistochemical staining for COX-2, and correlated the results of immunohistochemical staining with the clinicopathologic parameters such as pTNM staging, cell type of tumor, involvement of resection margin and patient's survival.

PATIENTS AND METHODS

Patients

Except for one case of postoperative mortal course (within 30 days) and 8 cases of short-term follow-up (less than 13 months), 46 patients of ampulla of Vater cancer who underwent curative surgical resection between January 1995 and August 2000 at our institution were included in our study. The median age of patients was 60 yr (range: 36-76 yr), and included 27 male (58.7%) and 19 female (41.3%) patients. The mean duration of follow-up was 35 months (range: 14-82 months). Twenty three (50%) patients were deceased during the follow-up period and 23 (50%) patients were still alive until the completion of follow-up.

Immunohistochemical Staining and Interpretation

The specimens obtained from surgical resection were fixed in 10%-buffered formalin, processed routinely, and embedded in paraffin. Five-μm sections were stained with hematoxylin and eosin, and histopathological diagnosis was confirmed by at least two pathologists. In each case, accompanying normal mucosa was collected for comparison.

Tissue immunohistochemical staining was done with LSAB kit (DAKO, Carpinteria, CA, U.S.A.) with avidin-biotin peroxidase complex method. The deparaffinized slide was immersed for 45 min in 0.3% hydrogen peroxide in methanol solution to deplete endogenous peroxide. Nonspecific binding sites were saturated with 0.3% bovine serum albumin and normal goat serum diluted to 1:66.7 in PBS for 20 min. After they were washed with PBS, separate sections were incubated with primary antibodies against COX-2 (1 μg/mL, Santa Cruz, Arbington, U.K.), Ki-67 (Signet Laboratories, Dedham, MA, U.S.A.) and CD34 (Immunotech, Marseille, France) at dilution of 1:50 in a humidified chamber at room temperature for 30 min. The sections were then washed with PBS for 10 min. Biotinylated goat antirabbit IgG were applied onto the tissue sections, and incubated at room temperature for 30 min. After washing with PBS for 10 min, these tissues were incubated with avidin-biotinylated peroxidase for 45 min. Finally, color was developed by the immersion of the sections in a peroxidase substrate solution with aminoethyl carbamazole. The sections were counterstained with Mayer's hematoxylin and were examined under light microscope.

For each tissue specimen, the presence or absence of staining with COX-2 was divided as positive or negative, and the observer assessed all tissues on the slide to assign the positive/negative status. Immunostaing for COX-2 appeared to be localized in cytosolic and perinuclear region (Fig. 1).

For evaluation of proliferation index (PI), the area with a high number of immunolabelled neoplastic nuclei were chosen. Only distinctly immunoreactive tumor cell nuclei were included, and the areas with necrosis and hemorrhage, section borders and vascular endothelium were omitted. In each specimen, approximately 1,000 tumor cells were counted in high-power fields using an eye grid. PI was defined as the ratio of labeled cells to total number of cells counted, represented in percentages.

The number of apoptotic bodies was determined and was expressed in relation to the total number of cells within 20 random high-power fiels (area of each field 0.07 mm^2). Strict criteria were used to define a cell as apoptotic on the H&E and TUNEL stained sections. These were: the presence of a condensed and often fragmented nucleus together with a surrounding halo. Cells containing weakly to moderately TUNEL positive nuclei in the absence of these additional morphologic features were not assessed as apoptotic. The apoptotic index (AI) was calculated as the percentage of tumor cell apoptotic bodies per total number of tumor cells counted for each case. Microvessel density was determined as mean number of CD34 positive microvessels in four $\times$ 200 fields.

Statistical Analysis

Comparison between the clinicopathological factors and immunohistochemical staining was performed using the chi-square test, Student t-test, one-way ANOVA and the linear

