Correlation between COX-2 and APC Expression in Left *Versus* Right-sided Human Colon Cancer

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Abstract. *Background: Because of clinicopathologic and genetic differences between left-sided colorectal cancer (LSCRC) and right-sided colon cancer (RSCC), cyclooxygenase-2(COX-2) and adenomatous polyposis coli (APC) expression may be of clinical relevance. Materials and Methods: Clinicopathologic information for 72 primary colon tumors, 44 left and 28 right, from 72 patients (34 F, 38 M) were analyzed. COX-2 and wild- type APC (W-APC) immunohistochemical expressions were determined for each case. The data were analyzed using the Chi-square test and exact binomial confidence intervals. Results: Overall, 31 out of 44 (70%) LSCRC were W-COX-2 positive vs. 13 out of 28 (46%) RSCC (p-value=0.042). When evaluated independently of the anatomic location, COX-2 expression showed a borderline statistical correlation with the lack of W-APC protein (p-value=0.054). When considering location of tumors, the inverse correlation between COX-2 and W-APC expression became statistically significant (p- value=0.024). Conclusion: We report a strong inverse correlation between COX-2 and W-APC expression, with COX-2 being more frequently as expressed in LSCRC. These data may be useful to stratify colorectal cancer patients into right- and left-sided and COX-2 expressor and non-expressor subsets, when evaluating COX-2 inhibitor and other targeted therapies in colon cancer.*

Colon cancer is the second most common fatal malignancy in the Western world, with more than 150,000 new cases accounting for 55,000 deaths in the United States every year (1). The relationship between colorectal tumors and increased cyclooxygenase-2 (COX-2) activity provides a rationale for the use of selective COX-2 inhibitors to prevent the formation of polyps (2). Similarly, *COX-2* and its gene product may be attractive targets for therapeutic and chemoprotective strategies in colon cancer patients (3).

Colorectal cancers involving the distal colon are more likely to have aneuploid DNA, to harbor mutations in adenomatous polyposis coli (*APC*), *p53* and *K-ras* genes, and to behave more aggressively, while proximal CRCs are more likely to have diploid DNA, to possess microsatellite instability (MSI), to harbor mutations in the mismatch repair genes, and to behave less aggressively as in hereditary non-polyposis colorectal cancer (HNPCC) (4). Because of these clinicopathologic and genetic differences between left CRCs (LCRC) and right-sided colon cancer (RSCC), determination of COX-2 status may be of clinical relevance in these subsets of patients, especially with reference to COX-2 inhibitor therapy. Furthermore, wild-type APC (W-APC) has been reported to play a role in the transcriptional regulation of COX-2 expression in HT-29 human colorectal carcinoma cells (5). In a recent report combined APC gene therapy and COX-2 inhibitor therapy was shown to be synergistic in reducing colorectal polyp formation in Min mice (2). These findings suggest an interaction between APC and COX-2, and their participation in colon carcinogenesis.

Therefore, in order to elucidate the relationship between the expression of W-APC and COX-2 proteins and their distribution in LSCRC *vs*. RSCC, we analyzed a series of 72 primary human colon tumors for the expression of COX-2 and W-APC proteins using the avidin-biotin complex immunohistochemical method.

Materials and Methods

This study was carried out in accordance with the research protocol approved by the Institutional Review Board at our Institution.

Study group. This study encompasses 72 patients, 34 females and 38 males, who underwent resection of their primary colorectal tumor at the Moffitt Cancer Center, Tampa, FL, USA. Pertinent clinical data were compiled on each case, including patient age, sex,

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tumor size, and location. The tumors arising proximal to the splenic flexure were designated right-sided colon tumors, while those located distal to the splenic flexure were designated as left-sided (6). Pertinent pathologic data were tabulated including histologic tumor type, histologic grade, nodal status and pathologic stage.

Methods. H&E slides of all of the tumors were reviewed by two GI pathologists (AN, DC) for confirmation of the histopathologic findings and for the selection of blocks to be used for COX-2 and W-APC immunostaining.

Immunohistochemistry. In this study, rabbit anti-COX-2 (H-62) and rabbit anti-W-APC (C-20) antibodies were used that react with the corresponding antigens of human origin in paraffin-embedded sections. Immunohistochemical staining was performed using 3-um-thick paraffin sections from each of the representative tumor blocks selected. Briefly, slides were deparaffinized on an automated system with EZ Prep solution (Ventana, Tucson AZ, USA). Following heat-induced antigen retrieval, the sections were incubated with the anti-COX-2 primary antibody (1:20 dilution; Santa Cruz Biotech, CA, USA) for 60 minutes following the manufacturer's instructions, and with the anti-W-APC antibody (1:25 dilution in PSS Diluent and incubated for 60 min, Ventana). The slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems) as per manufacturer's protocol with proprietary reagents. The detection system used was the Ventana DABMap kit and slides were then counterstained with Harris' hematoxylin. Slides were then dehydrated and coverslipped as per normal laboratory protocol. The sections were then examined under a light microscope (Olympus BX51).

