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Expression of Cyclooxygenase-2 and its Relationship to p53 Accumulation in Colorectal Cancers

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Purpose: Cyclooxygenase (COX)-2 is an inducible isoform responsive to cytokines, mitogens, and growth factors, and is believed to be an important enzyme related to colorectal cancer (CRC). Existing evidence suggests that COX-2 expression is normally suppressed by wild-type p53 but not mutant p53, suggesting that loss of p53 function may result in the induction of COX-2 expression. The aim of this study was to determine the relationship between COX-2 expression and p53 levels in CRC. **Materials and Methods:** Patients with sporadic colorectal adenocarcinoma (n = 161) who underwent curative surgery in Chosun University Hospital were enrolled in this study. Expression of COX-2 and p53 proteins was examined by immunohistochemistry in paraffin-embedded cancer tissue blocks, and the relationship between COX-2 and/or p53 expression with clinicopathologic parameters was analyzed. **Results:** Expression of COX-2 was positive in 47.8% of colorectal cancers, and significantly associated with the depth of tumor invasion (p = 0.042). In contrast, p53 was positive in 50.3% of the cases, and was associated with both age (p = 0.025) and the depth of tumor invasion (p = 0.014). There was no correlation between COX-2 expression and p53 expression (p = 0.118). **Conclusion:** These results suggest that COX-2 expression might play an important role in the progression of colorectal cancer. However, COX-2 expression was not associated with mutational p53. Further studies are needed to clarify the regulatory mechanisms governing COX-2 overexpression in colorectal cancers.

Key Words: Cyclooxygenase-2, p53, colorectal cancer, immunohistochemistry

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INTRODUCTION

Colorectal cancer is among the most common malignant diseases worldwide.[1] In Korea, it is the fourth most common cancer, and its incidence has increased steeply in recent decades.[2] Although the adenoma-carcinoma sequence in the pathogenesis of colorectal cancer is well established,[3] the etiology of this cancer remains poorly understood.

In recent years, much attention has been focused on the involvement of cyclooxygenases (COX) in tumor development and progression. COX is the rate-limiting enzyme in the biosynthesis of prostaglandin from arachidonic acid, and two isoforms have been characterized, COX-1 and COX-2. COX-1 is constitutively expressed in a wide variety of tissues, where it serves a homeostatic function. In contrast, COX-2 is an inducible enzyme that is up-regulated in response to various stimuli, including cytokines, growth factors, and tumor promoters; its pathophysiologic role has been connected to inflammation, wound healing, and carcinogenesis.[4-6] Enhanced COX-2 expression has been found in many tumors, including lung, breast, esophageal, gastric, and colorectal cancers. An association between overexpression of COX-2 in colorectal cancer has been suggested with tumor growth, angiogenesis, lymphatic invasion, and metastasis.[6]

Although COX-2 is highly and constitutively expressed in colorectal cancer and may have many important roles in the pathogenesis of colorectal cancer, the mechanisms for regulation of COX-2 expression remain unclear. Subbaramaiah et al. reported that expression of COX-2 was repressed by wild-type p53 in an *in vitro*

study.[7]

In this work, our goal was to examine COX-2 expression and its association with p53 accumulation in clinical colorectal cancer tissues. Both COX-2 and p53 protein expression in colorectal cancers were investigated immunohistochemically, and the association between COX-2 expression and p53 accumulation was analyzed in the context of various clinicopathologic parameters.

