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Molecular Analysis of Sulindac-Resistant Adenomas in Familial Adenomatous Polyposis

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ABSTRACT

Purpose: Sulindac causes the reduction of adenomas in familial adenomatous polyposis (FAP) patients, but complete regression is unusual, and breakthrough of colorectal carcinoma during sulindac treatment has been described. The molecular features related to sulindac resistance are unknown. Therefore, we investigated molecular alterations in adenomas from FAP patients with complete adenoma regression on sulindac (responsive patients) and from FAP patients with sulindac-resistant adenomas (resistant patients).

Design: Fourteen baseline adenomas (removed before sulindac treatment) from six responsive patients were studied. Also, 9 baseline adenomas and 34 resistant adenomas (removed during sulindac treatment) from three resistant patients were analyzed. Using immunohistochemistry, we evaluated the expression of β-catenin, cyclooxygenase-2 (Cox-2), p53, Bcl-2, and Bax. K-rar codon 12 mutations, loss of heterozygosity at 5q (4PC locus), and microsatellite instability were studied with PCR-based techniques.

Result: There were no significant differences between baseline adenomas from sulindac-responsive and -resistant patients (P > 0.05). There was less loss of membranous β-catenin staining and less nuclear β-catenin accumulation in resistant adenomas compared with baseline adenomas from the same (sulindac-resistant) patients (P < 0.01) or baseline adenomas from responsive patients (P < 0.01). Epithelial Cox-2 expression was less, though not significant, in resistant adenomas compared with baseline adenomas from resistant patients, but was significantly less in baseline adenomas from responsive patients (P < 0.01). K-ras mutations were found in 8 of 34 resistant adenomas (24%) and in none of the baseline adenomas (P < 0.05). Stromal Cox-2 expression, staining of p53 and Bcl-2, and loss of heterozygosity at 5q were comparable in both groups. Loss of Bax staining and microsatellite instability were not found in any adenoma.

Conclusions: Sulindac-resistant adenomas display less alteration in β-catenin staining and less epithelial Cox-2 expression when compared with adenomas removed before sulindac treatment. K-ras mutations may contribute to sulindac-resistance. Continued research is needed to investigate molecular alterations related to sulindac resistance.

INTRODUCTION

Epidemiological data, animal studies, and in vitro experiments have established the potential chemopreventive value of NSAIDs against colorectal adenocarcinoma (1). In addition, the NSAID sulindac can induce adenoma regression in patients with FAP, an autosomal dominant disorder characterized by the development of hundreds of colorectal adenomas and eventual carcinoma at a young age (2–4). The chemopreventive action of NSAIDs seems to be mediated by the induction of apoptosis (5–9). However, the mechanisms underlying these observations are not completely understood.

The best-known target of NSAIDs is the Cox enzyme (9). Two Cox genes are known, COX-1 and COX-2, which regulate the conversion of arachidonic acid to prostaglandins. Increased Cox-2 expression and elevated prostaglandin levels have been found in both adenomas and carcinomas of the colon (10–12). Thus, inhibition of Cox-2 may provide one likely explanation for the chemopreventive properties of NSAIDs. However, Cox-2-independent mechanisms of action might exist (8, 9, 13–15), possibly through inhibition of the transcriptional activity of the nuclear hormone receptor peroxisome proliferator-activated receptor δ (PPARS), a potential downstream target of the APC/β-catenin/T-cell factor 4 pathway (16). In addition, animal studies and in vitro experiments suggest that NSAIDs decrease the nuclear accumulation of β-catenin, restoring part of the tumor-suppressor effects of the wild-type APC gene (17, 18).

