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Reduced COX-2 Protein in Colorectal Cancer with Defective Mismatch Repair[1]

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ABSTRACT

Most colorectal adenomas and carcinomas arise in the setting of chromosomal instability characterized by progressive loss of heterozygosity. In contrast, approximately 15–20% of colorectal neoplasms arise through a distinct genetic pathway characterized by microsatellite instability (MSI) associated with frequent loss of expression of one of the DNA mismatch repair enzymes, most often hMLH1 or hMSH2. These distinct genetic pathways are reflected by differences in tumor histopathology, distribution in the colon, prognosis, and dwell time required for progression from adenoma to carcinoma. To determine whether these two groups of tumors differ in their expression of cyclooxygenase-2 (COX-2), a putative chemopreventative target, immunostaining for this protein was performed in colorectal cancers categorized by the presence (n = 41) and absence (n = 66) of defective mismatch repair. Defective mismatch repair was defined by the presence of tumor microsatellite instability (MSI-H, ≥40% of markers demonstrating instability) and by the absence of protein expression for either hMLH1 or hMSH2. Overall, our results showed that low or absent COX-2 staining was significantly more common among tumors with defective mismatch repair (P = 0.001). Other features predictive of low COX-2 staining included marked tumor infiltrating lymphocytosis, and solid/cribiform or signet ring histological patterns. These observations indicate that colorectal cancers with molecular and phenotypic characteristics of defective DNA mismatch repair express lower levels of COX-2. The clinical implications of this biological distinction remain unknown but should be considered when assessing the efficacy of COX-2 inhibitors for chemoprevention in patients whose tumors may arise in the setting of defective DNA mismatch repair.

INTRODUCTION

CRC[3] ranks second behind lung cancer as a cause of cancer deaths in the United States (1). Understandably, current efforts are focused on identifying and developing novel chemopreventative agents that block the formation or progression of the precursors of CRC, adenomatous polyps. NSAIDs effectively reduce polyp burden in cases of FAP (2) and may reduce adenoma incidence and colon cancer mortality in the general population (3, 4).

The chemopreventative effects of NSAIDs may be attributable to their inhibitory activity against COX-2, which catalyzes the conversion of arachidonic acid to prostaglandins (5). COX-2 and prostaglandin levels are increased in sporadic colorectal adenomas and carcinomas (6–7) and in FAP (8) in humans. COX-2 and prostaglandin levels are also increased in colonic tumors originating from azoxymethane-treated rats (9) and mouse models of FAP that have heterozygous mutations of the *APC* gene (10). Like NSAIDs, novel selective inhibitors of COX-2 suppress polyp formation in mouse models of FAP (11, 12) and in azoxymethane-treated rats (13). Similarly, knockout of the *COX-2* gene in APC(±) mice significantly suppresses adenoma formation (12). These observations suggest a critical role of COX-2 for the genesis, maintenance, and perhaps malignant progression of colorectal adenomas.

The majority of sporadic colorectal neoplasms, like those that arise in FAP, are triggered by functional loss of both *APC* alleles (14). These similar genetic origins suggest that sporadic neoplasms will show comparable sensitivity to COX-2-directed chemopreventative strategies. However, as many as 15–20% of sporadic colorectal neoplasms seem to arise through an alternative genetic mechanism involving defective DNA mismatch repair (15, 16). These tumors are characterized by the presence of tumor MSI mediated by alterations in one or more of a family of DNA mismatch repair enzymes. The two most commonly affected genes in these sporadic cases and in HNPCC are *hMSH2* and *hMLH1* (17–21), the functional absence of which can be detected by immunohistochemistry (20–22). CRCs with MSI exhibit distinct clinicopathological features including improved prognosis, reduced dwell time required for adenoma to carcinoma transition, right-sided predominance, and cribiform or signet cell histology and/or numerous tumor-infiltrating lymphocytes (23–33).

The molecular, clinical, and pathological distinctions between tumors with and without MSI suggest that they are biologically distinct and may respond differently to therapeutic and chemopreventative agents. To address this possibility, we focused on COX-2 protein expression because of its putative role as a chemopreventative target and because of its high level of expression in most colorectal carcinomas. Our findings indicate a significant reduction in COX-2 levels in tumors with MSI and in those with loss of hMLH1 and/or hMSH2 protein expression. We speculate that this biological distinction may be a direct reflection of differences in the molecular genesis of these tumors.

