SHORT REPORT

Mlh1 deficiency enhances several phenotypes of Apc\textsuperscript{Min/+} mice

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Defects in A\textsubscript{PC} and DNA mismatch repair genes are associated with a strong predisposition to colon cancer in humans, and numerous mouse strains with mutations in these genes have been generated. In this report we describe the phenotype of Min\textsuperscript{+/} + Mlh1\textsuperscript{-/-} mice. We find that these doubly mutant mice develop more than three times the number of intestinal adenomas compared to Min\textsuperscript{+/} + Mlh1\textsuperscript{+/+} or +/+ mice but that these tumors do not show advanced progression in terms of tumor size or histological appearance. Full length Apc protein was not detected in the tumor cells from Min\textsuperscript{+/} + Mlh1\textsuperscript{-/-} mice. Molecular analyses indicated that in many tumors from Min\textsuperscript{+/} + Mlh1\textsuperscript{-/-} mice, Apc was inactivated by intragenic mutation. Mlh1 deficiency in Min\textsuperscript{+/} + mice also led to an increase in cystic intestinal crypt multiplicity as well as enhancing desmoid tumorogenesis and epidermoid cyst development. Thus, Mlh1 deficiency influences the somatic events involved in the development of most of the phenotypes associated with the Min mutation. Oncogene (2000) 19, 2774–2779.

Familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) are two distinct forms of autosomal dominantly inherited intestinal cancer in humans. In addition to colon tumors, both syndromes predispose affected individuals to tumorogenesis in various other tissues (Peckham et al., 1995). FAP, less common than HNPCC, can lead to the formation of up to several thousand colonic polyps and has been shown to result from germline mutation of the A\textsubscript{PC} gene (Groden et al., 1991; Kinzler et al., 1991). Individuals with HNPCC generally develop far fewer tumors with a later age of onset. However, HNPCC is believed to account for approximately 5% of all colorectal cancer in humans and this disease results primarily from germline mutations in the DNA mismatch repair (MMR) genes MSH2 and MLH1 (Broner et al., 1994; Fishel et al., 1993; Kolodner, 1996).

Although the precise cellular role(s) in suppressing neoplasia remain(s) to be elucidated, the APC protein has been implicated in apoptosis, cell adhesion and transcriptional regulation of gene expression (Polakis, 1997). Defects in MMR functions lead to an increased accumulation of mutations that result from unrepaired replication errors, or other forms of DNA damage (Kolodner, 1996). Elevated tumor predisposition in HNPCC individuals is thought to be at least in part a direct consequence of an increased frequency of mutations in tumor suppressor genes and/or oncogenes. Somatic A\textsubscript{PC} mutations are frequently found in the tumors from HNPCC patients, as well as in the majority of sporadically occurring colonic tumors (Huang et al., 1996), suggesting that A\textsubscript{PC} plays a central role in maintaining intestinal homeostasis.

