Down-Regulation of the Down-Regulated in Adenoma (DRA) Gene Correlates with Colon Tumor Progression

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ABSTRACT

The down-regulated in adenoma (DRA) gene was originally identified as a gene that was down-regulated in colon tumors. It encodes a protein with anion transporter function that is expressed predominantly in the mucosa of the lower gastrointestinal tract. In this study, expression of DRA and its cellular distribution have been investigated in a series of benign adenomatous polyps and malignant colorectal tumors and in corresponding normal colonic mucosa. We show that DRA mRNA and protein are expressed in all normal colon tissue specimens with the protein restricted primarily to the terminally differentiated columnar epithelium and some goblet cells. Apical membrane localization was especially apparent in the columnar epithelium. The levels of DRA mRNA transcripts were down-regulated in all colon tumors examined relative to matched normal mucosa, with most specimens showing undetectable levels of DRA mRNA (77 of 104 tumors). DRA down-regulation was positively associated with colon tumor progression according to Dukes' stage and was particularly significant in the early transition from normal mucosa to polyp to adenocarcinoma. DRA expression does not appear to be strictly associated with colonic cell differentiation; rather, its absence and down-regulation were associated with the proliferating component of the crypt epithelium and with neoplastic transformation, respectively.

INTRODUCTION

The accumulation of genetic and epigenetic mutations underlies colorectal tumor initiation and progression (1). Mutations in key genes that predispose an individual to cancer can be broadly categorized into two groups: (a) those that directly affect the integrity of the genomic DNA and its replication, such as the mismatch repair genes p53, RAS, and APC; and (b) those with a more dependent effect on interaction with environmental agents (e.g., dietary compounds) such as the phospholipase MOM1 (2, 3) and cyclooxygenases (4), both of which affect lipid metabolism.

DRA (for down-regulated in adenoma) was originally isolated from a subtractive hybridization library enriched for normal-specific cDNAs that are down-regulated in colon cancer (5). The gene encodes a membrane anion transporter. Both the mouse and human homologues have been shown to facilitate the inward transport of sulfate (6, 7). DRA is also strongly implicated as an inward transporter of chloride because mutation of DRA appears to be responsible for the failure to transport chloride in the colon in the genetic disease CLD3 (8). Although little is known about the role colonic ion transport may play in the development of digestive neoplasms, recent evidence does indicate that mutation of the cystic fibrosis transmembrane regulator, a chloride channel, is associated with an increased risk of colon cancer (9). However, the mechanism of this association may be epigenetic rather than by direct genetic means.

To investigate the role of DRA in colonic cancer progression, we have undertaken an examination of DRA expression in a large series of patients and compared the malignant tumor specimens with corresponding normal colonic mucosa from the same patients. Benign adenomatous polyps have been included, as well as tissues that are representative of the range of colorectal tumor progression up to metastasis. Because the best marker for colorectal tumor progression is still the histological stage, we have correlated DRA expression with the Dukes' histological stage of the tumor. Analysis of DRA expression and its cellular localization should provide insight into the significance of DRA in colorectal cancer.

MATERIALS AND METHODS

Patients and Tissue Specimens. Colonic tissue specimens were obtained from 108 patients undergoing either colectomy or partial hepatectomy for colorectal carcinoma. Tissue samples

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3 The abbreviations used are: CLD, congenital chloride diarrhea; NC, normal colon; TBS, Tris-buffered saline.
were excised fresh from operative specimens and snap-frozen in liquid nitrogen for RNA analysis. Care was taken to exclude any normal mucosal tissue from tumor samples by histological examination. Matched NC was obtained from a distal portion of the colon in patients undergoing colectomy for colon tumors. Specimens from the tumor-normal tissue junction were also obtained, embedded in OCT embedding compound (Miles Laboratories, Elkhart, IN), snap-frozen, and stored at −70°C for immunohistochemical analyses. Fifty-nine normal colonic mucosa specimens were obtained from the distal margins of the resected colon, along with paired tumor tissue. The matching colorectal tumor specimens were grouped according to pathological stage of progression into Dukes’ A (tumor confined to bowel wall; n = 8), Dukes’ B (tumor invasion through the bowel wall; n = 29), Dukes’ C (tumor with lymph node metastasis; n = 18), and Dukes’ D (distant metastasis; n = 6). Hepatic colorectal metastases (n = 38), along with paired normal liver tissues, were also examined because normal colonic mucosa was not always obtainable from patients who underwent liver resection for colorectal carcinoma metastasis.