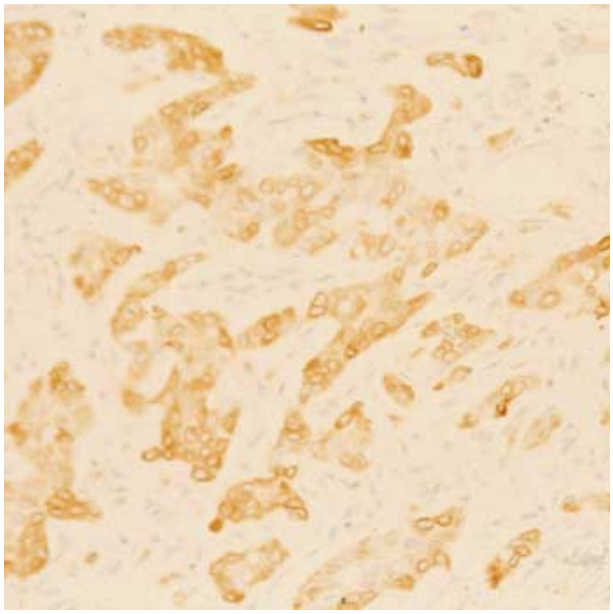

Fig. 1. Marked expression of immunoreactive COX-2 staining was noticed mainly in cytosolic and perinuclear region of cancer cells (×400).

regression analysis. The survival time and the cumulative survival rate were estimated using the Kaplan-Meier method. The significance level was set at 5% for each analysis, and the SPSS (version 10.0, SPSS Inc., U.S.A.) was used for statistical analysis.

RESULTS

Clinicopathologic Features of Patients with Ampulla of Vater Cancer

No patient had a familiy history of malignant gastrointestinal disease, including familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer syndrome. The 1, 2, 3, 4, and 5 yr cumulative survival rates of 46 patients with ampulla of Vater cancer were 97.8%, 77.9%, 57.0%, 42.2% and 35.2%, respectively, and median and mean survival were 39 and 48.9 months, respectively (Fig. 2).

Among 46 patients of ampullary cancer, 27 (58.7%) were male and 19 (41.3%) were female. There was no significant difference of cumulative survival rate between the group of male and female patients.

Among 46 patients of ampullary cancer, twenty four patients (52.2%) were pertained to pTNM stage II and followed by stage III (15 patients, 32.6%), stage I (5 patients, 7.8%) and stage IV (2 patients, 3.1%). There was no significant difference in the distribution of pTNM stage with reference to the presence or absence of COX-2 immunostaining (Table 1). However, there was significant difference of cumulative survival rate between the groups of pTNM stage (Fig. 3). Subdividing the pTNM staging to pT and pN staging, there were significant differences of cumulative survival rate between the groups of pT and pN stage (Fig. 3).

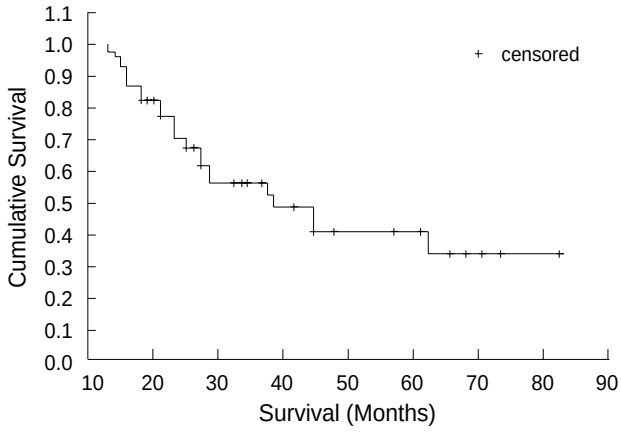

Fig. 2. The cumulative survival rate of 46 patients with ampulla of Vater cancer. The median and mean survival were 39 and 48.9 months, respectively.

Table 1. Comparisons of clinicopathologic features between COX-2 positive and negative group of patients with ampulla of Vater cancer

	COX-2 (+) (n=24)	COX-2 (-) (n=22)	*p*-value
Age (yr) (mean±SD)	60.4±9.5	56.3±7.1	NS
Sex (M/F)	18/6	9/13	0.035
pTNM stage			NS
I	2	3	
II	11	13	
III	10	5	
IV	1	1	
Differentiation			NS
well differentiated	10	13	
moderately differentiated	10	8	
poorly differentiated	4	1	NS
Complete resection (R0/R1)	24/0	22/0	NS
Lymphatic tumor emboli (+/-)	9/15	6/16	NS

NS: not significant.