MATERIALS AND METHODS

Patients and tissue samples

One hundred sixty-one patients who had undergone curative surgical resection for primary sporadic colorectal carcinoma at the Department of Surgery, Chosun University Hospital (Gwangju, Korea) between March 2002 and February 2005 were enrolled in this study. This group consisted of 69 cases of colon cancer and 92 cases of rectal cancer, with 83 males and 78 females, and a mean age of 61.54 ± 11.86 years. Exclusion criteria were as follows: a) patients who received preoperative chemoradiotherapy; b) those who had undergone emergency surgery; and c) those with evidence of hereditary non-polyposis colorectal cancer or of familial adenomatous polyposis. Samples were graded by a pathologist according to the pathological features of the tumors, which included histological grading, lymph node metastasis, distant metastasis, and tumor staging (AJCC TNM classification). In all cases, archival H&E slides of the primary tumors were retrieved and reviewed to confirm pathological features and to select suitable tissue blocks for immunohistochemical analysis.

Immunohistochemistry

The universal immunoenzyme polymer method was used for immunostaining. Four- μm thick sections were cut from formalin-fixed, paraffin- embedded tissue blocks, mounted onto poly-lysine-coated slides, dewaxed in xylene, and rehydrated through a graded series of ethanol washes. After deparaffinization, antigen retrieval treatment was performed at 121℃ (autoclave) for 15 minutes in 10 mmol/L sodium citrate buffer (pH 6.0), followed by treatment with 3% hydrogen peroxide in methanol solution for 20 minutes to quench endogenous peroxidase activity. Nonspecific binding was blocked by treating slides with 10% normal goat serum for 10 minutes. To block intrinsic avidin-biotin capabilities, the tissue slides were treated with avidin-biotin blocking kit reagents (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) for 15 minutes. The primary antibodies used were Anti-COX-2 mouse monoclonal antibody (Cayman Chemical Co., Ann Arbor, MI, USA) and anti-p53 mouse monoclonal antibody DO-7 (DAKO, Glostrup, Denmark), with working dilutions of 1:300 for anti-COX-2 and 1:100 for anti-p53. The final products were visualized using the 3-amino-9-ethylcarbazole (AEC) substrate system (DAKO, Glostrup, Denmark). Sections were counterstained with Mayer's hematoxylin for 20 sec before mounting. As a negative control for COX-2 and p-53 staining, tissue sections were treated with normal mouse serum IgG (Vector Laboratories, Burlingame, CA, USA) in place of the primary antibody. All experiments were performed in duplicate.

Evaluation of staining

One hundred sixty-one specimens immunostained for COX-2 and p53 were evaluated under a transmission light microscope by a pathologist who was blinded to the backgrounds of the patients. Scoring of COX-2 expression in tumor epithelial cells was done according to the methods of Remmele and Stegner.[8] The intensity of staining was scored as 0 (negative), 1 (weak), 2 (medium), or 3 (strong), and the extent of staining was scored as 0 (0%), 1 (1 - 25%), 2 (26 - 50%), 3 (51 - 75%), and 4 (76 - 100%), indicating the percentage of positive staining in the carcinoma tissue. Addition of an intensity score (0 - 3) and an extent score (0 - 4) resulted in a COX-2 immunoreactivity score (IRS-COX2), which ranged between 0 and 7. For statistical purposes, tumors having a final staining score of ≥ 3 were considered to be positive. Only nuclear localization of immunoreactivity was evaluated for p53. The extent of nuclear p53 protein was classified as either negative or positive nuclear immunoreactivity, corresponding

