Mouse Models for Hereditary Nonpolyposis Colorectal Cancer

Niels de Wind, Marleen Dekker, Agnes van Rossum, Martin van der Valk, and Hein te Riele
Division of Molecular Carcinogenesis, the Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands

ABSTRACT

Hemizygous germ-line defects in mismatch repair (MMR) genes underlie hereditary nonpolyposis colorectal cancer (HNPCC). Loss of the wild-type allele results in a mutator phenotype, accelerating tumorigenesis. Tumorigenesis specifically occurs in the gastrointestinal and genitourinary tracts; the cause of this tissue specificity is elusive. To understand the etiology and tissue distribution of tumors in HNPCC, we have developed mouse models carrying a deficiency in the MMR gene Msh2.

Most of the completely Msh2-deficient mice succumbed to lymphomas at an early age; lymphomagenesis was synergistically enhanced by exposure to ethynitrosourea. Lymphomas were absent in immunocompromised Tap1‘−/−;Msh2+/− mice; these mice generally succumbed to HNPCC-like tumors. Together, these data suggest that the HNPCC tumor spectrum is determined by exposure of MMR-deficient cells to exogenous mutagens, rather than by tissue-specific loss of the wild-type allele or by immune surveillance. Msh2 hemizygous mice had an elevated tumor incidence that, surprisingly, was rarely correlated with loss of the Msh2+ allele. To develop a model for intestinal tumorigenesis in HNPCC, we introduced the Min allele of the Apc tumor suppressor gene. We observed loss of the wild-type Msh2 allele in a significant fraction of intestinal tumors in Apc−/−;Msh2+−/− mice. In some of the latter tumors, one area of the tumor displayed loss of the Msh2+ allele, but not of the Apc+ allele, whereas another area displayed the inverse genotype. This apparent biclonality might indicate a requirement for collaboration between independent tumor clones during intestinal tumorigenesis.

INTRODUCTION

Loss of DNA MMR3 is a rate-limiting step in the etiology of tumors associated with HNPCC (Lynch syndrome) in humans (1). HNPCC families are characterized by a high incidence of cancers of mainly the gastrointestinal and female reproductive tracts, which often develop at an early age (1–3). Affected HNPCC family members have inherited a mutant allele of one of four MMR-related genes called MSH2, MLH1, PMS1, and PMS2 (4–12). In tumors, the wild-type allele is lost through mutational inactivation or LOH (13, 14). This condition is manifested by instability of the length of simple-sequence repeats [e.g., A(n) or (CA)m], a phenomenon referred to as the RER+ phenotype and a hallmark of HNPCC-related tumors (15–17).

MMR seems to be highly conserved throughout evolution (18, 19). In the prototypic mutS system of Escherichia coli, the MutS protein recognizes and binds to a mismatch in DNA, whereas MutL is believed to act as a molecular matchmaker, binding the DNA-MutS complex and triggering excision and resynthesis of the error-containing DNA strand. In eukaryotes, the bacterial MutS protein is represented by two heterodimeric protein complexes of MutS homologues. One of these, MutSc, a dimer composed of MSH2 and MSH6/GTBP, binds to base-base mismatches and small loops of one or two displaced nucleotides (e.g., resulting from slippage errors at mononucleotide or dinucleotide tracts; Refs. 20–23). Similarly, the mammalian equivalent of bacterial MutL is a heterodimer of MLH1 and PMS2 called MutLo (24).

In all systems studied, MMR is involved in at least two types of mutation avoidance reactions: (a) it recognizes and corrects bp mismatches and slippage errors made by DNA polymerase during replication and is always directed against the newly synthesized strand (25); and (b) it counteracts recombination between homologous but nonidentical DNA sequences (26–28). Furthermore, in mammalian cells, MMR mediates toxicity of methylating agents (28–30). Thus, loss of MMR results in a pleiotropic phenotype characterized by an increased accumulation of base substitutions and frame shifts (the RER+ phenotype), enhanced recombination between diverged DNA sequences, and tolerance to the cytotoxic effects of methylating agents (28).

