Microadenomatous Lesions Involving Loss of Apc Heterozygosity in the Colon of Adult Apc\(^{Min/+}\) Mice

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Abstract

Mutations in the human adenomatous polyposis coli (APC) gene are causative for familial adenomatous polyposis (FAP), a rare condition in which numerous colonic polyps arise during puberty and, if left untreated, lead to colon cancer. Mouse model for human FAP, Apc\(^{Min/+}\) mouse, contains a truncating mutation in the APC gene and spontaneously develops intestinal adenomas. However, the distribution of tumors along the intestine found in Apc\(^{Min/+}\) mice is different from that in human FAP. A majority of intestinal polyps in the Apc\(^{Min/+}\) mouse is known to be located in the small intestine but rarely detected in the colon. We report here that adult Apc\(^{Min/+}\) mice develop dozens of microadenomatous lesions in the colon (>20 lesions/colon). Surprisingly, the vast majority of such adenomatous lesions consisting of colonic crypts were <300 \(\mu\)m in their greatest dimension, whereas lesions in the small intestine showed various sizes. The allelic loss analysis revealed that these adenomatous crypts in the colon have already lost the remaining allele of Apc. Such findings suggest that, in contrast to tumorigenesis in the small intestine, loss of heterozygosity of the APC gene is not sufficient for tumor development in the colon of Apc\(^{Min/+}\) mice. Our results may give an account for the low incidence of colonic tumors in Apc\(^{Min/+}\) mice.

Introduction

The colorectal carcinogenesis is known to have multistep processes (1). Because of its gradual evolution toward malignancy, colorectal cancer might serve as an excellent paradigm to examine the genes involved in tumorigenesis. In humans, APC, \(\beta\)-catenin (CTNNB1), Ki-ras (KRAS1), and p53 (TP53) genes play important roles at different stages of colorectal carcinogenesis (2, 3). Of these, mutations in APC gene found in the earliest stages of the adenoma-carcinoma pathway are considered to play a gate-keeping role in the carcinoma pathway (4). Such findings suggest that, in contrast to tumorigenesis in the small intestine, loss of heterozygosity of the APC gene is not sufficient for tumor development in the colon of Apc\(^{Min/+}\) mice. Our results may give an account for the low incidence of colonic tumors in Apc\(^{Min/+}\) mice.

Materials and Methods

Experimental Procedure. Apc\(^{Min/+}\) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). They were bred and maintained on a basal diet, CE-2 (CLEA Japan Inc., Tokyo, Japan), until termination of the study. Sixteen Apc\(^{Min/+}\) mice (9 males and 7 females) and 7 Apc\(^{-/-}\) mice (4 males and 3 females), which were >20 weeks of age (20–33 weeks of age), were used in the present study. The colonies were removed, cut open along their longitudinal axis, and fixed flat in 10% buffered formalin for 24 h at room temperature. Colon tumors identified macroscopically were also fixed in 10% buffered formalin and processed for histopathological examination by routine procedures (5). To identify small tumors, mucosal surface with methylene blue staining was observed under a microscope. In brief, fixed colon were placed in 0.5% solution of methylene blue in distilled water. They were then placed mucosal side up on a microscope slide and observed through a light microscope at a magnification of \(\times40\).

Tissue Preparation. To identify intramucosal lesions, colon mucosa was examined in histological sections. Colon (the length of 6 cm from the anal ring) were divided into three segments and were embedded in paraffin. The middle part of the small intestine was also cut into small segments and embedded in paraffin. A total of 69 segments from the colon and 23 segments from the small intestine were examined by using en face preparation and 3–5-\(\mu\)m thick serial sections (14, 15, 17). For each case, 10–20 serial sections were used to investigate the intramucosal lesions.

LMM and LPC. In the current study, DNA for the analysis of the Apc allelic loss was extracted from cells isolated with LMM and LPC (zref18). For laser capturing, the slides were put into xylene for 30 min to dissolve the paraffin that otherwise interferes with LMM. Next, the slides were washed for 10 min in 100% ethanol. After staining with H&E, sections were dehydrated.
in 100% ethanol, incubated 2 min in xylene, and dried at room temperature. Sections were captured in one session using the PALM Robot-MicroBeam system (P.A.L.M. Mikrolaser Technology AG, Bernried, Germany). The LPC-collected cells were solved in 20 μl of lysis buffer.

Apc Allelic Loss Analysis. A total of 62 microdissected tissues (28 normal-appearing crypts, 14 microadenomatous lesions in the colon, 5 tumors in the small intestine, 10 colonic tumors in Apcmin/+ mice, and 5 normal crypts in Apcmin/min mice) were selected at random. They were digested overnight at 50°C in 20 μl of lysis buffer containing 500 μg/ml proteinase K, 10 mmol/liter Tris-HCl (pH 8.0), 50 mmol/liter KCl, 0.45% NP40, and 0.45% Tween 20. The proteinase K was heat inactivated (10 min at 95°C). The tubes were centrifuged for 5 min, and the supernatant was transferred to new tubes. LOH of the Apc gene was checked using PCR with mismatched primers, as described previously (12). Briefly, the amplification of the Apcmin allele resulted in a 155-bp PCR product with one HindIII site, whereas the 155-bp product from the Apc+/+ allele contained two HindIII sites. HindIII digestion of PCR-amplified DNA from Apcmin/+ heterozygous tissue resulted in a 123-bp product from the Apc+/+ allele and a 144-bp product from the Apcmin allele. Therefore, PCR products from tissue with LOH displayed only one band (144-bp) from the Apcmin allele. Samples were assayed at least twice, independently.