**Northern Blot Analysis.** Total RNA was isolated from frozen tissue specimens using the acid/guanidinium isothiocyanate method of Chomczynski and Sacchi (10). RNA samples (20 µg) were electrophoresed on 1.2% denaturing agarose gels and blotted onto nylon membranes (Hybond N, Amersham; or Duralon, Stratagene). The blots were probed with a 32P-labeled EcoRI fragment encoding a portion of the *DRA* cDNA (nucleotides 1062-1881). The membranes were hybridized as described (11) and washed to a final stringency of 0.1X SSC-0.1% SDS at 65°C. The membranes were exposed to Kodak XK-1 film between Dupont Cronex intensifying screens at −70°C for 1–3 days. Paired specimens from individual patients were blotted onto the same filter to control for variation in hybridization conditions and exposure. Each Northern blot was rehybridized with a radio-labeled oligonucleotide that was complementary to 18S rRNA as a measure of total RNA loaded in each lane. To control for RNA loading, the mRNA signal intensity was measured relative to 18S rRNA in each sample. The percentage reduction in *DRA* mRNA expression was calculated as the difference between the normalized signals in the mucosa and in the tumor divided by the mucosal expression of *DRA* as a percentage. Statistical comparisons were made using the Wilcoxon signed ranks test for paired values. The potential significance in the reduction in *DRA* mRNA expression across progression from polyps to metastases was determined using the one-way ANOVA. Significance was assumed at *P* ≤ 0.05.

**Immunohistochemistry.** Affinity-purified rabbit polyclonal antibody to human DRA (DRA 120F PAb) was produced in the laboratory of C. W. S. and recognizes the COOH-terminal amino acid sequence (INTNGGLRNRVYEVPVETKF). Frozen sections (6–8 µm) were affixed to sialinized slides, air-dried overnight, and wrapped in plastic cling film for storage at −20°C until use. Tissue sections were brought to room temperature, fixed in 10% formalin in TBS (pH 7.4) for 5–10 min, and then washed 3 times in TBS. In some cases, endogenous peroxidase activity in the sections was quenched by incubation in 0.3% H2O2 and 0.1% sodium azide in TBS for 10 min. After thorough washing in TBS, sections were immersed in 4% commercial nonfat skim milk powder in TBS for 15 min to inhibit nonspecific antibody binding before being transferred to a humidified chamber. To block endogenous biotin-like activity, sections were incubated with 0.1% avidin in Tris-HCl (pH 7.4), washed, and then incubated with 0.01% β-biotin (Dakopatts, Carpinteria, CA), followed by a further wash in TBS. The sections were then covered with 10% normal (nonimmune) goat serum for 15 min. Excess serum was decanted from the sections, and the primary anti-DRA antibody (DRA 120F PAb) was applied at a 1:100 dilution for 90 min at room temperature. Following the primary antibody and subsequent incubations, the sections were washed thoroughly in three changes of TBS each for 5 min. Sections were incubated for 15 min with biotinylated goat antirabbit immunoglobulins (Zymed, San Francisco, CA) and then with streptavidin-horseradish peroxidase conjugate (Zymed) for 12 min. Antigenic sites were revealed by incubating sections in 0.05% 3,3′-diaminobenzidine in Tris-HCl (pH 7.4) buffer with H2O2 as substrate. After washing in water, the sections were lightly counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene, and mounted with DePeX. Negative controls were stained as above but with TBS substituted for the primary antibody.

**RESULTS**

**DRA mRNA Is Progressively Down-Regulated with Dukes’ Stage in Colon Cancer.** Northern blot analyses (Fig. 1) were performed on specimens from NC, polyps, primary cancer of Dukes’ classification A–C, and metastases to the liver (Dukes’ D). In all cases, tumor tissue was compared to distal normal colonic mucosa from the same patient. This comparison is important because, although *DRA* mRNA was found to be expressed in all normal colonic tissues examined (59 of 59), the absolute level of *DRA* expression varies among individuals. A clear pattern of down-regulation is observed in all colonic tumor tissues examined relative to the adjacent normal colonic mucosa, with a marked loss of *DRA* mRNA expression in the majority of cases. Specifically, the percentage of tissues that were positive for *DRA* mRNA (irrespective of the level of expression) significantly decreased as a function of tumor progression according to the Dukes’ stage classification (Table 1). Polyps, Dukes’ A, B, and C, and liver metastases were all less likely to be positive for *DRA* mRNA than normal colonic mucosa: approximately one-half of adenomatous polyps express *DRA* mRNA expression (P < 0.001; Fisher’s two-tailed exact test), whereas only about one-quarter of Dukes’ A and B cancers (P < 0.001) and one-sixth of Dukes’ C and D cancers (P < 0.001) express detectable levels of *DRA* mRNA. *DRA* mRNA was not detected in normal liver tissue and was rarely detected in colonic liver metastases. The differences among the neoplastic stages are also significant: polyps versus Dukes’ A or B cancers (P < 0.001) and one-sixth of Dukes’ C and D cancers (P < 0.001) express detectable levels of *DRA* mRNA. *DRA* mRNA was not detected in normal liver tissue and was rarely detected in colonic liver metastases. The differences among the neoplastic stages are also significant: polyps versus Dukes’ A and B (P < 0.001) and Dukes’ A and B versus Dukes’ C and liver metastases (P < 0.001).

