Cyclooxygenase-2 Expression: A Significant Prognostic Indicator for Patients With Colorectal Cancer

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ABSTRACT

Purpose: **Recent studies have shown that cyclooxygenase (Cox)-2 may be involved in colorectal carcinogenesis. We aimed to determine whether Cox-2 expression in itself can predict outcome of colorectal cancer patient after surgery. In addition, the expression of Cox-1 was also evaluated.**

Experimental Design: **Tissue samples of primary and secondary tumors from 288 patients undergoing surgical resections for colorectal adenocarcinoma were immunohistochemically examined for Cox-2 and Cox-1 expressions. The specimens were graded based on the intensity and extent of staining; then, the correlations between Cox-2 and Cox-1 expressions with clinicopathologic parameters and survival time were analyzed.**

Results: **Expression of Cox-2 was positive in 70.8% of primary tumor, 92.0% of lymph node metastases, 100.0% of hepatic metastases, and was significantly associated with tumor size, depth of invasion, lymph node metastasis, vessels invasion, stage and recurrence. In contrast, Cox-1 was positive in 42.7% of primary tumor, 84.0% of lymph node metastases, 37.5% hepatic metastases, and was associated with only tumor size. Patients with Cox-2–positive tumors had a significant shorter survival time than those with negative tumors did ($P = 0.0006$ by log-rank test); and, in a multivariate analysis, Cox-2 was an independent prognostic factor ($P = 0.0103$; relative risk 4.114; 95% confidence interval, 1.397–12.120). Cox-1 status had no statistically effect on patient survival time.**

Conclusions: **Elevated Cox-2 expression, but not that of Cox-1, was significantly associated with reduced survival and recognized as an independent prognostic factor in our cohort of colorectal cancer patients.**

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INTRODUCTION

Colorectal cancer is the third most common cancer in the world and the second most common cause of cancer-related death (1). During 2000, in the United States, 130,200 new cases of colon cancer and rectal cancer were reported (2). In Japan, ~36,000 patients dead of this cancer in 2001 (3). Despite numerous attempts to detect cancer at an early stage, the overall long-term outcome of patients curatively resected has not significantly changed in the last decade, the 5-year survival rate being ~60% (4). Assessment of molecular prognostic factors associated with a distinct prognostic outcome would, therefore, be of great help for identification of patients who are likely to benefit from adjuvant therapies, leading to an improvement in prognosis.

Recent studies have suggested that an increase in the expression of cyclooxygenase (Cox)-2, a key-inducible enzyme involved in the production of prostaglandins and other eicosanoids, may play a significant role in carcinogenesis in addition to its well-known role in inflammatory reactions (5–10).

Interestingly, Cox-2 has been reported to be significantly increased in up to 85% of human sporadic colorectal carcinoma, predominantly within neoplastic epithelial cells (5, 6, 11–18), in which Cox-2 may induce resistance to apoptosis, alter extracellular matrix adhesion, modulate tumor angiogenesis, and increase metastatic potential (7, 9, 19). In addition, a specific cause-effect relationship between the overexpression of Cox-2 and colon cancer has been determined in studies on intestinal carcinogenesis in the adenomatous polyposis coli $(APC)^{\Delta 716}$ (8, 20).

Moreover, Kawamori *et al.* (21) have reported that celecoxib, a specific Cox-2 inhibitor suppressed both incidence and multiplicity of azoxymethane-induced colon cancer in rats by ~93 and 97%, respectively. In a previous clinical trial, we also showed that rofecoxib, another specific Cox-2 inhibitor, significantly decreased the number and size of rectal polyps in familial adenomatous polyposis patients (22).

However, it is controversial whether Cox-2 expression in itself is a prognostic factor for local recurrence and/or survival of patients with colorectal cancer (23).

Therefore, to clarify additionally the prognostic role of Cox-2 in colorectal carcinoma and to evaluate the possible contribution of isoenzyme Cox-1, we immunohistochemically determined the expression of both proteins in a series of patients with primary tumors, who were treated at our institution. In addition, sections of adjacent normal mucosa, lymph node metastases, and hepatic metastases samples were also evaluated.