MATERIALS AND METHODS

Patient Population. Patient samples (n = 107) for the present study were selected from a larger group of consecutive surgical patients who consented to participate in a prospective cancer risk assessment study. Patients with FAP were excluded, but family history was not an exclusion criterion; therefore, some cases of HNPCC may have been included in the study population. Patients with (n = 53) and without MSI (n = 54) involving at least 7 microsatellite loci were randomly selected from the larger group to provide an approximately equal number in each group. Additional data available on all of the tumors include age at diagnosis, gender, and location of primary tumor in the colon.

MSI. Paired normal and tumor DNA were analyzed for the presence of MSI with up to 7 markers, including *BAT26*, *D5S346*, *TP53*, *D18S61*, *D18S34*, *D18S49*, and *ACTC* as described previously (20). Tumors from each patient were placed into one of three separate groups: MSS (none of the markers showing instability, n = 54); MSI-L (1–39% of markers demonstrating instability, n = 12); and MSI-H (≥40% of markers demonstrating instability, n = 41; Refs. 20, 21).

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[3] The abbreviations used are: CRC, colorectal cancer; NSAID, nonsteroidal anti-inflammatory drug; FAP, familial adenomatous polyposis; COX-2, cyclooxygenase-2; APC, adenomatous polyposis coli; TIL, tumor-infiltrating lymphocyte; MSI, microsatellite instability; MSS, microsatellite stability; MSI-L, low-frequency MSI (<40 markers showing instability); MSI-H, high-frequency MSI (≥40 markers showing instability); MSI-Path, MSI with solid/cribiform pattern, signet cell histology, and/or moderate to marked TILs (TILs score of 3–4); HNPCC, hereditary nonpolyposis colon cancer; TBS, Tris-buffered saline; TGF, transforming growth factor; TCF, T-cell factor.

Tumor Characteristics. All tumors were assessed according to location, histological pattern, and the degree of tumor infiltrating lymphocytosis in a blinded fashion by a pathologist (L. J. B) as described previously (32, 33). Right-sided lesions were defined as those confined to the cecum or ascending or transverse colon, whereas left-sided lesions were defined as being confined to the descending colon, sigmoid colon, or rectum. Five histological patterns were defined: (*a*) ordinary type (glandular, desmoplastic), low grade; (*b*) ordinary type, high grade; (*c*) solid/cribiform; (*d*) colloid; or (*e*) signet ring. TILs were graded on a 1–4 scale: 1, rare; 2, few infiltrating lymphocytes involving a minority of malignant glands; 3, few infiltrating lymphocytes involving the majority of malignant glands; and 4, numerous infiltrating lymphocytes involving the entire tumor area. "MSI-Path" was assessed if any of the following features were present in the tumor: (*a*) solid/cribiform pattern; (*b*) signet ring type; and/or (*c*) TIL score of 3 or 4.

Immunohistochemistry. Immunohistochemical staining for hMSH2 and hMLH1 was performed essentially as described previously (22). For the assessment of COX-2 protein expression, paraffin-embedded tumor tissue was cut in 5-μm sections and mounted on superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were deparaffinized in xylene, rehydrated in ethanol and water, and then washed in TBS-0.05% Tween-20 and TBS. To block endogenous peroxidase activity, the slides were treated with 0.6% hydrogen peroxide in methanol. Immunohistochemical staining was performed after microwave antigen retrieval in 100 mM sodium citrate (pH 6.0). The sections were blocked with 1% BSA and 5% normal goat serum in 10 mM Tris (pH 7.4), 100 mM $MgCl_2$, and 0.5% Tween-20 for 1 h at room temperature. COX-2 was detected with an antibody specific for COX-2 (Cayman). The primary antibody, diluted with blocking solution, was incubated with the sections overnight at 4°C in a humid environment. The slides were then washed with TBS + 0.05% Tween-20 and incubated with the secondary biotinylated antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The antibody complex was then detected with ABC amplification according to the manufacture's recommended procedure (Vector Laboratories, Burlingame, CA). The horseradish peroxidase-labeled complex was detected using the substrate True Blue (Kirkegaard and Perry Laboratories) and counterstained with Contrast Red (Kirkegaard and Perry Laboratories). For the negative control, no primary antibody was added. Similar results were obtained with a COX-2-specific antibody from Santa Cruz Biotechnology (C-20; data not shown).

