Introduction

Inflammatory bowel disease, which includes ulcerative colitis (UC) and Crohn’s disease, affects approximately one million individuals in the USA each year. Patients with UC have a relative risk that is eight times higher than the normal population for developing colorectal cancer (CRC) (1). The risk of CRC and dysplasia in patients with UC increases with the extent of colonic involvement, age of onset, duration of disease (2) and histological severity of inflammation (3). Recent data suggest that the cumulative risk for CRC in UC patients is below 1% in the first 8–10 years and rises in annual increments of 0.5–1.0%, thereafter reaching 5–10% after 20 years and 15–20% after 30 years (4,5). After 40 years of colitis, 25–40% of all patients who have not had a prophylactic colectomy will develop CRC (5).

Recent studies suggest that loss of p53 function (mutation and loss of heterozygosity) is an early event in the development of colitis-associated neoplasia (6–10). The frequency of G:C to A:T transition (codons 5–8) by sequencing and for cellular localization of β-catenin by immunohistochemistry. The incidence of neoplastic lesions was 57, 20 and 20% in p53+/–, p53−/− and p53+/+ mice, respectively (P = 0.013). p53−/− mice had a greater number of total lesions (P < 0.0001), cancers (P = 0.001) and dysplasias (P = 0.009) per mouse than either p53+/− or p53+/+ mice. Flat lesions were associated with the p53−/− genotype, whereas polypoid lesions were associated with the p53+/− and p53+/+ genotypes (P < 0.0001). β-Catenin mutations were present in 75% of lesions of p53−/− mice and absent in lesions from p53+/− mice (P = 0.055). Nuclear expression of β-catenin was seen only in polypoid lesions (91%). No β-ras or p53 mutations were detected. These data indicate that loss of p53 enhances the induction of colitis-associated neoplasia, particularly flat lesions, and dysregulation of β-catenin signaling plays an important role in the formation of polypoid lesions in this mouse model. As observed in humans, p53 plays a protective role in colitis-associated neoplasia in the DSS model.

Loss of p53 enhances the induction of colitis-associated neoplasia by dextran sulfate sodium

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Loss of p53 function is an early event in colitis-associated neoplasia in humans. We assessed the role of p53 in a mouse model of colitis-associated neoplasia. Colitis was induced in p53+/–, p53−/− and p53+/+ mice using three or four cycles of dextran sulfate sodium (DSS) followed by 120 days of water. Mice were examined for incidence, multiplicity and types of neoplastic lesions. Lesions were examined for mutations in β-catenin (exons 5–8) by sequencing and for cellular localization of β-catenin by immunohistochemistry. The incidence of neoplastic lesions was 57, 20 and 20% in p53+/–, p53−/− and p53+/+ mice, respectively (P = 0.013). p53−/− mice had a greater number of total lesions (P < 0.0001), cancers (P = 0.001) and dysplasias (P = 0.009) per mouse than either p53+/− or p53+/+ mice. Flat lesions were associated with the p53−/− genotype, whereas polypoid lesions were associated with the p53+/− and p53+/+ genotypes (P < 0.0001). β-Catenin mutations were present in 75% of lesions of p53−/− mice and absent in lesions from p53+/− mice (P = 0.055). Nuclear expression of β-catenin was seen only in polypoid lesions (91%). No β-ras or p53 mutations were detected. These data indicate that loss of p53 enhances the induction of colitis-associated neoplasia, particularly flat lesions, and dysregulation of β-catenin signaling plays an important role in the formation of polypoid lesions in this mouse model. As observed in humans, p53 plays a protective role in colitis-associated neoplasia in the DSS model.

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Recent studies suggest that loss of p53 function (mutation and loss of heterozygosity) is an early event in the development of colitis-associated neoplasia (6–10). The frequency of G:C to A:T transition (codons 5–8) by sequencing and for cellular localization of β-catenin by immunohistochemistry. The incidence of neoplastic lesions was 57, 20 and 20% in p53+/–, p53−/− and p53+/+ mice, respectively (P = 0.013). p53−/− mice had a greater number of total lesions (P < 0.0001), cancers (P = 0.001) and dysplasias (P = 0.009) per mouse than either p53+/− or p53+/+ mice. Flat lesions were associated with the p53−/− genotype, whereas polypoid lesions were associated with the p53+/− and p53+/+ genotypes (P < 0.0001). β-Catenin mutations were present in 75% of lesions of p53−/− mice and absent in lesions from p53+/− mice (P = 0.055). Nuclear expression of β-catenin was seen only in polypoid lesions (91%). No β-ras or p53 mutations were detected. These data indicate that loss of p53 enhances the induction of colitis-associated neoplasia, particularly flat lesions, and dysregulation of β-catenin signaling plays an important role in the formation of polypoid lesions in this mouse model. As observed in humans, p53 plays a protective role in colitis-associated neoplasia in the DSS model.

