PRELIMINARY COMMUNICATION

# The Relationship Between Cyclooxygenase-2 Expression and Colorectal Cancer

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**Context** Epidemiological studies have implicated the inducible form of cyclooxygenase (COX-2) in the pathogenesis of colorectal cancer; however, its role is not fully understood.

**Objective** To examine the relationship between the expression of COX-2 in human colorectal cancer and patient survival.

**Design** Patients diagnosed as having colorectal cancer were evaluated and followed up for up to 9.4 years (median follow-up, 2.7 years). Tumor sections were stained for COX-2 using a rabbit polyclonal antibody raised against human COX-2. The extent of COX-2 staining was graded by 2 observers blinded to outcome. Preabsorption of the anti–COX-2 antibody with a COX-2 peptide abolished the staining, demonstrating the specificity of the assay.

**Setting** Gastrointestinal unit of a large general teaching hospital in Dublin, Ireland.

**Participants** Seventy-six patients (median age, 66.5 years) with colorectal cancer (Dukes tumor stage A, n = 9; Dukes B, n = 30; Dukes C, n = 25; Dukes D, n = 12) whose diagnosis was made between 1988 and 1991. Fourteen normal colon biopsies were stained for COX-2 as controls.

**Main Outcome Measures** Survival in years following diagnosis compared by extent of COX-2 epithelial staining (grade 1, <1%; grade 2, 1%-19%; grade 3, 20%-49%; grade 4, ≥ 50%), Dukes stage, tumor size, and lymph mode metastasis.

**Results** COX-2 was found in tumor epithelial cells, inflammatory cells, vascular endothelium, and/or fibroblasts. The extent of epithelial staining was heterogeneous, varying markedly among different tumors. Normal tissue adjacent to the tumors also stained weakly for COX-2. No COX-2 was detected in control tissue samples. The Kaplan-Meier survival estimate was 68% in patients who had grade 1 tumor epithelial staining compared with 35% in those with higher grades combined (log-rank $\chi^2 = 5.7$; $P = .02$). Greater expression of COX-2 correlated with more advanced Dukes stage (Kendall $\tau$-b, 0.22; $P = .03$) and larger tumor size (Kendall $\tau$-b, 0.21; $P = .02$) and was particularly evident in tumors with lymph node involvement (Kendall $\tau$-b, 0.26; $P = .02$).

**Conclusions** Our data indicate that COX-2 expression in colorectal cancer may be related to survival. These data add to the growing epidemiological and experimental evidence that COX-2 may play a role in colorectal tumorigenesis.

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COLORECTAL CANCER IS THE third most common cancer in both men and women in most Western countries, although incidence and mortality rates are decreasing in the United States.[1] Numerous studies have shown that the level of spread through the bowel wall and adjacent lymph node involvement are primary predictors of long-term outcome in patients with colorectal cancer.[2] Other features known to be related to survival include vascular and perineural invasion, tumor necrosis, character of invasive margin, and differentiation.[3]

Epidemiological studies have shown a lower than expected rate of colorectal adenomas and carcinomas in subjects who have taken nonsteroidal anti-inflammatory drugs (NSAIDs) for extended periods,[4-8] which suggests a pathogenic role for cyclooxygenase (COX) in colonic tumorigenesis. Consistent with these findings, the NSAID sulindac causes regression in polyp size and number in patients with familial adenomatous polyposis,[9] a precancerous lesion. Two forms of COX derived from separate genes have been described: COX-1, which is constitutively expressed in cells, and an inducible form, COX-2, which is usually absent or present only in low amounts in most normal cells.[10] COX-2 is induced by cytokines and growth factors[11,12] and has been found in human colorectal cancers.[13] Disruption of the *COX-2* gene or selective COX-2 inhibition in the *Min-1* mouse models of polyposis coli dramatically reduces polyp growth, which provides evidence of a role for COX-2 in the development of colonic tumors in mice.[14] However, it is not known what role, if

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any, COX-2 plays in human colon cancer. In this study, we demonstrate that the expression of COX-2 is particularly evident in aggressive tumors and correlates with a reduced survival.

