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Advances in Brief

Enhanced Intestinal Adenomatous Polyp Formation in *Pms2*$^{-/-}$;*Min* Mice[1]

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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[3] (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).[4] In contrast, we find that deficiency for *Pms2* does not predispose mice to intestinal tumor development during the first 17 months of life.[4]

FAP is associated with germ-line mutation in the tumor suppressor gene, *APC*. APC appears to function as a "gatekeeper" protein to regulate cell proliferation within intestinal epithelium (1). Unlike HNPCC, 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*$^{+/min}$) mice were obtained commercially (The Jackson Laboratory, Bar Harbor, ME) and were set up for breeding *Pms2*$^{+/-}$ female mice, which had been back-crossed to C57BL/6 for two generations. *Pms2*$^{+/-}$;*Min* mice born to these parents (hence, back-crossed for three generations to C57BL/6 strain) were further used in breeding with *Pms2*$^{+/-}$ female mice. These breedings generated *Pms2*$^{+/+}$;*Min*, *Pms2*$^{+/-}$;*Min*, and *Pms2*$^{-/-}$;*Min* offspring used in this study. Genotyping of the *Min* and *Pms2* mutations are as described previously (7, 14). All animals were fed food (9% total fat by weight) and water *ad libitum* and maintained on a 12-h light/dark schedule.

Intestinal Analysis. Starting at 2 months, the intestines from the mice were collected, flushed with 10% formalin, and opened longitudinally. The open intestines were fixed overnight in 10% formalin. The following morning, the intestines were rinsed in water and transferred to 70% ethanol. The polyps were counted along the entire gastrointestinal tract under a low power dissecting microscope.

Histological Evaluations. Tumor tissue was obtained from the intestines and prepared for histology by embedding in paraffin wax. Sections, 4 μm thick, were mounted onto slides and stained with H&E.

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 ($\alpha^* = 0.05$). 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 GTTCTCGTTCTGAGAAAGAC and CTTCCATAACTTTGGCTATC for 5′ and 3′ primers, respectively. PCR was performed, 95°C for 50 s, 58°C for 45 s, and 72°C for 60 s, for 35 cycles in the presence of [^{33}P]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.

[4] T. A. Prolla, S. M. Baker, A. C. Harris, J-L. Tsao, X. Yao, C. E. Bronner, B. Zheng, M. Gordon, J. Reneker, N. Arnheim, D. Shibata, A. Bradley, and R. M. Liskay. Tumor susceptibility and spontaneous mutation in mice deficient in Mlh1, Pms1, and Pms2 DNA mismatch repair genes. Nat. Genet., in press.

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).

Immunohistochemical 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.[4]

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.

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

Age ranges for the mice for the groups *Pms2*$^{-/-}$;*Min*, *Pms2*$^{+/-}$;*Min*, and *Pms2*$^{+/+}$;*Min* were 2–5 months, 2–7 months, and 2–7 months, respectively. Mean numbers in the third column represent adenomas in the intestinal tract. In parentheses are indicated the average numbers (per animal) of colonic adenomas for each genotype.

Genotype	No. of mice	Mean no. of adenomas
Pms2$^{-/-}$;*Min*	11	207[a] (5.6)
Pms2$^{+/-}$;*Min*	32	90[a] (1.2)
Min	30	72[a] (1.4)

[a] 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).

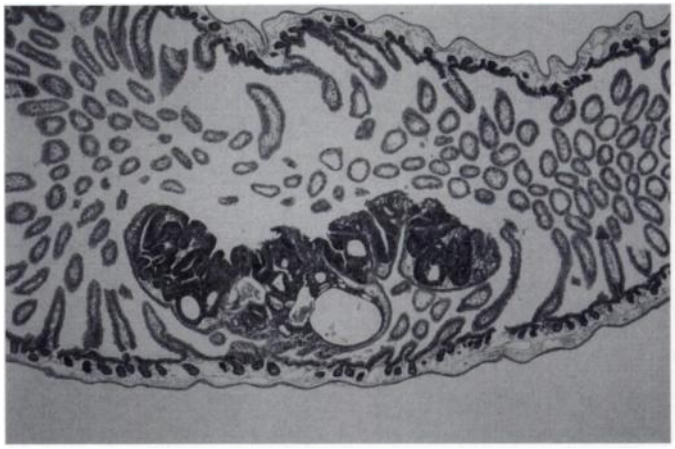

Fig. 1. Typical adenoma protruding into the lumen of the small intestines of a *Pms2*$^{-/-}$;*Min* mouse. H&E, ×40.

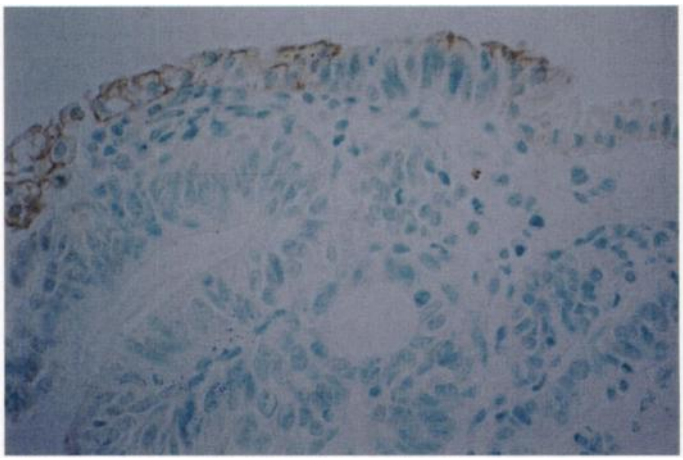

Fig. 2. Immunostaining showing loss of APC protein expression in the adenoma. APC expression is present in the normal epithelium overlying the adenomatous glands.

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 antiserum 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

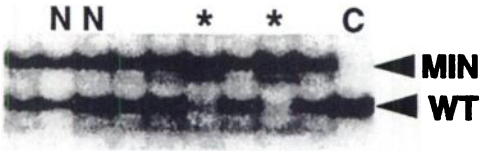

Fig. 3. Autoradiograph after SSCP electrophoresis of the PCR products from adenomas and normal tissues from a *Pms2*$^{-/-}$;*Min* mouse. The control (*C*) demonstrates the location of the *WT Apc* allele, whereas the *Min* allele migrates slower. Normal mucosa (*N*) shows both *WT* and *Min* alleles. Only two of the adenomas (*) show evidence of *WT* allelic loss.

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 Apc 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 Apc staining in the majority of these polyps is due to somatic inactivation via point mutation. DNA sequence analysis of *Apc* 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) has been identified as a modifier of *Min* polyp formation in different genetic mouse strains (14, 20). Perhaps if longer lived, *Pms2*$^{-/-}$;*Min* mice would readily develop adenocarcinomas.

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 indicates 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 studies, 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 Apc protein, presumably due to somatic inactivation of the normal *Apc* 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[4] 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,[4] suggests the possibility of differing but overlapping roles for *Msh2*, *Mlh1*, and *Pms2* in cellular processes in the intestine.

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