MATERIALS AND METHODS

Patients and Tissue Samples. A total of 288 consecutive patients who had undergone surgical resections for primary sporadic colorectal carcinoma at the Department of Surgical Oncology, Tokyo Medical and Dental University (Tokyo, Japan), between January 1986 and December 1996 was included in this study. There were 174 colon cancers and 114 rectal

Table 1 Distribution of primary tumors according to the extent and intensity of staining for Cox-2

	Extent of staining					
Staining intensity	0%	1–25%	26–50%	51–75%	76–100%	Total
Negative	21*					21
Weak		10*	16*	9	15	50
Medium		37*	12	5	29	83
Strong		11	23	14	86	134
Total	21	58	51	28	130	288

* Cox-2–negative tumors.

cancers; 183 males and 105 females; mean age, 61 ± 10 years. None of them had a history of hereditary colon cancer syndromes and regular use of aspirin-like drugs. There was no preoperative chemotherapy or radiotherapy; however, after surgery, for both colon and rectal cancer, patients with stage III tumor received oral 5-fluorouracil, and those with stage IV tumor had 5-fluorouracil–based systemic chemotherapy without any radiation. Patients were followed up until death or December 31, 2002, with a median postoperative follow-up duration of 63 ± 33 months.

In all cases, archival H&E slides of the primary and secondary tumors were retrieved and reviewed to confirm pathological features and to select suitable tissue blocks for immunohistochemical analysis.

The samples included 288 primary tumors, 25 available lymph node metastases, 16 available hepatic metastases. In all 25 metastatic lymph nodes and 4 of 16 hepatic metastases, concurrent primary tumors were examined. Additionally, for comparison, sections of 10 adjacent normal mucosa, 10 normal lymph node, 10 normal hepatic parenchyma, were also included in this study.

The clinicopathologic findings were determined according to the International Union Against Cancer Tumor-Node-Metastasis (TNM) classification of malignant tumors (24).

Immunohistochemical Staining. A universal immunoenzyme polymer method was used for immunostaining. Three-μm thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-lysine coated slides, dewaxed in xylene, and rehydrated through a graded series of ethanol. After deparaffinization, antigen retrieval treatment was performed at 121°C (autoclave) for 5 minutes in 10 mmol/L sodium citrate buffer (pH 6.0), then treated with 3% hydrogen peroxide in methanol solution for 20 minutes to quench endogenous peroxidase activity. Nonspecific bindings were blocked by treating slides with 10% normal goat serum for 10 minutes. To block intrinsic biotin-binding capabilities, hepatic tissue slides were treated with avidin-biotin blocking kit reagents (Vector Laboratories, Inc., Burlingame, CA) for 15 minutes. Thereafter, the slides were incubated with mouse monoclonal antibodies against human Cox-2 (dilution 1:250; Cayman Chemical Co., Ann Arbor, MI) for 2 hours at room temperature for Cox-2 and rabbit polyclonal antibodies against human Cox-1 (dilution 1:200; Cayman Chemical Co.) for 2 hours at room temperature for Cox-1. Next, slides were incubated with labeled polymer (N-Histofine Simple Stain MAX PO MULTI; Nichirei Co., Tokyo, Japan) for 30 minutes at room temperature. Color development was done with 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, Poole, United Kingdom) and 0.06% hydrogen peroxide in 50 mmol/L Tris-HCl (pH 7.6) for 5 minutes. Finally, the slides were counterstained with 1% Meyer's hematoxylin.

As a negative control for Cox-2 and Cox-1 staining, tissue sections were treated with normal serum instead of each primary antibody.

Evaluation of Staining. All sections were scored blind by two investigators (L.T.S. and Y.T.) under a light microscope; and, in cases of occasional scoring discrepancy, consensus was always achieved after discussion of findings. For Cox-2 and Cox-1 assessment, the entire tissue section was scanned to assign the scores. The staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). Extent of staining was scored as 0 (0%), 1 (1 to 25%), 2 (26 to 50%), 3 (51 to 75%), and 4 (76 to 100%), according to the percentages of the positive staining areas in relation to the whole carcinoma area or entire section for the normal samples. The sum of the intensity and extent score was used as the final staining score (0 to 7) for Cox-2 or Cox-1. This relatively simple, reproducible scoring method that gives highly concordant results between independent evaluators has been used in past studies (14, 17).

For the purpose of statistical evaluation, tumors having a final staining score of ≥3 were considered to be positive.

