Enhanced Intestinal Adenomatous Polyp Formation in Pms2−/−;Min Mice

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Abstract

Analysis of two human familial cancer syndromes, hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis, indicates that mutations in either one of four DNA mismatch repair gene homologues or the adenomatous polyposis coli (APC) gene, respectively, are important for the development of colorectal cancer. To further investigate the role of DNA mismatch repair in intestinal tumorigenesis, we generated mice with mutations in both ApC and the DNA mismatch repair gene, Pms2. Whereas Pms2-deficient mice do not develop intestinal tumors, mice deficient in Pms2 and heterozygous for Min, an allele of ApC, develop approximately three times the number of small intestinal adenomas and four times the number of colon adenomas relative to Min and Pms2+/−; Min mice. Although Pms2 deficiency clearly increases adenoma formation in the Min background, histological analysis indicated no clear evidence for progression to carcinoma.

Introduction

Germ-line mutation in the DNA mismatch repair gene homologues, MSH2, MLH1, PMS1, and PMS2, are associated with hereditary nonpolyposis colon cancer, HNPCC (1-3). In addition to colon cancer, HNPCC patients are susceptible to endometrial, ovarian, and stomach cancers (4) and possibly breast cancer (5). Tumors from HNPCC patients show frequent changes in the length of microsatellite DNA sequences consistent with a defect in DMR (6). The presence of microsatellite instability is also observed in a variety of sporadic cancers (1-3), suggesting a broader role for the involvement of DMR deficiency in the development of human cancers. Mice deficient for either Msh2, Mlh1, or Pms2 display the expected microsatellite instability and are susceptible to the early onset of lymphomas and sarcomas (7-9). In addition, Msh2−/− and Mlh1−/− mice frequently develop intestinal tumors during the first year of life (10). In contrast, we find that deficiency for Pms2 does not predispose mice to intestinal tumor development during the first 17 months of life.

FAP is associated with germ-line mutation in the tumor suppressor gene, APC. APC, APC appears to function as a "gatekeeper" protein to regulate cell proliferation within intestinal epithelium (1). Unlike HNPPC, mutation in APC results in the development of multiple polyps in the intestine, some of which progress to carcinoma (1). In addition, APC mutations have been found frequently in sporadic cases of colorectal cancer (1). Multiple intestinal neoplasia (Min) in mice resembles FAP in humans and is the result of a single point mutation in the murine ApC gene (11). Mice heterozygous for Min develop numerous adenomatous polyps, become anemic, and die around 5 months (12). Interestingly, in mice carrying the Min mutation and deficient for the DMR gene Msh2 results in increased numbers of adenomas without causing detectable progression to adenocarcinoma (13). Because we have found that mice deficient for the DMR gene Pms2 are not predisposed to intestinal tumors, it was of interest to examine the effect of Pms2 deficiency in mice carrying the Min mutation. Here we report that Pms2 deficiency in combination with Min significantly increases adenoma formation in both the small intestine and colon. However, Pms2 deficiency in combination with the Min mutation did not result in obvious progression from adenoma to adenocarcinomas.

Materials and Methods

Generation of Mice. The generation of Pms2 knockout mice has been described (7). C57BL/6 male Min (ApC−/−/−) mice were obtained commercially (The Jackson Laboratory, Bar Harbor, ME) and were set up for breeding Pms2−/−/−; Min mice. These breedings generated Pms2−/−/−; Min offspring used in this study. Genotyping of the Min and Pms2 mutations are as described previously (12, 14). All animals were fed food (9% total fat by weight) and water ad libitum and maintained on a 12-h light/dark schedule.

Statistical Analysis. To evaluate the differences in the number of intestinal and colon polyps occurring in Pms2−/−/−; Min, Pms2−/−/−; Min, and Pms2−/−/−; Min mice, one-way analyses of variance were performed. If found to be statistically significant (P < 0.05), these were followed up using the Tukey's Studentized Range Test at a procedure-wise error-rate equal to 0.05 (a* = 0.05). The analyses were performed on log-transformed data.

