• COLORECTAL CANCER •

COX-2 expression and tumor angiogenesis in colorectal cancer

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Abstract

AIM: Cyclooxygenase-2 (COX-2) is one of the rate-limiting enzymes in metabolism of arachidonic acid, and COX-2 inhibitors demonstrate preventive effects on cancer, especially on colorectal cancer. The underlying mechanism remains unclear. The aim of this study was to illustrate the relationship between angiogenesis and COX-2 in carcinogenesis of colorectal cancer.

METHODS: One hundred and seventy patients with colorectal cancer were enrolled in our study from January 1993 to September 2001 in School of Oncology, Peking University. COX-2 and VEGF expression were detected with the immunohistochemistry (IHC) technique. IHC assays were carried out with the aid of tissue microarray (TMA) procedure. Specimens from 35 of these patients were examined with reverse transcriptase PCR (RT-PCR).

RESULTS: COX-2 and VEGF expressions were stronger in colorectal cancer than those in the corresponding normal tissues, at both protein and mRNA levels. One hundred patients were eligible for analysis after IHC assay of COX-2 and VEGF. The positive rate of VEGF was much higher in COX-2 positive group (47/85) than in COX-2 negative group ($\chi^2 = 4.181$, $P = 0.041$). The result was further verified by the result of RT-PCR ($\chi^2 = 8.517$, $P = 0.003$). Correlation coefficient was 0.409 after Spearman correlation analysis ($P = 0.015$).

CONCLUSION: COX-2 may be involved in the course of tumor angiogenesis of colorectal cancer and acts through VEGF.

INTRODUCTION

Cyclooxygenase (COX) is one of the rate-limiting enzymes in metabolism of arachidonic acid, which catalyzes 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 superoxidants and transforms arachidonic acid into PGG$_2$, and then into PGH$_2$. 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 on cancer, especially on colorectal cancer[4-6]. Cellular and animal experimental studies have also indicated its relevance to tumor invasion, metastasis, cell apoptosis, cell cycle, and body immunity[7], and the role of COX-2 in the development of colorectal cancer.

Angiogenesis is among the most important characteristics of tumors and plays an important role in the course of cancer invasion and metastasis. Recent studies have confirmed the hypothesis of tumor growth, which is generally dependent on tumor angiogenesis. Any significant increase in tumor mass must be preceded by an increase in vascular supply to deliver nutrients and oxygen to the tumor[8]. Experimental studies from Seed and Tsujii[9,10] suggested that COX-2 might be involved in angiogenesis. Few reports on the role of COX-2 in tumor angiogenesis of colorectal cancer in clinical settings are available.

MATERIALS AND METHODS

Patients and tissues

A total of 170 patients after surgical treatment in School of Oncology, Peking University, from January 1993 to September 2001 were studied retrospectively. Specimens from these patients were prepared as tissue microarray and then underwent immunohistochemical assays. Specimens from thirty-five patients were prepared for reverse transcriptase PCR (RT-PCR), including 22 male patients and 13 female patients.

Tissue array preparation and immunohistochemical staining

Formalin-fixed and paraffin-embedded tissues were subjected to routine sectioning of 3-5 µm thickness and HE staining. Tissue array block was completed for subsequent sectioning according to the predetermined scheme described before[11]. Two-step immunohistochemical staining was used for COX-2 and VEGF detection. The titer of COX-2 (Cayman Chemical, USA) and VEGF (Zhongshan Biological Inc., Beijing, China) antibody were 1:200 and 1:50 respectively. Human colon adenocarcinoma with strong COX-2 staining served as a positive control, whereas PBS instead of antibody served as a negative control. Two pathologists independently reviewed slides with immunohistochemical staining. Microscopically, the slides with no staining in the negative control and the specific dark yellow staining of cytoplasm and neuclear membrane in the positive control were eligible for a 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 distributions. The mean score was used for statistical analysis and the threshold for positivity was 2[11].

Specimen preparation and RT-PCR

Tumor tissues and the corresponding normal mucosae at least 10 cm away were collected 30 min after removal of the specimens. The tissues were then stored under -70 °C for RNA extraction. Total RNA was prepared from the specimens with Trizol reagent

(Gibco BRL Co.) according to the manufacturer's instructions. cDNA synthesis was carried out with 1μg of total RNA. Sense and antisense primers of 0.5 μL were mixed with 11 μL dd H_2O, 5 μL PCR buffer, 3 μL $MgCl_2$ (25 mmol/L), 2 μL dNTP (10 mmol/L), 2 μL cDNA, and 0.8 μL Taq DNA polymerase for PCR. The primers for PCR were 5' TTC AAA TGA GAT TGT GGG AAA AT 3' (sense primer) and 5' AGA TCA TCT CTG CCT GAG TAT CTT 3' (antisense primer). PCR reactions were processed in a PTC 100 thermocycler under the following conditions: at 94 °C for 5 min, at 94 °C for 30 s, extension at 55 °C for 30 s, then at 72 °C for 30 s for 40 cycles, and then at 72 °C for 5 min. The RT-PCR products were then analyzed on 15 g/L agarose gels. Colon cancer cell line HT-29 and β-actin served as a positive and inner control, respectively. PCR reactions without cDNA were used as blank controls.