Statistical Analysis. All statistical analyses were carried out with the StatView Software (version 5.0). The correlations between expression of Cox-2 or Cox-1 and several clinicopathologic parameters were assessed with the χ^2 test or the Spearman rank test as indicated. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed with the log-rank test. The Cox proportional hazards model was used for multivariate analysis of prognostic factors. $P < 0.05$ was considered to be significant.

RESULTS

Cox-2 and Cox-1 Expression in Normal Mucosa and Primary and Secondary Tumors. Both cyclooxygenase proteins were mainly detected in the cytoplasm of the epithelial cells, vascular endothelial cells, and some stromal cells probably fibroblast and mononuclear cells. The allocation of staining score by the two investigators was concordant in >94% of Cox-2 samples and >96% of Cox-1 samples. In addition, as we stated in Materials and Methods, the discrepant cases were reevaluated and scored by consensus. The distribution of primary tumors according to the extent and intensity of Cox-2 and Cox-1 immunoreactivity are presented in Tables 1 and 2.

The expression of Cox-2 was positive (grade 3 to 7) in

Table 2 Distribution of primary tumors according to the extent and intensity of staining for Cox-1

	Extent of staining					
Staining intensity	0%	1–25%	26–50%	51–75%	76–100%	Total
Negative	75*					75
Weak		31*	40*	4	36	111
Medium		19*	6	10	30	65
Strong		2	9	5	21	37
Total	75	52	55	19	87	288

* Cox-1–negative tumors.

Table 3 Positivity rates for Cox-2 and Cox-1 expressions

Samples	Cox-2 expression		Cox-1 expression	
	Negative	Positive	Negative	Positive
Primary tumors	84 (29.2%)	204 (70.8%)	165 (57.3%)	123 (42.7%)
Lymph node metastases	2 (8.0%)	23 (92.0%)	4 (16.0%)	21 (84.0%)
Hepatic metastases	0 (0%)	16 (100.0%)	10 (62.5%)	6 (37.5%)
Normal mucosa	9 (90.0%)	1 (10.0%)	2 (20.0%)	8 (80.0%)
Normal lymph node	7 (70.0%)	3 (30.0%)	1 (10.0%)	9 (90.0%)
Normal hepatic parenchyma	5 (50.0%)	5 (50.0%)	3 (30.0%)	7 (70.0%)

70.8% of primary tumors, 92.0% of metastatic lymph nodes, and all of hepatic metastases (Table 3). The normal mucosa adjacent to the Cox-2–positive tumor and the normal samples showed a little or no stained for Cox-2. In contrast, the positivity rate for Cox-1 was 42.7, 84.0, 37.5, and, 80.0% in the primary tumors, metastatic lymph nodes, hepatic metastases, and normal samples, respectively (Table 3). Moreover, we found an elevated expression of both Cox-1 and Cox-2 in the normal liver tissue and biliary epithelium adjacent to the metastatic lesion, even after endogenous avidin-biotin blocking treatment. Representatives of the staining patterns are shown in Fig. 1.

When compared primary and secondary tumors *versus* normal samples, a significant difference was observed for Cox-2 expression: primary tumor *versus* normal mucosa ($P < 0.0001$), metastatic lymph node *versus* normal lymph node ($P = 0.0001$), and hepatic metastases *versus* normal hepatic parenchyma ($P = 0.0016$); and for Cox-1 expression, primary tumor *versus* normal mucosa ($P = 0.0195$), metastatic lymph node *versus* normal lymph node ($P = 0.6468$), and hepatic metastases *versus* normal hepatic parenchyma ($P = 0.1069$).

Correlation With Clinicopathologic Parameters. In Table 4, the association between Cox-2 and Cox-1 expression