COX-2 Scoring. On the basis of the intensity and the number of cells staining for COX-2, four different staining scores were defined (representative cases shown in Fig. 1): 0, no chromagen deposition; 1, weak to moderate chromagen deposition affecting less than 50% of the tumor area; 2, weak to moderate chromagen deposition in the majority of the tumor or strong chromagen deposition present in a minority of the tumor; and 3, strong chromagen deposition in the majority of the tumor area. A single pathologist (L. J. B.) scored COX-2 staining without knowledge of the results of MSI analysis or immunostaining for hMLH1 and hMSH2. Initial scoring by a nonpathologist (R. S-B.)—blinded to all of the parameters including pathological category, MSI analysis, and immunostaining for hMLH1 and hMSH2—provided essentially identical results. Scores provided by the pathologist were used for the final analysis.

Statistical Analysis. COX-2 expression was assessed for associations with the following parameters: (*a*) patient age at diagnosis; (*b*) gender; (*c*) location in the colon; (*d*) staining for hMLH1 and hMSH2; (*e*) histological pattern; (*f*) TILs; and (*g*) MSI-Path phenotype. MSI was also assessed for associations with: (*a*) patient age at diagnosis; (*b*) gender; (*c*) location in the colon; (*d*) staining for hMLH1 and hMSH2; and (*e*) MSI-Path phenotype. Associations with categorical variables were assessed using the Mantel-Haenszel χ^2 test for linear trend. Fisher's exact test was used when the sample sizes were insufficient for χ^2 analysis. The distribution of age at diagnosis was compared across the levels of COX-2 expression using Kruskal-Wallis test, and between MSHI-H and MSS using the Wilcoxon rank-sum test. All of the analyses were performed using SAS software.

RESULTS

Table 1 shows the three groups selected for study (MSS, MSI-L, and MSI-H) and a summary of the other patient and tumor characteristics. Not unexpectedly, the MSI-H (≥40% markers with MSI) and MSS (0% markers with MSI) groups differed significantly with respect to factors known to correlate with MSI, including location in the colon, staining for hMLH1 and hMSH2, and MSI-Path phenotype (20). No significant difference in age was seen between the groups. With the exception of a single case in which hMSH2 staining was inadequate for interpretation, all of the MSI-H tumors showed absent

