Cyclooxygenase 2 Expression in Colorectal Cancer with DNA Mismatch Repair Deficiency

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Abstract  Background: Cyclooxygenase 2 (COX-2) overexpression is a frequent but not universal event in colorectal cancer. It has been suggested that COX-2 protein expression is reduced in colorectal cancer with a defective mismatch repair (MMR) system, a phenomenon commonly associated with hereditary nonpolyposis colorectal cancer (HNPCC) but also present in up to 15% of sporadic tumors.

Aim: To assess COX-2 expression in a large series of fully characterized colorectal cancer patients with respect to the MMR system and to dissect the mechanisms responsible for altered COX-2 expression in this setting.

Patients and Methods: MMR-deficient colorectal cancer were identified in a nationwide, prospective, multicenter study (EPICOLON project). Control MMR-proficient colorectal cancer patients were randomly selected. COX-2 expression was evaluated by immunohistochemistry. Personal and familial characteristics, as well as MSH2/MLH1 expression and germ line mutations, were evaluated.

Results: One hundred fifty-three patients, 46 with MMR deficiency and 107 with MMR proficiency, were included in the analysis. Overall, tumor COX-2 overexpression was observed in 107 patients (70%). COX-2 overexpression was observed in 85 patients (79%) with a MMR-proficient system, but only in 22 patients (48%) with a MMR-deficient colorectal cancer (P<0.001). The lack of COX-2 overexpression was independently associated with a MMR-deficient system (odds ratio, 3.89; 95% confidence interval, 1.78-8.51; P=0.001) and a poor degree of differentiation (OR, 3.83; 95% CI, 1.30-11.31; P=0.015). In the subset of patients with a MMR-deficient colorectal cancer, lack of COX-2 overexpression correlated with a poor degree of differentiation, no fulfillment of Amsterdam II criteria, absence of MSH2/MLH1 germline mutations, presence of tumor MSH2 expression, and lack of tumor MLH1 expression. CpG island promoter hypermethylation of COX2 was observed in 6 of 18 (33%) tumors lacking COX-2 expression in comparison with 2 of 28 (7%) tumors expressing this protein (P=0.04).

Conclusions: Up to half of MMR-deficient colorectal cancer do not show COX-2 overexpression, a fact observed almost exclusively in patients with sporadic forms. COX2 hypermethylation seems to be responsible for gene silencing in one third of them. These results suggest the potential utility of nonsteroidal anti-inflammatory drugs in HNPCC chemoprevention and may explain the lack of response of this approach in some sporadic tumors.
Colorectal cancer is the second leading cause of cancer-related death in Western countries in spite of progressive refinements in preventive, diagnostic, and therapeutic approaches (1). In the last two decades, a tremendous advance has been experienced with respect to the knowledge of molecular mechanisms involved in its pathogenesis (2, 3). At present, two main pathways are well established: first, the “suppressor pathway,” involving activation of oncogenes and inactivation of tumor suppressor genes and responsible for most sporadic colorectal cancer forms (2), and second, the “mutator pathway,” in which an altered DNA mismatch repair (MMR) system leads to replication errors in tumor cells (4, 5). This alteration underlies the majority of hereditary nonpolyposis colorectal cancer (HNPCC) patients (6, 7), but it can also be observed in up to 15% of sporadic colorectal cancer cases (8). Whereas HNPPCC is caused by germ line mutations in MMR genes, mainly MSH2, MLH1, and MSH6 (9), defects on this pathway are due to MLH1 promoter methylation in sporadic forms (10).

A large body of evidence indicates that the use of nonsteroidal anti-inflammatory drugs (NSAID) can reduce the risk of colorectal cancer (11). Experimental studies have shown that NSAIDs decrease the incidence of carcinogen-induced colon tumors in rodents (12, 13), and several epidemiologic investigations have also shown a 40% to 50% reduction in the risk of colorectal adenoma and cancer development in patients taking NSAIDs (14–17). Moreover, individuals with familial adenomatous polyposis taking sulindac or celecoxib experience a reduction in adenoma size and number (18–20). These chemopreventive effects of NSAIDs may be largely related to inhibition of cyclooxygenase 2 (COX-2; refs. 21, 22), the inducible isoform of COX that catalyzes the conversion of arachidonic acid to prostaglandins (23).