Clinical trials with NSAIDs have shown a reduction in the size and number of adenomas (2–4, 19) and aberrant crypt foci (20) in FAP patients, but patient response to these agents is variable (21). In a randomized double-blind trial, an increased

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Sulindac-resistant adenomas

Table 1 Characteristics of patients with adenomas responsive and resistant to sulindac treatment

<table>
<thead>
<tr>
<th>Duration of treatment</th>
<th>No. of polyps studied</th>
<th>Age</th>
<th>Sex</th>
<th>APC mutation</th>
<th>Ileorectal anastomosis/ intact colon</th>
<th>Baseline polyp count/size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responsive patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>12 mo</td>
<td>32</td>
<td>F</td>
<td>Not tested</td>
<td>IRA*</td>
<td>19/2.2</td>
</tr>
<tr>
<td>II</td>
<td>36 mo</td>
<td>42</td>
<td>F</td>
<td>Codon 423</td>
<td>IRA</td>
<td>47/2.5</td>
</tr>
<tr>
<td>III</td>
<td>54 mo</td>
<td>52</td>
<td>F</td>
<td>Not tested</td>
<td>IRA</td>
<td>10/2.2</td>
</tr>
</tbody>
</table>

| IV                    | 45 mo                  | 24  | F   | Segment*     | IRA                                 | 29/3.5                        |
| V                     | 38 mo                  | 35  | F   | Not tested   | IRA                                 | 16/2.1                        |
| VI                    | 9 mo                   | 23  | F   | Codon 541    | IRA                                 | 11/2.2                        |
| Resistant patients    |                        |     |     |              |                                     |                               |
| VII                   | 48 mo                  | 36  | M   | Codon 827    | IRA                                 | 80/3.8                        |
| VIII                  | 30 mo                  | 25  | F   | Codon 1061   | IRA                                 | 7/3.1                         |
| IX                    | 9 mo                   | 23  | M   | Codon 625    | Impact colon                        | 34/4                          |

* IRA, ileorectal anastomosis.
* Segment 1, codons 1–804 of the APC gene.

The number of adenomas was noted between 6 and 9 months of sulindac therapy (2), suggesting the development or selection of resistant adenomas during long-term sulindac treatment. Moreover, breakthrough cancers in the rectal stump during sulindac chemopreventive therapy have been reported (22–24). This literature highlights potential limitations of NSAIDs as chemopreventive agents, and the need for biomarkers, which predict resistance to chemopreventive treatment. Recent in vitro data show that the loss of functional Bax results in resistance for NSAID-induced apoptosis (25). In addition, K-ras-transformed cells are relatively refractory to sulindac-induced apoptosis (26). These studies suggest that the lack of adenoma regression on sulindac may be related to BAX or K-ras mutations.

This study describes the immunohistochemical and molecular features of adenomas removed at baseline (before treatment with sulindac) from patients with complete polypl regression on sulindac (responsive patients) and adenomas removed at baseline and during sulindac treatment from individuals with sulindac-resistant adenomas (resistant patients). We investigated alterations possibly involved in the mechanisms underlying NSAID-induced chemopreventive of colorectal carcinoma, including B-raf expression, LOH at the APC locus Sq, and Cox-2 expression, or potentially related to resistance, such as K-ras mutations and the loss of Bax expression. In addition, apoptosis-related protein expression was assessed (p53 and Bcl-2). MSI was studied, because a relationship seems to exist between MSI and low Cox-2 expression (27).

MATERIALS AND METHODS

Study Population. The study population consisted of nine Caucasian FAP patients, of whom eight had undergone a colectomy and ileorectal anastomosis. Patient characteristics are shown in Table 1. All patients had adenomas in the rectum and were treated with sulindac 150 mg p.o. twice a day for at least 9 months. Informed consent was obtained in accordance with approval by the Johns Hopkins University Joint Committee on Clinical Investigation (Institutional Review Board). Medication compliance was assessed by pill count, and all patients took >80% of the scheduled doses. Patients underwent flexible sigmoidoscopy at baseline and every 3 months, using an Olympus flexible sigmoidoscope after preparation with a clear liquid diet and oral cathartic solution. Enemas that could influence mucosal biochemistry were not given. Biopsies taken from adenomas at baseline (before sulindac treatment) and during sulindac treatment were formalin fixed, paraffin embedded, and H&E stained for histological examination. Baseline adenomas from six FAP patients with complete polypl regression during treatment with sulindac (responsive patients) were studied (patients I-VI, Table 1). Three of the nine patients (patients VII-IX, Table 1) showed incomplete or no response to treatment with sulindac (resistant patients). Adenomas from those individuals, removed before treatment with sulindac (baseline) and during treatment with sulindac (resistant adenomas) were included. Adenomas from different groups were matched for size, architecture, and degree of dysplasia.

