

• COLORECTAL CANCER •

Role of COX-2 in carcinogenesis of colorectal cancer and its relationship with tumor biological characteristics and patients' prognosis

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Abstract

AIM: Recent clinical epidemiological studies have demonstrated the preventive effect of non-steroidal anti-inflammatory drugs (NSAIDs) against colorectal cancer. The underlying mechanism might be the inhibition of rate-limiting enzyme cyclooxygenase-2 (COX-2) in metabolism of arachidonic acid. The role of COX-2 in carcinogenesis of colorectal cancer and its relationship with tumor biological characteristics and patients' prognosis still remain unclear. This study was to investigate the role of COX-2 expression in carcinogenesis of colorectal cancer and its relationship with tumor biological characteristics and patients' prognosis.

METHODS: A total of 139 colorectal cancers and 19 adenomas surgically treated in School of Oncology, Peking University, from January 1993 to September 2001 were retrospectively studied. COX-2 expression was detected with tissue microarray (TMA) and immunohistochemistry (IHC) procedure. The association between COX-2 expression and clinicopathological features and its influence on patients' prognosis were studied.

RESULTS: COX-2 expression was strong in colorectal cancer, moderate in adenoma and weak in normal mucosa, which demonstrated statistically significant difference ($\chi^2=46.997$, $P<0.001$). COX-2 expression had no association with clinicopathological features such as gross type, differentiation, invasion depth, vessel emboli and TNM staging. Cox proportional hazards modeling analysis and Log rank test revealed no prognostic role of COX-2 expression in colorectal cancer patients.

CONCLUSION: COX-2 may play an important role in the early stage of carcinogenesis, and its expression in colorectal cancer is not associated with clinicopathological features and patients' prognosis.

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INTRODUCTION

Cyclooxygenase (COX) is one of the rate-limiting enzymes in metabolism of arachidonic acid that catalyzes the arachidonic acid into a series of products such as prostaglandins and other eicosanoids. It has two isoforms, COX-1 and COX-2. COX-2 acts as both superoxidase and peroxidase that can transform arachidonic acid into PGG₂, then PGH₂. COX-2 is inducibly expressed in many human tissues by cytokines, oncogenes and tumor promoters^[1-3]. Recent clinical epidemiological studies have demonstrated the preventive effect of COX inhibitors^[4-6]. Cellular and animal experimental studies indicated its relevance to tumor invasion, metastasis, cell apoptosis, cell cycle, and body immunity^[7]. Carcinogenesis and development of colorectal cancer are multistep and multistage processes involving cumulative effects of many genes^[8,9]. It would contribute to cancer prevention and treatment to illuminate the role of COX-2 in the carcinogenesis of colorectal cancer.

MATERIALS AND METHODS

Patients and tissues

A total of 170 patients underwent surgical treatment in School of Oncology, Peking University, from January 1993 to September 2001 were retrospectively studied. All the patients including 70 males and 69 females (M:F=1.01:1) with median age 59 years (22-89) received pathological examination. There were 4, 12, 4, 3, 4, 6, 21 and 85 cases of the cancer located in ileocecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon and rectum, respectively. As to the invasion depth, one case was in mucosa, 5 in submucosa, 29 in muscularis propria, 60 in serosa or adventitia, 40 beyond serosa or adventitia or in adjacent tissue. All the patients were followed up till October 2001.

Another 19 cases of colorectal adenomas and 29 normal colorectal tissues concurrently resected were selected for study, including 8 tubular adenomas, 7 villous adenomas and 4 tubulovillous adenomas.

Tissue array preparation

Formalin-fixed and paraffin-embedded tissues were subjected to routine sectioning of 3-5 μ m thickness and HE staining. Two typical tumor spots were chosen under microscopy for each case and marked on the corresponding spot on the tissue block. Then cylindrical tissue columns were punctured with tissue arrayer (Beecher Instruments, USA) in the marked area and transferred to corresponding receiver pore of the prepared block. The tissue array block was then completed according to the predetermined scheme. The block was heated at 40 °C for 15 min and the surface was flattened for subsequent section of 5 μ m thickness^[10].