Materials and methods

Animals

Ten-week-old wild-type (p53+/+) and p53-deficient (p53−/− and p53+/+) C57BL/6 mice were obtained from the Laboratory Animal Facility at Fox Chase Cancer Center. p53-deficient mice were originally developed by DOWNER et al. (21) and a colony was established in-house by inbreeding three male p53−/− mice (N4) with six female p53−/− mice (N5) (Taconic Farms, Hudson, NY). This colony has been maintained at Fox Chase Cancer Center for >10 years. Animals were housed in a temperature- and humidity-controlled room and received rodent chow (PFI Nutrition International, Richmond, IN) and tap water ad libitum. During the experimental period, no murine viruses, Mycoplasma pulmonis, parasites, pathogens (including Helicobacter hepaticus and Helicobacter bilis) or Pneumocystis carinii were detected. All animal treatments were approved by the Institutional Animal Care and Use Committee at Fox Chase Cancer Center.

Induction of colitis

Colitis was induced by providing animals with drinking water containing DSS (molecular weight 30–40 000; ICN, Costa Mesa, CA) as described previously (13,16). DSS was administered in a cyclic fashion, with each cycle consisting of 4 days of DSS and 17 days of untreated water (Figure 1). p53-deficient (p53−/− and p53+/−) and wild-type (p53+/+) mice received three and four cycles of DSS, respectively, followed by 120 days of untreated water. Results from previous experiments (22) indicate that four cycles of DSS are required to induce neoplasia in p53−/− mice on a C57BL/6 background. p53−/− and p53+/− mice without DSS treatment served as controls and were maintained in parallel until 183 days of age. A subset of mice from each treatment group was killed after three cycles of DSS treatment to assess the level of colorectal neoplasia.

Abbreviations: CRC, colorectal cancer; DSS, dextran sulfate sodium; PCR, polymerase chain reaction; UC, ulcerative colitis; PGE2, prostaglandin E2.
inflammation present. Animals were monitored for gross rectal bleeding and weight loss throughout the study.

**Histopathology**

At the time of killing, the colon and rectum were excised, opened longitudinally, rinsed with phosphate-buffered saline and examined for gross morphologic changes. All lesions were counted and recorded. The colon/rectum was divided into equal thirds (proximal, middle and distal) and fixed in 70% ethanol or 10% formalin for 12–72 h. The entire colon was cross-sectioned at 2 mm intervals, embedded in paraffin and submitted for histopathologic processing. All slides were stained with hematoxylin and eosin and reviewed in a blinded manner. Inflammation scores were determined as described previously (16). Briefly, the severity of inflammation was graded semi-quantitatively on a scale from 0 to 3 and multiplied by the extent of involvement (0–100%) to yield a score ranging from 0 to 300. Following the assignment of a score to each piece of tissue on the slide, the total score was computed by summing the scores of all pieces and dividing by the number of pieces of tissue evaluated.

The histopathologic evaluation of dysplasia was based on the classification scheme developed by the Inflammatory Bowel Disease–Dysplasia Morphology Study Group (23). Lesions were scored as negative, indefinite or positive for dysplasia. Carcinomas and dysplasias were classified as polypoid or flat. Polypoid lesions had an elevated growth pattern seen either grossly or microscopically. Flat lesions had no elevated growth component and were less than twice the height of the adjacent non-neoplastic mucosa. Lesions were diagnosed as adenocarcinomas when neoplastic cells had invaded into the submucosa or beyond and were classified according to the World Health Organization publication of the classification of tumors of the digestive system (24). ‘Pseudoinvasion’ was defined as displacement of non-neoplastic glands into the deeper layers of the intestinal wall as reported by Boquin et al. (25).

**Laser capture microdissection and DNA isolation**

Paraffin-embedded colon tissues were sectioned (5 μm thickness), dehydrated, incubated with Staining Solution® (Arcturus Bioscience, Mountain View, CA) and dehydrated with a series of gradient alcohols and xylene. Slides were air-dried for 5 min and laser capture microdissection was performed immediately. Two thousand cells from each lesion (dysplasia or cancer) were microdissected using the PixCell® II Automated System (Arcturus Bioscience). DNA was isolated from laser-microdissected samples using the PicoPure™ DNA kit (Arcturus Bioscience).