Numerous mouse strains with mutations in A\textsubscript{PC} and several of the known DNA MMR genes are now being used to analyse experimentally the process of intestinal neoplasia (Baker et al., 1995; de Wind et al., 1995; Edelmann et al., 1996, 1997; Prolla et al., 1998; Shoemaker et al., 1997a). Apc\textsuperscript{Min/+} (Min\textsuperscript{+/+}) is the most fully characterized of these mutants (Shoemaker et al., 1997a). Min is a germline nonsense mutation at codon 850 of Apc that predisposes heterozygotes to the development of dozens of neoplastic intestinal adenomatous tumors (primarily in the small intestine) as well as causing an increased susceptibility to mammary tumors, desmoid tumors and epidermoid cysts (Shoemaker et al., 1997a). Mice with targeted mutations in the MMR mut\textsubscript{S} homologs Msh2, Msh3, Msh5, and Msh6, as well as the mut\textsubscript{L} homologs Pms1, Pms2, and Mlh1 have also been created (Baker et al., 1995, 1996; de Vries et al., 1999; de Wind et al., 1995; Edelmann et al., 1996, 1997; Prolla et al., 1998; Reitmair et al., 1995). No evidence for enhanced tumorogenesis has so far been reported for the Msh3, Msh5, or Pms1 mutant strains (de Vries et al., 1999; Prolla et al., 1998; de Wind et al., 1999). Mice homozygous for a null allele of Pms2 show an enhanced susceptibility to lymphoma and sarcoma, but not intestinal neoplasia (Baker et al., 1995). In contrast, Msh2 and Mlh1 homozygous null mice are predisposed to intestinal neoplasia as well as a variety of other tumor types (Baker et al., 1996; de Wind et al., 1995; Edelmann et al., 1996, 1997; Prolla et al., 1998). There is conflicting data on the tumor susceptibility of Msh6 mutant mice that may reflect the effects of genetic background, but Msh3\textsuperscript{-/-} Msh6\textsuperscript{-/-} double mutants are predisposed to intestinal and other tumor types (de Wind et al., 1999; Edelmann et al., 1997).
Analysis of variations in genetic background, as well as mutations in several known genes, has demonstrated that the tumor phenotype of Min mice can be dramatically modified. These modifiers include Dmm, p53, Matrilysin, Mdm2 (which is believed to encode Pla2g2a), and two DNA MMR genes (Baker et al., 1998; Clarke et al., 1995; Cormier et al., 1997; Dietrich et al., 1993; Laird et al., 1995; Reitmair et al., 1996; Wilson et al., 1997). In this latter case, combining the Min mutation with homozygosity for a null mutation in either Msh2 or Pms2 increases tumor multiplicity 3–3.5-fold relative to Min/+ mice.

In this report we examine more broadly the effects of combining Mlh1 deficiency with the Min mutation. We present evidence for a strong enhancement of intestinal tumor multiplicity in these mice without an effect on tumor progression. In addition, Min/+ Mlh1−/− mice were more susceptible to the development of cystic intestinal crypts, desmoid tumors, and epidermoid cysts, but did not show elevated predisposition to mammary tumorigenesis. We also examined the status of Apc in intestinal tumors that arise in Min/+ Mlh1−/− mice. Our data indicate that elimination of normal Apc function is necessary for tumor formation and that an increase in somatic mutation frequency associated with loss of Mlh1 function largely accounts for the increase in tumor multiplicity observed in doubly mutant mice.

After backcrossing to generate a predominantly C57BL/6J (B6) genetic background, Mlh1 mutant mice were crossed to Min/+ mice and the phenotype of the Min−/− Mlh1−/−, Min−/− Mlh1+/−, and Min+/− Mlh1−/− mice was assessed (Figure 1). The total average tumor number from the scored regions of the intestine was 139 ± 25 for Min−/− Mlh1−/− mice versus approximately 40 for each of the other two groups (Figure 1a). Thus, intestinal tumor multiplicity was increased more than threefold in Min−/+ Mlh1−/− mice while Mlh1 heterozygosity had no effect on tumor number. The increase in tumor number was seen throughout the length of the intestine and there was no difference between the genotypic groups in tumor distribution along the length of the tract (data not shown). Intestinal tumor sizes were also determined for each group (Figure 2). Mlh1 genotype had no effect on tumor size; the average maximum tumor diameters were 1.05, 1.03, and 1.13 mm for the Mlh1+/+, Mlh1−/−, and Mlh1−/− mice, respectively. A set of 15–30 tumors from each genotypic class for the Mlh1 genotype had no effect on tumor size; the average tumor number was seen throughout the length of the intestine and there was no difference between the genotypic groups in tumor distribution along the length of the intestine and there was no difference between the genotypic groups in tumor distribution along the length of the tract (data not shown).