## METHODS

We evaluated 76 patients (50% male; median age, 66.5 years) with colorectal cancer diagnosed between 1988 and 1991. Cases were selected on the basis of tissue availability from the archives. The tumor stages were as follows at diagnosis: Dukes A (n = 9), Dukes B (n = 30), Dukes C (n = 25), and Dukes D (n = 12). All tumors were adenocarcinomas. Forty-six percent (35/76) of tumors were in the rectum, 12% in the caecum (9/76), and 42% (32/76) in the colon. Three patients had a coexisting second bowel carcinoma, and 14 patients had a separate adenoma at diagnosis. Forty-nine percent had lymph node metastasis. All patients underwent surgical resection of their tumors. It is worth noting that prior studies using the same database found only 6 of 266 Dukes B and C patients were given chemotherapy during this period.[15,16] In addition, 14 normal colon biopsies were stained for COX-2 as a control. There were 8 women and 6 men, with a median age of 65 years (range, 44-80 years). Four biopsy specimens were from the rectum; 8, the colon; and 2, the cecum. The indications for colonoscopy included bleeding from rectum, altered bowel habits, abdominal pain, weight loss, and diverticular disease.

Immunohistochemistry was performed using an avidin-biotin peroxidase complex procedure as described previously[13] in which COX-2 stained a brown color (Vector Laboratories, Burlingame, Calif). Primary antibody against human COX-2 (Cayman Chemical, Ann Arbor, Mich) was applied to the sections at a dilution of 1:500. As a control, additional sections were incubated with normal rabbit serum. Specificity was determined by preabsorption of anti–COX-2 antibody with a COX-2 synthetic polypeptide (Cayman Chemical) prior to staining.

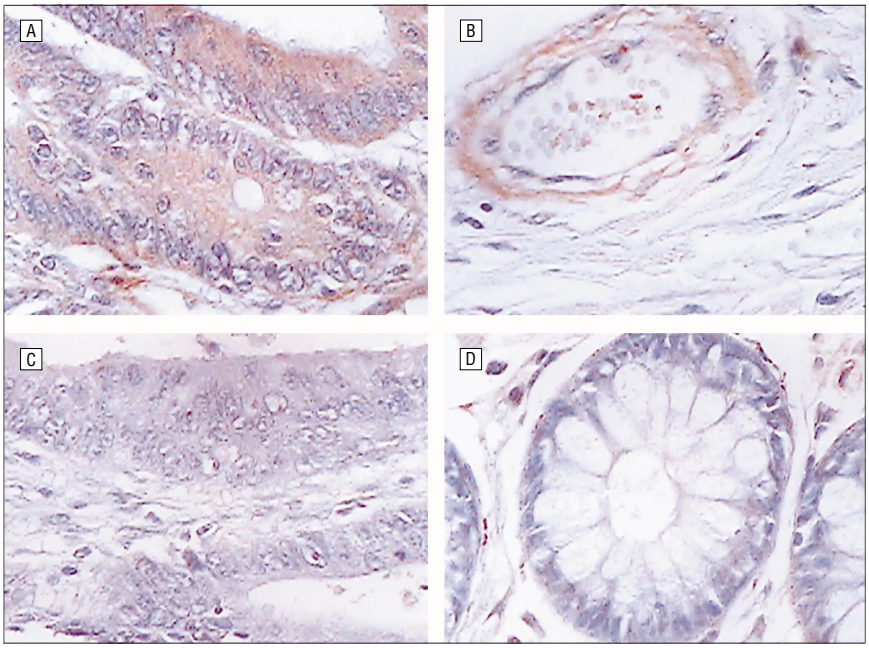
The extent of staining was recorded using 4 grades, depending on the percentage of tumor epithelial cells staining for COX-2: grade 1, less than 1%; grade 2, 1% to 19%; grade 3, 20% to 49%; and grade 4, 50% or more. For each tissue specimen, the extent of staining with COX-2 antibody was graded by 2 observers blinded to outcome, using coded slides ($\kappa = 0.91$ for COX-2 absent or present and 0.90 for COX-2 grades 1-4). The cellular location of COX-2 staining was also recorded. Furthermore, each tumor was scored by pathologists for 14 histological features and associated pathological parameters, many of which have been shown previously to predict prognosis in colorectal cancer.[17,18]

Survival was measured from the time of diagnosis until death. The median follow-up was 2.7 years, with 75% of patients followed up for 7.6 years or less. The probability of survival over time was estimated using Kaplan-Meier product limit survival curves. Differences in observed survival between groups were tested for statistical significance using the Mantel-Haenszel test. The survival analyses were corrected for cancer-related deaths only. Cox proportional hazards regression was used to simultaneously determine the effect of several potential confounders on the relation of COX-2 to survival. In addition, the relationship between COX-2 and pathological features was determined using Kendall $\tau$-b correlation.