**Mutational analyses**

Because >95% of k-ras, p53 and β-catenin mutations in human cancers are found at codons 12/13 (26), exons 5–8 (27) and exon 3 (28), mutational analyses focused on these regions.

**K-ras**. A 167 bp region of exon 1 of the mouse K-ras gene, containing codons 12 and 13, was amplified by polymerase chain reaction (PCR). Primers were designed based on genomic sequence obtained from GenBank (accession number S39586)—krasF, 5′-TTATTTTTATTTGTA-3′ and krasR, 5′-GCAGCAGACTGTTAGAG-3′. The reverse primer was 5′-biotinylated to facilitate single-strand DNA template isolation for the pyrosequencing reaction. Each PCR reaction contained 20 ng of genomic DNA, 10 pmol of each primer and 25 μl of Jumpstart ReadyMix RedTaq Polymerase (Sigma–Aldrich). The standard Ventana XT protocol was used except that biotinylated sheep anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) was substituted for the standard Ventana XT secondary antibody. Normal colon/rectum and neoplastic colorectal tissues from mice possessing a germ line mutation in the mouse p53 gene were aged in parallel with DSS-treated p53−/− and p53+/− mice.

**β-Catenin and p53**. Exon 3 of the mouse β-catenin gene and exons 5–8 of the mouse p53 gene were amplified by PCR in a 50 μl reaction with 20 ng of genomic DNA and 10 pmol of each primer using Jumpstart ReadyMix RedTaq Polymerase (Sigma–Aldrich). Primers for β-catenin and p53 were designed based on the gene sequence found in the Celera database (#mCG22702) and GenBank (#AF367373), respectively. Primers for β-catenin, β-cateninF, 5′-GCCCTTTTAAACAGTATT-3′ and β-cateninR, 5′-GCCCTTTTCAACCAACCA-3′, generated a 408 bp PCR product. Primers for p53 were as follows: exon5F, 5′-TACTTTCCTCCCTACATT-3′ and exon5R, 5′-GGGGAGATGGGAGGCTGC-3′; exon6F, 5′-GGGTTAGACTGGCAACC-3′ and exon6R, 5′-CTAAGACCAAAAAAACA-3′; exon7F, 5′-CTGTAGTGAGGTAGGAGGAG-3′ and exon7R, 5′-AGAGACGAAAGCACGGCAAG-3′; and exon8F, 5′-CTTACTGGCTTGTTGGTG-3′ and exon8R, 5′-GGAGTGACTTTTGAGTGA-3′.

**Immunohistochemistry of β-catenin**

Expression of β-catenin was evaluated by immunohistochemistry in paraffin-embedded tissues. A rabbit polyclonal antibody raised against β-catenin peptide amino acids 768–781 (C-2206) was purchased from Sigma–Aldrich and used at a dilution of 1:2000. Immunohistochemistry was performed on a Ventana XT automated immunohistochemistry stainer. Sections were incubated with the primary antibody for 32 min at 37°C. The standard Ventana XT protocol was used except that biotinylated sheep anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) was substituted for the standard Ventana XT secondary antibody. Normal colon/rectum and neoplastic colorectal lesions from mice possessing a germ line mutation in the adenomatous polyposis coli (APC) gene.
polyposis coli gene served as a positive control for β-catenin expression. The negative reagent control consisted of non-immune rabbit IgG at the same protein concentration. Cells were scored as positive or negative for membranous and nuclear staining of β-catenin.

**Statistical analysis**

The Wilcoxon signed rank test was used to compare differences between groups with respect to number of lesions and inflammation score. Fisher’s exact test was used to compare tumor incidence, type of lesions, mutation status of genes and staining pattern of β-catenin between groups. All tests were performed using SAS software (SAS Institute, Cary, NC).

**Results**

**Incidence, multiplicity and types of lesions**

The percentage of p53<sup>+/+</sup> and p53<sup>++</sup> mice completing the DSS treatment regimen (four or three cycles of DSS, respectively, plus 120 days of untreated water) was 77% (10/13) and 71% (89/125), respectively. However, only 34% (14/41) of p53<sup>−/−</sup> survived the DSS treatment (mean of 26 days after cycle 3 of DSS; range from 3 to 120 days), with the remainder euthanized prematurely due to sickness. Necropsy results indicated that 50% of the p53<sup>−/−</sup> mice had developed lymphoma, whereas an additional 14% had developed sarcoma. The incidence of cancer in DSS-treated p53<sup>−/−</sup> (42.9%, 6/14) mice was significantly higher than that of p53<sup>+/+</sup> (5.6%, 5/89) and p53<sup>++/−</sup> (0%, 0/10) mice (P = 0.0006). DSS-treated p53<sup>−/−</sup> mice had a significantly higher incidence of colorectal dysplasia (50.0%, 7/14) as compared with DSS-treated p53<sup>++/−</sup> and p53<sup>++/++</sup> mice [15.7% (14/89) and 20% (2/10), respectively] (P = 0.02). The incidence of colorectal neoplasia (dysplasia and cancer) was 57% (8/14), 20% (18/89) and 20% (2/10) in p53<sup>−/−</sup>, p53<sup>++/−</sup> and p53<sup>++/++</sup> mice, respectively (P = 0.013). No colorectal lesions were found in control mice not receiving DSS.