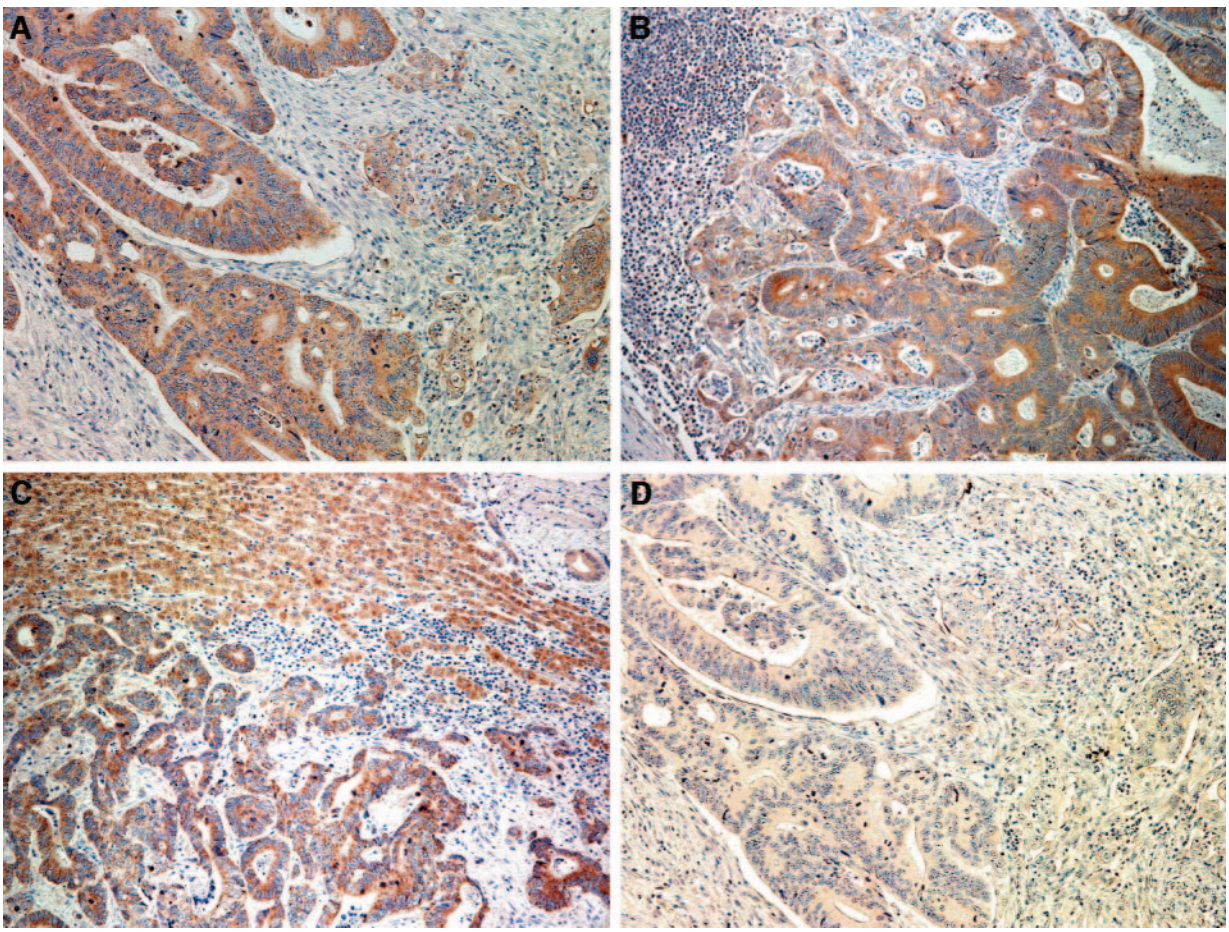

Fig. 1 Strong (score 7) immunohistochemical staining for Cox-2 is shown in a representative section of primary tumor (*A*), metastatic lymph node (*B*), and metastatic hepatic tissue (*C*). The same primary tumor is shown with moderate (score 4) staining for Cox-1 (*D*).

Table 4 Correlation of clinicopathologic parameters with Cox-2 and Cox-1 expressions

Clinicopathologic parameters	Number of cases	Cox-2 expression		*P*	Cox-1 expression		*P*
		Negative	Positive		Negative	Positive	
All cases	288	84	204		165	123	
Age (y)	288	Rho = 0.066		0.2605*	Rho = 0.006		0.9198*
Gender							
Male	183	51	132		110	73	
Female	105	33	72	0.5224	55	50	0.2019
Tumor site							
Colon	174	49	125		93	81	
Rectum	114	35	79	0.6427	72	42	0.1033
Tumor size	288	Rho = 0.411		<0.0001*	Rho = 0.208		0.0004*
Histology							
Well	163	45	118		86	77	
Moderate	112	35	77		70	42	
Poor	13	4	9	0.8012	9	4	0.1858
Depth of invasion							
pT_1	39	17	22		23	16	
pT_2	48	21	27		28	20	
pT_3	174	45	129		98	76	
pT_4	27	1	26	0.0004	16	11	0.9814
Lymph node metastasis							
Yes	103	22	81		60	43	
No	185	62	123	0.0296	105	80	0.8057
Lymphatic invasion							
Yes	197	49	148		114	83	
No	91	35	56	0.0183	51	40	0.7711
Venous invasion							
Yes	211	53	158		117	94	
No	77	31	46	0.0123	48	29	0.2957
TNM stage							
I	78	35	43		46	32	
II	97	20	77		54	43	
III	96	22	74		52	44	
IV	17	7	10	0.0013	13	4	0.3715
Recurrence							
Yes	59	9	50		39	20	
No	229	75	154	0.0084	126	103	0.1250

