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Original Article

Expression and significance of cyclooxygenase-2 in colorectal cancer and the colorectal adenomas tissue

Xiaofeng Li[1*], Lixia Kong[1*], Suhuan Liao[1], Jing Lu[1], Lin Ma[2], Xiaohua Long[1]

Departments of [1]Gastroenterology, [2]Oncology, The Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai 519000, China. [*]Co-first authors.

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Abstract: Objective: This study aims to investigate the expression and significance of cyclooxygenase-2 (COX-2) in colorectal cancer and colorectal adenomas tissues. Methods: Fresh tissue samples were collected from the Fifth Affiliated Hospital of Sun Yat-sen University, and divided into three group: colorectal cancer tissues (n = 43), colorectal adenomas tissues (n = 84) and normal tissues (n = 18). The mRNA expression of COX-2 was examined with reverse transcriptase polymerase chain reaction (RT-PCR), and the expression of COX-2 protein was examined with immunohistochemistry. The relationship between COX-2 expression and clinical pathological features were evaluated, compared the difference of the two methods too. Results: The positive rate of COX-2 mRNA in colorectal cancer was 80.0% (32/40), which was significantly higher than colorectal adenomas 59.0% (46/78), and normal tissues 11.1% (2/18), difference being significant between these three groups (χ^2 = 24.324, P = 0.000). The positive rate of COX-2 protein in colorectal cancer was 79.1% (34/43), which was significantly higher than colorectal adenomas 57.1% (48/84), and normal tissues 0.00% (0/18), difference being significant between these three groups (χ^2 = 32.314, P = 0.000). There were no significant association between COX-2 expression and various clinical pathological features of colorectal cancer and colorectal adenomas (P > 0.05). It showed good consistency between RT-PCR and immunohistochemical method to detect the expression of COX-2 (P > 0.05). Conclusion: COX-2 positive rate is higher in colorectal adenomas and colorectal cancer tissues, and COX-2 plays an important role in the development of colorectal adenomas to colorectal cancer.

Keywords: Cyclooxygenase-2, colorectal cancer, colorectal adenomas

Introduction

Cyclooxygenase (COX), also call prostaglandin peroxide synthetase, is a key regulatory enzyme in the synthesis of prostaglandins that catalyzes the arachidonic acid into various products. It has been confirmed that there are at least two kinds of isoenzymes [1], COX-1 and COX-2, COX-1 plays an important role in regulates normal physiological function. However, COX-2 is generally undetectable under physiological conditions, but it can be induced by a variety of factors [1, 2] such as growth factors, cytokines, carcinogens, oncogenes and nitric oxide. Up-regulated expression of COX-2 has been found in many human benign precancerous lesions and malignant tumor (eg, colonic polyp, Barrett oesophagitis, intestinal metaplasia, adenomas, colorectal cancer, gastric cancer, liver cancer, pancreatic cancer, etc) [3, 4]. In recent years, various studies found that COX-2 plays an important role in the development of colorectal cancer, and clinical epidemiology demonstrates that COX-2 inhibitors have the preventive effect in cancer, especially colon cancer [5-7]. Lobo et al. [8] study indicated that COX-2 was found to be expressed in 93% of colon cancers and 87% of rectal cancers by immunohistochemistry. Zhang and Sun [9] found that the expression level of COX-2 was up-regulated from normal cells to primary tumors and to metastases tissue in turn, and related to proliferative activity, tumor location, Dukes' stage, and differentiation. Various studies examined the relationship between COX-2 expression with the clinical outcome in patients with colorectal cancer, but yielded conflicting results. Elzagheid et al. [4] reported that high levels of COX-2 expression were associated with higher TNM class, and higher Dukes' stage, in contrast, there was no significant correlation with age, gender, tumor grade or lymph node

Table 1. Demographic and clinic feature between colorectal cancer and colorectal adenomas

Group		Colorectal cancer	Colorectal adenomas	P (χ^2)
Gender	Male	25	50	0.864 (0.029)
	Female	15	28	
Age	≥ 50	28	47	0.298 (1.084)
	< 50	12	31	
Location	colon	30	47	0.111 (2.535)
	rectum	10	31	
Tumor size	≥ 5 cm	16	33	0.810 (0.058)
	< 5 cm	24	45	