MMR genes are believed to be expressed in all proliferating cells (31–33). It is therefore surprising that in HNPCC patients, tumorigenesis is mainly restricted to the proximal colon and the endometrium (1–3) and, in patients with the related Muir-Torre syndrome, the sebaceous glands (34). This tissue specificity can be explained in several ways: (a) cells that have lost MMR may have a growth advantage with respect to MMR-proficient cells. This may either be intrinsic to specific tissues or be imposed by selective conditions, e.g., an increased exposure to methylating agents in the intestine (29, 30). Also, a T cell-mediated immune response against novel peptides generated by frameshift mutagenesis in transcribed genes in MMR-deficient cells (35) might suppress tumor development in tissues that are submitted to efficient control by the immune system; (b) oncogenic mutations in MMR-deficient cells accumulate more rapidly in tissues with a high cell turnover, and this may be further enhanced by exposure to exogenous mutagens; and (c) the proto-oncogenes or tumor suppressor genes that control growth and differentiation of susceptible tissues may have hot spots for mutation by MMR deficiency (like mononucleotide tracts), e.g., as was found for the transforming growth factor β type II receptor in HNPCC-related intestinal tumors (36).

Current mouse models carrying disruptions of MMR genes have not provided insights into the contribution of each of these factors to the etiology and tissue specificity of tumorigenesis in HNPCC (28, 37–41). The finding that the majority of completely MMR-deficient mice succumb to T-cell lymphomas (Ref. 41 and this work), whereas most tumors developing in hemizygous mice (having the HNPCC genotype) have retained the wild-type Msh2 allele (this work), has urged us to develop more informative mouse models for HNPCC. In the present report, we compared the tumor spectrum in immunoproliferative and immunocompromised Msh2-deficient mice and studied the synergism between MMR deficiency and ENU-induced mutagenesis. Furthermore, we targeted tumorigenesis to the intestine by introducing the Min allele (43) of the Apc tumor suppressor gene (44) in Msh2 mutant backgrounds. These tumor models provide valuable tools to identify genetic and environmental factors that influence the development of MMR-deficient tumors.
MOUSE MODELS FOR HNPCC

MATERIALS AND METHODS

Generation of Mice. MMR-deficient mice carrying a targeted disruption in exon 12 of the Msh2 gene were described previously (28). 129OLA Msh2"/" mice were generated by intercrossing of pure 129OLA Msh2 heterozygotes; 129OLA/FVB Msh2"/" mice were generated by intercrossing of Msh2 heterozygous F1 hybrids of 129OLA and FVB; chimeric Msh2"/" mice were generated by injection of Msh2"/" 129OLA-derived ES cells into C57BL/6-derived blastocysts. Tapi" mice were generated from three backcrosses of the original pure 129OLA background (45) to C57BL/6. In our analyses of Apc"mice, only animals carrying a reporter allele were included. The intestinal tumor load was assessed macroscopically and by examination of H&E-stained longitudinal sections.

Analysis of LOH and Microsatellite Length Variations. Loss of the wild-type Msh2 and Apc alleles was investigated using primer pairs (mappairs) amplifying polymorphic anonymous microsatellites on chromosomes 17 and 18, respectively (48). DNA was obtained from frozen tumor material and tails or from tumor cells and adjacent normal cells that were scraped off histological sections. PCRs were performed with end-labeled primers (48) and analyzed by electrophoresis on denaturing 6% polyacrylamide gels. The intensities of allele-specific PCR products were analyzed by densitometry. LOH was ascertained by the ratio in tumors between intensities of both alleles differed from that in normal tissue more than two times the SD of the ratio in normal tissue.

The same mappairs and mappairs amplifying additional microsatellites on other chromosomes were used to assess microsatellite instability. In each case, DNA isolated from normal tissue served as a control.

Generation of an Anti-Msh2 Antibody and Immunohistochemistry. The mouse Msh2 gene (33) was expressed in E. coli as a GST fusion. Inclusion bodies containing insoluble protein were isolated and washed in 4 M urea, as described previously (49). This material (400 μg) was used for the primary immunization of rabbits and for two monthly boosts. The fourth and last boost was performed with 200 μg of the same protein after purification from a preparative 8% polyacrylamide gel. Before use, the antiserum was purified by adsorption to inclusion bodies containing (unfitted) GST protein and subsequent protein A-Sepharose chromatography (50).

A polyclonal antibody preparation against a COOH-terminal peptide of Apc was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

For immunohistochemical staining, 5-μm paraffin-embedded intestinal sections were incubated with the Apc antibody or, after antigen retrieval, with the Msh2 antibodies. Bound antibody was visualized by incubation with biotinylated goat antirabbit antisera followed by staining with diaminobenzidine tetrahydrochloride as a chromophore, using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

RESULTS

Spontaneous Tumorigenesis in Msh2-deficient Mice. Msh2-deficient mice were generated in the inbred 129OLA background and in a hybrid background of mouse strains 129OLA and FVB. In addition, Msh2"/" chimeric animals were generated by blastocyst injection of Msh2"/" 129OLA-derived ES cells (28).