Responsive Patients. The control group consisted of 14 adenomas from the rectal stump of six patients (patients I-VI) with complete regression of adenomas within 6 months of treatment with sulindac. Adenomas removed before treatment with sulindac (baseline) were studied.

Resistant Patients. Patient VII had a baseline sigmoidoscope (before drug treatment) revealing 80 adenomas with an average size of 3.8 mm in the rectal stump. Initially, sulindac administration induced a regression in both number and size of adenomas. However, after 2 years on drug treatment, the number of adenomas began to increase. After 4 years of sulindac therapy, this patient was withdrawn from the trial because of acceleration of adenoma development and referred for surgical removal of the rectal stump to prevent rectal cancer. From patient VII, one adenoma removed before treatment with sulindac was studied. In addition, 31 adenomas from seven randomly chosen time points between 5 months and 4 years of treatment with sulindac were analyzed (resistant adenomas).

Patient VIII initially had seven adenomas with an average size of 3.1 mm in the rectal stump. After 3 months of sulindac treatment, there was complete adenoma regression. At 2 years of sulindac treatment, an ulcerative lesion was seen in the rectal stump at sigmoidoscopy. Histological examination at the ulcer margin revealed a tubular adenoma, which was analyzed in the present study (resistant adenoma). One adenoma removed before treatment with sulindac was studied.

Patient IX had an intact colon and was treated with sulin-
Sulindac did not affect number and size of the adenomas. Seven rectal adenomas removed before treatment with sulindac and two adenomas removed after 3 and 6 months of treatment with sulindac (resistant adenomas) were analyzed.

**Tissue Preparation.** Formalin-fixed, paraffin-embedded samples were cut into 5-μm sections and mounted on glass slides. For DNA isolation, slides were stained with hematoxylin, and dysplastic and normal epithelium were microdissected. A standard proteinase-K digestion was used to isolate DNA.

**Immunohistochemistry for β-Catenin; Cox-2; p53; Bcl-2; and Bax.** Immunohistochemistry was performed on unstained 5-μm sections as described previously (28). For antigen enhancement, the slides were submerged in citrate buffer and heated for 10 min at 100°C. Immunostaining was done using primary monoclonal antibodies against β-catenin, clone 14 (Transduction Laboratories, Lexington, KY), at a dilution of 1:1000; Cox-2, no. 160112 (Cayman Chemical, Ann Arbor, MI), at a dilution of 1:100; p53, DO7 (Dako, Glostrup, Denmark), at a dilution of 1:200; Bcl-2, clone 124 (Dako), at a dilution of 1:12.5; and against Bax, the polyclonal rabbit IgG p-19 (Santa Cruz Biotechnology, Santa Cruz, CA), at a dilution of 1:100. Primary antibodies against β-catenin and Cox-2 were incubated overnight at 4°C; incubation with antibodies against p53, Bcl-2, and Bax was done for 1 h at room temperature. Primary antibodies were replaced by PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄) in negative control slides. A known p53-positive colorectal carcinoma was used as a positive control for p53 staining. The staining pattern in the adjacent normal mucosa was used as a marker for the specific staining of β-catenin, Cox-2, Bcl-2, and Bax. To assess the specificity of the Cox-2 antibody, a subset of adenomas was stained with the primary antibody after preadsorption of a human Cox-2 control peptide (Cayman Chemical) for 1 h at room temperature. This resulted in the blocking of the Cox-2 staining pattern described below.

Immunostained slides were scored by two observers (G. J. A. O. and J. J. K.) in a coded fashion. For β-catenin, membranous, cytoplasmic, and nuclear staining were assessed separately. Membranous and cytoplasmic staining were compared with the normal mucosa and assessed as decreased expression (loss) or overexpression, respectively; nuclear staining (absent in normal tissue) was scored semiquantitatively using a scale from 1 to 4 (1, no expression; 2, <5% positive nuclei; 3, <25% positive nuclei; and 4, >25% positive nuclei). Cox-2 staining in the stroma underlying the epithelium and in the epithelium was assessed separately in a semiquantitative manner on a scale from 1 to 4 (1, no expression; 2, weak staining; 3, moderate staining; and 4, intense staining). p53 immunostaining was considered positive when >10% of nuclei stained positive (29). Bcl-2 staining was compared with the normal mucosa for assessment of overexpression, and Bax-stained adenomas were evaluated for loss of expression. Immunohistochemical staining was completely absent in one responsive adenoma, possibly because of a fixation error. Also, there was limited tissue available from another responsive adenoma, precluding assessment of Bax and Cox-2 staining.