Fourfold continuity correction chi-square test, no statistical difference ($P > 0.05$).

status. But some studies indicated that the level of COX-2 in colorectal cancer correlated with stage, lymph node metastasis [10]. The results of the studies are inconclusive and no consensus has been reached among COX-2 with clinical pathological features, which need further study to confirm it. Most of the research adopted the method of immunohistochemical and less by RT-PCR to detect the levels of COX-2 in tissues. So we employed reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemical technology to detect the expression level of COX-2 mRNA and protein in colorectal cancer, colorectal adenomas and normal tissues, and compared the differences of the two methods. We also analyzed the relationships between the levels of COX-2 with clinical pathological features.

Materials and methods

Subjects and sample

A total of 145 fresh tissue samples (2 × 2 × 1 cm) were collected from consecutive patients at the Fifth Affiliated Hospital of Sun Yat-sen University between April 2009 and April 2010. The subjects were divided into colorectal cancers (n = 43), colorectal adenomas (n = 84) and normal tissues (n = 18). There were 43 patients with colorectal cancer (40 cases were extract the RNA) and confirmed by pathology after surgery without radiation and chemotherapy and other adjuvant treatment before operative, the average age of the 40 patients was 59.6 years (range 32-86), including twenty-five males and fifteen females; the patients consisted of 30 cases of colon cancer and 10 cases of rectum cancer; there were 16 cases of higher than 5 cm and 24 cases of less than 5 cm according to the size. According to the level of tumor: 14 cases of high level (5 cases of moderately-low differentiated adenocarcinoma, 4 cases of poorly differentiated adenocarcinoma, 5 cases of mucus gland carcinoma), 26 cases of low level (16 cases of high-differentiation adenocarcinoma, and 10 cases of moderately differentiated adenocarcinoma); there were 22 cases of negative metastasis and 18 cases of positive metastasis; there were 32 cases of no distant metastasis and 8 cases of distant metastasis; according to Dukes staging, there were 4 cases, 11 cases, 17 cases, 8 cases in stage A, B, C, D respectively. 84 cases of colorectal adenomas specimens (78 cases were extracted RNA) were collected in colonoscopy room which were confirmed by pathology after polypectomy. The average age of the 78 patients were 53.8 years (range 28-81), including fifty males and twenty-eight females; there were 47 cases of more than 50 years and 31 cases of less than 50 years; there were 47 cases of colon adenomas and 31 cases of rectum adenomas; 44 cases of tubular adenoma, 14 cases villous adenoma and 20 cases of villous tubular adenoma. 18 patients cases of irritable bowel syndrome and colorectal inflammatory as normal control group, they were no obvious pathological changes through electronic colonoscopy biopsy (excluding colorectal cancer and colorectal polyps).

There were no significant different between colorectal cancer and colorectal adenomas in demographic and clinic feature, including age, gender, tumor location, and tumor size ($P > 0.05$, **Table 1**).

The samples were stored into the liquid nitrogen in -80°C refrigerator immediately after collection from the patients, part for total RNA extraction, another part for immunohistochemical staining. This study was conducted with approval from the Ethics Committee of Sun Yat-sen University. Written informed consent was obtained from all participants.

RT-PCR

Total RNA were extracted from colorectal cancer, colorectal adenomas and normal tissues by Trizol reagent (Takala, Dalian, China) follow-

Table 2. Positive rate of COX-2 in colorectal cancer and colorectal adenomas

Clinical index	RT-PCR			P (χ^2)	Immunohistochemical			P (χ^2)
	(+)	(-)	Positive rate (%)		(+)	(-)	Positive rate (%)	
Colorectal cancer	32	8	80.0	0.000 (24.324)	34	9	79.1	0.000 (32.314)
Colorectal adenomas	46	32	58.9		48	36	57.1	
Normal tissue	2	16	11.1		0	18	0.0	

Notes: RXC the chi-square test, difference were statistically significant ($P < 0.05$). COX-2 mRNA in colorectal cancer compare to colorectal adenoma: $\chi_1^2 = 5.30$, $P_1 = 0.025$; COX-2 mRNA in colorectal cancer compare to normal tissues: $\chi_2^2 = 9.14$, $P_2 = 0.000$; COX-2 mRNA in colorectal adenoma compare to normal tissues: $\chi^2 = 6.89$, $P = 0.0016$; COX-2 protein in colorectal cancer compare to colorectal adenoma: $\chi^2 = 5.977$, $P = 0.018$.