Concerning the postoperative histopathology, well-differentiated tumor was most frequent (23 patients, 50.0%), followed by moderately differentiated (18 patients, 39.1%) and poorly differentiated tumor (5 patients, 10.9%). There was no significant difference in the distribution of tumor histopathology with reference to the presence or absence of COX-2 immunostaining (Table 1). There was a different tendency of cumulative survival rate between the groups of tumor histopatholgy, however, did not reach the statistical significance (Fig. 3).

In our study, 31 patients (67.4%) of ampullary cancer had lymphatic tumor emboli in postoperative histopathology. There was no significant difference of the presence or absence

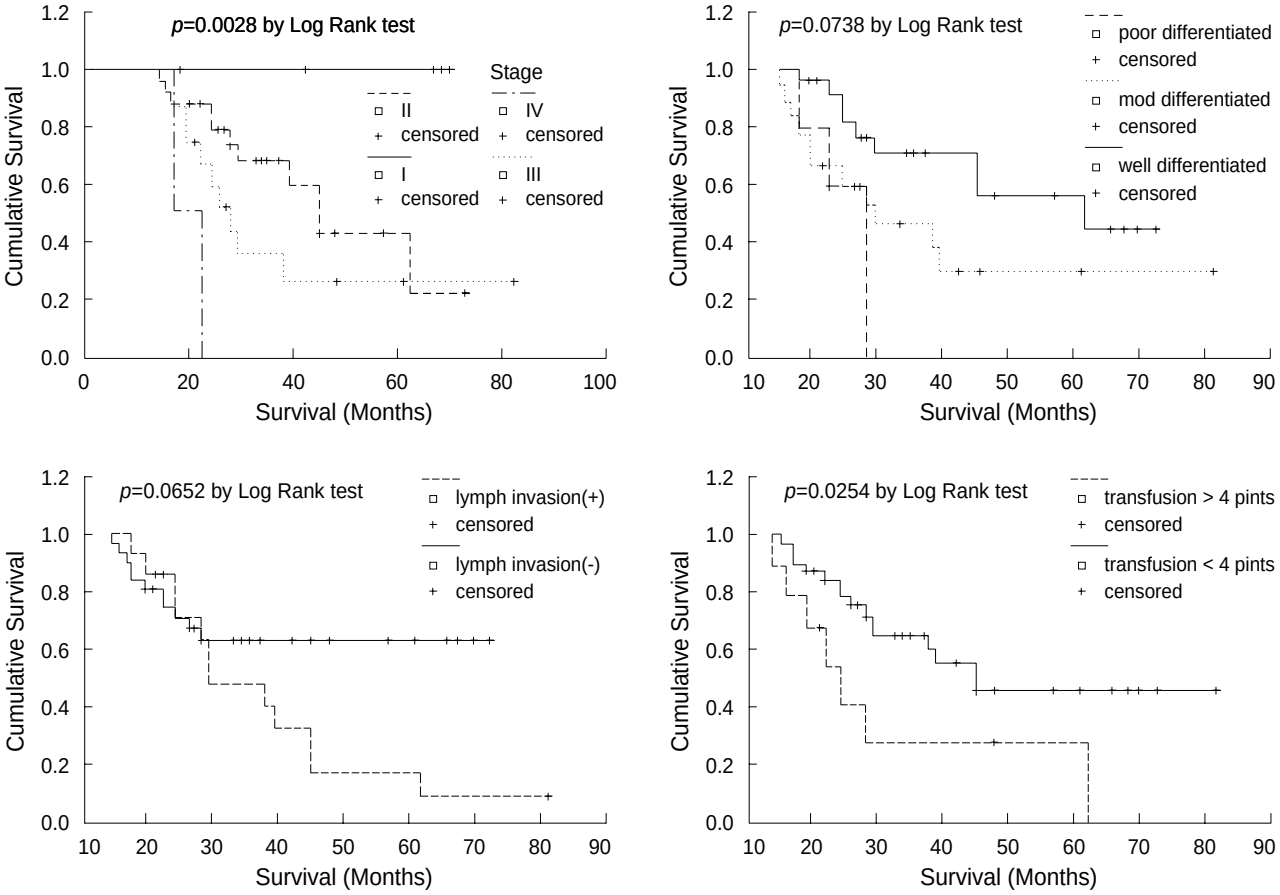

Fig. 3. The comparisons of cumulative survival rate between the groups of pTNM stage, tumor histopathology, lymphatic tumor emboli, and blood transfusion.