COX-2 overexpression is a frequent but not universal event in colorectal neoplasms. Indeed, ~50% of adenomas and 80% of colorectal cancer express high levels of COX-2 mRNA and protein in neoplastic tissue (24). Interestingly, a reduced COX-2 protein expression has been shown in colorectal cancer with defective MMR system (25) in a similar manner as in other MMR-deficient neoplasms (26, 27). In a seminal study (28), it was suggested that this reduced expression might affect HNPPCC patients, although the mechanism underlying this phenomenon was unclear. Moreover, a parallel investigation from the same group showed promoter hypermethylation of COX2 in colorectal cancer samples, thus suggesting that the reduced expression of COX-2 may also affect sporadic MMR-deficient forms (29). In addition to these apparently opposing results, it is important to note that COX2 hypermethylation did not correlate with the presence of microsatellite instability in this latter investigation (29). Altogether, these results emphasize the fact that the putative low COX-2 expression in MMR-deficient colorectal cancer, a relevant issue with potential implications in prevention and prognosis, is poorly understood. Specific characteristics of previous studies and a possible selection bias in the recruitment of patients might have precluded to elucidate this issue.

In 2001, a prospective, multicenter, nationwide, population-based study was launched to ascertain the incidence of HNPPCC and other familial colorectal cancer forms in Spain (30), as well as to establish the most effective and efficient strategy for the detection of MSH2/MLH1 gene carriers in newly diagnosed colorectal cancer patients (31). Besides these primary goals, the EPICOLON study provided a unique opportunity to prospectively assess the expression of COX-2 in a large series of colorectal cancer patients fully characterized with respect to the MMR system and to dissect the mechanisms responsible for the altered COX-2 expression in this setting.

**Patients and Methods**

**Patient population.** Between November 2000 and October 2001, all newly diagnosed colorectal cancer patients in 25 hospitals were included in the EPICOLON study (30). Eighteen of these 25 centers agreed to participate in a nested case-control study to evaluate COX-2 expression in tumors with MMR deficiency. Exclusion criteria were familial adenomatous polyposis, personal history of inflammatory bowel disease, and patient refusal to participate in the study. The study was approved by the institutional ethics committee of each participating hospital, and written informed consent was obtained from all patients.

Cases were identified among those patients with MMR-deficient tumors, shown by microsatellite instability and/or lack of MSH2/MLH1 protein expression. Two control colorectal cancer patients with MMR proficiency were randomly selected per each case subject, after stratification by center. Patients receiving aspirin or other NSAIDs were excluded.

Demographic, clinical, and tumor-related characteristics of probands, as well as a detailed family history, were obtained using a preestablished questionnaire, as described elsewhere (31).

**Evaluation of the MMR system.** Microsatellite instability testing and immunostaining for MMR proteins were done in all patients regardless of age, personal or family history, and tumor characteristics. Microsatellite instability testing was done using BAT-26 mononucleotide marker based on its high sensitivity, as described elsewhere (31). Immunostaining was done using mouse monoclonal antibodies against MLH1 protein (clone G168-15, dilution 1:40; PharMingen, San Diego, CA) and MSH2 protein (clone FE11, dilution 1:35; Oncogene Research Products, Boston, MA) as it is described elsewhere (31).

Patients found to have tumors with microsatellite instability and/or lack of protein expression of either MSH2 or MLH1 underwent germline genetic testing for MSH2 and MLH1 by both multiple ligation probe amplification analysis and sequencing as described elsewhere (31).