Cystic crypts are intestinal lesions of unknown neoplastic potential that are morphologically and histologically distinct from adenomas. Found predominantly in the proximal small intestine, the multiplicity of these lesions is increased dramatically following somatic ethynilnitrosourea (ENU) mutagenesis of B6 Min−/+ mice (Shoemaker et al., 1995). The effects of Mlh1 deficiency on cystic crypt multiplicity was determined using the same intestinal samples scored for intestinal adenomas (Figure 1b). An average of 70 ± 22 cystic crypts was found for the Min−/− Mlh1−/− mice compared to 20 ± 11 and 24 ± 10 for the Min−/+ Mlh1−/− and Min+/− Mlh1−/− mice, respectively. Thus, as for intestinal adenomas, Mlh1 deficiency led to an approximately threefold increase in cystic crypt multiplicity.

Epidermoid cysts are pigmented lesions of the subdermal layer of the skin that are a common extracolonic manifestation of FAP. We have shown previously that ENU treatment dramatically increases the incidence of these lesions in B6 Min−/+ mice (Shoemaker et al., 1995). Epidermoid cysts were found in 7/14 Min−/+ Mlh1−/− mice but were not observed in mice of the other two genotypic classes (22 animals of each genotype). Another extracolonic lesion seen in FAP patients, desmoid tumors, are fibromatoses that occur in the peritoneal wall (Peckham et al., 1995). In Min−/+ mice, respec-
the normal tissue controls was 0.74. However, loss of the Apc allele (average Min+/+ Apc−/−, Min+/+ Mlh1−/−, and Min+/+ Mlh1+/+ mice. The percentage of total intestinal adenomas within the indicated range of tumor sizes is shown for each genotypic class. Tumor sizes and multiplicities were obtained from the same intestinal tissue samples. The maximum tumor diameter was determined for every tumor from nine mice from each genotypic class. These nine mice consisted of three mice with the lowest, the median, and the highest tumor number for each genotypic class. Tumor diameters were determined using a calibrated eye piece reticle on a dissection microscope. There is no statistically significant difference in tumor sizes between the three groups (P > 0.05, Wilcoxon Rank Sum test).

Increased intestinal tumor multiplicity in Min+/+ Mlh1−/− mice may reflect an increased frequency of somatic inactivation of Apc and/or other loci important for tumor initiation. It has been demonstrated previously that tumor formation in Min+/+ mice invariably involves somatic inactivation of the wild type Apc allele (Levy et al., 1994; Luongo et al., 1994). On the B6 genetic background, Apc inactivation occurs by allele loss, most likely by a chromosomal event. However, several studies have shown that the mechanism of Apc inactivation can be influenced by genetic background or by treatment with mutagens (Luongo and Dove, 1996; Shoemaker et al., 1997b, 1998). We used several methods to examine Apc status in tumors from mice of each genotypic class. Immunohistochemical analysis indicated a lack of normal Apc expression in cells from all tumors examined from mice of each class (Figure 3a). Apc expression was also absent in the anaplastic cells found in the cystic intestinal crypts (Figure 3b). These results are consistent with numerous studies suggesting that inactivation of APC/Apc is commonly an early and necessary event for adenoma formation. In order to examine the mechanism of Apc inactivation in more detail, we first employed a quantitative PCR assay to examine allele loss. A total of 36 tumor and seven normal intestinal tissue samples were examined (Table 1). Seven tumors (19%) showed loss of the wild type Apc allele (average Apc+/ApcMin ratio = 0.15 ± 0.07). However, loss of the Apc+ allele was not observed in the remaining 29 tumors (81%; average Apc+/ApcMin ratio of 0.67 ± 0.14). The average Apc+/ApcMin ratio for the normal tissue controls was 0.74 ± 0.06. The fraction of tumors reported here that retained the Apc+ allele is roughly consistent with the increase in tumor number caused by Mlh1 deficiency. These results suggest that Mlh1 deficiency leads to inactivation of Apc+ expression by mechanisms other than allelic loss, such as by intragenic mutation. We have shown previously that treatment of B6-Min+/+ mice with ENU leads to a significant increase in tumor number and that many of these tumors have acquired somatic Apc truncation mutations, similar to the types of APC mutations often observed in human intestinal tumors (Shoemaker et al., 1997b). Tumors from the Min+/+ Mlh1−/− mice that failed to show Apc+ allelic loss were examined for Apc truncation mutations by the in vitro synthesized protein (IVSP) assay. Truncation mutations were found in six of these 29 tumors in a region of the gene spanning approximately codons 680–1230 (Figure 4). These six truncations occurred in the same region of Apc and at a similar frequency as previously identified ENU-induced truncating mutations (Shoemaker et al., 1997b). This segment of the gene is slightly upstream from the corresponding somatic mutation cluster region of human APC. Due to limiting amounts of tumor sample, we were not able to identify the nature of these mutations by sequence analysis.