Statistical analysis

All statistical analyses were carried out with the 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 statistically significant. Correlation analysis was processed via the Spearman method.

RESULTS

Expression of COX-2 protein in colorectal cancer tissues

Of the 170 colorectal cancer patients, 139 were eligible for analysis after COX-2 immunohistochemical assays. Immunohistochemical assays demonstrated that COX-2 protein was located in 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 a microscope (Figure 1A). A weak staining of COX-2 was observed in normal tissue with a positive rate of 24.1% (7/29). COX-2 expression was much stronger in tumor cells with dark yellow or brown staining with occasional granular distributions than in normal tissues. The positive rate of COX-2 in colorectal cancer was 84.9% (118/139).
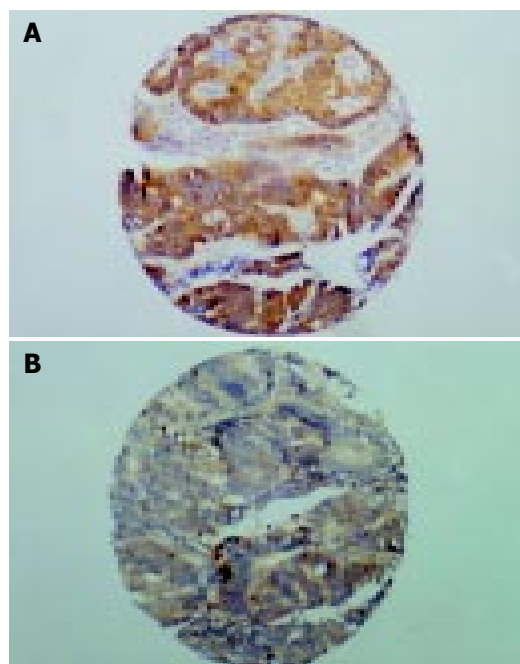

Figure 1 COX-2 and VEGF expressions in colorectal cancer tissues (IHC assay). A: Well-differentiated colonic adenocarcinoma, COX-2 immunostaining, ×100. B: Well-differentiated colonic adenocarcinoma, VEGF immunostaining, ×100.

Expression of VEGF protein in colorectal cancer tissues

For some reasons, only 100 colorectal cancer patients were eligible for further analysis after immunohistochemical assays. VEGF protein was located in cytoplasm of tumor cells and endothelial cells (Figure 1B). About 51.0% (51/100) of the tumors presented with strong VEGF staining, and the rate was much higher than that of normal tissues.

Expression of COX-2 and VEGF mRNA in colorectal cancer tissues

In consistent with the results of IHC assay, RT-PCR revealed stronger expressions of COX-2 and VEGF in tumors than in corresponding normal tissues (Figure 2). Among the 35 paired specimens of colorectal cancer tissues undergoing RT-PCR assays, 27 (77.1%) and 28 (80.0%) were positive with VEGF and COX-2, respectively, compared with 9 (25.7%) and 11 (31.4%) in normal tissues ($P<0.01$).
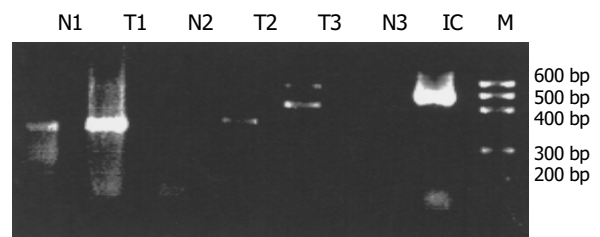

Figure 2 COX-2 and VEGF expressions in colorectal cancer tissues (RT-PCR assay). N: normal T: tumor IC: internal control M: marker. The Arabic number represents the number of samples. The marker has six lanes from 100 bp to 600 bp. The left four lanes stand for the 304 bp products of COX-2 while the right two lanes are the VEGF products of 541 and 408 bp respectively. The internal control of β-actin produced a 496 bp product.

Correlation between COX-2 and VEGF expression in colorectal cancer tissues

For the reason of TMA, a total of 100 cases were eligible for IHC analysis. Among these, 47 COX-2 positive cases presented with a positive VEGF staining (55.3%). The positive rate of VEGF was much higher than that in COX-2 negative group (26.7%). The difference between two groups had a statistical significance ($\chi^2 = 4.181$, $P = 0.041$). Among the 27 cases with COX-2 mRNA expression, 25 had a strong VEGF expression while only 2 in COX-2 negative group ($\chi^2 = 8.517$, $P = 0.003$) (Table 1). COX-2 and VEGF expression demonstrated a tendency to having a positive correlation with correlation coefficient of 0.409 ($P = 0.015$).