**Tumor COX-2 protein expression.** One block of formalin-fixed, paraffin-embedded tumor tissue was selected per case. Four-micrometer-thick sections were dewaxed and rehydrated using xylene and alcohol. Before immunostaining, antigen retrieval was done by immersing sections in a 10 mmol/L concentration of citrate buffer (pH 6.0) and boiling in a pressure cooker for 3 minutes. Sections were then incubated for 20 minutes at room temperature with mouse monoclonal antibodies against COX-2 (clone PG 27b, dilution 1:400, Oxford Biomedical Research, Oxford, MI). The Ultra-Vision streptavidin-biotin peroxidase detection kit (DAKO, Carpinteria, CA) was used as secondary detection system. The peroxidase reaction was developed using diaminobenzidine tetrachloride as chromogen.

Staining was scored for intensity (0, 1+, 2+, or 3+) and percentage of cytoplasm staining in malignant cells (1, 0-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%; Fig. 1). The sum of intensity and percentage counts was used as the final score (32). Cytoplasm staining in normal epithelial cells (discrete positivity near the nucleus on the apical side of epithelial cells both in the normal crypt and the surface epithelium; ref. 33) in each slide served as internal positive controls. Pathologists (C. Alenda and A. Payá) were blinded to the results of microsatellite instability testing and MSH2/MLH1 immunostaining.
**Cox2 CpG island methylation analysis.** Cox2 CpG island methylation status was determined by PCR analysis of bisulfite-modified genomic DNA, which induces chemical conversion of unmethylated, but not methylated, cytosine to uracil, using methylation-specific PCR with primers specific for either the methylated or modified unmethylated DNA (34). Primer sequences of Cox2 for the unmethylated reaction were 5'-GAGAGGGGATTTTTTGTTTTT-3' (sense) and 5'-CCCACAACACTCCAAAAACC-3' (antisense) and for the methylated reaction, 5'-GAGGGGATTTTTTGCGTTTTC-3' (sense) and 5'-CCGAACGCTTCCGAAAAC-3' (antisense). Primers were located encompassing the transcription start site. The annealing temperature for both unmethylated and methylated reactions was 60°C. DNA from normal lymphocytes treated in vitro with SssI methyltransferase was used as a positive control for methylated alleles. DNA samples from normal lymphocytes and adrenal medulla were used as a positive control for unmethylated alleles. PCR products were loaded onto nondenaturing 3% polyacrylamide gels, stained with ethidium bromide, and visualized under a UV transilluminator.

**Statistical analysis.** Continuous variables were expressed as mean ± SD. Differences in qualitative variables were evaluated by means of χ² test or the Fisher's exact test when necessary. Continuous variables were compared by means of the Student’s t test.

Tumor Cox2 expression was analyzed both qualitatively and quantitatively. For qualitative analyses, patients were classified as overexpressing Cox2 (immunostaining score from 3 to 7) or nonoverexpressing Cox2 (immunostaining score from 0 to 2).

To identify variables associated with Cox2 expression, a multivariate analysis was done. Variables achieving a P value of <0.2 in the univariate analysis were subsequently included in a stepwise backward logistic regression procedure to identify those factors independently associated with Cox2 overexpression.

All P values are two sided. A P value of <0.05 was considered to indicate a statistically significant difference. All calculations were done by using the 10.0 SPSS software package (SPSS, Inc., Chicago, IL).

**Results**

**Characteristics of patients.** From among the centers that agreed to participate in this nested, case-control study, 55 patients with a MMR-deficient colorectal cancer were identified. Simultaneously, 110 colorectal cancer patients matched by center were randomly selected among those with a proficient MMR system. Cox2 immunostaining led to ambiguous results in 12 patients and, consequently, they were excluded from the analysis. Thus, 153 patients, 46 of them with a deficient MMR system and 107 with MMR proficiency, constitute the basis of the present investigation. Demographic, clinical, and tumor-related characteristics of these patients are summarized in Table 1.