![Figure 2: Intestinal adenoma sizes in Min+/+ Mlh1−/−, Min+/+ Mlh1+/+, and Min+/+ Mlh1+/+ mice. The percentage of total intestinal adenomas within the indicated range of tumor sizes is shown for each genotypic class. Tumor sizes and multiplicities were obtained from the same intestinal tissue samples. The maximum tumor diameter was determined for every tumor from nine mice from each genotypic class. These nine mice consisted of three mice with the lowest, the median, and the highest tumor number for each genotypic class. Tumor diameters were determined using a calibrated eye piece reticle on a dissection microscope. There is no statistically significant difference in tumor sizes between the three groups (P > 0.05, Wilcoxon Rank Sum test).](image)

![Figure 3: Analysis of Apc expression in intestinal tissue samples from Min+/+ Mlh1−/− mice. Periodate-lysine-paraformaldehyde (PLP) fixed tissues were stained with rabbit polyclonal antibody 3122, raised against amino acids 8–347 of human APC, at a dilution of 1:500 (Shoemaker et al., 1998). (A) An intestinal adenoma and surrounding tissue from a Min+/+ Mlh1−/− mouse. Brown Apc staining can be seen in the normal intestinal crypts and no Apc expression is detected in the neoplastic cells of the adenoma. (Scale bar = 100 µm) (B) A cystic crypt and surrounding tissue from a Min+/+ Mlh1−/− mouse. The arrowhead indicates the Apc-negative anaplastic cells. (Scale bar = 100 µm).](image)
inactivation occurs by other mechanisms, including chromosomal loss of heterozygosity event (Luongo et al., 1994). This assay has been shown to be linear over a wide range of input DNA quantities. All samples were independently PCR amplified at least two times. Only samples that gave repeatable values (within 10%) with signals at least 10-fold above background were included in the results. Numerous comparisons of normal and intestinal tumor samples have allowed us to conclude that an average Apc/ ApcMin ratio of less than or equal to 0.30 is indicative of extensive Apc loss. Examination of Apc for truncation mutations was carried out using the in vitro synthesized protein (IVSP; Figure 4). NA: not applicable.

Table 1 Analysis of Apc allelic status in intestinal tumors for Min+/Mlh1−/− and Min+/+ mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tumor multiplicity</th>
<th>Apc+/ApcMin ratio [mean ± s.d. (N/total) for tumors; mean ± s.d. (n) for normal tissue]</th>
<th>Tumors that maintained Apc+</th>
<th>Tumors that lost Apc+</th>
<th>Normal intestinal tissue</th>
</tr>
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<tbody>
<tr>
<td>Min+/+</td>
<td>139.4 ± 25.0</td>
<td>0.67 ± 0.14 (29/36)</td>
<td>0.15 ± 0.07 (7/36)</td>
<td>0.74 ± 0.06 (7)</td>
<td></td>
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<tr>
<td>B6-Min+/+</td>
<td>29.2 ± 9.4</td>
<td>NA</td>
<td>0.17 ± 0.07 (12/2)</td>
<td>0.70 ± 0.15 (54)</td>
<td></td>
</tr>
</tbody>
</table>