## RESULTS

COX-2 was present in all of the tumors studied. The extent of staining and the cell types that stained for COX-2 varied. COX-2 was found mainly in the tumor epithelial cells, where it was localized within the cytoplasm and in the endothelial cells of tumor vessels (FIGURE 1, A, B). There was also staining of inflammatory mononuclear cells and fibroblasts. However, in 14 tumors, COX-2 expression was limited to tumor vascular endothelium only. Immunostaining with anti–COX-2 antibody preabsorbed with the synthetic COX-2 peptide was completely negative (Figure 1, C). Normal colonic tis-

**Figure 1.** Cells Stained for Cyclooxygenase-2 (COX-2)




A, COX-2 expression in colon cancer epithelial cells; B, COX-2 expression in tumor vascular endothelium and stroma; C, inhibition of COX staining in colon cancer epithelial cells after preabsorption of anti–COX-2 antibody with COX-2 polypeptide; and D, normal colonic epithelium cells. COX-2 staining appears brown. Following antibody incubation, color was developed by immersion of the sections in diaminobenzidine tetrahydrochloride/hydrogen peroxide solution for 2 minutes. Sections were counterstained with hematoxylin (original magnification, ×40).

**Table 1.** Distribution of Individuals in Each COX-2 Grade by Dukes Stages and Lymph Node Involvement*

| | COX-2 Grade | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Total |
| Dukes Stage† | | | | | |
| A | 3 | 0 | 3 | 3 | **9** |
| B | 9 | 3 | 4 | 14 | **30** |
| C | 2 | 2 | 3 | 18 | **25** |
| D | 0 | 3 | 2 | 7 | **12** |
| **Total** | **14** | **8** | **12** | **42** | **76** |
| Lymph node involvement‡ | | | | | |
| Yes | 2 | 5 | 5 | 25 | **37** |
| No | 12 | 3 | 7 | 17 | **39** |
| **Total** | **14** | **8** | **12** | **42** | **76** |

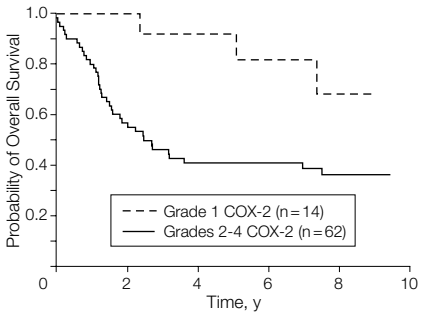
*COX indicates cyclooxygenase. Staining is the percent of tumor epithelial cells staining for COX-2: 1, <1%; 2, 1%-19%; 3, 20%-49%; and 4, ≥50%.
†Kendall τ-b, 0.22; $P = .03$.
‡Kendall τ-b, 0.26; $P = .02$.

**Table 2.** Relationship Between Extent of Epithelial Staining (Grades 1-4) for COX-2 and Clinical and Pathological Features of Colorectal Tumors*

| Pathological Feature | Kendall τ-b | *P* Value |
|---|---|---|
| Lymph node involvement | 0.26 | .02 |
| Maximum tumor diameter | 0.21 | .02 |
| Dukes stage | 0.21 | .03 |
| Crohnlike reaction | −0.21 | .06 |
| Neural invasion | 0.20 | .08 |
| Primary site | 0.18 | .10 |
| Differentiation | 0.12 | .27 |
| Level of spread | 0.09 | .42 |
| Necrosis | 0.07 | .50 |
| Tumor type | 0.06 | .58 |
| Vascular invasion | 0.03 | .77 |

*COX indicates cyclooxygenase. See footnote to Table 1 for definition of COX-2 grades.