* Spearman rank test *P* value.

and the clinicopathologic parameters is shown. Cox-2 expression was significantly correlated with primary tumor size (Rho = 0.411, $P < 0.0001$), depth of invasion ($P = 0.0004$), lymph node metastasis ($P = 0.0312$), lymphatic invasion ($P = 0.0253$), venous invasion ($P = 0.0186$), TNM stage ($P = 0.0013$), and tumor recurrence ($P = 0.0097$). There was no significant correlation between Cox-2 expression and age, gender, tumor site, and histologic type. Cox-1 was significantly correlated with only the primary tumor size (Rho = 0.208, $P = 0.0004$).

Correlation With Patients' Survival. Within a median postoperative follow-up duration of 63 ± 33 months, 57 cancer-related deaths occurred: 10 in patients with Cox-2–negative tumors and 47 in the positive group ($P = 0.0311$) and 30 in patients with Cox-1–negative tumors and 27 in the positive group ($P = 0.4271$).

In the entire cohort, the overall survival rates of patients with Cox-2–negative tumors were significantly higher when compared with those of the Cox-2–positive group ($P = 0.0006$, log-rank test; Fig. 2*A*). The same significant difference in survival between the two groups of patients was also observed in combined stage I and stage II ($P = 0.0271$; Fig. 2*B*) and in stage III cases ($P = 0.0081$; Fig. 2*C*). The 5-year survival rate of Cox-2–negative and Cox-2–positive cases in stage I and stage II was 97 and 82%, respectively, and that in stage III was 88 and 67%, respectively. Stage IV was not considered alone because of the limit number of cases.

The Kaplan-Meier curves demonstrated no association between Cox-1 expression status and patients survival ($P = 0.4016$; log-rank test).

Furthermore, in the multivariate analysis, potential prognosis factors such as age and gender of the patient, tumor site, size, depth of invasion, lymphatic invasion, venous invasion, degree of differentiation, lymph node metastasis, TNM stage, and Cox-1 and Cox-2 expression were included in the Cox proportional hazards model. The results indicated that, in addition to lymph node metastasis and TNM stage, Cox-2 expression status was an independent prognostic factor in our cohort ($P = 0.0103$; Table 5).

DISCUSSION

In this study of human colorectal carcinoma, the expression of Cox-2 protein showed a markedly increased from normal