*A data taken from Shoemaker et al., 1997b. The ratio of the Apc+ to ApcMin alleles was determined by a quantitative PCR assay (Luongo et al., 1994). This assay has been shown to be linear over a wide range of input DNA quantities. All samples were independently PCR amplified at least two times. Only samples that gave repeatable values (within 10%) with signals at least 10-fold above background were included in the results. Numerous comparisons of normal and intestinal tumor samples have allowed us to conclude that an average Apc+/ ApcMin ratio of less than or equal to 0.30 is indicative of extensive Apc loss. Examination of Apc for truncation mutations was carried out using the in vitro synthesized protein (IVSP; Figure 4). NA: not applicable.

In this report we demonstrate that elimination of Mlh1 function leads to a 3.5-fold increase in intestinal tumor number in Min+/+ mice. Furthermore, the magnitude of increase in tumor number is correlated with the fraction of these tumors that fail to show Apc+ allelic loss. Many of the tumors that failed to show detectable levels of Apc expression (Figure 3). LOH was found to be the mechanism of Apc inactivation in approximately 20% of these tumors (Table 1). Apc truncation mutations were found in six of the 29 tumors (21%) with MOH at Apc (Figure 4). This frequency of truncation mutations is similar to the 25% truncation frequency previously reported for ENU-induced tumors in B6 Min+/+ mice (Shoemaker et al., 1997b). For the remaining tumors with MOH, Apc inactivation must be the result of other types of Apc mutations and/or other somatic events that lead to silencing of Apc expression.

Importantly, although both FAP and HNPCC are autosomal dominantly inherited diseases in humans, mice heterozygous for mutations in MMR genes show no pronounced tumor phenotype, even on the sensitized Min+/+ genetic background (Edelmann et al., 1996, 1999; Prolla et al., 1998). Our finding that Min+/+ Mlh1−/− and Min+/+ Mlh1−/− mice have similar tumor multiplicities is consistent with other studies involving Apc and MMR mutant mouse strains (Baker et al., 1998; Reitmair et al., 1996). The reason for this difference in tumor susceptibility between humans and mice is not understood but may reflect the relatively short life span and smaller size of mice. The total number of intestinal mitoses during an approximately 1.5 year mouse life span is estimated at 1 × 1011. This is several orders of magnitude less than the number of mitoses that are expected to occur in the colon of humans by the average age of onset of HNPCC (approximately 41 years) (Potten and Morris, 1988). Alternatively, other factors such as environmental differences may result in a higher probability of somatic mutations occurring in human intestinal stem cells compared to that of laboratory mice.
Initial characterization of Mlh1 and Pms2 mutant mice indicates that Mlh1−/− but not Pms2−/− mice develop intestinal tumors (Prolla et al., 1998). This difference may reflect the different functions of these proteins in MMR. Genetic and biochemical analyses of MMR in yeast have shown that Mlh1 can form heterodimers with either Pms1 (mouse Pms2) or Mlh3 (mouse Msh1) and that these different protein complexes are each involved in the repair of distinct types of mismatches (Flores-Rozas and Kolodner, 1998; Kolodner, 1996). Indeed, detailed examination of mutation frequencies in mouse tissues has shown that the mononucleotide mutation frequency is 2–3-fold higher in Mlh1−/− mice compared to Pms2−/− mice (Yao et al., 1999). Interestingly, the effect of Mlh1 deficiency on tumor multiplicity in Min/+ mice is only slightly greater than that previously reported for Min/+ Pms2−/− mice (3.5 vs 2.9-fold increase relative to MMR−/− Min/+ mice). These results suggest that there may be different pathways of intestinal tumorigenesis in MMR−/− Apc+/+ vs MMR−/− Apc−/− mice and that the mutator phenotype associated with Mlh1 deficiency influences both pathways, whereas Pms2 deficiency may have a stronger effect on the latter pathway. Rigorous analysis of this possibility will require the concurrent examination of intestinal tumor and mutator phenotypes of these strains on identical genetic backgrounds and environmental conditions.