 Virtually all Msh2"/" mice died before 1 year of age (Fig. 1a); inbred 129OLA mice died more rapidly (one-third survival at 19.5 weeks) than the 129OLA/FVB hybrids (one-third survival at 30 weeks). Of all Msh2"/" animals (both 129OLA and hybrid backgrounds) that succumbed before 30 weeks, 80% presented with a lymphoid tumor, mostly of T cell origin. These included all but one of the 129OLA animals and 63% of the hybrids. Lymphomas generally consisted of CD4/CD8 double-positive cells and CD8 single-positive cells (data not shown). In 129OLA animals surviving beyond 19.5 weeks and in hybrid background animals surviving beyond 30 weeks, a variety of other tumor types were detected on autopsy. Table 1 presents an overview of all tumor types identified. Half of these older animals carried multiple, some even up to four, independent nonlymphoid tumors of different origin. These included intestinal tumors, skin tumors (of which half showed sebaceous gland cell differentiation), and uterine tumors (of which half were of endometrial origin).

Thus, most of the older Msh2"/" animals developed tumors that correspond to those generally found in HNPCC and Muir-Torre patients (Table 2). Depending on the genetic background, a number of other tumor types were seen as well. In the hybrid background, erythroleukemias and tumors of the mammary gland and lung were seen, whereas a brain tumor was found in 13% of the Msh2-deficient 129OLA and chimeric mice (Table 1).

As expected, all of the Msh2-deficient tumors tested showed the RER phenotype, as evidenced by length alterations of microsatellite markers (data not shown).

ENU-induced Tumorigenesis in Msh2"/" Mice. To investigate whether the exposure to mutagenic agents influences oncogenic transformation of MMR-deficient cells, mice were exposed to ENU by transplacental administration. Whereas about 40% of the nontreated Msh2"/" 129OLA/FVB mice did not develop lymphomas (as described above), all ENU-treated Msh2"/" mice succumbed to lymphomas within 14 weeks (Fig. 1c). In contrast, ENU treatment induced lymphomas in only 25% of wild-type mice between 13 and 27 weeks; the remainder survived beyond 45 weeks and ultimately succumbed to lung and liver tumors (data not shown). The mean latency period of lymphomagenesis was reduced from 18 weeks in Msh2"/" or ENU-treated wild-type mice to 11 weeks in ENU-treated Msh2"/" mice. Because Msh2-deficient cells and wild-type cells are equally sensitive to the cytotoxic effects of ENU, this result shows that endogenous mutagenesis due to MMR deficiency and mutagenesis induced by exogenous mutagens synergistically collaborate in tumorigenesis.

Msh2-deficient Immunocompromised Mice. The tumor spectrum in Msh2-deficient mice in the third of the cohort that survived relatively long largely corresponds to that found in HNPCC patients. To investigate whether this tumor spectrum is controlled by the immune system, we followed 13 Msh2"/" mice that also carried a homozygous disruption in Tapi. This gene encodes a subunit of the TAP complex (51). Tapi-deficient mice are impaired in peptide transport and therefore in normal MHC class I-restricted antigen presentation and have very few CD8+ T cells (42, 45). Indeed, Tapi"";Msh2"/" mice did not develop T-cell lymphomas, and most of them (10 of 13) survived

4 N. Claij, M. Dekker, and H. te Riele, unpublished observations.
MOUSE MODELS FOR HNPCC

Fig. 1. Survival of Msh2 mutant mice. a: 129Msh2^-/-, pure inbred 129OLA; 129FVB/Msh2^-/-, 1:1 hybrids of 129OLA and FVB; chimeric Msh2^-/-; chimeric mice generated by injection of Msh2^-/- 129OLA-derived ES cells into C57BL/6-derived blastocysts. Msh2^-/- and Msh2^+/^- cohorts were a mixture of inbred 129OLA animals and F1 (129OLA/FVB) hybrids. b, survival of immunocompromised Tap^-/-;Msh2^-/- mice. These animals were hybrids of 129OLA and FVB at a ratio of approximately 1:2. c, ENU-induced lymphogenesis; Msh2^+/^- and Msh2^-/- mice were 1:1 hybrids of 129OLA and FVB. ENU was administered 1 or 2 days prenatally.