Interpretation of immunohistochemical findings. The sections stained for COX-2 and W-APC protein were independently evaluated by both gastrointestinal pathologists (AN, DC) without prior knowledge of the clinicopathologic data. These evaluations were made in the most representative and viable areas of the stained tumor sections. Both qualitative and semi-quantitative results were determined for each case based on cytoplasmic expression of COX-2 and W-APC proteins. The tumors were considered COX-2 and W-APC positive if ≥10% tumor cells showed unequivocal immunostaining. This cut-off was used in order to avoid interpreting cases with borderline/focal (<10% tumor cells with positive immunostaining) immunoreactivity as being positive. Such an approach has previously been utilized by other investigators for good reproducibility and clinical correlations in similar studies (7). In addition, a semi-quantitative system was also used to score the cytoplasmic expression of COX-2 and W-APC proteins based on the intensity of immunostaining from 0 to 3 (negative, weak, medium and strong) and the proportion of immunostained tumor cells from 0 to 5. An overall immunostaining score was calculated by adding the intensity and proportion scores, as previously described (8). Any difference of interpretation was resolved by joint immunhistochemical review by the pathologists.

Positive immunostains for W-APC indicated the presence of wild-type APC. Negative stain indicated the presence of mutated APC protein.

Tissue controls. Known COX-2-positive and -negative tumors were used as controls. Non-neoplastic colonic epithelium served as the W-APC internal control. Tumor tissue sections incubated with normal rabbit serum were also utilized as negative control.

Statistical analyses. The Chi-square test of independence was used to analyze the associations between the expression of COX-2 and W-APC in right *vs*. left tumors (DB).

Results

Clinicopathological data. When comparing LSCRC *vs*. RSCC, the mean patient age was 72.5 years (range 43-83 years) *vs*. 69.2 years (range 41-87 years), and the male to female ratio was 38/34. Forty-four out of 72 (61%) tumors were located in the left colon and 28 out of 72 (39%) in the right colon as defined above. The mean tumor size was 4.2 cm for LSCRC and 5.3 cm for RSCC.

Immunostaining results. Both COX-2 and W-APC proteins were localized in the cytoplasm of the cancer cells in all cases studied (Figure 1B and C). Thirty-one out of 44 (70%) LSCRC and 13 out of 28 (46%) RSCC were COX-2 positive, while 13 out of 44 (30%) LSCRC and 15 out of 28 (54%) RSCC were COX-2 negative (p=0.042). Major findings of this study follow.

Inverse correlation between COX-2 and W-APC protein expression in colon tumors: Twenty-nine out of 41 (71%) W-APC negative tumors were COX-2 positive (Figures 1A and B; and Figure 2) and 16 (52%) out of 31 W-APC positive tumors were COX-2 negative (Figures 1C-1D; and Figure 3). Without taking the anatomic location of tumors into consideration, the association between expression of COX-2 and the lack of expression of W-APC protein reached a borderline statistical significance (p=0.054). Even though the overall rate of COX-2 positivity was lower in the RSCCs (46%) as compared to the LSCRCs (70%), the inverse correlation between COX-2 and W-APC expression was still evident in some of the RSCCs.

Differential expression of COX-2 and W-APC proteins in LSCRC vs. RSCC: When the location of tumors was taken into consideration, there was a statistically significant inverse correlation between the COX-2 and W-APC protein expression (p=0.024).

Discussion

While overexpression of COX-2 has been demonstrated in several types of human tumors (8), COX-2 and prostanoid production have also been implicated in the pathogenesis of colorectal carcinoma (1). Convincing epidemiological data have been presented supporting the hypothesis that non-steroidal inflammatory drugs (NSAIDs) reduce the risk of colorectal tumorigenesis (1, 9-14). This is believed to be mediated, at least in part, by inhibition of COX activity (13). Furthermore, convincing evidence has been presented in cell culture (15) and animal (8, 14, 16-19) experiments supporting the inhibition of COX-2 as a potential

chemopreventive and chemotherapeutic approach in the control of colorectal neoplasia. Therefore, in recent years COX-2 has emerged as one of the most promising potential target for the antineoplastic activity of NSAIDs (20).