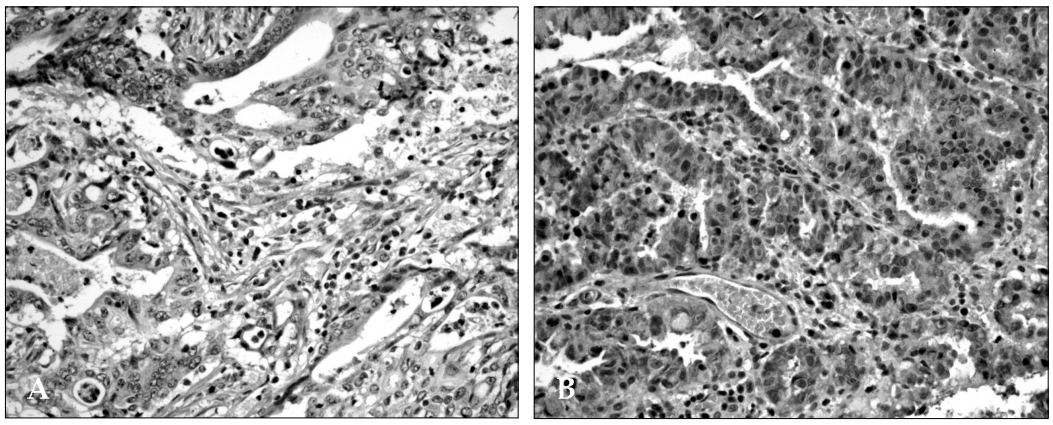

Fig. 1. Immunohistochemical staining for COX-2 shown in a representative section. (A) Negative COX-2 staining. (B) Positive staining for COX-2 in colorectal cancer cells.

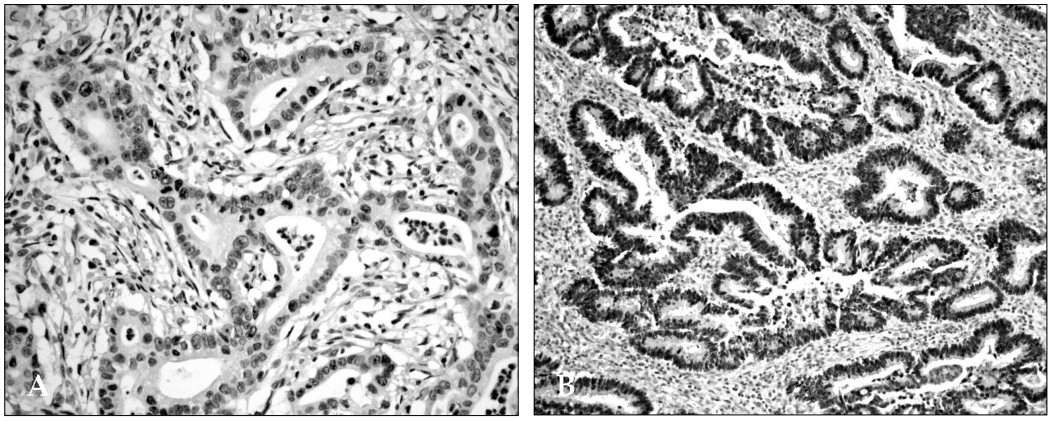

Fig. 2. Immunohistochemical staining for p53 shown in a representative section. (A) Negative staining (less than 10%) for p53. (B) Positive staining for p53.

to positive nuclei composing less than 10% of total nuclei or greater than 10% in tumor cells, respectively. Representative examples of COX-2 and p53 immunohistochemical staining are shown in Fig. 1 and 2.

Statistical analysis

For statistical analysis, the χ^2 test of significance and Fisher's exact probability calculation were used to analyze the distribution of COX-2 expression and p53 accumulation with clinicopathologic characteristics. The relationship between COX-2 and p53 expression was evaluated using the χ^2 test of significance. Results were considered statistically significant if $p < 0.05$. The SPSS version 12.0 software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

The distribution of the tumors according to the extent and intensity of COX-2 expression are presented in Table 1. The expression of COX-2 was positive (grade 3 to 7) in 47.8% of the patients. Weak staining of COX-2 (grade 1 to 2) was found in 10.4% of the group, which is considered negative for expression of COX-2, and no staining of COX-2 was seen in 41.8%. The expression of p53 was positive in 50.3% (81/161) of the cancers. When associations between COX-2 or p53 expression and the clinicopatholgic parameters were examined (Table 2), COX-2 expression was significantly correlated with only the depth of invasion ($p = 0.042$). There were no significant correlations between COX-2 expression and age, gender, tumor site, lymph node status,

Table 1. Distribution of Tumors according to the Extent and Intensity of Staining for COX-2

Staining intensity	Extent of staining (n)					
	0%	1 - 25%	26 - 50%	51 - 75%	76 - 100%	Total
Negative (0)	67*					67
Weak (1)		17*	19	12	2	50
Moderate (2)		11	11	10	3	35
Strong (3)			1	2	6	9
Total	67	28	31	24	11	161

*COX-2 negative tumors.