Table 1 Tumorigenesis in Msh2^-/- mice

<table>
<thead>
<tr>
<th>Genotype (background)</th>
<th>Msh2^-/- (129OLA)</th>
<th>ChimMsh2^-/- (129OLA)</th>
<th>Msh2^-/- (hybrid)</th>
<th>Tap^-/-; Msh2^-/- (hybrid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at death (wk)</td>
<td>&lt;19.5</td>
<td>19.5</td>
<td>19.5</td>
<td>30</td>
</tr>
<tr>
<td>No. of mice</td>
<td>23</td>
<td>59</td>
<td>59</td>
<td>30</td>
</tr>
<tr>
<td>No. of tumors</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymphoid</td>
<td>22</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Erythroid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Intestine</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Skin (sebaceous gland)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6 (3)</td>
</tr>
<tr>
<td>Uterus (endometrium)</td>
<td>1</td>
<td>0</td>
<td>1 (1)</td>
<td>6 (3)</td>
</tr>
<tr>
<td>Brain</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Lung</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Nonhematopoietic</td>
<td>5</td>
<td>9</td>
<td>6</td>
<td>35</td>
</tr>
<tr>
<td>HNPCC-like</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>19</td>
</tr>
</tbody>
</table>

129OLA: pure strain 129OLA; hybrid: genetic mix of mouse strains 129OLA and FVB.

Moreover, the high incidence of HNPCC-like tumors in completely Msh2-deficient mice suggests that the HNPCC tumor spectrum is not determined by positive selection for loss of the wild-type MMR allele in susceptible tissues.

Tumorigenesis in Msh2 Hemizygous Mice. No effect on survival was observed for hemizygosity of Msh2 (Fig. 1a). However, in the group of Msh2^+/^- animals that succumbed within 100 weeks, 40% more tumors were seen than in the control group of wild-type animals (Table 3). None of these tumors, however, originated from the intestine. To investigate whether loss of MMR had contributed to tumorigenesis in Msh2^+/^- mice, the Msh2 and RER status of 65 tumors was determined. The entire chromosome 17 carrying the wild-type Msh2
Apc tumor suppressor gene is an early and rate-limiting step in confirmed by a Msh2-specific PCR assay (data not shown). In agreement with this, microsatellite length alterations were detected at 5 of 10 loci (Fig. 2). In none of the other tumors could we find evidence for allelic loss of Msh2 or intragenic Msh2 mutations, as assayed by a protein truncation test (data not shown). A weak RER+ phenotype (microsatellite length alterations at 1 of 10 loci) was observed in a skin tumor and a Schwann cell tumor; however, these tumors showed no loss of Msh2. No strong or weak RER+ cases or loss of Msh2 were detected among 24 tumors from Msh2+/-- mice.

Intestinal Tumorigenesis in Msh2-deficient Mice. Loss of the Apc tumor suppressor gene is an early and rate-limiting step in intestinal tumorigenesis in both mice and humans (44, 52). Mice carrying one mutated Apc allele, e.g., the ApcMin allele, develop adenomas of (predominantly) the small intestine in which the wild-type Apc allele is generally lost by LOH (53). To study the effect of MMR deficiency in intestinal tumorigenesis, the ApcMin allele was introduced into Msh2+/-- and Msh2+/+ mice. Because susceptibility to intestinal tumorigenesis in mice is strongly influenced by the Mom-1 (modifier of Min) locus (47), care was taken that all animals carried at least one resistance allele of Mom-1. Complete Msh2 deficiency reduced the survival of Apc+/-- mice 4-fold (Fig. 3). Note that only lymphoma-free animals that succumbed to intestinal tumors were included. All of these Apc+/--/Msh2+/-- animals died within 5 months, presenting with an intestinal tumor load at least 8-fold in excess of that in MMR-proficient Apc+/--Min mice (see also Table 4).

The status of the wild-type Apc allele in the tumors was investigated by amplifying polymorphic microsatellite markers located on chromosome 18 (which carries the Apc gene). Intestinal tumorigenesis in Apc+/--/Msh2+/-- mice resulted from LOH of Apc, which is in agreement with previous results (53). In contrast, in Apc+/Min, Msh2+/-- animals, LOH of Apc was only found in 2 of 30 intestinal tumors, confirming a recent report (54). This observation suggests that in the vast majority of tumors developing in MMR-deficient Apc+/Min mice, the wild-type Apc allele was lost by mutational inactivation, although we cannot exclude the possibility that the wild-type Apc allele had been retained in these tumors (see “Discussion”).