**Cox2 expression in patients with colorectal cancer.** In the whole series, 107 patients (70%) had colorectal cancer overexpressing Cox2, whereas in the remaining 46 patients (30%) the tumor did not show Cox2 overexpression. In this latter subset of patients, Cox2 expression was absolutely abolished in 33 of them.

With respect to the MMR status, Cox2 overexpression was observed in 85 patients (79%) with a proficient system, but only in 22 patients (48%) with a deficient MMR system (P < 0.001; Table 2). Other characteristics associated with tumor Cox2 expression were site of tumor, degree of differentiation, and tumor-node-metastasis stage (Table 2).

The multivariate analysis showed that the lack of Cox2 overexpression was independently associated with a MMR-deficient system (odds ratio, 3.89; 95% confidence interval,
COX-2 expression in patients with MMR-deficient colorectal cancer. In the subset of patients with a MMR-deficient colorectal cancer, 24 tumors (52%) did not show COX-2 overexpression (Table 3). The lack of COX-2 overexpression correlated with a poor degree of differentiation, no fulfillment of Amsterdam II criteria, absence of MSH2/MLH1 germ line mutations, presence of tumor MSH2 expression, and lack of tumor MLH1 expression (Table 3).

CpG island promoter hypermethylation of COX2. Considering that the lack of tumor COX-2 overexpression correlated with characteristics commonly associated with MMR-deficient sporadic colorectal cancer (i.e., absence of MSH2/MLH1 germ line mutations, no fulfillment of Amsterdam II criteria, and lack of tumor MLH1 expression), promoter hypermethylation of COX2 seemed as the most attractive explanation for gene silencing in patients with MMR-deficient colorectal cancer. In colorectal cancer cell lines, COX2 hypermethylation was associated with loss of COX-2 expression, while treatment with a DNA demethylating agent restored COX-2 expression (Fig. 2).

Tumor COX-2 expression decreased in cases with COX2 hypermethylation with respect to unmethylated lesions (immunostaining score: 1.00 ± 1.93 versus 3.29 ± 2.80, respectively; P = 0.01). Indeed, COX2 hypermethylation was observed in 6 of 18 (33%) tumors lacking COX-2 expression in comparison with 2 of 28 (7%) tumors expressing this protein (P = 0.04; Fig. 2).

Discussion

The results of this study indicate that although COX-2 overexpression was observed in most MMR-proficient colorectal cancer, half of those tumors with a deficient repair system did not overexpress this protein. More importantly, lack of COX-2 overexpression was observed almost exclusively in patients with MMR-deficient sporadic forms, thus suggesting an epigenetic mechanism responsible for gene silencing. This was confirmed by the observation that promoter hypermethylation of COX2 was significantly associated with the lack of tumor COX-2 expression, although this mechanism can only explain one third of them. The strength of this study relies on the fact that it was carried out on a general population basis, which ensures an unbiased selection of patients; it involved a large number of subjects, thus guaranteeing that both HNPCC-related and sporadic MMR-deficient tumors are well represented; all patients were fully characterized with respect to the MMR system, including microsatellite instability testing, immunohistochemistry, and germ line mutational analysis; finally, tumor COX-2 immunostaining was done in a blinded fashion and results were analyzed both qualitatively and semiquantitatively.

Results of the present study suggest that HNPCC patients may benefit from chemoprevention using COX-2 inhibitors because five of six MSH2/MLH1 gene carriers exhibited tumor COX-2 overexpression, although the relatively low number of HNPCC patients included precludes to draw definitive conclusions in such subset of patients. This fact was already proposed by Sinicrope et al. (28), although in their study the frequency and intensity of COX-2 expression was significantly reduced in HNPCC with respect to sporadic forms. Conversely, half of those sporadic MMR-deficient tumors did not show COX-2 overexpression. This difference may also have important implications in terms of colorectal cancer chemoprevention because the lack of COX-2 immunostaining would theoretically imply a less relevant pathogenic role of this molecule (11, 35). In that sense, this observation could explain the lack of benefit from COX-2 inhibition in a significant