Results

Microadenomatous Lesions in theColon of Apcmin/+ Mice. Unlike the frequency of tumors in the small intestine (30–60 tumors/small intestine), only small numbers of tumors (0–5 tumors/colon) were evident in the colon of adult Apcmin/+ mice. Histological sections of these colonic tumors were examined for tumor types, and they were classified as adenomas or adenocarcinomas. In the sections stained with H&E, dysplastic crypts were detected frequently in the colonic mucosa of all of the Apcmin/+ mice (Fig. 1A), whereas the lesions were absent in the colons of Apc+/+ mice. Adult Apcmin/+ mice developed at least 20 lesions/colon. The histological features of such dysplastic crypts resembled those of adenomas; the crypts bore basophilic cytoplasm and hyperchromatic nuclei. Mucin production was almost absent in those crypts. F, a cross-section of a single adenomatous crypt in the small intestine, which have been described as the earliest lesion in the small intestine (13, 24). Bars. 300 μm (A and B), 100 μm (C, D, and F), 50 μm (E).

![Fig. 1. Microadenomatous lesions found in the colon of aged Apcmin/+ mice. A, at lower magnification of en face sections, intramucosal adenomatous crypts are frequently detectable in the colonic mucosa of aged Apcmin/+ mice (arrowheads). Note that all lesions are <300 μm in their greatest dimension. B, in the small intestine, various sizes of adenomatous lesions are seen in cross-sections. C–E, higher magnifications of microadenomatous crypts in the colon. The crypts bear basophilic cytoplasm and hyperchromatic nuclei. Mucin production is almost absent in those crypts. F, a cross-section of a single adenomatous crypt in the small intestine, which have been described as the earliest lesion in the small intestine (13, 24). Bars. 300 μm (A and B), 100 μm (C, D, and F), 50 μm (E).](cancerres.aacrjournals.org)
six adenomatous crypts. The greatest dimensions of the adenomatous lesions, including microadenomatous lesions, adenomas, and adenocarcinomas, in both colon and small intestine are summarized in Fig. 2. In contrast to the colon, adult Apc\(^{Min+}\) mice developed a number of adenomatous lesions with a variety of sizes in the small intestine (Fig. 1, B and F; Fig. 2). The numbers of the lesion/area in the colon and small intestine were 17.85 ± 9.86/cm\(^2\) and 8.07 ± 3.06/cm\(^2\), respectively (Table 1). The incidence of colonic lesions was significantly higher than that of the small intestine (P < 0.001). The mean dimensions of colonic lesions and small intestinal lesions were 176.04 ± 410.84 \(\mu\)m and 1286.96 ± 1069.78 \(\mu\)m, respectively (Table 1). The size of colonic lesions was smaller than that of the small intestine (P < 0.001).

**Apc Allelic Loss in Colonic Lesions.** Twenty-eight normal-appearing crypts, 14 microadenomatous lesions in the colon, and 15 intestinal tumors were randomly selected, and picked individually from the histological sections of adult Apc\(^{Min+}\) mice. Fig. 3 represents the results of nondenatured acrylamide gel electrophoresis. After HindIII digestion of PCR-amplified DNA, two DNA bands (144-bp and 123-bp) appeared on gels from phenotypically normal crypts of Apc\(^{Min+}\) mice, whereas only one band at 123-bp was evident in normal crypts of the Apc\(^{+/-}\) mice (Fig. 3). All of the PCR products from tumors revealed a single band (144-bp) deriving from the Apc\(^{Min}\) allele (Fig. 3; Table 2). Remarkably, the majority of microadenomatous lesions (12 of 14 lesions) showed a single band at 144 bp, suggesting that such small lesions have lost Apc\(^+\) allele already (Fig. 3; Table 2).

**Discussion**

Min mice are heterogeneous for a nonsense mutation in the Apc gene. They develop spontaneously Mins similarly to the FAP syndrome in humans. However, it is well demonstrated that the distribution pattern of intestinal tumors in Apc\(^{Min+}\) mice is different from that in human FAP. Most adenomatous polyps in FAP patients arise in the colon and, if left untreated, lead to colonic cancers. In contrast, the highest frequency of tumors in Apc\(^{Min+}\) mice is seen in the small intestine, whereas lower numbers are located in the colon (19). In the current study, we found that there are many microadenomatous lesions that are hardly identified in the whole mount preparations in the colonic mucosa of the Apc\(^{Min+}\) mice. It is noteworthy that adult Apc\(^{Min+}\) mice developed a number of lesions not only in the small intestine but also in the colon.