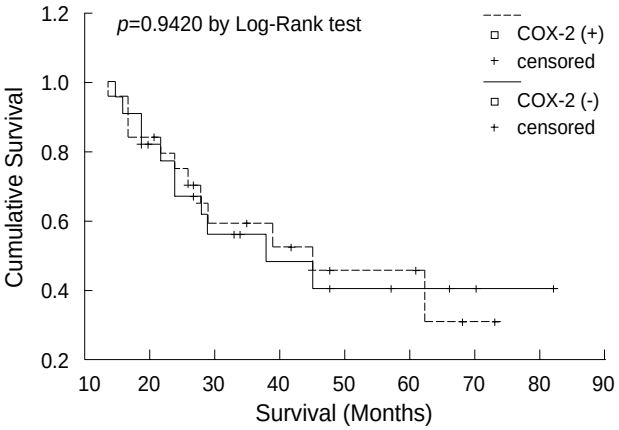

Fig. 4. The comparison of cumulative survival rate between COX-2 positive and negative group of patients with ampulla of Vater cancer.

of lymphatic tumor emboli with reference to the COX-2 immunostaining (Table 1). There was a different tendency of cumulative survival rate between the positive and negative group of lymphatic tumor emboli, however, did not reach the statistical significance (Fig. 3).

Among 46 patients of ampullary caner, 9 patients (19.6%) had more than 4 pints of blood transfusion during the operation. There was significant difference of cumulative survival rate between the group of patients whether they had received more than 4 pints of blood transfusion or not (Fig. 3).

Immunohistochemical Staining of COX-2 and its Correlation with Proliferation Index (PI), Apoptotic Index (AI) and Microvessel Density

In the tissues of ampulla of Vater cancer, we found marked expression of immuno-reactive COX-2 in inflammatory mononuclear cells, vascular endothelial cells and cancer cells (Fig. 1). The cytosolic and perinuclear immunostaining for COX-2 was appeared in 24 (52.2%) patients with ampulla of Vater cancer. There was no significant difference of cumulative survival rate between the COX-2 positive and COX-2 negative group (p=0.9420 by Log Rank test, Fig. 4).

There were no significant differences of PI (p=0.326), AI (p=0.764), and microvessel density (p=0.135) in reference to the presence or absence of COX-2 immunostaining (Fig. 5). Also, there were no impacts on survival for PI, AI, and microvessel density in patients with ampulla of Vater cancer.

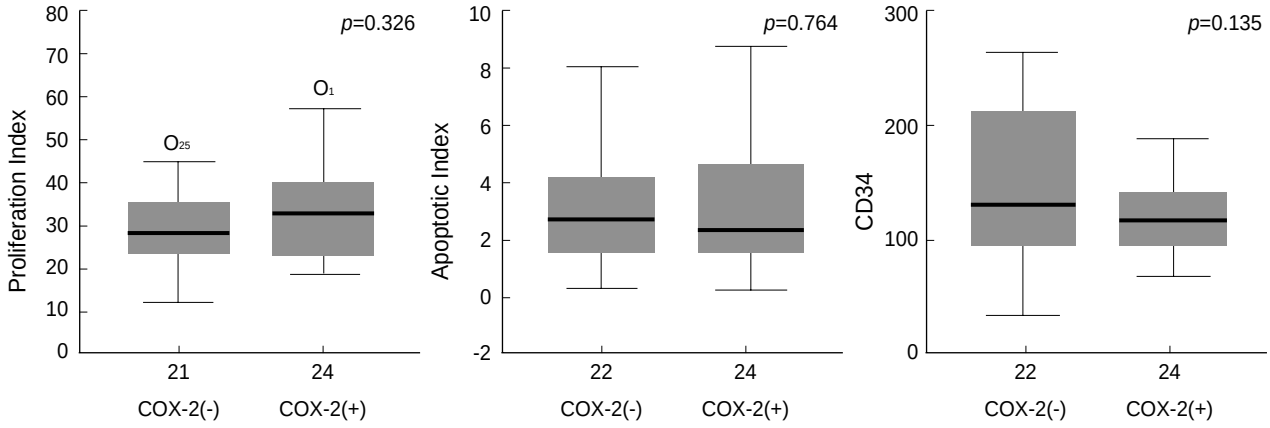

Fig. 5. The comparisons of PI, AI, and microvessel density between the COX-2 positive and negative group of patients with ampulla of Vater cancer. Each data contains median, quartiles and extreme values.