**K-Ras Codon 12 Analysis.** K-ras codon 12 analysis was performed as described previously (30). DNA samples were used for amplification of K-ras codon 12-specific sequences by PCR. PCR products were then digested with MvaI, which only recognizes wild-type K-ras codon 12. Subsequently, a second-round PCR was performed on both the digested and undigested first-round PCR products. After denaturation, the undigested and digested (mutant-enriched) PCR products were spotted onto a nylon membrane and hybridized to each of the K-ras codon 12 mutation-specific oligodeoxynucleotides. Final stringency washes were carried out at 63°C before autoradiography. K-ras codon 12 mutational analysis was performed twice, in independent experiments.

**LOH and MSI Analysis.** Analysis of LOH and screening for MSI was done as described previously (31), comparing microdissected tumor tissue with normal tissue from the same patient. LOH analysis of the APC locus at chromosome 5q was performed with the markers DSS82, DSS107, and DSS346. MSI was assessed with the BAT-26 marker. Cycling was performed in a PTC 100 cycler (MJ Research, Inc., Waltham, MA), and the PCR products were analyzed using an automated ABI377 sequencer and the Genescan 2.1 software (PE Biosystems, Foster City, CA).

**Statistics.** Nonparametric tests were used. Comparisons between groups were made by Mann-Whitney test for nuclear β-catenin staining and epithelial and stromal Cox-2 expression. The nonparametric Fisher's exact test was used for analysis of differences in β-catenin (loss of membranous staining and overexpression of cytoplasmic staining), Cox-2 (moderate/strong compared with weak/absent staining), p53, Bcl-2, K-ras mutations, and 5q LOH. The relationship between membranous and nuclear β-catenin staining was analyzed by Mann-Whitney test. The relationship between stromal and epithelial Cox-2 staining was evaluated by Spearman's rank correlation test. A P <0.05 was considered statistically significant. All Ps were two-sided.

**RESULTS**

All adenomas were tubular or tubulovillous lesions, <1 cm, with mild to moderate dysplasia. No morphological differences were noted between adenomas from responsive patients (n = 14) and adenomas from resistant patients removed before (n = 9) or during (n = 34) treatment with sulindac. The results of immunohistochemistry (Fig. 1) for β-catenin, Cox-2, p53, Bcl-2, and Bax, K-ras codon 12 mutational analysis (Fig. 2) and 5q LOH and MSI are listed in Table 2.

**Baseline Adenomas from Sulindac-responsive and Sulindac-resistant Patients.** There were no significant differences in any molecular parameter analyzed in baseline adenomas (removed before treatment with sulindac) from sulindac-responsive patients compared with sulindac-resistant patients (P > 0.05; Table 2). In general, baseline adenomas displayed reduced membranous β-catenin staining compared with the normal mucosa and an accumulation of cytoplasmic and nuclear β-catenin (Fig. 1.A–B) in accordance with literature reports (32, 33). Very little Cox-2 staining was found in the normal mucosa. The Cox-2 staining pattern in adenomas (Fig. 1, D–E) was

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4 For primer sequence see the Genome Database; Internet address: http://www.gdb.org.
similar to that described by Chapple et al. (12), i.e., increased epithelial staining and stromal staining beneath dysplastic surface epithelium. In baseline adenomas, there was a positive correlation between stromal and epithelial staining ($P = 0.003$). Although epithelial Cox-2 overexpression seemed more pronounced in baseline adenomas from sulindac-responsive patients compared with baseline adenomas from sulindac-resistant patients ($P = 0.06$), the frequency distribution of Cox-2 assessed as moderate or strong ($\geq 3$) compared with negative or weak was not significantly different in both groups ($P = 0.12$). Stromal Cox-2 expression, positive staining for p53 (Fig. 1J), Bcl-2 overexpression (Fig. 1K), and LOH at the APC-locus 5q were comparable in both groups. Loss of Bax expression (Fig. 1G–H), K-ras codon 12 mutations, and MSI were not found in any baseline adenoma.