Mouse genotype affected not only tumor incidence but also the multiplicity of colorectal lesions and types of lesions (flat versus polypoid). DSS-treated p53<sup>−/−</sup> mice had significantly greater numbers of total lesions (P = 0.0001), dysplasias (P = 0.009) and carcinomas (P = 0.001) per mouse compared with DSS-treated p53<sup>++/−</sup> and p53<sup>++/++</sup> mice (Table I). In the DSS-treated p53<sup>++/−</sup> mice, 84.6% of the colorectal lesions were flat, whereas 15.4% of the lesions were polypoid. In contrast, 83.3% of the colorectal lesions in DSS-treated p53<sup>++/−</sup> mice were polypoid and only 16.7% of the lesions were flat and 100% of the colorectal lesions in DSS-treated p53<sup>++/++</sup> mice were polypoid (P < 0.0001) (Figure 2). Irrespective of mouse genotype, 8 of 11 cancers (72.7%) were flat and 3 of 11 cancers (27.3%) were polypoid.

**Pathology of colitis-associated neoplasia**

Polypoid lesions tended to be larger in size than flat lesions. All polypoid cancers arose from a background of polypoid dysplasia (Figure 3), whereas flat cancers tended to invade from the bottom of the crypts, often without dysplastic changes in the full thickness of the overlying mucosa (Figure 3). This histopathology and pattern of invasion from the crypt base is identical to that of low-grade tubuloglandular adenocarcinomas seen in human inflammatory bowel disease (29). Flat cancers exhibited small numbers of invasive malignant glands (Figures 3 and 4), which occasionally disappeared upon deeper sectioning (see below). There were 11 invasive adenocarcinomas, six of which were associated with contiguous acellular mucin pools. Nine cancers invaded into the submucosa and two into the muscularis propria.

A few DSS-treated mice (one p53<sup>−/−</sup>, three p53<sup>++/−</sup>, but no p53<sup>++/++</sup>) had pools of acellular mucin within the submucosa and muscularis propria. These findings are similar to colitis cystica profunda as seen in patients with UC (30). Serial and deeper histological sections (hematoxylin and eosin) were obtained from all cases with acellular mucin pools; in one of the cases, deeper sections revealed a carcinoma. In another case of adenocarcinoma, the carcinoma was absent in deeper hematoxylin and eosin sections, leaving only acellular mucin pools.

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**Table 1. Colorectal tumor multiplicity in DSS-treated mice**

<table>
<thead>
<tr>
<th>p53 genotype</th>
<th>N</th>
<th>Cancer</th>
<th>Dysplasia</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>10</td>
<td>0 ± 0</td>
<td>0.40 ± 0.32</td>
<td>0.40 ± 0.32</td>
</tr>
<tr>
<td>+/−</td>
<td>89</td>
<td>0.06 ± 0.03</td>
<td>0.17 ± 0.04</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>−/−</td>
<td>14</td>
<td>0.50 ± 0.18&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>0.57 ± 0.18&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>1.07 ± 0.28&lt;sup&gt;∗∗∗&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>∗</sup>Significantly different from p53<sup>+/+</sup> and p53<sup>+/-</sup> mice, P = 0.001.<n>∗∗</n>Significantly different from p53<sup>±/-</sup> mice, P = 0.009.<n>∗∗∗</n>Significantly different from p53<sup>−/−</sup> and p53<sup>++/−</sup> mice, P < 0.0001.

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**Fig. 2. Colorectal lesions in DSS-treated mice categorized by subtype of lesion.** In DSS-treated p53<sup>−/−</sup> mice, 38.5% of cancers and 46.1% of dysplasias were flat, 15.4% of cancers were polypoid and 0% of dysplasia was polypoid. In DSS-treated p53<sup>++/−</sup> mice, 16.7% of cancers were flat, 0% of dysplasias were flat, 5.6% of cancers were polypoid and 77.8% of dysplasias were polypoid. In DSS-treated p53<sup>++/++</sup> mice, 100% of the lesions were polypoid dysplasias.