**Figure 2.** Kaplan-Meier Survival Estimates by Cyclooxygenase (COX-2) Epithelial Staining in Colorectal Cancer Tumors




Relative risk, 3.77; log-rank $\chi^2 = 5.7$ ($P = .02$). See the "Methods" section for an explanation of COX-2 grades.

sue adjacent to the COX-2 positive tumors also stained weakly for COX-2 (Figure 1, D). The number of tumors in each COX-2 epithelial staining group is represented in TABLE 1, which shows that most tumors had extensive staining for COX-2. In contrast, no COX-2 was detected in the set of 14 normal samples from patients without cancer.

We examined the relationship between extent of COX-2 expression and various clinical and pathological features that may affect prognosis (TABLE 2). The extent of COX-2 staining was greater in more advanced Dukes stage tumors ($P = .03$), in larger tumors ($P = .02$), and in those patients who had lymph node involvement ($P = .02$). Patients with high COX-2 expression (grade 4) were 4 times more likely to be classified as Dukes C and D and to have lymph node metastasis than patients with low levels of COX-2 expression (grade 1) (Table 1). Interestingly, there was no correlation between COX-2 expression and level of local tumor spread.

Survival analyses were carried out with a maximum follow-up of 9.4 years (median follow-up, 2.7 years). We observed a relationship between the presence of COX-2 expression and patient survival, although there was no evidence of a graded relationship. The Kaplan-Meier survival estimate of those patients with less than 1% COX-2 tumor epithelial staining was significantly better than patients with higher grades of COX-2 combined (log-rank $\chi^2 = 5.7$; $P = .02$) (FIGURE 2). Furthermore, the 5-year survival rate in the absence of COX-2 (grade 1) was 91.6% compared with 40.5% in patients with tumors that expressed COX-2 (grades 2-4) (log-rank $\chi^2 = 8.3$; $P = .004$). Cox proportional hazards regression showed a hazard ratio of 3.8 for COX-2 unadjusted for other variables. The assumption of proportionality for the Cox model was tested and confirmed that the 2 survival functions were approximately parallel. Adjustment for Dukes stage and lymph node involvement reduced the hazard ratio associated with COX-2 expression from 3.8 to 2.1, and while the latter figure is not statistically significant, the confidence interval for the estimate is wide (0.6-7.3). Furthermore, a Wald test showed no significant difference between the coefficients for COX-2 in the unadjusted and adjusted models ($P = .34$). Addition of age, sex, tumor size, differentiation, TNM staging, vascular invasion, neural invasion, character of invasive margin, Crohnslike reaction,[19] and necrosis had no further effect on the model. There was no significant difference ($P = .10$) between COX-2 expression in colonic and rectal carcinomas and survival.

## COMMENT

The major observations in this study are that the level of COX-2 expression in colorectal cancer varies among tumors and the extent of COX-2 staining correlates with a poorer prognosis. The colorectal cancer database used in this study was established in 1983 and contains prospectively gathered data on all patients presenting with colorectal tumors to a large gastrointestinal service in a Dublin teaching hospital. The database provides a unique means to prospectively examine the factors that determine outcome and has been applied in other areas of colorectal cancer research, particularly in relation to pathological characteristics of prognosis[20] and the identification of new prognostic markers.[16]

In this small study, we demonstrated that the extent of COX-2 expression in colorectal tumor epithelial cells is related to survival. We also showed a relationship between COX-2 staining and advancing Dukes tumor stage. This is not surprising because the Dukes staging system, which is based on level of spread and lymphatic involvement, is also related to survival.[2] Colon cancer cells expressing COX-2 are more invasive, possibly due to enhanced expression of metalloproteinases.[21] It also has been reported that COX-2 is present in epithelial cells of more invasive carcinomas[22,23] but not in epithelial cells of adenomas.[14] Consistent with these findings, our data show a relationship between lymph node metastasis, tumor size, and COX-2 expression. Two additional studies have shown that COX-2

expression correlates with the size and invasiveness of colorectal carcinomas and adenomas, although no survival data were provided.[22,23] In a study of 43 specimens, Fujita et al[22] identified a size- and invasion-dependent increase in COX-2 levels in human colorectal carcinomas. Yang et al[23] demonstrated a size-dependent increase in prostanoids in adenomas of familial adenomatous polyposis patients. Another study[24] did not find such a relationship in 25 cases of colorectal cancer, although that study had only 1 Dukes A and 1 Dukes D patient.