Table 1. Characteristics of patients with colorectal cancer included in the study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MMR deficiency (n = 46)</th>
<th>MMR proficiency (n = 107)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)*</td>
<td>69 ± 14</td>
<td>67 ± 11</td>
<td>0.24</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>Male</td>
<td>21 (46%)</td>
<td>65 (61%)</td>
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</tr>
<tr>
<td>Female</td>
<td>25 (54%)</td>
<td>42 (39%)</td>
<td></td>
</tr>
<tr>
<td>Site of tumor, n (%)</td>
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<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Distal to splenic flexure</td>
<td>14 (30%)</td>
<td>80 (75%)</td>
<td></td>
</tr>
<tr>
<td>Proximal to splenic flexure</td>
<td>32 (70%)</td>
<td>27 (25%)</td>
<td></td>
</tr>
<tr>
<td>TNM tumor stage, n (%)</td>
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<td></td>
<td>0.004</td>
</tr>
<tr>
<td>I</td>
<td>1 (2%)</td>
<td>22 (20%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>31 (67%)</td>
<td>42 (39%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>11 (24%)</td>
<td>32 (30%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>3 (7%)</td>
<td>11 (10%)</td>
<td></td>
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<tr>
<td>Degree of differentiation, n (%)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Well</td>
<td>9 (19%)</td>
<td>69 (64%)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>28 (61%)</td>
<td>29 (27%)</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>9 (19%)</td>
<td>9 (8%)</td>
<td></td>
</tr>
<tr>
<td>Fulfillment of Amsterdam II criteria, n (%)</td>
<td>7 (15%)</td>
<td>1 (1%)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Abbreviation: TNM, tumor-node-metastasis.

*Expressed as mean ± SD.
proportion of patients at risk for colorectal cancer (15–17). It is important to mention, however, that the lack of COX-2 expression does not absolutely exclude the response to NSAIDs because they may also act through non-COX-2-dependent mechanisms (11, 35). Moreover, whether a reduced COX-2 expression in MMR-deficient colorectal cancer is reflecting events occurring during premalignant adenoma stages is also unknown and, therefore, may have no bearing on the preventive efficacy of COX-2 inhibitors in this setting (11).

Regulation of COX-2 expression is not completely uncovered. Data obtained in cancer cell lines suggest that COX-2 overexpression is mainly determined by transcriptional activation (36). In animal models, COX-2 expression increases coincidentally with loss of adenomatous polyposis coli, suggesting that TCF/β-catenin complexes may also regulate COX2 gene transcription (21, 37). In MMR-deficient colorectal cancer, mutations in the transforming growth factor-β type II receptor gene have been suggested as a potential regulatory mechanism (37), but results obtained thus far seem to discard this possibility (28). On the contrary, according to results of the present study as well as other previously published investigations in patients with colorectal cancer (29) or gastric cancer