The relative level of *DRA* mRNA expression in 58 paired samples (normal and tumor) and 38 unpaired liver metastases was quantitated by densitometric scanning of Northern blots and normalized to the signal obtained by hybridizing to 18S rRNA. Pairwise comparisons were made for each patient using the Wilcoxon signed ranks test for paired values (Fig. 2 and Table 2). These results indicate that, relative to NC, the median *DRA* expression is decreased 95% in adenomatous polyps, 95.5% in Dukes’ A cancer, 100% in Dukes’ B cancer, 100% in
Dukes' C cancer, and 92.5% in Dukes' D cancer. Compared to NC, the percentage reduction of DRA mRNA expression in polyps and adenocarcinomas is highly significant (Table 2). Furthermore, although the reduction in expression was large in all categories from polyps up to liver metastases, there is a significant trend in decrease in DRA expression as tumors progress ($P = 0.015$, ANOVA; Fig. 2).

**DRA Protein Expression Does Not Correlate with Colonic Tumor Cell Differentiation.** The distribution of the DRA protein was examined directly by immunohistochemistry and is illustrated by the panels in Fig. 3. In normal mucosa, DRA protein is expressed primarily in the columnar epithelium with the most protein localized to the apical surface and the brush border (Fig. 3A). Such a location is consistent with DRA's function as a sulfate and chloride anion transporter (6-8). DRA protein expression is also seen in some goblet cells but does not extend down into the crypt to include the proliferating precursors of the terminally differentiated epithelium (Fig. 3A and B). A typical example of an adenomatous polyp is seen in Fig. 3C. Here, the epithelial cells in the polyp show no DRA protein, whereas the adjacent normal mucosa is positive for DRA (arrow). The Northern blot from this polyp (Fig. 1, patient 302) is also negative for DRA mRNA. The great majority of Dukes' B specimens tested showed no DRA protein expression, as typified in Fig. 3F. However, exceptions to this observation were found, as shown in Fig. 3, D and E. Fig. 3D shows a moderately well-differentiated tumor that retains considerable DRA protein expression but also has areas where DRA expression is reduced (compare arrows). Fig. 3E shows a well-differentiated tumor with low DRA expression. The sample in Fig. 3E was also weakly positive for DRA mRNA expression in the Northern blot (Fig. 1, patient 372), consistent with the positive staining by immunohistochemistry. Fig. 3, D and E, illustrates that the pattern of DRA staining in tumors was similar to that found in normal colonic mucosa, i.e., the apical surface of the columnar epithelium expressed the greatest amount of DRA. Fig. 3G shows a section of Dukes' C colon cancer containing the junction of both normal and adenocarcinoma tissue. It is noteworthy that DRA expression significantly decreases and, ultimately, disappears in the transition from normal to neoplastic regions (arrows). Also, there is no apparent "field effect" for DRA protein expression. That is, DRA expression is normal in morphologically normal tissue adjacent to the tumor. Fig. 3H shows a section from the middle of the same tumor as in Fig. 3G. No DRA staining is apparent in this tumor. Interestingly, this part of the tumor is well-differentiated compared to the distal part of the tumor seen in Fig. 3G, yet DRA expression is absent in both. This illustrates that DRA expression, although charac-
Expression in Colon Tumor Progression

Table 1  DRA mRNA expression in colorectal cancer tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of specimens</th>
<th>No. of specimens with detectable DRA mRNA</th>
<th>% with detectable mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal colonic mucosa</td>
<td>59</td>
<td>59</td>
<td>100a</td>
</tr>
<tr>
<td>Adenomatous polyps</td>
<td>17</td>
<td>2</td>
<td>122bc</td>
</tr>
<tr>
<td>Dukes’ A</td>
<td>9</td>
<td>2</td>
<td>22d</td>
</tr>
<tr>
<td>Dukes’ B</td>
<td>29</td>
<td>8</td>
<td>27</td>
</tr>
<tr>
<td>Dukes’ C</td>
<td>18</td>
<td>8</td>
<td>16c</td>
</tr>
<tr>
<td>Liver metastases (Dukes’ D)</td>
<td>31</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Adjacent normal liver</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The difference between detectable DRA mRNA expression in normal mucosa versus polyps, Dukes’ A, B, and C, and liver metastases is significant (P < 0.001; Fisher’s two-tailed test).