DISCUSSION

COX-2, a key enzyme required for the conversion of arachidonic acid to prostaglandins, plays an important role in the promotion of intestinal tumorigenesis in animal models, but the underlying mechanism of action is still poorly understood. Eberhart et al. (22) have reported that COX-2 expression is clearly up-regulated in the majority of colorectal carcinoma compared with levels of expression in accompanying normal mucosa. More recently, a subset of colorectal adenomas, the precursor lesions in colorectal carcinogenesis, show up-regulation of COX-2 mRNA.

Recently, several epidemiologic studies have suggested that NSAIDs decrease the incidence of colorectal cancer (12, 13). COX and PGs may play a role in this mechanism because the main function of NSAIDs is in the inhibition of COX-1 and COX-2 enzymatic activity. Shiff et al. (26) reported that NSAIDS could inhibit the proliferation, alter morphological appearance, modify the cell cycle phase distribution and induce cell death by apoptosis of HT-29 colon adenocarcinoma cells.

There have been several immunocytochemical studies in localization of COX in the gastrointestinal tracts. The immunolocalization of COX in normal human colon and colorectal caner were reported by Mikkelson et al. (27) and Sano et al. (28), respectively. They detected the expression of immunoreactive COX-2 in colorectal cancer cells, inflammatory mononuclear cells, vascular endothelial cells, and fibroblasts, but, immunoreactive COX-1 was very weak. In normal colonic tissues, immunoreactive COX-1 and -2 were weakly expressed in mucosal epithelial cells and vascular endothelial cells. In our study for ampulla of Vater cancer, the result is similar, and immunoreactive COX-2 staining was mainly localized in cytosolic and perinuclear region of cancer cells.

Kokawa et al. (29) have reported that marked COX-2 expression was observed in 57% of pancreatic ductal adenocarcinomas, 58% of adenomas, and in 70% of adenocarcinomas of intraductal pancreatic mucinous tumors (IPMTs) (38). Likewise, COX-2 expression is known to be increased in human gastric cancers (30) and esophageal cancers (31). Overexpression of COX-2 in malignancies does not, however, seem to be a general phenomenon, since COX-2 protein was not found in human breast carcinoma (32).

In the current study, we found that immunoreactive COX-2 expression was noticed in 52.2% of patients with ampulla of Vater cancer. To our knowledge, there has been no report with a large number of patients with ampulla of Vater cancer focusing on COX-2 expression and its correlation with clinicopathologic features. We found no significant difference in the cumulative survival rate between COX-2 positive and negative group, which was controversial points in previous reports for COX-2 and its relation with survival (33-37). Also, we have correlated the COX-2 expression with proliferative index (PI), apoptotic index (AI), and microvessel density of ampulla of Vater cancer and could not find significant correlation between them. Our results of no correlation between COX-2 and proliferation, apoptosis, and angiogenesis for ampullary cancer were contradictory to the previous report.

Cao et al. (38) have reviewed the many actions of COX-2 in cellular dynamics and in cancer. They indicate that the prostaglandin products of the COX-2 pathway enhance cell proliferation and growth in both normal and tumor cells. Other important actions of COX-2 on cellular process which they reviewed, are inhibition of apoptosis, promotion of tumor cell migration, cell adhesion, tumor invasiveness and role of tumor angiogenesis. So far, it is not certain whether or not the dissension is due to the peculiar biologic properties of ampulla of Vater cancer. Also, it is not certain whether or not the relatively small number of cases included in our study is the probable cause of statistical insignificance. Clinical studies of prospective design with a large number of samples for elucidating the biologic and therapeutic role of COX-2 in ampulla of Vater cancer will be warranted.

In our study, initial pTNM stage and blood transfusion

more than 4 pints during operation were independent prognostic factors for patients with ampulla of Vater cancer. Although lymphatic tumor emboli and tumor differentiation in postoperative histopathology appeared as statistically insignificant prognostic factors, they showed distinctively discrepant tendency of survival.

In conclusion, our study suggests that COX-2 expression in ampulla of Vater cancer is not correlated with the clinicopathologic and biologic features of tumor, such as length of survival, proliferation, apoptosis and angiogenesis of tumor cells.

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