The *APC* gene is a tumor suppressor gene that has been termed the gatekeeper gene for colon cancer (21). The gene plays a major role in directing epithelial growth and differentiation (22). The normal function of W-APC protein is to bind the key effector molecule beta-catenin. When the *APC* gene is inactivated, beta-catenin translocates from the lateral cell membrane to the nucleus, where it drives the transcription of multiple genes implicated in tumor growth and differentiation (23). In addition to the 100% mutation rate in patients with familial adenomatous polyposis, *APC* gene mutations have been reported in 50-80% of sporadic colon and intestinal cases (16, 24-26).

Recent studies have reported the correlation between up-regulation of COX-2 protein and the presence of *APC* mutation (9-11, 27). Beta-catenin, a member of the PEA family of transcription factors and of the WNT-1 pathway is believed to be the mediator of the link between APC and COX-2 (3, 27) Here we further investigated the association between COX-2 and W-APC proteins expressions in a series of primary human tumor. We found an inverse correlation between the immunohistochemical expression of COX-2 and W-APC proteins. This difference became statistically significant when tumor location (right *vs*. left) was considered. In addition, our data show a differential COX-2 expression between LSCRC and RSCC. Since the demographic information and pathologic features of the LSCRC and RSCC subsets in our study were essentially matched, the findings appear to reflect true pathobiologic/ genetic differences rather than a patient selection bias. These differences may have potential therapeutic implications as the stratification of patients into COX2-positive and COX2-negative cohorts will allow targeted inhibition of COX2 activity.

Our observation of a higher rate of COX-2 positivity in LSCRC (70%) as compared to RSCC (46%) further substantiates our preliminary observations in a series of primary tumors (34), and it is in agreement with a recent study reporting higher COX-2 mRNA expression in distal colorectal adenomas as compared to the proximal lesions (31).

While the inverse correlation between COX-2 and W-APC expression may partially explain the observed distribution of COX-2 positivity between LSCRC and RSCC, this finding also substantiates the observations that a full-length *APC* promoter down-regulates COX-2 protein expression in HT-29, a human colon cancer cell line (5). The observation that a good proportion of RSCCs derive from alterations in the serrated pathway (BRAF, cyclin, TGFRbeta, mismatch repair genes) and not from the APC pathway (APC/Ras/p53) may also account for the different COX-2 protein distribution between the two cohorts.

Our finding of a greater frequency of COX-2 expression in LSCRC has potential clinical implications suggesting a greater clinical efficacy of COX-2 inhibitors in these patients as compared to those with RSCC. Preliminary data to support our finding is the reported higher chemopreventive efficacy of COX-2 inhibitors in therapy of recto-sigmoidal and large colorectal adenomas (28). Our finding of lower frequency of COX-2 expression in RSCC suggests that the inhibition of other molecular targets, such as BRAF, cyclin, TGF-beta and mismatch repair genes may be biologically more relevant than COX-2 inhibition in the RSCC.

Interestingly, recent studies have shown that COX-2 inhibitor NS-398 (29) and sulindac (30) increase the expression of tumor suppressor *APC* gene in azoxymethane-induced rat colorectal tumors. Furthermore, the combined effect of restoration of normal APC expression through gene therapy and COX-2 inhibition in Min mice has been found to be additive (2). Validation of such data in human cases, would propose COX-2 /APC testing as a predictive marker of therapeutic response.

Conclusion

The data presented here are in line with previously reported *in vitro* data, and indicate the inverse correlation between COX-2 and W-APC expression in archival human colorectal tumors. We also report the differential distribution of COX-2 protein expression between RSCC and LSCRC.

The demonstration of higher COX-2 positivity in LSCRC as compared to RSCC in our series suggests that these two subsets may have different levels of clinical responsiveness to COX-2 inhibitor therapy and, depending on the expression of other molecular targets, may be sensitive to different combinations of targeted therapies.