A limitation of this study is the use of immunohistochemistry alone as a method for evaluating the expression of COX-2. However, staining for COX-2 was abolished by preabsorption with the signal peptide against which the COX-2 antibody was raised, confirming its specificity. A second limitation is the small study size. Few of the patients (14/76) had tumors in which the epithelial cells failed to express COX-2, which is consistent with previous data demonstrating COX-2 in the majority of colon cancers.[25] However, the database is unique in that it combines histological and clinical data with an extended follow-up of survival. There are no data available on the use of NSAIDs in our population. However, because NSAIDs do not suppress COX-2 expression (these drugs reduce COX-2 activity), use of NSAIDs would have lessened rather than exaggerated the relationship between COX-2 and survival.

It is not clear from our study whether COX-2 plays a primary role or is simply a coincidental finding. A primary role for COX-2 is consistent with the epidemiological data linking reduced incidence of colorectal tumors and NSAID use, including aspirin.[4-6] The NSAID sulindac reduces polyp number by 44% and polyp size by 35% of pretreatment values in patients with familial adenomatous polyposis.[9] In animals, indomethacin, ibuprofen, piroxicam, sulindac, and aspirin all suppress colon tumorigenesis.[26-29] The results of these studies are impressive, but because these compounds inhibit both isoforms, it is not clear which isoform of COX is involved. Our study provides evidence that COX-2 expression is associated with a worse prognosis in colorectal cancer. Prostaglandins promote cell proliferation and tumor growth[7,30] and COX-2 inhibitors induce apoptosis in cell lines derived from colonic tumors and in familial adenomatous polyposis.[31-33] Moreover, COX-2 regulates angiogenesis in colorectal cancer.[34] These studies and those relating tumor COX-2 and invasiveness described above[22,23] suggest that COX-2 may contribute to colonic tumorigenesis by promoting cell growth, new vessel formation, invasiveness, and metastatic potential.

In conclusion, we have demonstrated that expression of COX-2 in tumor epithelial cells is related to lymph node metastasis, advanced Dukes staging, and poorer long-term outcome for patients with colorectal cancer.

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seling session with simulated patients. Two independent observers rated the sessions using a 10-point checklist based on the Agency for Health Care Policy and Research (now called the Agency for Healthcare Research and Quality [AHRQ]) criteria. Student scores ranged from 6.5 to 10. Only 10% of the scores were below 8, with a high level of interobserver agreement ($r$ = 0.847).

Our data show that students can readily learn cessation counseling skills and such performance can be reliably evaluated.

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**In Reply:** While conducting our survey, we learned from faculty at several medical schools that clinical training and evaluation of nicotine dependence is most commonly incorporated in the primary care clerkships, as described by Dr Wadland and colleagues. Objective structured clinical examinations (OSCEs) are increasingly used in the third and fourth years to evaluate mastery of interviewing, physical diagnosis, and counseling skills. We know of a few medical schools that currently incorporate nicotine dependence treatment into their OSCE panel of cases, similar to those at the Michigan State University College of Human Medicine. Their encouraging outcome is consistent with other studies that show that medical students can develop skills in counseling nicotine-dependent patients.[1,2] Unfortunately, schools with such commitments to nicotine dependence education remain in the minority.

Since our 1997 survey, other schools may have improved their nicotine dependence curricula. We are soliciting any teaching material, (eg, curriculum goals and objectives, OSCE case descriptions, videotapes, teaching manuals, handouts, interactive computer training program, role playing, problem-based learning, and evaluation tools) to create a syllabus of the best examples for distribution to all medical school faculty, and would be happy to receive any such material.