**Pathology of colitis and inflammation score**

At the end of three cycles of DSS, all three groups of mice (p53<sup>+/+</sup>, p53<sup>++/−</sup> and p53<sup>++/++</sup>) showed similar classic histopathologic changes of chronic active colitis with chronic inflammation, crypt distortion, ulceration and regenerative epithelium. p53<sup>−/−</sup> mice had higher inflammation scores than p53<sup>+/+</sup> and p53<sup>++/−</sup> mice for both the entire colon/rectum (P = 0.02 and 0.06 for p53<sup>+/+</sup> and p53<sup>++/−</sup> mice, respectively) and for the distal colon/rectum (P = 0.01 and 0.02 for p53<sup>+/+</sup> and p53<sup>++/−</sup> mice, respectively). Inflammation scores of p53<sup>++/−</sup> and p53<sup>++/++</sup> mice did not differ significantly. At the end of three cycles of DSS (four cycles for p53<sup>++/−</sup> mice) plus 120 days of water, the p53<sup>+/+</sup> and p53<sup>++/−</sup> mice showed almost total healing and normalization of the mucosa with focal areas of minimal crypt changes. However, these changes could not be compared with those of p53<sup>−/−</sup> mice since p53<sup>−/−</sup> mice had to be killed early (mean—26 days following completion of cycle 3). The only p53<sup>−/−</sup> mouse that completed 120 days of water showed histopathologic changes identical to those of p53<sup>+/+</sup> and p53<sup>++/−</sup> mice killed after 120 days of water.

**Mutation of K-ras, p53 and β-catenin**

Tumor cells were laser capture microdissected from 21 colorectal lesions (4 p53<sup>+/+</sup>, 12 p53<sup>++/−</sup> and 5 p53<sup>++/++</sup> mice) for mutation analyses. The mutational status of K-ras and β-catenin was evaluated in all 21 samples, whereas similar analyses for p53 were performed only on lesions from p53<sup>+/+</sup> and p53<sup>++/−</sup> mice. Mutation of K-ras (codons 12/13) or p53 (exons 5–8) was not detected in any of the samples. β-Catenin mutations (exon 3) were detected in p53<sup>−/−</sup>, p53<sup>+/+</sup> and p53<sup>++/−</sup> mice at an incidence of 0% (0/5), 33.3% (4/12) and 75% (3/4), respectively.
respectively ($P = 0.055$). Hot spots included codons 32 ($n = 1$), 33 ($n = 1$), 34 ($n = 3$) and 37 ($n = 2$) of exon 3 of $\beta$-catenin (Table II). Irrespective of the $p53$ genotype, 7 of 16 (43.7%) polypoid lesions had $\beta$-catenin mutations compared with 0 of 5 (0%) flat lesions. However, this difference was not statistically significant ($P = 0.12$).

**Immunohistochemistry of $\beta$-catenin**

Nuclear expression of $\beta$-catenin was observed in 18 of 19 (91.3%) polypoid lesions versus 0 of 7 flat lesions (0%) ($P < 0.0001$) (Figure 4). The number of neoplastic cells positive for nuclear $\beta$-catenin varied from 5 to 50%. Both cancers and dysplasias exhibited a similar nuclear localization of $\beta$-catenin. No significant relationship was observed between mouse genotype and the nuclear expression of $\beta$-catenin in polypoid lesions ($P = 0.84$). Of the seven flat lesions with membranous $\beta$-catenin, five had sufficient material for $\beta$-catenin mutational analysis, and no $\beta$-catenin mutations were detected. Of the 18 polypoid lesions expressing nuclear $\beta$-catenin, 15 had sufficient material for mutational analysis. Six cases had $\beta$-catenin mutations and nine cases lacked $\beta$-catenin mutations. All lesions with $\beta$-catenin mutations expressed nuclear $\beta$-catenin.
Table II. β-Catenin mutation (exon 3) in colorectal lesions obtained from DSS-treated mice