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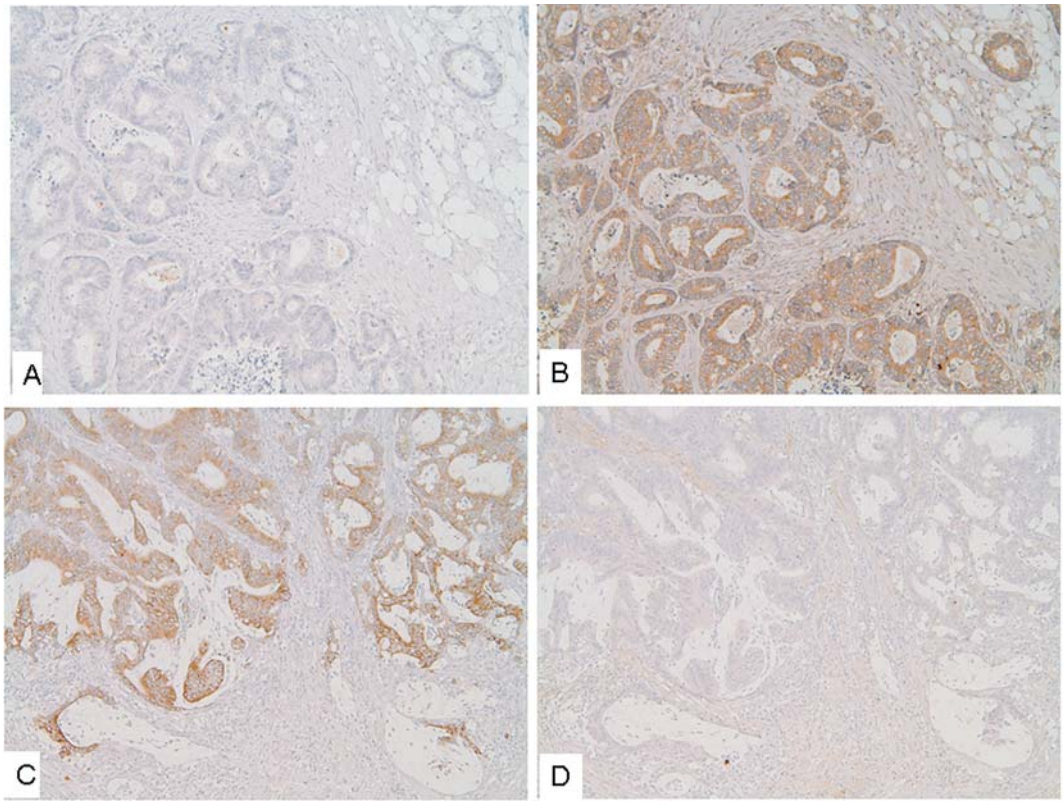

Figure 1. *Differences in APC/COX2 staining pattern in two moderately differentiated left-sided colonic adenocarcinomas.. For both invasive carcinoma cases, the same microscopic field is shown. A and B depict an APC-negative/COX-2-positive tumor, while that shown in C and D is APC-positive/COX-2-negative. Immunoperoxidase staining for APC and COX-2 proteins (original magnification for all ×100).*

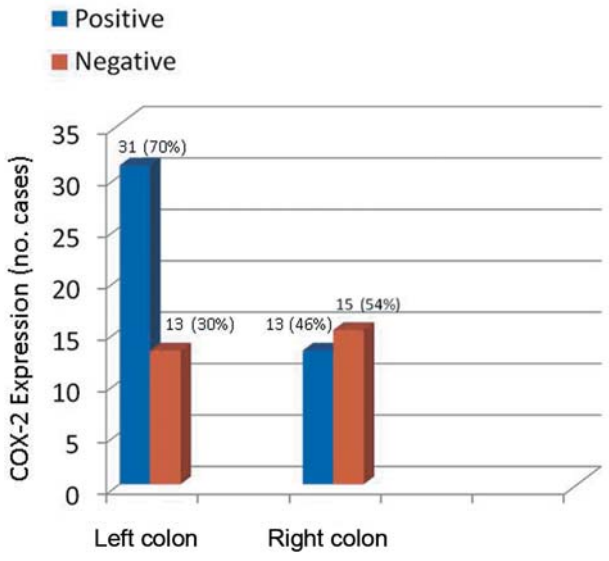

Figure 2. *Higher rate of COX-2 positivity in LSCRC (70%) vs. RSCC (46%) (p=0.042) (N=72).*

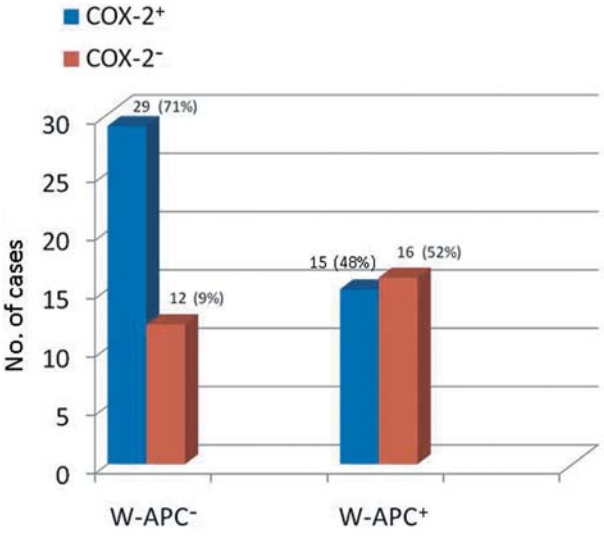

Figure 3. *Immunohistochemical expression of COX-2 protein correlates with the lack of expression of W-APC-P in surgically resected human colorectal cancer (p=0.054) (N=72).*

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