TNM stage, histologic type, or pre-operative carcinoembryonic levels. p53 was significantly correlated with age ($p = 0.025$) and depth of invasion ($p = 0.014$), and no other clinicopathologic parameters. The relationship between COX-2 expression and p53 accumulation status in colorectal adenocarcinomas is shown in Table 3. Although the COX-2 positive rate was higher in p53 positive cases (53.1%), there was no significant positive correlation between COX-2 expression and p53 accumulation status ($p = 0.118$, χ^2 test).

DISCUSSION

In this study, we found that the COX-2 protein was over-expressed in patients with colorectal adenocarcinoma, which was consistent with studies reporting that overexpression of COX-2 has been found in 65% to 80% of colorectal cancers.[9-11] In our study, the expression rate of COX-2 was found to be 47.8% by immunohistochemical analysis, a result slightly lower than that found in other studies. However, other studies have reported that overexpression of COX-2 protein has been found in 21% to 25% of colorectal cancers.[12-14] The discrepancies in the positive rate of COX-2 protein expression among the different studies may be explained by several factors, including variability in population size and characteristics, antibodies, lack of standardization in testing methods, and especially the differences in the choice of the scoring system or cutoff levels.

Although COX-2 is overexpressed in most colorectal cancers compared to normal mucosa, and may be related to the development of colorectal adenocarcinoma, the precise role of COX-2 in colorectal cancer is not completely known. Yamauchi et al. reported that COX-2 expression in the primary lesion may be a useful marker for evaluating the prognosis and liver metastasis in patients with colorectal cancer. COX-2 expression was significantly correlated with histologic type, depth of invasion, pathologic stage, and metachronous liver metastases of colorectal cancer. Multivariate analysis for factors associated with metachronous liver metastasis of colorectal cancer showed that COX-2 expression was a significant independent risk factor, second only to lymph node metastasis. Patients exhibiting COX-2 expression had a poorer outcome compared with those without COX-2 expression.[9]

Clinically, regional lymph node status proximal to a distant metastasis is the strongest prognostic factor for colorectal cancer. In patients with regional lymph node involvement (Stages I or II), the 5 year survival rate is greater than 75%, but this rate decreased steeply to about 45% in patients with lymph node involvement (Stage III), in spite of adjuvant therapy.[15] As for the association between COX-2 expression and lymph node status, Soumaoro et al. recently reported that COX-2 expression in colorectal cancer was correlated with lymph node metastasis and lymphatic invasion, and that COX-2 expression even functioned as an independent prognostic factor.[16] In contrast, our study only found the depth of tumor invasion (T-staging), and no other clinicopathologic parameters, to be correlated with COX-

Table 2. Immunohistochemical Expression of COX-2 and p53 in Colorectal Adenocarcinomas