The release of the revised AHRQ clinical practice guidelines for smoking cessation in 2000 will be an opportunity for medical schools to reevaluate their basic and clinical science curriculum for the content areas suggested as requisite for all physicians.[3] If the clinical training skills recommended by the AHRQ panel are followed, the next survey of medical school curricula would ideally find no medical school reporting less than 1 hour of nicotine dependence training in the clinical years. In our survey, this minimal level was not met in 46.6% of the third-year curricula and in 79.3% of fourth-year curricula.

The most costly and lethal health behavior in the United States is still overlooked in most medical school curricula. The simple inclusion of didactic presentations, without evidence that students can demonstrate skills and application of the knowledge, is not the answer. Curricula like those described by Wadland and colleagues demonstrate the effective training physicians will need for nicotine dependence interventions in the 21st century.

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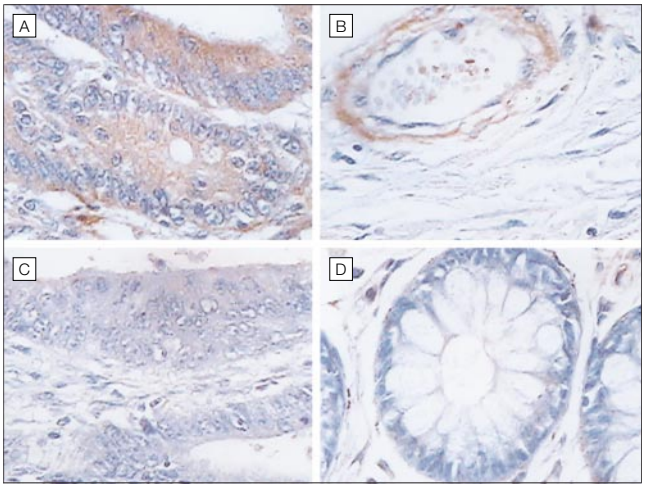
## CORRECTIONS

**Omission:** In the Preliminary Communication entitled "The Relationship Between Cyclooxygenase-2 Expression and Colorectal Cancer" in the October 6, 1999, issue of THE JOURNAL (1999;282:1254-1257), Ms Theresa Keane was omitted from the acknowledgment. The authors thank Ms Keane for her technical assistance.

**Incorrect Color Reproduction:** In the same Preliminary Communication, the colors of the 4 photomicrographs in Figure 1 on page 1255 were incorrectly reproduced. The correct image is shown below.

**Figure 1.** Cells Stained for Cyclooxygenase-2 (COX-2)




A, COX-2 expression in colon cancer epithelial cells; B, COX-2 expression in tumor vascular endothelium and stroma; C, inhibition of COX staining in colon cancer epithelial cells after preabsorption of anti–COX-2 antibody with COX-2 polypeptide; and D, normal colonic epithelium cells. COX-2 staining appears brown. Following antibody incubation, color was developed by immersion of the sections in diaminobenzidine tetrahydrochloride/hydrogen peroxide solution for 2 minutes. Sections were counterstained with hematoxylin (original magnification ×40).

seling session with simulated patients. Two independent observers rated the sessions using a 10-point checklist based on the Agency for Health Care Policy and Research (now called the Agency for Healthcare Research and Quality [AHRQ]) criteria. Student scores ranged from 6.5 to 10. Only 10% of the scores were below 8, with a high level of interobserver agreement ($r$ = 0.847).

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**3.** US Department of Health and Human Services. *Smoking Cessation, Clinical Practice Guideline No. 18.* Rockville, Md: US Dept of Health and Human Services, Agency for Health Care Policy and Research; 1996. AHCPR publication 96-0692.
**4.** Mavis BE, Henry RC, Ogle KS, Hoppe RB. The emperor's new clothes: the OSCE reassessed. *Acad Med.* 1996;71:447-453.

**In Reply:** While conducting our survey, we learned from faculty at several medical schools that clinical training and evaluation of nicotine dependence is most commonly incorporated in the primary care clerkships, as described by Dr Wadland and colleagues. Objective structured clinical examinations (OSCEs) are increasingly used in the third and fourth years to evaluate mastery of interviewing, physical diagnosis, and counseling skills. We know of a few medical schools that currently incorporate nicotine dependence treatment into their OSCE panel of cases, similar to those at the Michigan State University College of Human Medicine. Their encouraging outcome is consistent with other studies that show that medical students can develop skills in counseling nicotine-dependent patients.[1,2] Unfortunately, schools with such commitments to nicotine dependence education remain in the minority.