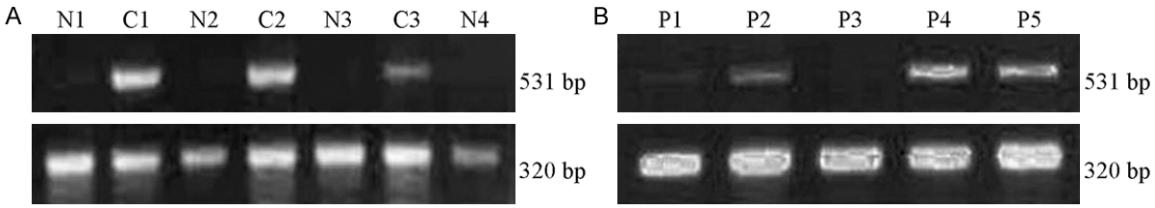

Figure 1. Expression of COX-2 mRNA in colorectal cancer, colorectal adenomas and normal tissue detected by RT-PCR. A: Colorectal cancer tissues and normal tissues; B: Colorectal adenomas tissues; C: Colorectal cancer; N: Normal tissue; P: Colorectal adenomas. Bp: The amplified fragments of COX-2 were 531 bp, and the amplified fragments of β-actin were 320 bp.

ing the manufactuters' instructions, and its content and purity were measured by ultraviolet spectrophotometry. 1.0 ug RNA was reverse transcribed in cDNA by Avian Myeloblastosis Virus (AMV) Reverse Transcriptase Kit (Takala, Dalian, China). cDNA was used as the template in PCR amplification with primers for COX-2 (Forward: 5'-CCACCTCTGCGATGCTCTTC-3', and Reverse: 5'-ACATTCCCCACGGTTTTGAC-3') and β-actin (Sangon Bitotech, Shanghai, China). The PCR cycle consisted of the following steps: denaturing at 94°C for 5 min, than denaturing at 94°C for 30 s, annealing at 55°C for 45 s and elongation at 72°C for 45 s, which was repeated for 35 cycles. Finally, the results were interpreted by agarose electrophoresis analysis.

Immunohistochemical staining

Immunohistochemical staining was employed Streptomyces avidin-peroxide enzyme (S-P method) (Maxim, Fuzhou, China) following the manufactuters' instructions. Randomly selected 10 vision (400 ×) at high magnification, count of positive cells in 500 cells, calculate the percentage of positive cells (reference the Labile's calculation: [11] 1 = 1-10% of cells; 2 = 11-50% of cells; 3 = 51-80% of cells, and 4 = 81-100% of cells). Staining intensity was scored as 0 = negative, 1 = weak, 2 = medium, and 3 = strong. The sum of the intensity and extent of the score was used as the final staining score. A score of 0 (-) was considered negative, 1-4 (+) was considered weak, 5-8 (++) was considered moderate, and 9-12 (+++) was considered strong expression.

Statistical analysis

Statistical analysis were performed with SPSS-19.0 software, and qualitative data was described by frequency and rate; the comparison between groups of qualitative data was made using the χ^2 test and χ^2 test with Yates' continuity correction, $P < 0.05$ was considered significant.