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type of lesion</th>
<th>β-catenin mutation</th>
<th>Gene alteration</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1 Flat cancer</td>
<td>No mutation</td>
<td>GAT → GTT</td>
<td>Asp → Val</td>
</tr>
<tr>
<td></td>
<td>2 Flat cancer</td>
<td>No mutation</td>
<td>GGA → GAA</td>
<td>Gly → Glu</td>
</tr>
<tr>
<td></td>
<td>3 Flat dysplasia</td>
<td>No mutation</td>
<td>TCT → TT</td>
<td>Ser → Phe</td>
</tr>
<tr>
<td></td>
<td>4 Polypoid dysplasia</td>
<td>No mutation</td>
<td>GGA → GAA</td>
<td>Gly → Glu</td>
</tr>
<tr>
<td></td>
<td>5 Polypoid dysplasia</td>
<td>No mutation</td>
<td>TCT → TT</td>
<td>Ser → Phe</td>
</tr>
<tr>
<td>p53&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>1 Polypoid dysplasia</td>
<td>32 codon</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 Polypoid dysplasia</td>
<td>34 codon</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 Flat dysplasia</td>
<td>No mutation</td>
<td>TT</td>
<td>Ser → Phe</td>
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<tr>
<td></td>
<td>4 Polypoid dysplasia</td>
<td>37 codon</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
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<td>5 Polypoid dysplasia</td>
<td>No mutation</td>
<td>TT</td>
<td>Ser → Phe</td>
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<td>6 Polypoid dysplasia</td>
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<td>TT</td>
<td>Ser → Phe</td>
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<td>7 Polypoid dysplasia</td>
<td>37 codon</td>
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<td>8 Polypoid dysplasia</td>
<td>No mutation</td>
<td>TT</td>
<td>Ser → Phe</td>
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<td>9 Polypoid cancer</td>
<td>No mutation</td>
<td>TT</td>
<td>Ser → Phe</td>
</tr>
<tr>
<td></td>
<td>10 Polypoid dysplasia</td>
<td>No mutation</td>
<td>TT</td>
<td>Ser → Phe</td>
</tr>
<tr>
<td></td>
<td>11 Polypoid dysplasia</td>
<td>No mutation</td>
<td>TT</td>
<td>Ser → Phe</td>
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<tr>
<td></td>
<td>12 Polypoid dysplasia</td>
<td>No mutation</td>
<td>TT</td>
<td>Ser → Phe</td>
</tr>
<tr>
<td>p53&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>1 Polypoid dysplasia</td>
<td>34 codon</td>
<td>GGA → GAA</td>
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<td>2 Polypoid dysplasia</td>
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<td>4 Polypoid dysplasia</td>
<td>No mutation</td>
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Discussion

In this study, we have shown that loss of function of p53 is a critical event in the DSS mouse model of colitis-associated neoplasia, similar to that reported in human colitis-associated neoplasia (8,10). An increased frequency of G:C to A:T transition mutations in p53 have been observed within the non-neoplastic inflamed colorectal tissues of colitis patients as compared with normal colorectal tissue from healthy controls (8). Takaku et al. (7) found a high percentage (50%) of p53 mutations in non-neoplastic mucosa near UC-associated cancers. Yoshida et al. (6) microdissected individual crypts from patients with long-standing UC and found mutations in 66.7 and 33.3% of crypts that were regenerative or indefinite for dysplasia, respectively. Holzmann et al. (11) reported that p53 mutations were present in 19% (40/212) of colon specimens without dysplasia, 41% (17/29) of specimens indefinite for dysplasia, 38% (9/24) of specimens of low-grade dysplasia and 63% (5/8) of specimens of high-grade dysplasia. In addition, loss of heterozygosity of p53 was observed in ~25% of samples that were non-dysplastic, actively inflamed and indefinite for dysplasia, 45% of informative dysplastic lesions and 70% of cancers (9,10).

