**MSH2 Deficiency Contributes to Accelerated APC-mediated Intestinal Tumorigenesis**

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**Abstract**

Accelerated intestinal tumorigenesis is probable in hereditary non-polyposis colorectal cancer, a condition associated with germ line DNA mismatch repair (MMR) gene defects, and is believed to be caused by rapid accumulation of replication errors in critical genes, such as the APC (adenomatous polyposis coli) tumor suppressor gene. To study the potential contribution of MMR genes to accelerated intestinal tumorigenesis, we crossed the Min mouse, heterozygous for a germ line mutation of Apc, with an MMR gene (Msh2)-deficient mouse. MSH2 deficiency resulted in the development of many colonic aberrant crypt foci, as well as reduced survival of the mice, secondary to both a greater number and more rapidly developing adenomas. The mechanism of inactivation of the wild-type Apc allele depended on MSH2 status. In the presence of functional MSH2, all tumors demonstrated loss of heterozygosity. In contrast, whereas all adenomas were Apc negative by immunostaining, only 34 adenomas from Apc+/−/Msh2−−/− mouses demonstrated loss of heterozygosity of the wild-type Apc allele, suggesting that somatic Apc mutations are responsible for the additional tumors. These findings provide evidence for the important role of MMR genes in accelerated intestinal tumorigenesis, thus supporting more aggressive surveillance strategies to prevent colorectal cancer in hereditary non-polyposis colorectal cancer.

**Introduction**

Approximately 5% of cases of colorectal cancer can be attributed to two autosomal dominant inherited syndromes, FAP and HNPCC (reviewed in Ref. 1). FAP is caused by germ line mutations of the APC tumor suppressor gene (2), and most patients with HNPCC have germ line mutations of one of the MMR genes (3). Both FAP and HNPCC are characterized by the development of colorectal adenomas and carcinoma at an early age. In FAP, and in most sporadic colorectal cancers, inactivation of the APC gene appears to be critical. APC mutations are among the earliest detectable lesions in potentially premalignant microscopic ACF (4), whereas it is likely that mutations of MMR genes, such as MSH2, are important during the apparent rapid progression of the adenoma-to-carcinoma sequence in HNPCC (5, 6). Yet, the combined molecular contribution of the APC and MMR proteins to colorectal polyp development and progression is not known. In the present study, we have taken advantage of mouse models harboring germ line mutations of Apc and Msh2 to investigate the relationship of APC and MMR genes to intestinal tumorigenesis.

**Materials and Methods**

Mice. Min mice (Apc−−−−; Ref. 7) have been bred on the C57BL/6J strain in our facility (original breeding pair from The Jackson Laboratory, Bar Harbor, ME) for the past 18 months. The generation of our Msh2 knockout mouse (Msh2−−−−) has been described recently (8). Male Min mice were crossed with female Msh2−−−− mice to generate Apc+/−/Msh2−−−− mice. Male Apc+/−/Msh2+/− mice were then bred with female Msh2−−−− mice to generate Apc+/−/Msh2+/−, Apc+/−/Msh2−−−−, and Apc+/−/Msh2+/− offspring.

Enumeration of Intestinal Adenomas and Colonic ACF. Mice were sacrificed by cervical dislocation, and intestines were immediately removed and flushed with Ringer's solution to remove food and fecal debris. The intestines were opened longitudinally, laid flat on Whatman paper, and fixed for at least 3 h in 10% neutral buffered formalin. Fixed intestines were stained with methylene blue and examined for tumors and ACF by gross inspection and light microscopy, as described (4).

**Mathematical Models of Adenoma and ACF Accumulation.** Adenomas were assumed to result from a Poisson-distributed (with mean 1) number of mutant clones present at birth and from postnatal somatic mutations that occur randomly throughout life at rate λ. The mutant clones are assumed to behave independently and to require a random period of time to develop into adenomas (with cumulative density function F(t) assumed to be a γ distribution). With these assumptions, the number of visible adenomas at time t is Poisson-distributed with the mean

\[ N(t) = \lambda \int_0^t F(x) \, dx \]