Results

The positive rate of COX-2 in colorectal cancer, colorectal adenomas and normal tissues between the two methods

The positive rate of COX-2 mRNA in normal tissue, colorectal adenomas and colorectal cancer, was 11.1% (2/18), 59.0% (46/78) and 80% (32/40) respectively, which showed an increas-

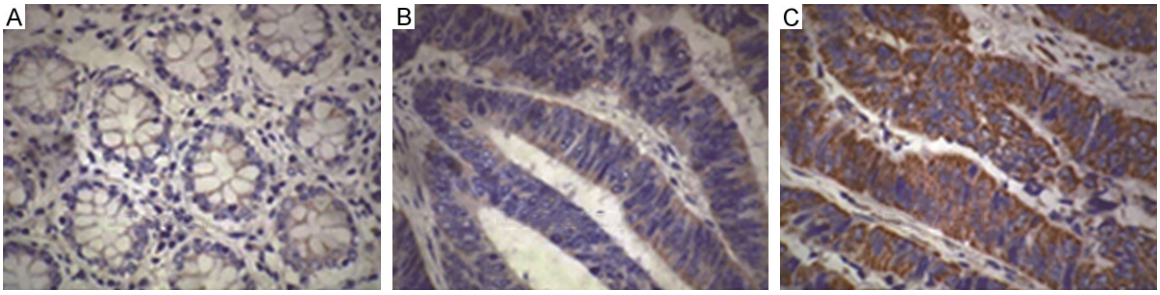

Figure 2. The staining results of COX-2. A: In normal tissues: the staining was no yellow in cytoplasm; B: In colorectal adenomas tissues: the staining was yellow in cytoplasm; C: In colorectal cancer tissues: the staining was dark in cytoplasm.

Table 3. Relationship between the positive rate of COX-2 mRNA by RT-PCR and clinical pathological parameters of colorectal cancer

Clinical pathological parameters	Case	COX-2 mRNA		Positive rate (%)	P (χ^2)
		(-)	(+)		
Differentiation					
Well	14	4	10	71.4	0.320 (0.989)
Poor	26	4	22	84.6	
Tumor size					
≥ 5 cm	16	5	11	68.8	0.146 (2.109)
< 5 cm	24	3	21	87.5	
Dukes stages					
A+B	15	2	13	86.7	0.414 (0.667)
C+D	25	6	19	76.0	
Lymphatic metastasis					
Positive	18	5	13	72.2	0.266 (1.237)
Negative	22	3	19	86.4	
Distant metastasis					
Positive	8	2	6	75	0.930 (0.156)
Negative	32	6	26	81.3	

Fourfold continuity correction chi-square test or RXC the chi-square test, no statistical difference ($P > 0.05$).

ing trend among them, difference are statistically significant ($\chi^2 = 24.324$, $P = 0.000$, **Table 2**). The positive rate of COX-2 mRNA in colorectal cancer were significantly higher than colorectal adenoma and normal tissues, difference being statistically significant ($\chi_1^2 = 5.30$, $P_1 = 0.025$; $\chi_2^2 = 9.14$, $P_2 = 0.000$). The positive rate of COX-2 mRNA in colorectal adenoma was higher than normal tissues too ($\chi^2 = 6.89$, P = 0.016; **Table 2**; **Figure 1**).

The positive rate of COX-2 protein in colorectal cancer, colorectal adenomas and normal mucous was 79.1% (34/43), 57.1% (48/84) and 0% (0/18) respectively (**Table 2**). COX-2 protein were high express in colorectal cancer and colorectal adenomas, the difference being significant between the two groups ($\chi^2 = 5.977$, $P = 0.018$) (**Table 2**; **Figure 2**).

Relationship between COX-2 expression and clinical pathological factors

There were no significant association between COX-2 expression in colorectal cancer tissue with clinical pathological factors, including differentiation, tumor size, Dukes stage and distant metastasis ($P > 0.05$, **Table 3**). There were also no significant association between COX-2 expression in colorectal adenomas with clinical pathological factors, like age, gender, tumor location, and histological type ($P > 0.05$, **Table 4**).

Differences expression by two detect methods

The positive rate of COX-2 detected by RT-PCR and immunohistochemical have good consistency, there were 80.0% and 79.1% in colorectal cancer, 59.0% and 57.1% in colorectal adenomas, and 11.1% and 0% in normal tissue respectively (**Table 1**), there were no statistically difference ($P = 0.916$, $P = 0.056$, **Table 5**).