Clinicopathologic parameters	Cases (n)	COX-2 (%)	*p* value	p53 (%)	*p* value
All cases	161	77 (47.8)		81 (50.3)	
Sex					
Male	83	44 (53.0)	0.230	45 (54.2)	0.387
Female	78	33 (42.3)		36 (46.2)	
Age (yrs)					
< 50	29	17 (58.6)	0.289	9 (31.0)	0.025
≥ 50	132	60 (45.5)		72 (54.5)	
Site					
Colon	69	31 (44.9)	0.632	33 (47.8)	0.585
Rectum	92	46 (50.0)		48 (52.2)	
Depth of invasion					
pT1/pT2	49	17 (34.7)	0.042	17 (34.7)	0.014
pT3/pT3	112	60 (53.6)		64 (57.1)	
Lymph node metastasis					
No	99	49 (49.5)	0.709	48 (48.5)	0.672
Yes	62	28 (45.2)		33 (53.2)	
TNM stage					
I	40	16 (40.0)	0.289	14 (35.0)	0.093
II	57	33 (57.9)		32 (56.1)	
III	59	26 (44.1)		31 (52.5)	
IV	5	2 (40.0)		4 (80.0)	
Histologic differentiated					
Well/Moderate	133	65 (48.9)	0.958	66 (49.6)	0.573
Poor/Mucinous	24	11 (45.8)		14 (58.3)	
Pre-operative CEA level					
Normal	70	36 (51.4)	0.944	35 (50.0)	0.845
Elevated	37	18 (48.6)		17 (45.9)	

Tested by chi-square test.

2 protein expression. Hence, our results suggest that COX-2 expression in colorectal cancer may be partly associated with the early phases of tumor progression.

p53 is a tumor suppressor that plays an important role in the suppression of cellular growth and transformation. p53 controls numerous downstream targets involved in apoptosis, transient growth arrest, and sustained growth arrest or senescence.[17] Alterations in the p53 gene were found in 50-60% of colorectal cancers, and most mutations occur as missense mutations within the conserved regions of the gene (exons 5-8). Mutant p53 proteins generally have a longer half-life than

Table 3. Relationship between COX-2 Expression and p53 Accumulation Status in Colorectal Adenocarcinoma

	n	COX-2 protein expression	
		Negative (%)	Positive (%)
All cases	161	84 (52.2)	77 (47.8)
p53 accumulation			
Negative	80	46 (57.5)	34 (42.5)
Positive	81	38 (46.9)	43 (53.1)

$p = 0.118$

wild-type p53 proteins, which leads to their nuclear accumulation.[18] It is now widely accepted that tumors with elevated levels of p53 protein frequently possess mutant p53 genes.[19] In the present study, we used immunohistochemistry to demonstrate the accumulation of mutant p53 protein in colorectal cancer patients. In agreement with other studies, p53 overexpression was found in 50.3% (81/161) of the colorectal cancers. Many other studies have demonstrated that p53 overexpression in colorectal cancer was closely related to clinicopathologic parameters and prognosis.[12,19] In our study, p53 overexpression was correlated with age and depth of tumor invasion.

Subbaramaiah et al. initially demonstrated that wild-type p53 suppressed COX-2 expression by inhibiting its promoter activity. COX-2 protein and mRNA levels were found to be markedly decreased in cells expressing wild-type p53, but not mutant p53. Electrophorectic mobility shift assays showed that p53 competed with TATA-binding protein (TBP) for binding to the COX-2 promoter.[7]

To our knowledge, only three studies have attempted to evaluate the correlation between COX-2 overexpression and p53 accumulation status in colorectal cancer tissues.[20-22] In a study with 73 colorectal cancer tissue samples, Cressey et al. reported that overexpression of COX-2 was frequently associated with p53 protein accumulation and HDM2 overexpression. Therefore, COX-2 overexpression observed in colorectal cancer cells may result, in part, from the dysfunction of p53.[20] However, in the other two studies with 21 and 114 colorectal cancer patients, respectively, the correlation between COX-2 overexpression and p53 accumulation status was not found.[21,22] With our larger sample size (n = 161), we found no significant correlation between COX-2 expression and p53 accumulation status, suggesting that expression of COX-2 might not be influenced by p53 status in colorectal cancer.

In conclusion, we have demonstrated that COX-2 protein was overexpressed in colorectal cancer and might play an important role in the early phases of tumor progression. However, COX-2 expression was not associated with mutated p53 levels. Further work is needed to clarify the regulatory mechanisms for COX-2 overexpression in colorectal cancer.

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