**Sulindac-resistant Adenomas.** Loss of membranous $\beta$-catenin expression was found in 13 of 34 (38%) sulindac-resistant adenomas, which was significantly less than in baseline adenomas from the same patients (88%; $P = 0.001$) and baseline adenomas from responsive patients (85%; $P = 0.008$; Fig. 1A–C). In addition, there was significantly less nuclear accumulation of $\beta$-catenin in resistant adenomas compared with baseline adenomas from the same patients ($P < 0.001$) and baseline adenomas from responsive patients ($P = 0.002$; Fig. 1A–C). Cytoplasmic $\beta$-catenin staining was present in 33 of 34 (97%) resistant adenomas and did not differ from baseline adenomas.

There was less epithelial Cox-2 expression (Fig. 1D–F) in resistant adenomas compared with baseline adenomas from the same patients, however, this difference was not significant ($P = 0.32$). There was significantly less epithelial Cox-2 expression in sulindac-resistant adenomas than in baseline adenomas from responsive patients ($P = 0.002$). Moderate or strong epithelial Cox-2 staining (assessed as $\geq 3$) was found in 13 of 34 (38%) resistant adenomas compared with 5 of 9 (55%) baseline adenomas from resistant patients ($P = 0.46$) and 11 of 12 (92%) baseline adenomas from responsive patients ($P = 0.002$). There was also less stromal Cox-2 staining (Fig. 1D–F) in resistant adenomas compared with baseline adenomas, however, the difference was not significant ($P = 0.14$ when compared with baseline adenomas from the same patients; and $P = 0.098$ when compared with baseline adenomas from responsive patients). There was a positive relationship between epithelial and stromal Cox-2 expression in resistant adenomas ($P = 0.045$), as was also found in the baseline adenomas (see above).

Resistant adenomas showed positive staining for p53 (Fig. 1J) and overexpression of Bcl-2 (Fig. 1K), comparable with baseline adenomas from resistant and responsive patients. Loss of Bax expression (Fig. 1G–I) was not found in resistant adenomas. K-ras codon 12 mutations were found in 8 of 34 (24%) resistant adenomas (Fig. 2), whereas no mutations were found in the nine baseline adenomas from the same patients ($P = 0.17$) nor in the 14 baseline adenomas from responsive patients ($P = 0.09$). When baseline adenomas from resistant and
resistant adenomas with and without nuclear accumulation of DISCUSSION

make-up of sulindac-resistant tumors. The timing of the occurrence of nuclear accumulation of colorectal cancer, literature reports reveal that these agents do not completely prevent the occurrence of colorectal carcinoma. This emphasizes the importance of understanding the mechanisms of action of these drugs and identifying biomarkers, which predict resistance to chemopreventive treatment.

The present study found less loss of membranous β-catenin, less nuclear accumulation of β-catenin, and less epithelial COX-2 in sulindac-resistant adenomas compared with adenomas from the same patients, removed before treatment with sulindac (baseline), and baseline adenomas from patients who showed a complete response after sulindac administration. In addition, K-ras codon 12 mutations were only noted in sulindac-resistant adenomas. These findings may indicate a role for these factors in the mechanism underlying NSAID-induced chemoprevention. Alternatively, our results might reflect a distinct molecular make-up of sulindac-resistant tumors. The timing of the occurrence of sulindac-resistant adenomas seems unpredictable, inasmuch as two of three sulindac-resistant patients were initially in complete remission during the first years of treatment. The results need to be interpreted with caution because of the small number of resistant patients from whom adenomas were available for study. The vast majority of resistant adenomas are from one patient, and potential confounding factors related to this patient cannot be excluded. Also, our study design precludes definitive conclusions between the possibilities that the observed differences are caused by treatment with sulindac or are related to sulindac resistance. Ideally, individual polyps could be marked and biopsied before and during sulindac therapy, comparing molecular alterations in polyps that ultimately regress to those that remain. Barriers to this methodology in FAP include the small size of adenomas and the difficulty in obtaining patient consent for such a protocol.