Immunohistochemical staining

Two step immunohistochemical staining was used for COX-2 detection. Tissue sections were dewaxed in xylene for 30 min, rehydrated through graded alcohol to PBS, then immersed in

3 % hydrogen peroxide at room temperature for 10 min to quench endogenous peroxidase activity. After washed with PBS, the sections were subjected to antigen retrieval in boiling sodium citrate buffer (0.01M, pH 6.0) for 10 min (microwave 450W). After cooled at room temperature, and washed with PBS and distilled water sequentially, 100 μ l of diluted anti-COX-2 murine antibody from Cayman Chemical, USA (1:200) was applied for each section. Then the slides were incubated overnight in a humidified chamber. After washed with PBS, each of the sections was incubated at 37 °C for 45 min with 100 μ l of goat anti-mouse IgG from Zhongshan Biological Corp, Beijing, China. After washed with PBS again, the sections were subjected to sequential 3, 3-diaminobenzidine (DAB Kit, Zhongshan Biological Corp, Beijing, China) for immune complex visualization and then counterstained with haematoxylin for 30 seconds. Formalin-fixed and paraffin-embedded sections of human colon carcinoma with strong staining served as positive control whereas PBS instead of antibody as negative control. The COX-2 staining was independently reviewed by two immunohistochemistry experts. Microscopically, the slides with no staining in negative control and specific dark yellow staining of cytoplasm and nuclear membrane in positive control were eligible for further analysis. Semiquantitative scoring system was adopted according to the staining intensity: 0 for no staining, 1 for weak yellow, 2 for dark yellow and 3 for brown staining with granular distribution. The mean score was used for statistical analysis and the threshold for positivity was 2^[11].

Statistical analysis

All statistical analyses were carried out with SPSS software, 10.0, USA. The relationship between COX-2 expression and categorical variables was compared with χ^2 test or Fisher two-sided exact test. Continuous variables were analyzed with *t* test and *P* < 0.05 was considered significant. Cox proportional hazards model and Log rank test as well as Kaplan-Meier were used for multivariate analysis of prognostic factors and survival estimation^[12].

RESULTS

Localization of COX-2 protein

Immunohistochemical assay demonstrated that COX-2 protein was located in the cytoplasm and nuclear membrane. The staining was weak yellow, dark yellow and brown at a low power field and diffuse or granular staining at a high power field under microscopy (Figures 1A-C).

Expression of COX-2 in colorectal tissues

A weak staining of COX-2 was observed in normal tissue with a positive rate of 24.1 % (7/29). COX-2 expression was relatively stronger in adenoma and the rate of positivity was 57.9 % (11/19) (Figure 1D). COX-2 expression was much stronger in the tumor cells with dark yellow or brown staining with occasional granular distributions. Some sections revealed gradual staining escalation from normal mucosa, adenoma to carcinoma. 84.9 % of the cancer tissues scored not lower than 2 were considered positive, which much higher than those in normal and adenomatous tissues (*P* < 0.01).

Relationship between COX-2 expression and clinicopathological factors of colorectal cancer

The study failed to find the correlation between COX-2 expression in tumor and factors such as age and gender of the patients, tumor location, size, gross type, differentiation, invasion depth, vessel emboli, lymph node metastasis, haematogenous metastasis and TNM staging (Table 1).