Table 1 Expression of COX-2 and VEGF in colorectal cancer tissue (RT-PCR/IHC)[1]

VEGF expression	COX-2 positive	COX-2 negative	Total
Positive	25/47	2/4	27/51
Negative	3/38	5/11	8/49
Total	28/85	7/15	35/100

[1]Fisher double-side exact test ($P = 0.003/0.041$).

DISCUSSION

It has been documented that COX-2 plays an important role in the development of human tumors[12-16]. COX-2 could promote the growth and invasion of tumors[17,18] and increase invasiveness and potential of tumor metastasis[19,20]. Recent research results indicated that COX-2 could also induce tumor angiogenesis and was correlated with hematogenous metastasis of tumors[10,21].

Tumor angiogenesis is one of the important characteristics of tumors involving their growth and metastasis. The formation of primary or metastatic lesions and even their further development are dependent on tumor angiogenesis. Newly-formed vessels were in need of nutrition and oxygen when the tumor grew to 2-3 mm in size[8].

It had been found many factors are involved in tumor angiogenesis including bFGF, aFGF, PGE_1, PGE_2, VEGF, TGFα, TGFβ, and TNF[22]. The most important is vessel endothelial growth factor (VEGF), a highly specific mitogen of vessel endothelial cells. The biological functions of VEGF included selective promotion of mitosis of endothelial cells, stimulation of their proliferation and angiogenesis, an increase in vessel transparency and extravasculization of plasma large molecules[23-26]. It was reported that COX-2 might interact with VEGF[27,28]. Therefore, the relationship of COX-2 and angiogenesis (VEGF as the marker of angiogenesis) was then investigated at protein and mRNA levels.

The positive rate of COX-2 mRNA and COX-2 protein were 80% (28/35) and 84.9% in colorectal cancer. Both were much higher than those in normal tissues ($P<0.01$), which indicated the role of COX-2 in carcinogenesis of colorectal cancer. The expressions of COX-2 and VEGF were studied through RT-PCR and IHC assays. The result of RT-PCR showed that 25 were VEGF positive among the 28 COX-2 positive patients, and only 2 were VEGF positive in the COX-2 negative group. Correspondingly, of the 85 patients with COX-2 positive staining, 47 (55.3%) expressed VEGF while only 26.7% in the COX-2 negative group, indicating the correlationship between the expressions of COX-2 and VEGF, which was further verified by the Spearman correlation analysis. Cianchi[28] recently found a close correlation between COX-2 and VEGF as well as microvessel density (MVD) in their studies on 31 colorectal cancer patients by Western blot and Northern blot analysis, and immunohistochemical assays, which was consistent with our results.

The mechanism underlying the role of COX-2 in tumor angiogenesis has been unclear[29]. Preliminary results showed that angiogenesis occurred when endothelial cells were cultured with cancer cells over-expressing COX-2[10]. It could be inhibited by selective COX-2 inhibitors in endothelial cells *in vitro*[29]. It was proved that the activity of COX-2 in interstitial cells was necessary for secretion of VEGF, proliferation of endothelial cells and new vessel formation[30]. These experimental results suggested that such metabolic products might induce the expression of VEGF via pathways of paracrine, autocrine or intracrine, and stimulate proliferation of vessel endothelial cells and formation of tumor vessels[29].

The regulatory effect of COX-2 on VEGF production and tumor angiogenensis suggested that COX-2 expression might be an upstream event in tumor angiogenesis[10,29]. We also found that COX-2 expression was positively correlated with expression of VEGF, one of the most important factors for tumor angiogenesis. The expression rate of VEGF was high in patients with positive COX-2 expression (25/28) while low in COX-2 negative patients (2/7). In addition, staining intensity of COX-2 was correlated with that of VEGF. Management of the murine animal model of lung and prostate cancers with the gene knockout technique or COX-2 inhibitor resulted in not only tumor shrinkage but also inhibition of tumor angiogenesis. Endothelial cells were scattered without formation of an ordered tubular structure. The MVD of tumor and expression of VEGF were significantly decreased, further suggesting the role of COX-2 on VEGF expression and tumor angiogenesis[31,32].

Great progresses have been made in the research of tumor angiogenesis. Trials on targeted therapy against angiogenesis have been ongoing to block or postpone tumor angiogenesis so as to cure cancer[33,34]. More than twenty drugs are in their phase I or II and even III clinical trials in the United States of America. Though preliminary results were promising, there is still a long way to go. Therefore, as an upper-stream event, COX-2 might be a potential target of the cancer gene therapy worthy of further research.

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