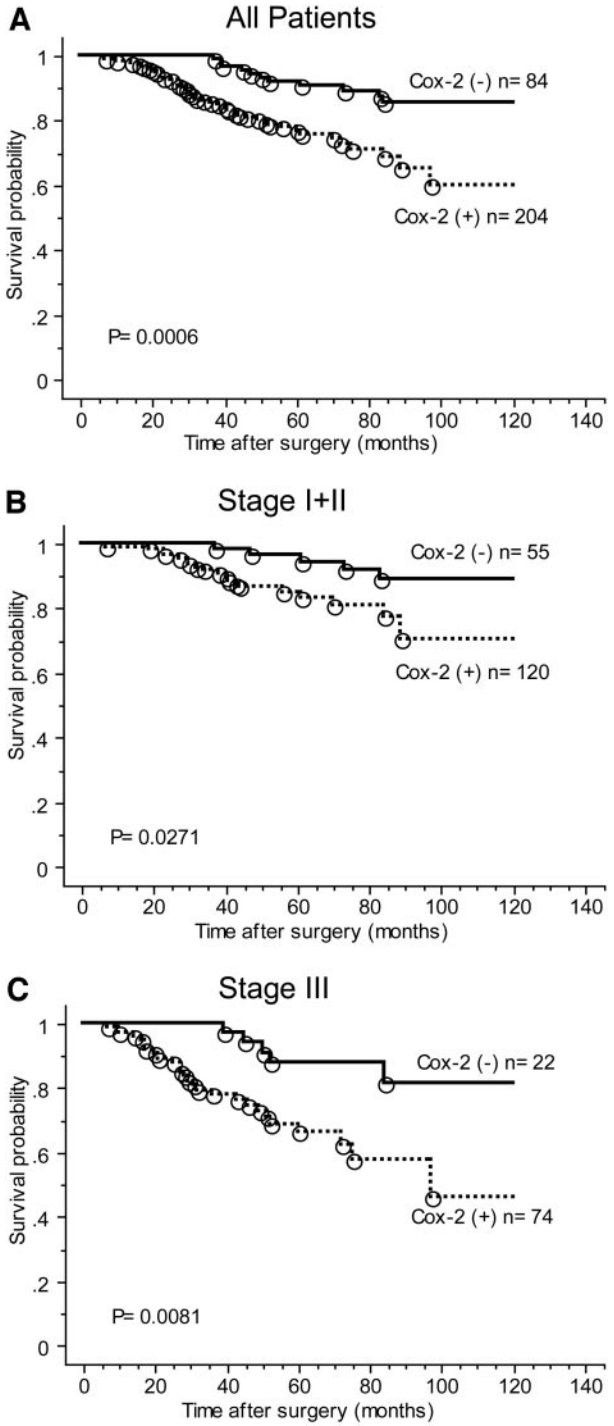

Fig. 2 The overall 10-year survival curves of patients with Cox-2 negative and Cox-2 positive are shown for the entire cohort, $P = 0.0006$ (*A*), as well as for patients with stage I/II, $P = 0.0271$ (*B*), or stage III, $P = 0.0081$ (*C*) disease.

samples to primary tumors and metastatic samples. We found that elevated expression of Cox-2 was significantly associated with prognostically worse clinicopathologic variables, including tumor size, depth of invasion, lymph node metastases, and vessels invasion. These findings are consistent with our previous reports that tumors with higher Cox-2 gene expression grow larger and in a more invasive manner in colorectal (25) and gastric carcinoma (26). Contrary to the results from some recent studies (14, 17), we could not prove a statistically significant relationship between Cox-2 expression with the degree of tumor cells differentiation.

Interestingly, we showed a significant correlation between high-grade Cox-2 expression with a shortened survival of patients and Cox-2 status as well as lymph node metastasis and TNM stage was an independent prognostic factor.

Actually, the negative prognostic value of Cox-2 overexpression has been reported in several studies on various carcinomas such as those of colon and rectum (12, 14, 17, 27, 28), stomach (29), esophagus (30), breast (31), lung (32, 33), and ovaries (34).

However, there is a disagreement over the independent prognostic capability of Cox-2 as compared with other known prognostic factors, including tumor features and molecular characteristics (14, 27). Also, some authors that used immunohistochemical analysis method claimed that Cox-2 may be involved in tumorigenesis but lack a prognostic significance for colorectal cancer patients (18, 23, 35–37). At this point, it is considered that tumor invasiveness, frequent metastasis, and expression in larger tumors are responsible for the worse prognosis for patients bearing Cox-2–positive tumors (14). We believe that variability in population size and characteristics, antibodies, lack of consensus on and standardization of methods, and differences in the choice of cutoff levels may explain the discrepancies between the different studies. In fact, our findings were obtained by analysis of a relatively large number of consecutively resected cases in a single institution and, thus, should be of considerable interest. Moreover, the monoclonal antibodies we used have been shown in several excellent studies (13, 14, 30, 31, 38) and is considered to give the most sensitive, specific, and reproducible immunoreactivity of Cox-2 protein. Nevertheless, as proposed by Buecher *et al.* (28), it will be of critical importance to standardize the methods used for the evaluation of tumor Cox-2 status and to apply common interpretative criteria to allow direct comparison of results between laboratories.

To date, several molecular pathways have been postulated to elucidated the mechanism by which up-regulation of the Cox-2 gene can contribute to tumor development and progression. Among them, the hypothesis of an inhibition of apoptosis (7, 39), an increased in cell proliferation and angiogenesis (14, 15, 19, 20, 39, 40), and a local immunosuppression (16) as carcinogenic properties of Cox-2 have in the recent years received more attention. These possible pathways may all influence the prognosis of patients with colorectal cancer to worse.