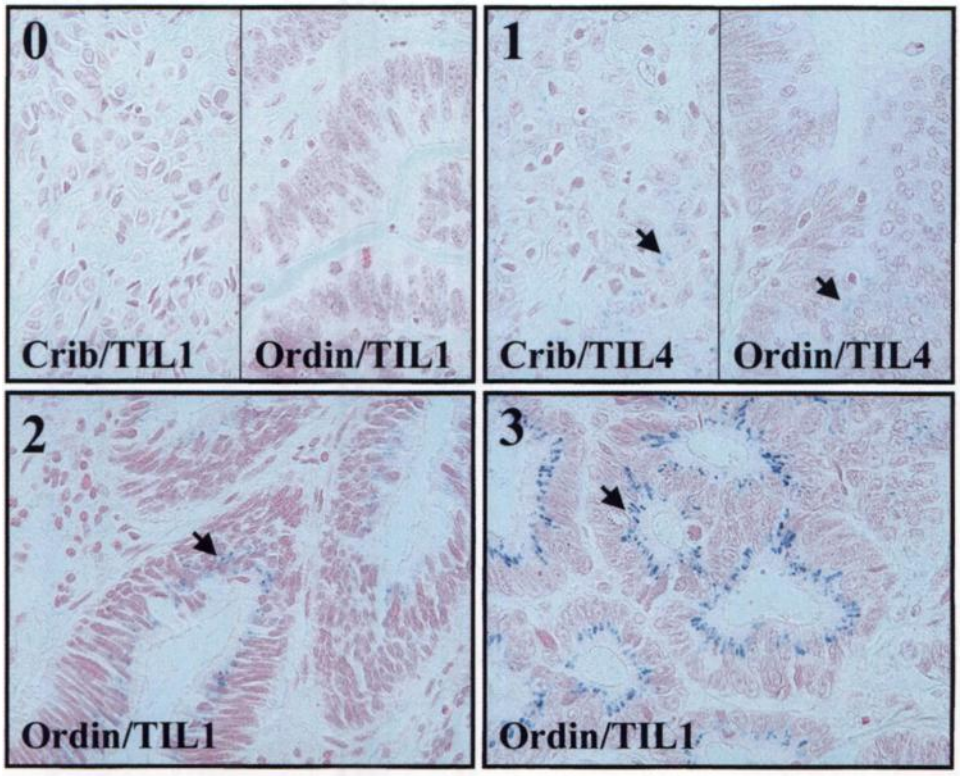

Fig. 1. Representative photomicrographs (×40) of colorectal tumor specimens immunostained for COX-2. Anti-COX-2 antibody was detected using True Blue chromagen (*arrowheads*), counterstained with Contrast Red as described in the "Materials and Methods" section. Numbers at the *upper left of each panel* indicate COX-2 scores (*0*, no chromagen deposition; *1*, weak to moderate chromagen deposition affecting less than 50% of the tumor area; *2*, weak to moderate chromagen deposition in the majority of the tumor or strong chromagen deposition present in a minority of the tumor; and *3*, strong chromagen deposition in the majority of the tumor area. Histopathological categories for each tumor are labeled at the *lower left of each panel*: *ordin*, ordinary type (glandular, desmoplastic); *crib*, solid/cribiform; *TIL1*, rare TILs; and *TIL4*, numerous TILs involving the entire tumor area.

Table 1 *Comparison of clinical and pathological parameters between MSI groups*

Values represent the numbers of patients (No.), median age (range), or percentages within each of three categories of MSI (minimum of six markers assessed): MSS (no markers with instability); MSI-L (<40% of markers with instability); and MSI-H (≥40% of markers with instability).

Category	No.	Age (range)	Male (%)	Right-sided[a] (%)	hMLH1(−)[b] (%)	hMSH2(−) (%)	MSI-Path[c] (%)
MSS	54	71 (36–87)	57	39	0	0	6
MSI-L	12	66 (39–85)	67	67	0	0	42
MSI-H	41	73 (35–91)	44	85	95	5	71
P[d]		NS[e]	NS	0.001	0.001	0.036	0.001

[a] Right-sided lesions were defined as those proximal to the splenic flexure.
[b] hMLH1(−) and hMSH2(−) indicate the percentage of tumors showing absent staining for each protein.
[c] MSI-Path was defined if a tumor exhibited any of the following: solid/cribiform pattern, signet cell histology, and/or moderate to marked TILs.
[d] Reported *P*s are from associations with MSS *versus* MSI-H.
[e] NS, not significant.

staining for either hMLH1 (39 cases) or hMSH2 (2 case), whereas none of the MSS or MSI-L tumors showed an absence of staining for either protein.

COX-2 expression was then examined for associations with a number of molecular (Table 2), clinical (Table 3) and pathological (Table 4) parameters. Of the 107 cases examined, 70 (65%) showed absent or weak staining for COX-2 (scores 0 and 1), whereas 37 (35%) cases showed moderate to strong staining (scores 2 and 3). Tumors with absent COX-2 staining (score = 0) were distributed primarily into the MSI-H (61%) and hMSH1/hMLH2(−) (61%) groups (Table 2). In contrast, tumors with high levels of COX-2 expression (score = 3) were distributed predominately into the MSS (88%) and hMSH1/hMLH2(+) (88%) groups (Table 2). Highly significant trends in the proportion of COX-2 scores were detected over the levels of MSI ($P = 0.001$), hMLH1 staining ($P = 0.001$), and MSI-Path phenotype ($P = 0.001$). Similarly, the distribution of tumor histological patterns and TIL scores among COX-2 scores revealed that each of the tumor characteristics used to define MSI-Path (solid/cribiform, signet ring, and/or TIL score of 3–4) were also significantly associated with low or absent COX-2 staining (Table 3). Given the strong inverse association between COX-2 staining and MSI, it was not surprising that tumors with negative COX-2 staining (score = 0) occurred predominately in the right colon (79%, $P = 0.003$) and in females (57%, $P = 0.20$; Table 4). No difference in age was observed between groups by COX-2 score.