Discussion

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and fourth most common cause of cancer-related mortality in the United States. Colorectal cancer is curable in early stages, but mortality is very high when be-

Table 4. Relationship between the positive rate of COX-2 mRNA by RT-PCR and clinical pathological parameters of colorectal adenomas

Clinical pathological parameters	Case	COX-2 mRNA		Positive rate (%)	P (χ^2)
		(-)	(+)		
Gender					
Male	50	24	26	52.0	0.094 (2.800)
Female	28	8	20	71.4	
Age					
≥ 50	47	22	25	53.2	0.201 (1.635)
< 50	31	10	21	66.7	
Location					
Colon	47	20	27	57.4	0.735 (0.114)
Rectum	31	12	19	61.3	
Histological types					
Tubular adenoma	44	23	21	47.7	0.063 (5.528)
Villous adenoma	14	3	11	78.6	
Tubulovillous adenoma	20	6	14	70.0	

Fourfold continuity correction chi-square test, no statistical difference ($P > 0.05$).

come metastatic. Therefore, increasing efforts are being focused on developing more effective screening and prevention measures for colorectal cancer. There were a number of studies have shown that COX-2 is involved in the development of a variety of tumors at home and abroad (such as colorectal cancer, gastric cancer, breast cancer, lung cancer, esophageal cancer, liver cancer and bladder cancer) [12-15]. Regular intake of OTC NSAIDs produced highly significant composite risk reductions of 43% for colon cancer, 25% for breast cancer, 28% for lung cancer, and 27% for prostate cancer [16]. The views of COX-2 involved in the development of tumors are as follow: 1, The catalytic product of COX-2-prostaglandin can promote the proliferation of tumor cells. 2, Influence the expression of oncogenes or tumor suppressor genes. Chiou et al. [17] research showed that destroy the gene of COX-2 or handle ACF mutant mice with COX-2 inhibitors, the number of the colon polyps were decrease. 3, Associated with cell apoptosis by changing the expression of some apoptosis-related gene, and lead to unlimited proliferation of tumor cells. Lewis et al. [18] study found that prostaglandin can increase the concentration of intracellular cAMP, sequentially inhibit cell apoptosis by increasing the expression of apoptosis suppressor genes of Bcl-2. 4, Associated with tumor angiogenesis. Du et al. [19] study showed that COX-2 were mainly express in newborn vascular endothelial cells, and consistent with the expression of endothelial growth factor (VEGF), the expression level of COX-2 is associated with the degree of tumor malignant, which indicated that COX-2 can promote tumor angiogenesis through affect the expression of VEGF. 5, Associated with the invasion and metastasis of tumor, Wu et al. [20] study proved this point. 6, Peng et al. [21] studied the hereditary non polyp colorectal cancer (HNPCC), also found that the expression of COX-2 were associated with mismatch repair (MMR) protein gene and microsatellite instability. 7, Associated with immunoregulation. Lee et al. [22] studies have shown that prostaglandin E2 can inhibit antitumor immune response which mediated by T and B lymphocyte, thus inhibits the activity of NK cells. PGE2 can also inhibit TNF production to reduce the body's immune surveillance and cell function. However, the exact mechanism of COX-2 with colorectal cancer is unclear.

In an effort to better understand the role of COX-2 in colorectal cancer. We used RT-PCR and immunohistochemical to assess the expression of COX-2 in colorectal cancer, colorectal adenomas tissues and normal tissues. The positive rate of COX-2 mRNA was 32/40 (80.0%), 46/78 (58.9) and 2/18 (11.1%), which suggest that COX-2 were high express in both colorectal cancer and adenomas tissues, and the level of COX-2 in colorectal cancer was obviously higher than that of adenoma and normal tissues, difference being significant between these three groups ($P < 0.05$). It has been reported that COX-2 is over expressed in 70-90% of colorectal cancer [23], our results were in agreement with the universally accepted and consistent with DuBois et al. [24] result, who also thought that the level of COX-2 mRNA in colorectal cancer was significantly higher than adenoma and normal mucosa. But the positive rate of COX-2 in colorectal adenomas (59.0%) was higher than the previous research, it may be related to hyperplasia and carcinogenesis of our adenoma tissue. The positive rate of

Table 5. Comparison of the positive rate of COX-2 between the two methods

Group	Colorectal cancer		P (χ^2)	Colorectal adenomas		P (χ^2)
	Positive	Negative		Positive	Negative	
Immunohistochemistry	34	9	0.916 (0.011)	48	36	0.813 (0.056)
RT-PCR	32	8		46	32	

Fourfold continuity correction chi-square test, no statistically significant difference ($P > 0.05$).