No significant differences were found between baseline adenomas from resistant patients and those with a complete response on sulindac. Thus, the studied markers could not predict which patients would completely respond to treatment with sulindac. The molecular alterations of baseline adenomas from sulindac-responsive and -resistant FAP patients were consistent with previously reported alterations in FAP-related adenomas. Loss of membranous β-catenin and translocation of β-catenin to the nucleus has been described in colorectal adenomas and carcinomas (32-34). Indeed, most baseline adenomas showed reduced membranous β-catenin staining and nuclear accumulation of β-catenin. Interestingly, there was less loss of membranous β-catenin and less nuclear β-catenin in sulindac-resistant adenomas.

FAP patients have an inherited mutation of the APC gene. Normally, wild-type APC regulates the degradation and nuclear export of β-catenin (35, 36), which is mainly localized at the cell membrane. Inactivation of APC results in increased cytoplasmic levels, loss of membranous expression, and nuclear accumulation of β-catenin. Nuclear β-catenin binds to T-cell factor 4 and activates the transcription of different oncogenic factors (35). Animal studies and in vitro experiments have addressed overexpression and nuclear translocation of β-catenin as potential targets for the anticarcinogenic action of NSAIDs. Mahmoud et al. (7) showed decreased expression of β-catenin after treatment with sulindac in the normal intestinal mucosa of Apcmin/+ mice, a murine model of FAP, and Oshima et al. (37) showed an increased membrane-bound fraction of β-catenin in adenomas of Apcmin/+ mice after 8 weeks of treatment with sulindac, whereas no change in nuclear accumulation was noted. In addition, McEntee et al. (38) reported decreased nuclear and cytoplasmic β-catenin expression in the small intestinal adenomas of Apcmin/+ mice after treatment with sulindac for 2-4 days, but not after 20 days treatment. Adenomas which persist after 20 days treatment with sulindac could be considered resistant. We found less nuclear β-catenin in sulindac-resistant adenomas, whereas nuclear accumulation of β-catenin was unaltered in sulindac-resistant adenomas of Apcmin/+ mice and Apcmin/+ mice (37, 38). This discordance might reflect differences between rectal adenomas in human FAP and small intestinal adenomas in these mice models. Recently, Brown et al. (17) reported decreased nuclear accumulation of β-catenin in carcinogen-induced tumors in rats after treatment with sulindac, supporting our present findings. Also, in vitro experiments have shown that the NSAID indomethacin decreases nuclear β-cate-

**Fig. 2** Autoradiogram of K-ras codon 12 mutation analysis. Displayed are the wild-type oligonucleotides (WT), the D mutation-specific oligonucleotides (valine mutation; D), and the E mutation-specific oligonucleotides (aspartic acid mutation; E). From each sample, nonenriched DNA (left) and mutant-enriched DNA (right) are hybridized next to each other in a paired way. Each row contains three pairs of spots' column with DNA from three different samples. Row 1, hybridization controls. DNA complementary to the labeled oligonucleotide. The eight detected mutations (6 D mutations and 2 E mutations) and a positive control (marked with *) are shown as paired spots. DNA was replaced by H2O as a negative control (blank spot in WT column marked with 2).
Molecular alterations in adenomas removed before treatment with sulindac (baseline) from patients with a complete response on sulindac (A) and sulindac-resistant patients (B) in sulindac-resistant adenomas (C). Nuclear β-catenin, and epithelial and stromal Cox-2 expression were assessed in a semiquantitative manner, and the median and range (lowest and highest scores) are presented.