Table 2 *COX-2 scores among molecular features of defective DNA mismatch repair*

Values represent the number and percentage of tumors within each COX-2 score that fell into one of three MSI groups (MSS, MSH-L, and MSI-H) or into one of two groups defined by the presence (+) or absence (−) of staining for hMLH1 or hMSH2.

		MSI group			hMSH1/hMLH2	
COX-2 score	Total no.	MSS no. (%)	MSI-L no. (%)	MSI-H no. (%)	(+) no. (%)	(−) no. (%)
0	28	8 (28)	3 (11)	17 (61)	11 (39)	17 (61)
1	42	18 (43)	6 (14)	18 (43)	24 (57)	18 (43)
2	21	14 (67)	3 (14)	4 (19)	17 (81)	4 (19)
3	16	14 (88)	0 (0)	2 (12)	14 (88)	2 (12)
			$P = 0.001$		$P = 0.001$	

DISCUSSION

CRCs arise through at least two distinct genetic mechanisms, one involving chromosomal instability and another involving defective DNA mismatch repair (34). The present study indicates that CRCs with molecular and phenotypic characteristics of defective DNA mismatch repair are biologically distinct with respect to immunohistochemical expression of COX-2 protein. Our findings are consistent with the isolated report that the DLD1/HCT15 colon cancer cell line, which exhibits microsatellite instability and a frameshift mutation of *hMSH6*, does not express COX-2 (35). We speculate that this biological distinction reflects underlying differences in the molecular genesis of these tumors and, therefore, may have implications related to the efficacy of COX-2 inhibitors for chemoprevention of colorectal neoplasms that arise in the setting of defective DNA mismatch repair.

COX-2 is ordinarily expressed at very low levels in the colonic mucosa. However, approximately 50% of adenomas and 80% of carcinomas of the colorectum in humans express high levels of *COX-2* mRNA and protein (6). The mechanisms responsible for increased COX-2 expression in colorectal neoplasms remain unknown. Like most early-response genes, COX-2 levels are regulated at the level of transcription (36) and mRNA stability (37). *COX-2* promoter-reporter genes are efficiently transcribed in CRC cell lines that overexpress COX-2 (38), which suggests that transcription activation plays an important role in *COX-2* overexpression in colorectal neoplasms.

The known inducers of *COX-2* expression include tumor promoters and receptor-mediated signals triggered by TGF-β, interleukins, and ligands of the epidermal growth factor receptor (39, 40). Colorectal adenomas and cancers frequently express high levels of a variety of growth factors including epidermal growth factor receptor ligands (41) and TGF-β (42), which potentially could stimulate constitutive *COX-2* expression.

During colorectal carcinogenesis in APC± mice, induction of

Table 3 *COX-2 scores among pathological features of tumors*

Values represent the numbers and percentages (parentheses) of tumors within each COX-2 score categorized by histologic pattern, TIL score, and MSI-Path.

		Histologic pattern				TIL score		MSI-Path	
COX-2 score	Total (*n*)	Ordin[a] *n* (%)	Crib *n* (%)	Signet *n* (%)	Other *n* (%)	1–2[b] *n* (%)	3–4 *n* (%)	(−)[c] *n* (%)	(+) *n* (%)
0	28	14 (50)	8 (29)	3 (11)	3 (11)	16 (57)	12 (43)	11 (39)	17 (61)
1	42	29 (69)	7 (17)	2 (5)	4 (10)	33 (79)	9 (21)	26 (62)	16 (38)
2	21	20 (95)	1 (5)	0 (0)	0 (0)	18 (86)	3 (14)	18 (86)	3 (14)
3	16	15 (94)	1 (6)	0 (0)	0 (0)	16 (100)	0 (0)	15 (94)	1 (6)
			$P = 0.001$			$P = 0.001$		$P = 0.001$	

[a] Ordin, ordinary; Crib, solid/cribiform; Signet, signet ring.
[b] TIL score: 1–2, rare to few TILs, and 3–4, moderate to high numbers of TILs.
[c] MSI-Path (−), tumors that lack all MSI-Path (+) features; MSI-Path (+), tumors with any of the following features: solid/cribiform pattern, signet cell histology, and/or moderate to marked TILs.