In this study, the incidence of colitis-associated neoplasia (dysplasias and cancers) was significantly higher in p53<sup>−/−</sup> mice (57%) as compared with both p53<sup>+/−</sup> and p53<sup>+/+</sup> mice (20%) (P = 0.013). This significance held true when dysplasias (P = 0.02) and carcinomas (P = 0.0006) were examined independently. p53<sup>−/−</sup> mice had significantly greater numbers of total lesions (P = 0.0001), dysplasias (P = 0.0009) and carcinomas (P = 0.001) per mouse compared with p53<sup>+/−</sup> and p53<sup>+/+</sup> mice. This difference may be underestimated because p53<sup>−/−</sup> mice were euthanized prematurely due to illness. Compared with the results for DSS-treated p53<sup>−/−</sup> mice reported recently by Fujii et al. (14), we report a higher incidence of invasive carcinoma (42.9 versus 3.0%), whereas they report a higher incidence of neoplasia (100 versus 57%) and numbers of lesions per mouse (5.0 versus 0.93). In addition, we observed that flat lesions were associated with the p53<sup>−/−</sup> genotype, whereas polypoid lesions were associated with the p53<sup>+/−</sup> and p53<sup>+/+</sup> genotypes (P < 0.0001). In contrast to our finding, Fujii et al. reported that flat lesions were associated with the p53<sup>−/−</sup> genotype. There are several possible explanations for these differences. First, the mice used in the present study were bred on a C57BL/6 background, whereas in the study of Fujii et al. (14), the p53<sup>−/−</sup> and p53<sup>+/−</sup> mice were on a C57BL/6 × CBA background and p53<sup>+/+</sup> mice were obtained by backcrossing p53<sup>−/−</sup> mice with wild-type C57BL/6 mice. It is well known that mouse strains differ in their susceptibility to DSS (31), which could lead to different levels of inflammation as well as incidence and numbers of neoplastic lesions (16). Second, although animals in both studies were maintained under specific pathogen-free conditions, the intestinal microflora is most likely different. Bacterial flora has been shown to affect the type and magnitude of the inflammatory response to DSS (32,33). Third, different DSS treatment regimens were used in these two studies. In the present study, mice were treated with DSS for three cycles (4 days of 4% DSS plus 17 days of untreated water per cycle), followed by 120 days of untreated water; whereas Fujii et al. (14) used two cycles of DSS (each cycle consisting of 7 days of 4% DSS followed by 14 days of water), followed by 84 days of water.

Nuclear β-catenin has been observed in DSS-induced tumors (16) and is usually attributed to the mutation of members of the Wnt pathway. Because of this, lesions from the present study were examined for mutation and subcellular localization of β-catenin by direct sequencing and immunohistochemistry, respectively. Mutations in exon 3 of β-catenin were detected in 0% of p53<sup>−/−</sup>, 36.4% of p53<sup>+/−</sup> and 75% of p53<sup>+/+</sup> mice (P = 0.055). This direct relationship between incidence of β-catenin mutations and increasing copy number of the wild-type p53 gene suggests that β-catenin mutations are probably important for colorectal tumorigenesis in mice with wild-type p53 but not in those mice with loss of p53 function. In the present study, β-catenin mutations were detected in codon 32 (one lesion), codon 33 (one lesion), codon 34 (three lesions) and codon 37 (two lesions) at or near the glycogen synthase kinase-3 beta phosphorylation sites. Similarly, β-catenin mutations have been detected in tumors induced by azoxymethane (at codons 33, 37 and 41) (34), DSS/azoxymethane (codons 32, 33 and 34) (15) and DSS/2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (codons 32 and 34) (35). To date, only one study has reported on mutation of β-catenin in human colitis-associated neoplasia (36), and no mutations (exon 3) were found in 30 UC-associated carcinomas. It should be noted that this study also reported a lower frequency of the adenomatous polyposis coli mutations in sporadic CRC (26%) than is usually reported in the literature. Four to 15% of all sporadic colorectal adenomas and cancers harbor β-catenin mutations primarily in exon 3 (37). Johnson et al. (38) recently reported that β-catenin mutations (exon 3) were present in 18.2% of cancers from hereditary non-polyposis colon cancer patients but absent in microsatellite stable (N = 78) and unstable (N = 34) sporadic CRCs. Immunohistochemical analysis of β-catenin expression revealed that 91.3% of polyloid lesions expressed