Note that for simplicity we have ignored potential effects of the crypt cycle in this analysis (9). It turns out to be a good approximation since most tumors arise early in life. ACF accumulation was modeled in the same way, except that it was assumed that ACF, after a random period of time, form adenomas. Then the number of ACF at time t is Poisson-distributed with the mean

\[ u(t) + \lambda \int_0^t r(x) \, dx \]

where \( r(t) = \int_0^t f_0(x)[1 - F_2(t - x)] \, dx \) and \( f_2(t) \) is the probability density function for the time spent before a mutant clone converts into an ACF and \( F_2(t) \) is the cumulative density function for the time required for an ACF to develop into a visible adenoma. Similarly, the number of adenomas expected is Poisson-distributed with the mean

\[ v(t) + \lambda \int_0^t s(x) \, dx \]

where \( s(t) = \int_0^t f_0(x)f_2(t - x) \, dx \)

The times required for ACF formation and adenoma conversion were assumed to be exponentially distributed.

**Microsatellite Instability and ACF LOH Assay.** DNA was extracted from paraffin blocks of normal liver and intestinal adenomas by proteinase K
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digestion (8). Care was taken to avoid nonneoplastic areas in adenomas. Microsatellite instability was detected by comparison of electrophoretic mobility of amplified normal and tumor DNA using primers from loci on mouse chromosomes 1, 2, 5, 6, and 10, as described (8).

The PCR-based Ape locus quantitation assay was performed as described by Luongo et al. (10). Briefly, following digestion with HindIII, the 144-bp PCR product from the Apc<sup>Min</sup> allele and 123-bp product from the Apc<sup>+</sup> allele were resolved on 6% polyacrylamide gels, followed by densitometric analysis. All samples were amplified in duplicate, and ratios of pairs differed by less than 10%. An Apc<sup>+</sup> allele:Apc<sup>Min</sup> allele ratio of 0.85 is expected in normal tissue (no LOH) based on the difference in the number of radioactive deoxycytosine residues in the two alleles following digestion with HindIII.

**Immunostaining of Intestinal Adenomas for the APC COOH Terminus.** Sections from intestinal adenomas were incubated with rabbit polyclonal antibodies specific for the COOH terminus of APC protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then stained with Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Controls, which were negative, included omission of the anti-APC antibody and competition with the peptide against which the antibodies were generated (human APC amino acids 2824-2843).

**Results and Discussion**

**MMR Deficiency Results in Accelerated Intestinal Tumorigenesis in Apc<sup>+</sup>/Msh2<sup>−/−</sup> Mice Compared to Apc<sup>+</sup>/Msh2<sup>+</sup>/+ and Apc<sup>+</sup>/Msh2<sup>+/−</sup> Mice.** Min mice bred in our facility developed approximately 100 small intestinal adenomas and 5 colorectal adenomas by 160-180 days, at which time they became moribund and died from anemia and intestinal obstruction. Msh2<sup>−/−</sup> mice are susceptible to the development of lymphoma by 2 months of age (8, 11), whereas Msh2<sup>+</sup>/+ mice are healthy up to at least 1 year of age. Min mice were bred with MSH2-deficient mice to examine the effects of MMR on intestinal adenoma formation. Animals were killed serially beginning at 10 days of age, and all adenomas in small and large intestines were counted. The number of adenomas were compared in Apc<sup>+</sup>/Msh2<sup>−/−</sup>, Apc<sup>+</sup>/Msh2<sup>+/−</sup>, and Apc<sup>+</sup>/Msh2<sup>+/+</sup> mice. Dysplastic adenomas were present from 10 days in all three genotypes, and the tumors were histopathologically similar. However, striking numbers of small and large bowel adenomas soon emerged in the Apc<sup>+</sup>/Msh2<sup>−/−</sup> mice in comparison with the other genotypes. For example, two of three Apc<sup>+</sup>/Msh2<sup>−/−</sup> mice had 8 colon adenomas at 27 days, whereas Apc<sup>+</sup>/Msh2<sup>+/−</sup> and Apc<sup>+</sup>/Msh2<sup>+/+</sup> mice never developed more than five colon adenomas until at least 70 days of age. Even more remarkable was the very large number of small bowel adenomas that arose in Apc<sup>+</sup>/Msh2<sup>−/−</sup> mice after 47 days. Between 47-78 days, an average of 333 adenomas were scored in 10 Apc<sup>+</sup>/Msh2<sup>−/−</sup> mice compared to a mean of 48 and 42 small bowel adenomas in similar age Apc<sup>+</sup>/Msh2<sup>+/+</sup> (n = 9) and Apc<sup>+</sup>/Msh2<sup>+/−</sup> (n = 8) mice, respectively (Fig. 1, A and B). No carcinomas were detected in any of the animals. The increased number of colon and small bowel adenomas in Apc<sup>+</sup>/Msh2<sup>−/−</sup> mice contributed to
their early death from anemia and bowel obstruction (mean age when moribund: Apc+/~/Msh2+/+, 163 days; Apc+/~/Msh2+/−, 159 days; Apc+/~/Msh2−/−, 82 days).