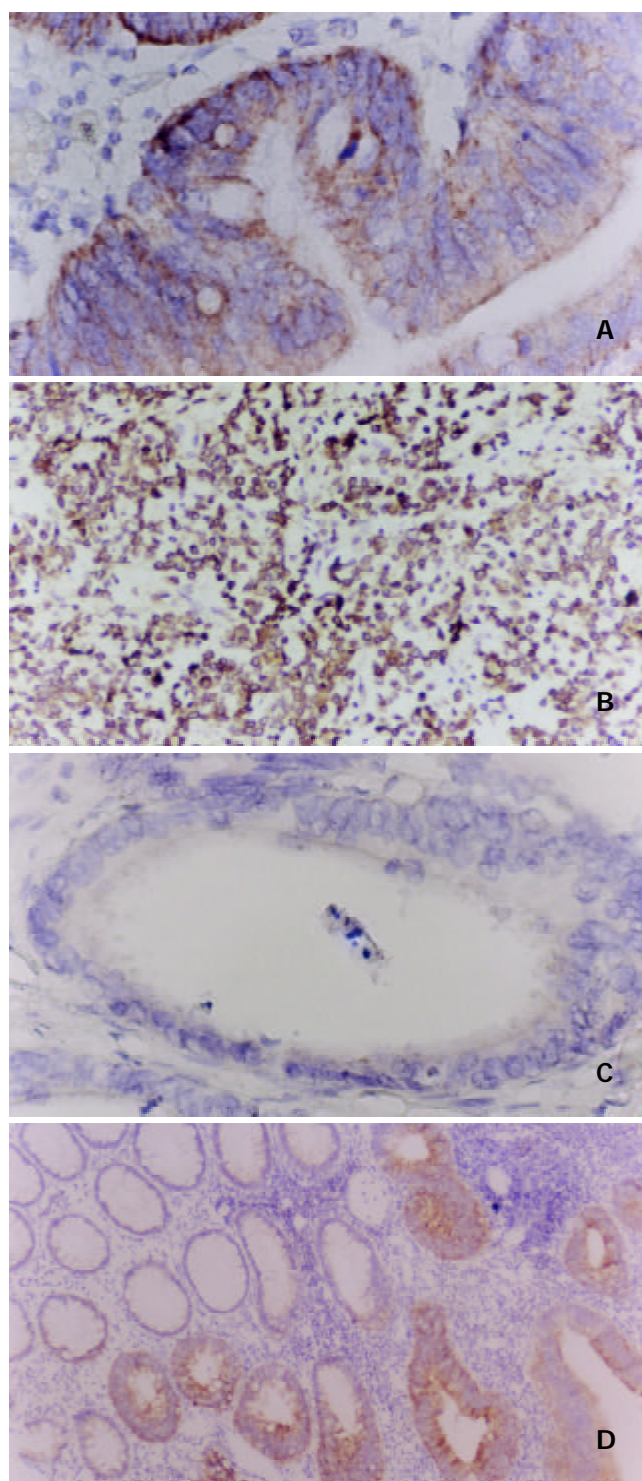


Figure 1 A: Well-differentiated adenocarcinoma of colon, COX-2 immunostaining, $\times 400$; B: Poorly-differentiated adenocarcinoma of colon, COX-2 immunostaining, $\times 400$; C: Negative control, COX-2 immunostaining, $\times 400$; D: Adenoma of colon, COX-2 immunostaining, $\times 200$.

Prognostic factor analysis of colorectal cancer patients

The median follow-up period was 55 months for the whole group (1-106 months). Two cases were missed and the follow-up rate was 98.5 %. Two and five-year survival rates of the whole group were 79.9 % and 61.6 %, respectively.

Eligible prognostic factors such as age and gender of the patients, tumor location, size, gross type, differentiation, invasion depth, vessel emboli, lymph node metastasis, COX-2 expression and TNM staging were introduced into the Cox proportional hazards model and analyzed using the backward:

wald method. The results demonstrated that TNM staging and vessel emboli were independent prognostic factors while COX-2 expression did not affect the prognosis of colorectal cancer patients (Figure 2).

Table 1 Relationship between COX-2 expression and clinicopathological factors in colorectal cancer