Table 4 *COX-2 scores among clinical features*

COX-2 score	Total (*n*)	Side of colon: Left[a] *n* (%)	Side of colon: Right *n* (%)	Gender: Male *n* (%)	Gender: Female *n* (%)	Age median (range)
0	28	6 (21)	22 (79)	12 (43)	16 (57)	71 (45–88)
1	42	16 (38)	26 (62)	24 (57)	18 (43)	71 (35–85)
2	21	11 (52)	10 (48)	10 (48)	11 (52)	69 (46–91)
3	16	10 (63)	6 (38)	11 (69)	5 (31)	72 (45–80)
		$P = 0.003$		$P = 0.20$		$P = 0.89$

[a] Right-sided lesions included those proximal to the splenic flexure. Left-sided lesions included those distal to and including the splenic flexure.

COX-2 expression occurs coincidentally or slightly after the loss of the wild-type *APC* allele during adenoma development in suggesting a direct role of APC loss in COX-2 overexpression (12). APC loss is associated with formation of β-catenin/TCF complexes that activate transcription of *TCF* promoter elements (43, 44). TCF-like promoter elements are present in the *COX-2* promoter (45). Although unproven, these observations raise the possibility that COX-2 may be transcriptionally regulated by TCF/β-catenin complexes that accumulate in tumors with APC loss.

The reduced COX-2 expression in CRCs with defective DNA mismatch repair suggests that mechanisms responsible for COX-2 induction are suppressed in these tumors. Of potential significance in this regard, tumors with MSI frequently have mutations at an instability locus in the *TGF-βII* receptor gene resulting in absent or nonfunctioning TGF-βII receptors (46, 47). Thus, CRCs with MSI and mutant TGF-βII receptor may lack a signal important for constitutive COX-2 induction. Alternatively, mechanistic links between APC loss and COX-2 expression, if they exist, may not be operative in many tumors that arise through defective DNA mismatch repair, which may have a lower incidence of APC mutations (48). Finally, reduced COX-2 expression in sporadic MSI-H tumors could be related to hypermethylation of its promoter. Although not yet demonstrated for the COX-2 promoter, this epigenetic mechanism is proposed to cause loss of expression of tumor suppressor genes and DNA mismatch repair enzymes in tumors with MSI (49–53).

The clinical significance of our findings is unknown. By generating prostanoids, regulating arachidonic acid levels, and promoting release of angiogenic factors, COX-2 activity may provide survival advantage for early neoplasms by promoting cell growth, survival, and angiogenesis (54–56). Our observations suggest that CRCs with defective DNA mismatch repair develop independently of COX-2 expression. However, our data do not necessarily imply that NSAIDs or selective COX-2 inhibitors will fail to prevent colorectal neoplasms arising in the setting of defective DNA mismatch repair: (*a*) debate continues concerning the role of COX-2 in the antitumor effects of NSAIDs (57); (*b*) we did not assess COX-1, which may be equally important for supporting neoplastic growth and angiogenesis (56); (*c*) defective DNA mismatch repair and low COX-2 levels may occur late in the sequence of molecular events leading to sporadic carcinomas and, therefore, may have no bearing on the preventative efficacy of COX-2 inhibitors; and (*d*) the absence of immunohistochemically detectable *COX-2* does not exclude the possibility that COX-2 activity may be sufficient to support the growth and survival of these tumors.

Despite these caveats, our observations raise the concern that COX-2 inhibitors may not be effective chemopreventative agents in patients whose tumors arise in the setting of defective DNA mismatch repair. Our observations encourage analysis of MSI, *COX-2* expression, and prostanoid levels in "breakthrough" adenomas as an integral part of future chemoprevention studies of COX-2 inhibitors in sporadic and HNPCC cases.

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