The difference between detectable DRA mRNA expression in Dukes’ A versus Dukes’ B (P = 1.0) and Dukes’ C versus liver metastases (P = 0.724) are not significant (Fisher’s two-tailed test).

The differences between detectable DRA mRNA expression in Dukes’ A and Dukes’ B versus Dukes’ C and Dukes’ D is significant (P < 0.001; Fisher’s two-tailed test).

DISCUSSION

The DRA gene encodes an anion transporter expressed predominantly in the mucosa of the lower gastrointestinal tract and is down-regulated in colon tumors and in inflammatory bowel disease. Here we show that DRA is ubiquitously expressed in normal colonic mucosal epithelium, with the protein expression restricted primarily to the terminally differentiated columnar epithelium and some goblet cells. Apical membrane localization is especially apparent in the columnar epithelium. The levels of DRA mRNA transcripts were down-regulated in all colon tumors examined relative to matched normal mucosa, with the majority of specimens showing undetectable levels of DRA (77 of 104 tumors). The down-regulation of DRA expression is particularly significant in the earliest stages of colon tumorigenesis: normal mucosa and adenomatous polyp formation.

DRA was originally identified in the colon and ileum, yet its tissue distribution is not restricted to these organs, although DRA expression does appear to be specifically linked to the apical surface of epithelium. DRA protein is expressed in the kidney proximal tubule epithelium in a pattern similar to the apical localization observed in normal colonic mucosa. However, DRA is not expressed in the normal fetal kidney, esophagus, duodenum, liver, skin, sweat glands, placenta, pancreas, or salivary glands. Höglund et al. (8) reported that DRA mRNA was expressed in normal prostate tissue using Northern blot analysis, but our immunohistochemical analyses show that DRA protein is expressed only on the apical surface of the intraepithelial seminal vesicle epithelium and not in the prostate tissue itself. These observations suggest that DRA plays an anion transport role in the kidney and seminal vesicle as well as in the intestine.

Because DRA expression was originally observed to be down-regulated in colon tumors, we undertook a systematic survey of all of the neoplastic stages of colon tumor progression. The results presented here demonstrate that all stages of colon tumor progression are significantly down-regulated for DRA mRNA and protein expression. Not only are polyps and adenocarcinomas less likely to express DRA, but the level of DRA expression, if detected, is much lower than that of normal colonic mucosa. Furthermore, the more advanced the stage of tumor progression, the more likely DRA expression was decreased (normal mucosa > polyp > Dukes’ A and B > Dukes’ C and D). Down-regulation of DRA expression did not show a field effect, as has been reported for alterations in sulfomucin composition (12) and for methyltransferase activity (13), because morphologically normally appearing mucosa expresses DRA even when it is directly adjacent to adenocarcinoma (see Fig. 3G). It is tempting to suggest that DRA down-regulation is related to the loss of differentiation of the colonic epithelium during tumor progression; however, the results presented here do not conform strictly to that notion (compare Fig. 3D versus Fig. 3H; Fig.

4 M. Willingham and C. W. Schweinfest, unpublished observations.
Table 2  % reduction in the median DRA expression in colorectal cancer tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of specimens (n)</th>
<th>DRA/18S mucosa (median)</th>
<th>DRA/18S tumor (median)</th>
<th>% reduction in DRA expression</th>
<th>Interquartile range (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenomatous polyps</td>
<td>15</td>
<td>1.4</td>
<td>0.069</td>
<td>95</td>
<td>76–100</td>
<td>0.001</td>
</tr>
<tr>
<td>Dukes’ A</td>
<td>8</td>
<td>1.3</td>
<td>0.027</td>
<td>95.5</td>
<td>90.95–98</td>
<td>0.012</td>
</tr>
<tr>
<td>Dukes’ B</td>
<td>29</td>
<td>1.4</td>
<td>0.020</td>
<td>100</td>
<td>98–100</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Dukes’ C</td>
<td>18</td>
<td>1.5</td>
<td>0.00</td>
<td>100</td>
<td>100</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Dukes’ D</td>
<td>6</td>
<td>0.7</td>
<td>0.00</td>
<td>92.5</td>
<td>46.75–97</td>
<td>0.028</td>
</tr>
<tr>
<td>Liver metastases</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Wilcoxon signed ranks test for paired values.