Assays for LOH for ApC and Microsatellite Instability. A 150-bp fragment containing the Min mutation (11) was amplified using primers GTTTCGTTCTGAGAAAGAC and CTTCCATAACTTTGGCTATC for 5' and 3' primers, respectively. PCR was performed, 95°C for 30 s, 58°C for 45 s, and 72°C for 60 s, for 35 cycles in the presence of [3P]dCTP. SSCP was performed on a 6% nondenaturing polyacrylamide gel containing 10% glycerol. Gels were run overnight at 10 W at room temperature. The different alleles (WT and Min) could be distinguished after SSCP, and loss of the WT

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3 The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; DMR, DNA mismatch repair; FAP, familial adenomatous polyposis; APC, adenomatous polyposis coli; Min, multiple intestinal neoplasia; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism; WT, wild type.
Microsatellite Instability and Mechanism for Loss of WT Apc Expression in Adenomas from Pms2−/−:Min Mice. To address the genetic changes within Pms2−/−:Min, Pms2+/−:Min, and Min adenomas, we examined the stability of microsatellite sequences from both the adenomas and surrounding normal tissue. Selective UV radiation fractionation procedures were performed on a limited population of cells from either normal mucosa or adenomas (15). Stable microsatellite sequences were observed in both adenomas and surrounding normal tissue from Min and Pms2+/−:Min intestines. In contrast, and as expected from previous examination of Pms2-deficient mouse tissues (7, 15), frequent mutations at several dinucleotide repeat loci were observed in both adenomas and normal mucosa from Pms2−/−:Min mice (data not shown).

An essential step for intestinal tumor development in both human FAP patients and Min mice is loss or inactivation of the remaining WT Apc allele (1). We tested for Apc protein in intestinal adenomas by immunohistochemical staining with antisera specific for the COOH terminus of Apc. Whereas staining was observed in the normal tissue surrounding the lesion, the adenomatous portions of the Pms2−/−:Min polyps consistently failed to stain for Apc protein (Fig. 2). A similar lack of Apc staining was observed in Pms2+/−:Min polyps.

The analysis of human colon cancers indicates that the WT Apc gene is either lost, as determined by LOH studies, or inactivated by somatic mutation (1). LOH is primarily responsible for loss of Apc expression in polyps from Min mice (19). To determine the Apc gene

allele was estimated to be present when the intensity was visually less than one-half of the Min allele. For microsatellite instability analysis, DNA from small 200–400 cell regions of polyps and normal mucosa was microdissected using selective UV radiation fractionation (SURF) procedures and subsequent PCR as described previously (15).

**Immunochemistry Staining of Intestinal Polyps for the Apc COOH Terminus.** Formalin-fixed, paraffin-embedded tissues were analyzed with an Apc antibody specific to the COOH terminus (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using the antigen retrieval method (16).

**Results and Discussion**

**Pms2 Deficiency Enhances Adenomatous Polyp Formation in Mice with the Min Mutation.** In our facility, Min mice developed a mean number of 72 polyps/intestine, whereas mice of the genotype Pms2+/−:Min developed a mean number of 90 intestinal polyps. In contrast, we observed a mean of 207 intestinal polyps in Pms2−/−:Min mice or 2.9 and 2.3 times more polyps than the number observed in the Pms2+/−:Min and Pms2+/−:Min mice, respectively (Table 1). Statistical analysis confirmed that the greater number of polyps in the Pms2+/−:Min animals was significant ($P < 0.001$). In addition, the mean number of colonic polyps in Pms2−/−:Min mice was increased approximately 3-fold over Min and Pms2+/−:Min mice. The greater number of polyps occurring in the Pms2+/−:Min intestine is likely the cause of early onset of morbidity in the Pms2+/−:Min mice. Pms2 deficiency alone did not predispose to adenoma formation during the course of this study or even in animals living to 17 months of age.

All intestinal polyps analyzed from Pms2+/−:Min mice were adenomatous in nature (Fig. 1). Using histological criteria for grading tumor development, we did not observe any evidence for adenocarcinoma, e.g., cell invasion into the underlying mucosa in tumors examined from either the small intestine or the colon of Pms2−/−:Min mice. We observed a similar pathology of adenomatous polyp development in Min and Pms2+/−:Min intestines. Therefore, Pms2 deficiency in combination with Min enhances adenomatous polyp formation without detectable progression to adenocarcinoma. As mentioned above, similar findings, including increases in the number of intestinal polyps without adenocarcinoma development, have been reported for Msh2−/−:Min mice (13).