Since our 1997 survey, other schools may have improved their nicotine dependence curricula. We are soliciting any teaching material, (eg, curriculum goals and objectives, OSCE case descriptions, videotapes, teaching manuals, handouts, interactive computer training program, role playing, problem-based learning, and evaluation tools) to create a syllabus of the best examples for distribution to all medical school faculty, and would be happy to receive any such material.

The release of the revised AHRQ clinical practice guidelines for smoking cessation in 2000 will be an opportunity for medical schools to reevaluate their basic and clinical science curriculum for the content areas suggested as requisite for all physicians.[3] If the clinical training skills recommended by the AHRQ panel are followed, the next survey of medical school curricula would ideally find no medical school reporting less than 1 hour of nicotine dependence training in the clinical years. In our survey, this minimal level was not met in 46.6% of the third-year curricula and in 79.3% of fourth-year curricula.

The most costly and lethal health behavior in the United States is still overlooked in most medical school curricula. The simple inclusion of didactic presentations, without evidence that students can demonstrate skills and application of the knowledge, is not the answer. Curricula like those described by Wadland and colleagues demonstrate the effective training physicians will need for nicotine dependence interventions in the 21st century.

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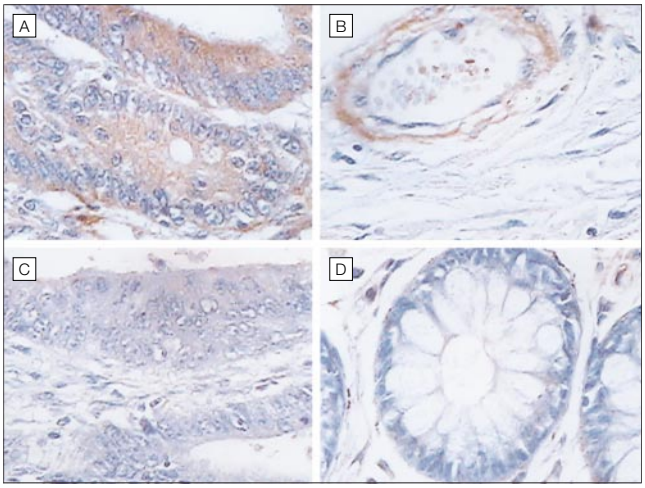
**1.** Coultas DB, Klecan DA, Whitten RM, et al. Training medical students in smoking-cessation counseling. *Acad Med.* 1994;69:S48-S50.
**2.** Allen SS, Bland CH, Dawson SJ. A mini-workshop to train medical students to use a patient-centered approach to smoking cessation. *Am J Prev Med.* 1990;6:28-33.
**3.** US Department of Health and Human Services. *Smoking Cessation, Clinical Practice Guideline No. 18.* Rockville, Md: US Dept of Health and Human Services, Agency for Health Care Policy and Research; 1996. AHCPR publication 96-0692.

## CORRECTIONS

**Omission:** In the Preliminary Communication entitled "The Relationship Between Cyclooxygenase-2 Expression and Colorectal Cancer" in the October 6, 1999, issue of THE JOURNAL (1999;282:1254-1257), Ms Theresa Keane was omitted from the acknowledgment. The authors thank Ms Keane for her technical assistance.

**Incorrect Color Reproduction:** In the same Preliminary Communication, the colors of the 4 photomicrographs in Figure 1 on page 1255 were incorrectly reproduced. The correct image is shown below.

**Figure 1.** Cells Stained for Cyclooxygenase-2 (COX-2)




A, COX-2 expression in colon cancer epithelial cells; B, COX-2 expression in tumor vascular endothelium and stroma; C, inhibition of COX staining in colon cancer epithelial cells after preabsorption of anti–COX-2 antibody with COX-2 polypeptide; and D, normal colonic epithelium cells. COX-2 staining appears brown. Following antibody incubation, color was developed by immersion of the sections in diaminobenzidine tetrahydrochloride/hydrogen peroxide solution for 2 minutes. Sections were counterstained with hematoxylin (original magnification ×40).