COX-2 in colorectal cancer and colorectal adenomas were 80% and 59.0%, difference being significant between them ($P < 0.05$), which further prompt that COX-2 plays an important role in the early stages of colorectal cancer development (adenoma to adenocarcinoma).

We found that there were no significant correlation between the level of COX-2 and clinical pathological parameters in colorectal cancer ($P > 0.05$), including differentiation, lymphatic and distant metastasis, Dukes staging etc, which were consistent with Yamac et al. [25] study. They found that there was no obvious correlation between COX-2 expression with age, sex, tumor location, differentiation, lymphatic and distant metastasis, Dukes stage, tumor size, tumor infiltration depth in colorectal adenocarcinoma. Suggesting that the expression of COX-2 is a universal event in the stage of adenocarcinoma. But there were also contrast results, Zhang and Sun [9] found that the expression of COX-2 were associated with tumor location, Dukes staging and differentiation. Sheehan et al. [26] found that high expression of COX-2 were associated with the Dukes stage, tumor size and lymph metastasis in the study of 76 cases of colorectal cancer patients. Tomozawa et al. [27] studied the patients of colorectal cancer, and found that there were no correlation between the expression of COX-2 and clinical pathological parameters (including Dukes' stage, venous and lymphatic invasion, lymph metastasis, tumor size and infiltration depth), but COX-2 was obviously associated with tumor recurrence, especially the hemorrhagic transfer, so they considered COX-2 not only involved in the development of tumor, but also participated the hemorrhagic tumor metastasis. As summing above, the relationship between COX-2 with clinical pathological parameters is still divided in colorectal cancer. The results of our research were inconsistent with Hong, the reason may be our sample is not big enough, or there were less sample in the early stages, or COX-2 itself was not associated with clinical pathological parameters of colorectal cancer. Further study is needed to confirm it.

We also found that the level of COX-2 was not obvious correlation with gender, age, location and histology in colorectal adenomas patients. But some studies have shown that the expression of COX-2 in colorectal adenomas were associated with clinical pathological parameters. In a study of 175 cases of sporadic colorectal adenomas by Wasilewicz et al. [3] found that the level of COX-2 were positively correlated with adenomatous hyperplasia, size, growth areas, but with the total number of adenomas, age, gender showed no significant correlation. Benamouzig et al. [28] found that the level of COX-2 in colorectal adenomas was related to the size of adenoma, organization type and degree of hyperplasia. Currently, the relationship between COX-2 expression and clinical pathological parameters in colorectal adenomas is still controversial, the reason may relate to sample size, testing methods and test reagents, the evaluation criteria and so on.

In our study, the positive rate of COX-2 is basically consistent between immunohistochemistry and RT-PCR, but higher in RT-PCR. The reason may be relate to COX-2 are expressed in interstitial. Zhan et al. [29] research suggests that COX-2 can be visible in interstitial macrophages (our study is defined as negative by immunohistochemical), and the process of extract fresh tissue is likely to sneak into the RNA of interstitial cells. In the aspects of statistical differences of COX-2 expression between colorectal cancer and colorectal adenomas, our study showed consistent results by the two methods too. Compared to RT-PCR, immunohistochemical is simpler.

In conclusion, our study showed that COX-2 is linked to the development of colorectal cancer, and the high expression of COX-2 of colorectal adenomas and colorectal cancer tissues is a common event. RT-PCR and immunohistochemical method showed good consistency

in detect COX-2 in colorectal adenomas and colorectal cancer. We hope our study can provide a new insight to explore this field in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lin Ma, Department of Oncology, The Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai 519000, China. Tel: +86 756 2528845; Fax: +86 756 2528845; E-mail: linmacn@163.com

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