Item	Total	COX-2 positive	Positive rate (%)	χ^2 value	P value
Gender				0.557	0.455
Male	70	61	87.1		
Female	69	57	82.6		
Tumor location				0.006	0.939
Colon	54	46	85.1		
Rectum	85	72	84.7		
Gross type				0.330	0.848 ^a
Protruding or fungoid	59	50	84.7		
Ulcerative	76	65	85.5		
Infiltrative	4	3	75.0		
Differentiation				3.917	0.417 ^a
Papillary	3	3	100.0		
Well-differentiated	56	49	87.5		
Moderately-differentiated	52	42	80.8		
Poorly-differentiated	11	8	72.7		
Mucinous/signet cell	17	16	94.1		
Invasion depth				0.188	0.910
Mucosa to muscularis propria	35	29	82.8		
Serosa or adventitia	60	51	85.0		
Extraserosa or extraadventitia	44	38	86.4		
TNM staging				0.985	0.811 ^a
Stage I	32	26	81.3		
Stage II	50	43	86.0		
Stage III	44	37	84.1		
Stage IV	13	12	92.3		
Vessel emboli				0.003	0.955
Yes	39	33	84.6		
No	100	85	85.0		
Lymph node metastasis				0.234	0.628
Yes	53	44	83.0		
No	86	74	86.0		
Haemotogenous metastasis				1.108	0.292
Yes	40	32	80.0		
No	86	75	87.2		
Age	57.2±3.1	57.3±1.2	0.962 ^b		
Maximal diameter (cm)	4.3±0.3	4.6±2.9	0.599 ^b		

^brefers to with *t* test, others with χ^2 test or Fisher two side exact test; ^arefers to that more than 25 % of the theoretical values were less than 5 and no statistical significance was reached even after the combination.

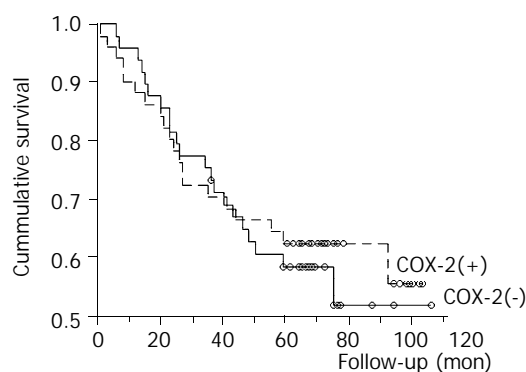


Figure 2 COX-2 expression and patients' survival (Kaplan-Meier curve).

DISCUSSION

Cumulative studies have demonstrated that COX-2 plays an important role in the carcinogenesis and development of many kinds of human cancers such as colorectal cancer^[7,13], gastric cancer^[14,15], lung cancer^[16] and esophageal cancer^[17]. Most of the colorectal cancers are derived from adenomas. Many genes are involved in the course from normal colorectal mucosa, adenoma to cancer such as APC, DCC, p53, etc^[9,18-23]. The rate of COX-2 expression increases with the course of cancer development. As identified in our study, we found that most normal tissues with negative or weak positive staining had a positive rate of 24.1 % (7/29), adenomatous tissues stained stronger had a positive rate of 57.9 % (11/19), while the positive rate in cancer reached 84.9 %. A statistically significant difference existed ($\chi^2=46.997$, $P<0.001$) among the three groups. In addition, some sections revealed a significant trend of gradual staining escalation from normal mucosa, adenoma to carcinoma. The present data indicated that COX-2 might be involved in early carcinogenesis, which was also supported by animal experiments. Oshima *et al*^[24] found that when the COX-2 gene in a familiar adenomatous polyposis mouse model Apc Δ 716 was knocked out, the number of colon polyps decreased and the size reduced significantly. The same inhibitory effect was noted when the specific COX-2 inhibitor was used^[13,25]. As stated in the prospective study on 60 000 people, the relative risk factor for colorectal cancer was reduced to 0.60 when more than 16 tablets of aspirin, a non-specific COX-2 inhibitor, was taken daily^[4]. This conclusion was verified by subsequently prospective randomized trials^[5,6,26,27].