<table>
<thead>
<tr>
<th>A: (n = 14)</th>
<th>B: (n = 9)</th>
<th>C: (n = 34)</th>
<th>A compared with C</th>
<th>B compared with C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responsive patients</td>
<td>Resistant patients</td>
<td>Resistant patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>Median (range)</td>
<td>Median (range)</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Nuclear β-catenin</td>
<td>3 (2-4)</td>
<td>3 (1-4)</td>
<td>2 (1-4)</td>
<td>P = 0.002</td>
</tr>
<tr>
<td>Epithelial Cox-2</td>
<td>4 (1-4)</td>
<td>3 (1-4)</td>
<td>2 (1-4)</td>
<td>P = 0.002</td>
</tr>
<tr>
<td>Stromal Cox-2</td>
<td>3 (1-4)</td>
<td>3 (1-4)</td>
<td>2 (1-4)</td>
<td>P = 0.25</td>
</tr>
<tr>
<td>Membranous β-catenin decreased</td>
<td>11/13 (85%)</td>
<td>9/9 (100%)</td>
<td>13/34 (38%)</td>
<td>P = 0.008</td>
</tr>
<tr>
<td>Cytoplasmic β-catenin overexpression</td>
<td>12/13 (92%)</td>
<td>7/9 (77%)</td>
<td>33/34 (97%)</td>
<td>P = 0.70</td>
</tr>
<tr>
<td>p53 overexpression</td>
<td>5/13 (38%)</td>
<td>2/9 (22%)</td>
<td>6/34 (18%)</td>
<td>P = 0.70</td>
</tr>
<tr>
<td>Bcl-2 overexpression</td>
<td>11/13 (85%)</td>
<td>6/9 (66%)</td>
<td>25/34 (74%)</td>
<td>P = 0.70</td>
</tr>
<tr>
<td>Bax loss of expression</td>
<td>0/12</td>
<td>0/9</td>
<td>0/34</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>K-ras codon 12 mutation</td>
<td>0/14 (0%)</td>
<td>0/9</td>
<td>8/34 (24%)</td>
<td>P = 0.17*</td>
</tr>
<tr>
<td>LOH at 5q (APC locus)</td>
<td>2/14 (14%)</td>
<td>0/9</td>
<td>7/32 (22%)</td>
<td>P = 0.70</td>
</tr>
<tr>
<td>MSI (Bat-26)</td>
<td>0/14</td>
<td>0/9</td>
<td>0/34</td>
<td>P = 0.42</td>
</tr>
</tbody>
</table>

* There were no statistically significant differences in any molecular parameter between A and B (P > 0.05).

Overall, the difference in K-ras mutation rate between baseline adenomas (from sulindac-resistant and sulindac-responsive patients together) and resistant adenomas was statistically significant: P = 0.02.

Mutant p53 was found in both groups, supporting in vitro findings that p53 is not involved in NSAID-induced chemoprevention (8). Recently, BAX mutations have been associated with sulindac resistance in vitro (35). We found Bax expression in all (resistant) adenomas studied. However, immunohistochemistry might fail to detect loss of functional Bax protein. Arber et al. (26) showed that K-ras-transformed cells were relatively resistant to sulindac-induced apoptosis. In this study, 8 K-ras codon 12 mutations were found in 34 sulindac-resistant adenomas (24%), whereas those mutations were not found in baseline adenomas from either sulindac-resistant or -responsive patients. K-ras codon 12 mutations account for the majority of ras mutations in human colorectal carcinogenesis and occur in <10% of small colorectal adenomas (<1 cm; Ref. 41). Thus, the frequency of K-ras codon 12 mutations in sulindac-resistant adenomas was higher than expected. In addition, when baseline adenomas from sulindac-resistant and -responsive patients were grouped, a significant difference in the K-ras mutation rate between baseline and resistant adenomas was noted, suggesting a role for K-ras mutations in sulindac resistance. Our study does not determine whether the observed differences are caused by sulindac or are an underlying cause of sulindac resistance. However, it is unlikely that sulindac treatment would induce ras mutations. More probably, K-ras mutations constitute a point of no return beyond which chemopreventive treatment with NSAIDs is ineffective. Of interest will be the effect of combinatorial chemopreventive therapy; in this regard, the work of Torrance et al. (42), describing almost complete adenoma regression in Apcmin mice after treatment with a combination of sulindac and a specific epidermal growth factor receptor kinase inhibitor seems promising.

In summary, our results show less oncogenic activation of β-catenin and less B-catenin in sulindac-resistant adenomas. One explanation for these findings is that nuclear β-catenin accumulation and Cox-2 expression are decreased with sulindac treatment. However, resistant adenomas may occur from limited suppression of these factors caused by drug delivery and/or involvement of an alternative (sulindac-resistant) tumor progression pathway. K-ras mutations might be involved in sulindac resistance. Additional research is needed to elucidate such an alternative tumor progression pathway and to investigate whether nuclear β-catenin staining, epithelial Cox-2 overexpression, and K-ras codon 12 mutations can be useful biomarkers.

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