Studies with Min mice treated with mutagen show that inactivation of the WT Apc allele must occur early in perinatal mouse development for intestinal polyps to arise (17). By analogy, the significant increases in the number of adenomas observed in intestines from both relatively young Msh2−/−:Min (13) and Pms2+/−:Min mice suggest a similar early inactivation of the WT Apc gene during adenoma development. Therefore, Apc is a target for the development of DMR-deficient intestinal tumors in the mouse. Similarly, the Apc gene is targeted in human colorectal tumors with a microsatellite instability phenotype, where a predominance of frame shift mutation is observed (18). The increased number of adenomas observed in Pms2+/−:Min intestines further suggests that Pms2 function is normally required for genetic stability in the intestinal mucosa.

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**Table 1. Effect of Pms2 genotype on Min-associated intestinal adenomas**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of mice</th>
<th>Mean no. of adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pms2+/−:Min</td>
<td>11</td>
<td>207 (5.6)</td>
</tr>
<tr>
<td>Pms2+/−:Min</td>
<td>32</td>
<td>90 (1.2)</td>
</tr>
<tr>
<td>Pms2−/−:Min</td>
<td>30</td>
<td>72 (1.4)</td>
</tr>
</tbody>
</table>

Note: SDs were calculated as follows: 104 for Pms2−/−:Min mice, 66 for Pms2+/−:Min mice, and 50 for Min mice. (Analysis of the log-transformed data indicates that the variance is similar among the three groups of mice).
status in the Pms2+/--:Min and Pms2+/--:Min adenomas, we used PCR-SSCP procedures with primers flanking the Min point mutation (Fig. 3). In Pms2+/--:Min polyps, LOH for the Min allele was observed in 25 of 29 (86%) of the samples analyzed. The percentage of Ace loss due to LOH is consistent with previous data published for Min mice (19). In contrast, only 12 of 37 (32%) of the Pms2+/--:Min polyps analyzed demonstrated LOH using this assay. Our results suggest that the lack of Ace staining in the majority of these polyps is due to somatic inactivation via point mutation. DNA sequence analysis of Ace will be required to confirm this postulated mechanism of inactivation.

As noted earlier, Pms2 deficiency enhanced adenoma formation in Min mice but did not obviously accelerate progression to adenocarcinoma. Because adenocarcinomas occur infrequently in Min mice (12), the progression to a stage beyond adenoma may be a rate-limiting step. Morbidity in the Min mouse occurred as a result of intestinal blockage and anemia. One possibility is that both the time necessary for mutations to accumulate and the metabolic events sufficient for tumor promotion may be limiting. Several lines of evidence have supported a link between intestinal physiology and tumor susceptibility in the Min mouse. The inhibition of cyclooxygenase activity in the intestines of Min mice by the administration of sulindac results in reduced polyp formation. Mom-1 (phospholipase A2) activity in the intestines of Min mice by the administration of sulindac results in reduced polyp formation. 

In humans, evidence for cooperativity between mutation in DMR genes and APC in colorectal cancer development has been reported (18). The analysis of HNPCC and sporadic tumors with microsatellite instability suggests that a significant proportion contain insertion/deletion mutations within short poly(A) tracts in APC (18). This observation suggests that a combination of DMR deficiency and APC mutation in humans can enhance the progression from polyp to carcinoma. In our mouse models, we found that the combination of Pms2 deficiency and Min results in a 2-3-fold increase in the number of adenomas compared to Min animals. The adenomas from the Pms2+/--:Min animals display microsatellite instability and a lack of Ace protein, presumably due to somatic inactivation of the normal Ace allele by point mutation. Notably, although Pms2 deficiency resulted in an increase in adenoma number in Min animals, we observed no evidence for progression to adenocarcinoma. The lack of obvious progression may be due to early morbidity and consequent insufficient time for the accumulation of the mutational events required for carcinoma. In total, our findings on combining Pms2 deficiency with the Min mutation are notably similar to findings reported for mice carrying the Min mutation and deficient in Msh2 (13). Finally, we stress that whereas Msh2 deficiency (10) or Mlh1 deficiency each predisposes to intestinal adenomas or carcinomas, Pms2 deficiency does not. The lack of intestinal tumors in Pms2-deficient mice, despite the increased mutation frequencies observed in all tissues studied (21), including intestinal mucosa, suggests the possibility of differing but overlapping roles for Msh2, Mlh1, and Pms2 in cellular processes in the intestine.

References

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