Having shown that MSH2 deficiency results in the development of more adenomas in Apc+/~/ mice, we sought to understand the dynamics of intestinal tumorigenesis and the genetic basis of our observations. We developed a mathematical model to obtain maximum likelihood estimates of the timing and frequency of initiating mutations, as well as estimates of the rate of adenoma growth, in each of the genotypes. The model assumes that some adenomas are present in nascent form perinatally (e.g., clones derived from prenatal somatic mutations), whereas others arise at random times throughout life. The analysis revealed three main conclusions that apply to both the large intestine and small intestine: (a) in all three genotypes, the bulk of adenomas arise from mutant clones that are present perinatally (few arise postnatally); (b) there are about three times more nascent tumors in Apc+/~/Msh2−/− mice than in the other genotypes; and (c) tumor progression is accelerated in the Apc+/~/Msh2−/− mouse (Fig. 1A; Table 1). Therefore, MSH2 deficiency leads to the development of more perinatal mutant clones, which expand into tumors more rapidly.

The observation that tumors arise primarily perinatally in mice supports the findings of others (12) but is surprising since MSH2 deficiency would be expected to contribute to ongoing postnatal replication errors in genes such as Apc, resulting in additional intestinal neoplasms during the life of Apc+/~/Msh2−/− mice. To address this issue, we used the ACF as an intermediate biomarker to determine the effects of MSH2 deficiency on postnatal genetic events. ACF have been proposed as precursors to colonic adenomas in man and carcinogen-treated rodents, based on their high frequency in colon from FAP patients, and by the presence of somatic APC and K-ras mutations (13, 14). We counted dysplastic ACF in methylene blue-stained colons from each of the genotypes. ACF were heterozygous and informative with a 2–8-bp separation between dinucleotide repeat loci/sample. Although the majority of these loci were heterozygous and informative with a 2–8-bp separation between the two alleles, only two RER+ patterns were observed in 2 colon adenomas from 2 mice of a total of 72 PCR reactions. Thymic lymphomas were observed in two of seven Apc+/~/Msh2−/− mice, which were 70–102 days old. These lymphomas were both positive at the D1 locus (Fig. 2). Our observation that the majority of intestinal adenomas from Apc+/~/Msh2−/− mice were RER negative is not too surprising since many human colorectal adenomas as well as normal tissues from Msh2−/− mice also do not display microsatellite instability when analyzed with standard (non-dilution) detection techniques (8, 17–19). Thus, widespread microsatellite instability is likely not a major factor leading to accelerated ACF and adenoma development in Apc+/~/Msh2−/− mice. It seems probable that perhaps only one or a few critical genes manifest replication errors and are thus responsible for the Apc+/~/Msh2−/− phenotype. This prompted us to investigate the contribution of APC to tumorigenesis in Apc+/~/Msh2−/− mice.

Loss of both copies of the APC gene, either by somatic mutations or by inactivation of the second allele, as demonstrated by LOH, appears to be a prerequisite for intestinal tumor development in humans and Min mice (10, 14, 20). Although both somatic APC mutations and LOH have been shown in human tumors, all adenomas from Min mice demonstrate LOH (10, 14, 20). We examined the frequency of LOH of Apc in small bowel and colon adenomas from each genotype. Ratios of Apc+ allele/ApcMut allele for normal liver in Apc+/~/Msh2−/− mice were 0.75 ± 0.08 (mean ± SD; n = 5), and for small bowel (n = 7) and colon adenomas (n = 7) together were 0.24 ± 0.13. Mean ratios for normal liver in Apc+/~/Msh2−/− mice. Shown are representative results for the DIM locus. Lane 1, spleen; Lane 2, liver; Lane 3, thymic lymphoma; Lanes 4–6, colon adenomas; Lanes 7–11, small bowel adenomas. Note that only the lymphoma is RER positive (arrow).