1 All values were 100%.

2 Paired samples with normal colonic mucosa.

3 Twenty-four of 38 values were 0.

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**Fig. 3** Photomicrographs of tissue sections stained with H&E and with DRA polyclonal antibody. A, NC; B, normal crypt (×400); C, juncional specimen of NC and polyp, patient 302; D, Dukes’ B adenocarcinoma, patient 375; E, Dukes’ B adenocarcinoma, patient 372; F, Dukes’ B adenocarcinoma, patient 340; G, Dukes’ C adenocarcinoma, patient 350 (×100); H, Dukes’ C adenocarcinoma, patient 350; I, Dukes’ D adenocarcinoma, patient 273. The apparent positive staining of tissue macrophages in the specimens is a result of endogenous peroxidase activity. Magnification, ×200 (unless otherwise indicated).

3C versus Fig. 3D). Rather, DRA protein expression is most consistently associated with absence of proliferation. *In vitro*, DRA is not expressed in any growing colon cancer cell line tested. However, it can be induced in differentiating postconfluent Caco-2 cells (6); yet, it is not inducible in differentiating HT-29 cells.6 Previously, we reported *in situ* RNA hybridization showing that DRA mRNA was present throughout the colonic crypt, including the proliferating component (7). This does not appear to be the case for neoplastic tissue, in that absence of DRA protein is well correlated with loss of

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5 G. D. Wu, personal communication.

6 N. Kondoh and C. W. Schweinfest, unpublished observations.
**DRA** mRNA. The most consistent association with DRA down-regulation in neoplastic tissue is renewed proliferation.

DRA protein from human and mouse has been shown to transport sulfate anions in heterologous systems such as insect SF9 cells (14) and microinjected Xenopus oocytes (6), respectively. Reduction of sulfomucin levels in the colon has been correlated with both premalignant and malignant conditions (12). Reduction of sulfate intake may well be the cause of undersulfated proteoglycans observed in some tumor cell lines (15, 16) and in transformed cell lines (17). This, in turn, may reduce cell-matrix and cell-cell adhesion (16), allowing mucosal epithelial cells to outgrow their normal architecture at the earliest stages of colon tumor progression, i.e., polyps.

Mutations in the DRA gene (primarily an in-frame deletion of the exon for Val-317) have been reported (8) as the cause for the disease CLD, which is characterized by a voluminous watery stool of high chloride content. Ostensibly, these mutant DRA proteins fail to transport chloride and water in the colon. Thus, DRA appears to be responsible for the inward transport of chloride as well as sulfate. If DRA down-regulation contributes to colon tumor progression, one would predict that CLD patients are at an increased risk of developing colon cancer, especially in conjunction with the presence of mutations in other genes, such as APC or RAS. In fact, a recent survey of such patients has indicated a slightly elevated risk of intestinal cancer (18). An increased risk of digestive tract cancer also exist in patients with cystic fibrosis (9). However, it is not yet clear how abrogation of anion transport bears on the risk of colon cancer. One hypothesis is that malabsorption (due to loss of cystic fibrosis transmembrane regulator or DRA) is associated with systemic deficiencies in antioxidants that may confer some protection from carcinogenic compounds (19, 20). Alternatively, DRA may be involved in other activities, in addition to its role in ion transport.

Other membrane ion channel proteins are reported to function frequently as G protein coupled effector molecules. Ga and Gβ subunits are differentially expressed along the crypt-villus axis in the rat small intestine, with Ga subunits abundantly expressed in the proliferating crypt cells and the Gβ₂, Gβ₃, and Gββ subunits preferentially expressed in the nonproliferating villus cells (21). G proteins have been reported to be involved in intestinal chloride channel activation on the apical surfaces of the intestinal epithelia (22). Thus, DRA would be well placed to mediate effects through G proteins. Furthermore, knockout mice deficient in the Ga₂, subunit develop a pathological condition resembling human ulcerative colitis, including the development of colonic adenocarcinoma in some animals. Clearly, the biological functions of DRA in relation to its role in ion transport and potential for signaling through the epithelium will be important to elucidate for our understanding of the biology of the colon and its associated diseases.

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