It has been demonstrated that loss of Apc function plays a pivotal role in colorectal carcinogenesis. It is also known that all of the intestinal tumors in mice heterozygous for a mutant allele of Apc have lost the Apc function by LOH (12, 13). In this study, microadenomatous crypts in the colon were found to have lost the remaining allele of Apc, indicating that loss of Apc function has already occurred in such crypts. Accordingly, it seems to be reasonable to apply Knudson’s “two-hit” theory (20) to the formation of microadenomatous lesions in the colon of Apc\(^{Min+}\) mice. Importantly, in this study, the number of colonic adenomatous lesions per area was much higher than that of lesions in the small intestine, suggesting that LOH of the Apc occurs frequently in the colonic crypts as well as epithelium in the small intestine.

It is also interesting that almost all of the intramuscosal adenomatous lesions in the colon were <300 \(\mu\)m in their greatest dimension. Because Apc\(^{Min+}\) mice used in this experiment were >20 weeks of age, such microadenomatous crypts are suggested to be self-limiting lesions and not grow into colonic tumors. Conditional knockout mice of the Apc are reported to develop only 6.7 colon tumors on average, although numerous colonic cells in the mice ought to be lacking in both alleles of Apc (21). The results in the present study, together with previous findings, suggest that inactivation of the Apc is not sufficient for development of colonic tumors. It is noteworthy that mutation of K-ras seems to be correlated with the development of large, dysplastic adenomatous polyps in humans (1, 22). K-ras mutation may be associated with the tumor development in the colon of Apc\(^{Min+}\) mice. However, no ras mutations have been found in intestinal tumors of Apc\(^{Min+}\) mice (23), indicating that the mutational activation of K- or H-ras is not a common event in the formation of intestinal adenomas in Apc\(^{Min+}\) mice. Therefore, it is possible that another genetic and/or epigenetic event except ras mutations occurs in those colonic tumors.

In contrast to colonic lesions, it is quite interesting to note that the adenomatous lesions in the small intestine had various sizes, and the mean size of the lesions was significantly larger than in the colon. We detected different adenomatous lesions, which have been reported in the stages of polyp development (13, 24), including a single adenomatous crypt. It has been demonstrated that loss of the Apc gene is also involved in the earliest lesions in the small intestine of mice heterozygous for a mutant allele of Apc (13). It is possible that, in the small intestine, Apc plays a crucial role in colorectal carcinogenesis.

**Table 1** Adenomatous lesions in the colon and small intestine

<table>
<thead>
<tr>
<th></th>
<th>Lesion/area (cm(^2))</th>
<th>Mean diameter ((\mu)m)</th>
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<tbody>
<tr>
<td>Colon</td>
<td>17.85 ± 9.86(^b)</td>
<td>176.04 ± 410.84(^c)</td>
</tr>
<tr>
<td>Small intestine</td>
<td>8.07 ± 3.06</td>
<td>1286.96 ± 1069.78</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SD.
\(^b\) The incidence of colonic lesions was significantly higher than that in the middle portion of the small intestine (P < 0.001).
\(^c\) The mean diameter of colonic lesions was significantly smaller than that in the small intestine (P < 0.001).

**Table 2** Apc allelic loss in the Apc\(^{Min+}\) mouse

<table>
<thead>
<tr>
<th></th>
<th>Normal-appearing crypts</th>
<th>Microadenomatous crypts</th>
<th>Tumors</th>
<th>Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>0/28 (0%)</td>
<td>12/14 (85.7%)</td>
<td>10/10 (100%)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>Small intestine</td>
<td></td>
<td></td>
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Fig. 3. Apc allelic loss assay. Two DNA bands (144-bp and 123-bp) appear on gels from phenotypically normal crypts of Apc\(^{Min+}\) mice, whereas only one band at 123-bp is evident in normal crypts of the Apc\(^{+/-}\) mice. PCR products from tumors revealed a single band (144-bp), which were derived from the Apc\(^{Min}\) allele. Remarkably, microadenomatous lesions also showed a single band at 144 bp, suggesting that such crypts already have lost Apc\(^+\) allele.
intestine, a dominant-negative mechanism by the loss of function of Apc is directly responsible for the formation of microadenomas that could develop into intestinal tumors. Thus, our results may explain why the Apc<sup>M<sub>Min</sub></sup> mouse develops intestinal tumors preferably in the small intestine and suggest that mechanisms of tumorigenesis involved in the small intestine may differ from those in the colon.

In conclusion, it was shown that there are a number of microadenomatous lesions together with a few tumors in the colon of aged Apc<sup>M<sub>Min</sub></sup> mice. Such microadenomatous lesions have already lost the remaining allele of Apc, indicating that LOH of the Apc gene has occurred in the crypts. These findings suggest that loss of Apc function is responsible for the formation of microadenomatous lesions in the colon but is not sufficient for the development of colonic tumors in Apc<sup>M<sub>Min</sub></sup> mice.

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References


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