The characteristics of invasion and metastasis are acquired when carcinogenesis of epithelial cells in colon mucosa occurs. Thus cancer cells might invade through the mucosa, submucosa, muscularis propria and even the adventitia to the adjacent tissues. When blood and lymphatic vessels are invaded, metastasis might occur. Invasion and metastasis are also a multistage process^[28] involving many genes including COX-2. Caco-2 cell line, when transfected with COX-2, acquires increased invasiveness. It was found that COX-2 overexpressing Caco-2 cells increased about 6 times in its ability of invasion and had more lateral extension. Furthermore, metalloproteinase-2 was activated and the expression of membrane-type metalloproteinase also increased 2.8 times than the control^[29], indicating the involvement of COX-2 and its metabolites in tumor invasion^[30]. Tomazawa *et al*^[31] injected mouse cell line colon-26 into the tail vein of VALA/C mice with simultaneous administration of JTE-52, a selective COX-2 inhibitor, into the abdominal cavity. The results showed that JTE-52 group with COX-2 overexpression had less lung metastasis than the control, but no difference was found in the group with low COX-2 expression. Therefore, COX-2 expression might be associated with metastasis of colorectal cancer, especially haematogenous metastasis.

Few clinical data are available on the relationship between COX-2 expression and clinicopathological characteristics of colorectal cancer. Thus we carried out this study in 170 colorectal cancer patients surgically treated in our institution with tissue microarray assay and tried to illustrate the relationship between COX-2 expression and clinicopathological factors of colorectal cancer. For certain reasons, only 139 cases could be analyzed and 84.9 % of the cases were positively stained according to our modified criteria of staining intensity, which combined the Masanaga's criteria^[11] with characteristics of tissue microarray technique. Among the 107 cases of advanced colorectal cancer, the expression rate of COX-2 was 86.1 %, 84.1 %, 92.3 % in stages II, III, and IV respectively. Even in stage I, COX-2 expression could reach 81.3 %. The data

indicated that COX-2 expressed high in cancer tissues. Further analysis showed that COX-2 protein expression increased with the invasion depth, its positive rate was 80 %, 82.8 %, 85 % to 86.4 % from submucosa, muscularis, serosa/adventitia to extraserosa/extraadventitia and adjacent tissues respectively, though it did not reach statistical significance ($P=0.910$).

Two pathways for colorectal cancer metastasis are the lymphatic and haemotogenous pathways. Masunaga *et al*^[11] reported that there was a significant difference between the two groups with less or more than 3 metastatic lymph nodes based on their study in 100 patients. However, no statistical difference was found between the two groups with/without or <3 or ≥ 3 lymph nodes metastasis in our study. It was inconsistent with the results of Masunaga *et al*^[11]. One hundred and twenty-two colorectal cancer patients had no haemotogenous metastasis preoperatively. Forty cases of haemotogenous metastasis were found at the end of follow-up, in which 32 COX-2 positive patients had carcinoma (32/107) while 8 out of 19 COX-2 negative patients had haemotogenous metastasis. Still no correlation was found between COX-2 expression and haemotogenous metastasis. Contrary to our results, Tomozawa *et al*^[32] from Japan demonstrated that COX-2 expression was correlated with haemotogenous metastasis especially in the recurrence of colorectal cancer both in clinical studies and in animal experiments. High COX-2 expression group showed a higher rate of haemotogenous metastasis. As an explanation, tumor invasion and metastasis are such a complex course that involves multiple genes. Thus, the role of one single gene should be carefully evaluated.

Few studies are available on COX-2's role as a prognostic factor, and there are still different opinions^[11,31]. Reports from Tomazawa *et al*^[32] indicated that COX-2 expression was the only significant prognostic factor while the traditional TNM staging did not reach statistical significance. Our study introduced 13 eligible prognostic factors such as age and gender of the patients, tumor location, size, gross type, differentiation, invasion depth, vessel emboli, lymph node metastasis, COX-2 expression and TNM staging into the analysis, and found that TNM staging and vessel emboli could serve as independent prognostic factors instead of COX-2 expression. This was consistent to the results from Masunaga *et al* that also demonstrated that prognosis involved many factors and many genes, and expression of one single gene was not sufficient to determine the patients' prognosis.

In conclusion, our study found that COX-2 protein expression increased gradually from normal tissue, adenoma to carcinoma and no association existed between COX-2 expression and clinicopathological factors as well as prognosis of colorectal cancer patients. Therefore, it is concluded that COX-2 may play an important role in the early stage of carcinogenesis. Though involvement of COX-2 has been established, further study is needed to clarify its role in the development of colorectal cancer.

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