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**Table 1 Maximum likelihood estimates of parameters for the adenoma accumulation model**

The application of the mathematical model of macroscopic adenoma accumulation (see "Materials and Methods") yielded maximum likelihood estimates of the average time required for a nascent tumor to develop into a macroscopically visible adenoma. In the model, some nascent tumors arise from mutant clones present at birth, whereas others arise from postnatal somatic mutations that occur randomly throughout life at rate \( \lambda \) mutant clones/day. The number of perinatal mutant clones was assumed Poisson-distributed with mean \( \lambda \) mutant clones. The analysis also provided maximum likelihood estimates \( \lambda \) and \( t \) for \( \lambda \) and \( t \), respectively. Note that for all genotypes, no evidence of postnatal tumor initiation was found (\( \lambda = 0 \)).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Avg. time to</th>
<th>1 (clones)</th>
<th>( \lambda ) (clones/day)</th>
</tr>
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<tr>
<td>Small intestine</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Apc+/~/Msh2+/−</td>
<td>42 days</td>
<td>350</td>
<td>0</td>
</tr>
<tr>
<td>Apc+/<del>/Msh2+/− and Apc+/</del>/Msh2+/+</td>
<td>66 days</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apc+/~/Msh2−/−</td>
<td>27 days</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Apc+/<del>/Msh2+/− and Apc+/</del>/Msh2+/+</td>
<td>100 days</td>
<td>5</td>
<td>0</td>
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</tbody>
</table>

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**Fig. 2. Intestinal adenomas in Apc+/~/Msh2−/− mice are RER negative.** Microsatellite analysis of normal tissues, lymphoma, and small and large bowel adenomas in Apc+/~/Msh2−/− mice. Shown are representative results for the DIM locus. Lane 1, spleen; Lane 2, liver; Lane 3, thymic lymphoma; Lanes 4–6, colon adenomas; Lanes 7–11, small bowel adenomas. Note that only the lymphoma is RER positive (arrow).
Apc+/~/Msh2~'~ mice were 0.77 ± 0.07 (n = 7), and ratios for small bowel adenomas were based on ratios of 0.04, 0.16, and 0.02 and two small bowel adenomas (n = 20) and colon adenomas (n = 14) together were 0.19 ± 0.13. Therefore, the lack of LOH in most adenomas from Apc+'~/Msh2~'~ mice prompted us to confirm APC deficiency in these tumors. Immunostaining of small bowel and colon adenomas from Apc+'~/Msh2~'~ mice, using antibodies generated against the COOH terminus of APC protein, demonstrated lack of staining of dysplastic epithelium, as did two colon adenomas from Min mice. Wild-type staining is abolished by competition with the COOH-terminal peptide, against which the antibody was generated (data not shown).

Msh2++/ mice were 0.72 ± 0.10 (n = 6), and for small bowel (n = 6) and colon adenomas (n = 7) together were 0.19 ± 0.13. Therefore, LOH was observed in all (27 of 27) adenomas from Apc+'~/Msh2++/ and Apc+'~/Msh2++/ mice. Mean ratios for normal liver in Apc+'~/Msh2++/ mice were 0.77 ± 0.07 (n = 7), and ratios for small bowel (n = 20) and colon adenomas (n = 14) together were 0.77 ± 0.14, except for 3 colon adenomas, which displayed LOH with ratios of 0.33 and 0.34. These data indicate that only 5 of 34 intestinal adenomas from 7 Apc+'~/Msh2~'~ mice had lost the wild-type Apc allele. Apc Min allele are indicated below each lane. B, absence of APC COOH terminus in adenomas. Confocal fluorescence micrograph of a section of a colon adenoma, from an Apc+'~/Msh2~'~ mouse, incubated with rabbit polyclonal antibodies specific for the COOH terminus of APC protein and then stained with Cy3-conjugated goat anti-rabbit IgG (bar, 50 µm). Note the wild-type staining of normal epithelium surrounding nonstaining dysplastic epithelium (top left). Similar sections from six small intestinal and eight colon adenomas, from Apc+'~/Msh2~'~ mice, demonstrated lack of staining of dysplastic epithelium, as did two colon adenomas from Min mice. Wild-type staining is abolished by competition with the COOH-terminal peptide, against which the antibody was generated (data not shown).

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**References**

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