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nuclear β-catenin compared with 0% of flat lesions (P < 0.0001). These data are identical to those of our previous study in DSS-treated Swiss Webster mice (16). In contrast, Fuji et al. (14) observed similar expression of nuclear β-catenin in polyoid and flat lesions of DSS-treated p53+/–, p53+/- and p53+/+ mice. One possible explanation for the lack of nuclear β-catenin in flat lesions in the present study is that the small size of these lesions hindered identification of foci positive for nuclear localization of β-catenin. All seven mice with β-catenin mutations expressed nuclear β-catenin, whereas nine mice expressed nuclear β-catenin but lacked β-catenin mutations. From the latter group, three were p53+/– mice, five were p53+/- mice and one was a p53+/+ mouse. Several studies (39,40) have shown that cellular expression of β-catenin can be regulated by p53. Cagatay et al. (41) convincingly demonstrated that nuclear accumulation of β-catenin in hepatocellular carcinomas is due to mutation of p53. Wild-type p53 has been shown to induce transcription of the Siah-1 gene, which encodes a protein involved in the ubiquitin-mediated degradation of β-catenin (39). The loss of regulation of β-catenin by Siah-1 could explain the observed presence of nuclear β-catenin in colorectal lesions from p53+/– mice lacking β-catenin mutations. Castellone et al. (42) recently reported that prostaglandin E2 (PGE2), through its prostaglandin E receptor type 2-binding site, can prevent degradation of β-catenin in cells with activated Wnt signaling, resulting in nuclear accumulation of β-catenin. Since PGE2 is up-regulated in UC (43), this could be another explanation for the nuclear localization of β-catenin in the absence of β-catenin mutations. In human studies, a significantly higher incidence of nuclear β-catenin staining has been observed in sporadic CRC as compared with UC-associated cancers (44,45). Wong et al. (46) reported nuclear localization of β-catenin in 45% of UC-associated CRC as compared with 85% of sporadic CRC. However, in none of these studies were comparisons made between polyoid and flat lesions. The results of this study suggest that flat and polyoid lesions arise from different molecular pathways. First, the presence of flat lesions was significantly associated with loss of p53 function. Second, nuclear localization of β-catenin was present in 91.3% of the polyoid lesions whereas none of the flat lesions showed this pattern. This result is in agreement with our previous study, which revealed a significant association between nuclear β-catenin and polyoid colitis-associated neoplastic lesions in DSS-treated Swiss Webster mice (16). Third, the differential response of flat and polyoid lesions to chemoprevention by different agents has been noted. In the azoxymethane/DSS model of colitis-associated neoplasia, treatment with celecoxib decreased the multiplicity of polyoid lesions but not flat lesions (47). Lastly, recent examination of the gene expression profile of flat and polyoid colitis-associated adenomas by cDNA array revealed the differential regulation of 27 genes (48). Sixteen genes (e.g., p21, Smad4 and IGF-2) were up-regulated and 11 genes (e.g., Cdc42, p120 and Ras-GAP) were down-regulated significantly in the flat as compared with the polyoid lesions. These data, when combined with the results from the present study, provide strong support for the emergence of flat and polyoid lesions via different molecular pathways.

No mutations were found in codons 12/13 of K-ras in any of the neoplastic lesions from DSS-treated p53+/–, p53+/- and p53+/+ mice. Similarly, K-ras mutations are uncommon in UC-associated neoplasia in the human (49) and have not been reported in DSS colitis-associated neoplasia in the mouse (14,17). In an attempt to explain the mechanism of colitis-associated neoplasia in those mice without β-catenin mutations or loss of p53 function (p53+/– mice), lesions from DSS-treated p53+/– and p53+/- mice were analyzed for p53 mutations (exons 5–8) by direct sequencing. However, no mutations were found. In other mouse models of colitis-associated neoplasia, no p53 mutations were found in the IL-10–/– mice, whereas C/G → T:A transitions at codon 229 have been observed in IL-2–/– × β-micro–/– mice (19,20). Although neither mutations of p53 were found in DSS-treated p53+/- and p53+/+ mice, we still cannot rule out the possibility of loss of function of p53 by methylation or mutation/methylation of other members of the p53 pathway (i.e., mouse double minute 2 over-expression (50)).

Levels of inflammation have been shown to play an important role in both the type (flat versus polyoid) and incidence of colitis-associated neoplasia in mouse models (16) and in humans (3). In order to investigate if the increased incidence and multiplicity of neoplasia in the p53–/– mice could be due to differences in inflammation, we studied inflammation scores in all three genotypes. After three cycles of DSS, p53+/- mice were found to have significantly higher inflammation scores than p53+/- and p53+/+ mice. No significant difference in the inflammation scores of p53+/- and p53+/+ mice was observed. At the end of 120 days of water, little if any inflammation was present in p53+/- and p53+/+ mice and the colorectal mucosa was healed. p53–/– mice of the same age could not be compared at this point in time, as 13 of 14 mice were killed (due to illness) at a mean of 26 days after the third cycle of DSS. These findings indicate that the higher incidence and multiplicity of colitis-associated neoplasia in p53–/– mice may not have any association with levels of inflammation.

In this study, we have characterized colitis-associated neoplasia in p53–/–, p53+/- and p53+/+ mice using the DSS model of mouse colitis. Our results show that loss of p53 enhances the induction of colitis-associated colorectal neoplasia, in particular flat lesions, and dysregulation of β-catenin signaling plays an important role in the formation of polyoid lesions in this mouse model of colitis-associated dysplasia. Because p53 function appears to play a protective role in colitis-associated neoplasia in the human and in this DSS mouse model, we believe this model provides an excellent system to study both the pathogenesis and chemoprevention of colitis-associated neoplasia. The contribution of β-catenin mutations and different molecular pathways to the formation of polyoid and flat lesions is worthy of further investigation in the human.

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References


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