| Table 2. Variables associated with tumor COX-2 expression in the whole series (n = 153) |
|-----------------------------------------------|------------------|------------------|------------------|------------------|
|                                              | n                | No COX-2 overexpression | COX-2 overexpression | P                | COX-2 expression (score)* |
| Site of tumor, n (%)                         |                  | (n = 46)               | (n = 107)           |                  |                            |
| Distal to splenic flexure                    | 94               | 21 (22%)              | 73 (78%)            | 0.009            | 4.46 ± 2.47              |
| Proximal to splenic flexure                  | 59               | 25 (42%)              | 34 (58%)            | 0.009            | 3.22 ± 2.59              |
| Degree of differentiation, n (%)             |                  |                      |                   |                  |                            |
| Well                                          | 78               | 14 (18%)              | 64 (82%)            | 0.001            | 4.53 ± 2.30              |
| Moderate                                      | 57               | 21 (37%)              | 36 (63%)            | 0.001            | 3.80 ± 2.62              |
| Poor                                          | 18               | 11 (61%)              | 7 (39%)             | 0.001            | 2.44 ± 2.91              |
| TNM tumor stage, n (%)                       |                  |                      |                   |                  |                            |
| I                                             | 23               | 2 (9%)                | 21 (91%)            | 0.07             | 5.26 ± 2.12              |
| II                                            | 73               | 27 (38%)              | 46 (63%)            | 0.07             | 3.48 ± 2.68              |
| III                                           | 43               | 13 (30%)              | 30 (70%)            | 0.07             | 4.02 ± 2.53              |
| IV                                            | 14               | 4 (29%)               | 10 (71%)            | 0.07             | 4.00 ± 2.48              |
| MMR status, n (%)                            |                  |                      |                   | 0.07             | 4.45 ± 2.35              |
| Proficient                                    | 107              | 22 (21%)              | 85 (79%)            | 0.07             | 2.89 ± 2.79              |
| Deficient                                     | 46               | 24 (52%)              | 22 (48%)            | 0.07             | 4.45 ± 2.35              |

*Expressed as mean ± SD.

| Table 3. Variables associated with tumor COX-2 expression in patients with MMR deficiency (n = 46) |
|---------------------------------------------------------------|------------------|------------------|------------------|------------------|
|                                                              | n                | No COX-2 overexpression | COX-2 overexpression | P                | COX-2 expression (score)* |
| Degree of differentiation, n (%)                              |                  | (n = 24)               | (n = 22)            |                  |                            |
| Well                                                          | 9                | 2 (22%)               | 7 (78%)            | 0.06             | 3.56 ± 2.35              |
| Moderate                                                      | 28               | 15 (54%)              | 13 (46%)           | 0.06             | 3.15 ± 2.82              |
| Poor                                                          | 9                | 7 (78%)               | 2 (22%)            | 0.06             | 1.78 ± 3.03              |
| Fulfillment of Amsterdam II criteria, n (%)                   |                  |                      |                   |                  |                            |
| Yes                                                           | 7                | 1 (14%)               | 6 (86%)            | 0.04             | 5.00 ± 1.83              |
| No                                                            | 39               | 23 (59%)              | 16 (41%)           | 0.04             | 2.51 ± 2.78              |
| MSH2/MLH1 germ line mutations, n (%)                          |                  |                      |                   |                  |                            |
| Presence                                                      | 6                | 1 (17%)               | 5 (83%)            | 0.09             | 4.83 ± 2.64              |
| Absence                                                       | 40               | 23 (58%)              | 17 (42%)           | 0.09             | 2.60 ± 2.73              |
| Tumor MSH2 expression, n (%)                                  |                  |                      |                   |                  |                            |
| Absence                                                      | 7                | 1 (14%)               | 6 (86%)            | 0.05             | 5.29 ± 2.43              |
| Presence                                                     | 38               | 22 (58%)              | 16 (42%)           | 0.05             | 2.47 ± 2.69              |
| Tumor MLH1 expression, n (%)                                  |                  |                      |                   |                  |                            |
| Presence                                                     | 9                | 2 (22%)               | 7 (78%)            | 0.07             | 4.56 ± 2.74              |
| Absence                                                      | 37               | 22 (60%)              | 15 (40%)           | 0.07             | 2.49 ± 2.68              |

*Expressed as mean ± SD.

† In one patient, MSH2 immunostaining was not evaluable.
COX-2 expression does not absolutely preclude the response to NSAIDs, because they may also act through non-COX-2-dependent mechanisms, our results justify the stratification of patients according to their MMR status in any study aimed at evaluating the potential utility of this chemopreventive strategy in colorectal cancer.

Appendix A. Investigators from the Gastrointestinal Oncology Group of the Spanish Gastroenterological Association who participate in the study

All participants listed below were fully involved in the study:

References

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