One distinctive feature in the present study is that cancer cells in 23 of 25 lymph node metastases and all hepatic metastases, as well as their corresponding primary tumors, were positive for Cox-2 protein. Hull *et al.* (37), who investigated 54 colorectal cancer liver metastases, reported that Cox-2 protein was detected in cancer cells in 100% of cases. Unfortunately, the authors were unable to obtain data regarding Cox-2 status in

Table 5 Multivariate survival analysis: Cox proportional hazards model

Parameters	β	SE	*P*	Relative risk ratio	95% Confidence interval
Age (<61 *versus* ≥61 y)	0.304	0.285	0.2870	1.355	0.775–2.370
Gender (female *versus* male)	0.080	0.308	0.7963	1.083	0.592–1.982
Tumor site (colon *versus* rectum)	0.249	0.277	0.3683	1.283	0.745–2.210
Tumor size (<4.0 *versus* ≥4.0 cm)	0.665	0.446	0.1358	1.945	0.811–4.662
Histology (well *versus* moderate, poor)	−0.093	0.274	0.7346	0.911	0.532–1.560
Depth of invasion ($pT_{1,2}$ *versus* $pT_{3,4}$)	0.869	0.634	0.1706	2.385	0.688–8.268
Venous invasion (no *versus* yes)	0.694	0.561	0.2160	2.002	0.666–6.016
Lymphatic invasion (no *versus* yes)	0.689	0.566	0.2240	1.991	0.656–6.045
Lymph node metastasis (no *versus* yes)	0.788	0.336	0.0189	2.199	1.139–4.247
TNM stage (I, II *versus* III, IV)	1.180	0.372	0.0015	3.255	1.570–6.750
Cox-1 status (negative *versus* positive)	0.458	0.297	0.1234	1.581	0.883–2.830
Cox-2 status (negative *versus* positive)	1.414	0.551	0.0103	4.114	1.397–12.120

the corresponding primary tumors. To our knowledge, this is the first investigation in which Cox-2 protein expression is evaluated in both primary and secondary tumors. This finding may indicate that Cox-2 expression by cancer cells probably occurred before metastasis, and only Cox-2 bearing cancer cells have more of a chance to metastasize to the lymph node and the liver. Additional studies are warranted to clarify this question and, more interestingly, to assess the utility of Cox-2 inhibitors in the treatment of metastatic tumors.

In contrast to Cox-2, the possible roles of Cox-1 expression in human tumorigenesis have received less attention. Our attempt to show the clinicopathologic significance of Cox-1 expression in colorectal cancer, as has been shown in ovarian cancer (41) and testicular tumors (42), showed only an association with tumor size.

However, in conjunction with a genetic study reporting that disruption of Cox-1 gene in APC^{Min} mice causes suppression of polyposis (43) and also given the fact that some nonsteroidal anti-inflammatory drugs such as aspirin that inhibit only Cox-1 can reduce colon cancer incidence and mortality (44–46), it is likely that both cyclooxygenases play critical roles in tumorigenesis at different stages. In support to this possibility, Takeda *et al.* (47), in studying intestinal polyp formation, suggested that, whereas Cox-2 induction is essential for polyps to expand beyond ~1 mm, nascent polyps are likely to develop to ~1 mm with prostaglandin E_2 supplied by Cox-1 alone. Additional studies are necessary to gain more insight into the functional role of Cox-1 in the development and progression of colorectal carcinoma.

Finally, evidence from several experimental and clinical studies has shown that selective Cox-2 inhibitors possess significant antitumor activity on their own with favorable safety profile (1, 8, 21, 22, 48) and can also enhance tumor response to radiation or cytotoxic agents (49, 50). However, recent clinical studies (51, 52) with rofecoxib in the treatment of metastatic colorectal cancer pointed out an increased toxicity and lack of efficacy. Nevertheless, although this is an interesting observation, the few patients in those studies precludes it from being presented as a definitive conclusion. More clinical trials with more patients are needed to really assess the therapeutic benefit of Cox-2 inhibitors in human colorectal cancer.

We conclude that elevated Cox-2 expression, but not that of its isoform Cox-1, is significantly associated with reduced survival in patients with colorectal adenocarcinoma. Accordingly, our results taken together with those of other prompted us to believe that assessment of Cox-2 level in colorectal cancer may be used as a marker to select not only high-risk patients group for recurrence and offer them more frequent follow-up but also patients who may likely benefit from Cox-2 inhibitors as adjuvant therapy either alone or in combination with established anti cancer drugs.

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