A striking increase in intestinal tumor multiplicity has also been reported in an independently derived Mlh1−/− mouse strain heterozygous for the 1638N allele of Apc (Edelmann et al., 1999). Neither Mlh1−/− nor Apc1638N−/− mice develop more than five tumors throughout the intestinal tract. However, the combination of these mutations results in an average tumor multiplicity of more than 30 per animal. The synergistic effect of this double mutant may reflect the unique nature of the Apc allele used in these studies (Smits et al., 1999). Although the fold increase in tumor number is greater than reported here for Min/+ Mlh1−/− mice (and for previous studies with Min/+ Pms2−/− and Min/+ Msh2−/− mice), the absolute increase in tumor number is still much smaller than found in any of the studies with the Min strain.

It has been proposed recently that MMR proteins may also function in apoptosis. Mammalian cells, including mouse intestinal crypt cells, deficient in either Msh2 or Mlh1 were shown to be defective in induction of apoptosis following treatment with several DNA damaging agents (Gong et al., 1999; Hickman and Samson, 1999; Toft et al., 1999). In addition, over expression of hMsh2 or hMLH1, but not hMsh3, hMsh6, or hPMS2, in human cell lines resulted in the induction of apoptosis (Zhang et al., 1999). Taken together, these results suggest that defective apoptosis could contribute to the enhanced tumor susceptibility observed in HNPCC patients. It is presently not known whether defective apoptosis contributes to the increased tumor multiplicity observed in MMR−/− Apc−/− mice.

Min/+ mice are predisposed to several types of extra-intestinal lesions. Simple haploinsufficiency at the Apc locus seems to be inadequate to explain the development of these lesions, which include cystic crypts, desmoid fibromas, and epidermoid cysts. Mlh1 deficiency caused an approximately threefold increase in the multiplicity of cystic intestinal crypts relative to Mlh1+/+ Min/+ mice. This increase in cystic crypt multiplicity is similar to the effect previously observed for ENU treated Min/+ mice (Shoemaker et al., 1995). Cystic crypts are morphologically and histologically distinct from intestinal adenomas and thus may represent a non-neoplastic endpoint lesion. The absence of detectable Apc expression in the cells from cystic crypts suggests that events beyond elimination of normal Apc function are needed for full adenoma formation. The incidence of epidermoid cysts and desmoid tumors was also increased in Min/+ Mlh1−/− mice. Our results indicate that, as for the formation of intestinal adenomas, loss of MMR influences the somatic events necessary for the formation of lesions in tissues other than the intestinal epithelium in Min/+ mice. Recent studies have shown that Apc−/− p53−/− Dpc4−/− mice are also strongly predisposed to epidermoid cyst formation (Takaku et al., 1998) and that desmoid tumorigenesis is enhanced in Min/+ p53−/− mice (Halberg et al., 2000). These findings suggest that Mlh1 deficiency may enhance the probability of acquiring Dpc4 and/or p53 mutations in these tissues.

Detailed examination of both tumor size and histology failed to reveal any significant evidence for tumor progression in Min/+ Mlh1−/− mice. These results are consistent with investigations of intestinal tumorigenesis in Min/+ Msh2−/− and Min/+ Pms2−/− mice (Baker et al., 1998; Reitmaier et al., 1996). The lack of tumor progression may reflect the shortened life span that results from the high adenoma burden in these animals. However, it is worth noting that aggressive intestinal tumor progression is also relatively uncommon in Mlh1−/− (and other mismatch repair defective strains) that develop a small number of tumors and routinely live longer than 1 year. These results argue that the mutator phenotype associated with compromised mismatch repair may have a stronger effect on intestinal tumor initiation than on tumor progression.

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