Loss of Msh2 during Intestinal Tumorigenesis. Hemizygosity for Msh2 did not significantly affect the survival of Apc+/Min mice (Fig. 3). To investigate whether loss of the Msh2+ allele had occurred in tumors developing in Apc+/Min/Msh2+/-- mice, we raised a polyclonal antiserum against the mouse Msh2 protein. This antiserum was specific for Msh2, as verified by Western blotting (data not shown). Histochemical staining of sections through the ileal mucosa of Msh2+/-- and Msh2+/-- mice showed strong Msh2-specific nuclear staining in the crypts of Lieberkühn and in the proximal part of the villi that vanished toward the lumenal part of the villi (Fig. 4, a and b). This expression pattern of murine Msh2 is similar to that of human MSH2 (31, 32).

All intestinal tumors developing in Apc+/Min/Msh2+/-- mice that were tested stained positive for Msh2 (Fig. 5a). However, 8 of 48 tumors from Msh2+/-- animals contained Msh2 nonstaining areas.
Table 4: Intestinal tumorigenesis in Apc<sup>+/-</sup>; Msh2 mutant mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of mice&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of tumors</th>
<th>Load&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Age&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Msh2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>3</td>
<td>78</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Msh2&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>6</td>
<td>17</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Msh2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>17</td>
<td>48</td>
<td>34</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of mice analyzed histologically.
<sup>b</sup> Based on histological analysis.
<sup>c</sup> Time of death of 50% of animals (months).

Forty-eight tumors: Msh2-negative-staining areas, 9/48 (18%); LOH of Msh2, 4/4 tested (8%); no demonstrated LOH Apc, 2/3 mixed tumors.

MOUSE MODELS FOR HNPCC

Both areas were negative for staining with an Apc antiserum, indicating that they consisted of crypt-like tumor cells (Ref. 55; data not shown). We determined the genetic status of Apc by PCR amplification of polymorphic chromosome 18 markers. In Msh2-positive areas, LOH for Apc was detected in all cases examined. Surprisingly however, in two of three mixed tumors that were informative, Msh2-negative areas did not show LOH for Apc (e.g., Tumor B in Fig. 6). This result indicates that loss of Msh2 was not a late event that had occurred after loss of Apc. Instead, these two tumors containing an Msh2-negative part were biclonal, resulting from loss of Apc and loss of Msh2 in separate ancestor cells.

DISCUSSION

Genetic instability is a hallmark of human cancer. It was therefore hypothesized that the early loss of systems that safeguard the integrity of the genome is mandatory to the multistep process of tumorigenesis (56). Considering the strong mutator phenotype of MMR-deficient cells in vitro (28), it is perhaps not surprising that inherited defects in MMR genes strongly predispose to cancer in humans. However, in view of the presumed ubiquitous role of MMR in mutation avoidance, the tissue distribution of tumorigenesis in HNPCC patients is remark-
ably limited. Here we describe the generation of mouse models that address this point and enable us to study the implications of MMR deficiency in the etiology and tissue specificity of HNPCC-related cancer.

**Tumorigenesis in Msh2-deficient Mice.** The early onset and 100% incidence of tumorigenesis in Msh2-deficient mice corroborate the increased risk for MMR-deficient cells to undergo oncogenic transformation. Lymphoid tumors predominated, probably because the extensive expansion and high turnover of cells during the development of the T-cell compartment promoted the rapid accumulation of oncogenic mutations. However, the high lymphoma incidence in Msh2<sup>−/−</sup> mice is probably also related to the intrinsic propensity of mice to develop sarcomas rather than tumors of epithelial origin. Lymphomas in mice can be readily induced by irradiation and carcinogen treatment and also predominate in p53<sup>−/−</sup> mice (57). Clearly, tumor susceptibility of different organs is strongly species dependent, and even large variations within one species exist (58). With this in mind, it is striking that most Msh2<sup>−/−</sup> mice that did not succumb to a lymphoma at an early age developed epithelial tumors within 1 year, and that more than 60% of these were reminiscent of the predominant tumor types in HNPCC and Muir-Torre patients. The intestinal tumors in mice were mainly located in the small intestine, whereas in HNPCC patients, colon tumors predominate. This difference probably reflects a species-dependent preference for specific tumor locations in the intestinal tract, because a similar difference was observed between familial adenomatous polyposis coli patients and mice with defects in the APC/Apc tumor suppressor gene (44, 52, 53).

In humans, a substantial fraction of RER<sup>+</sup> colorectal tumors lost antigen presentation due to mutations in the B2m gene (59). It was therefore suggested that MMR-deficient tumors can elicit an immune response against novel peptides generated by frameshift mutagenesis in transcribed genes. Immune surveillance may therefore suppress the accumulation of MMR-deficient cells and tumors in most tissues, and this process may be involved in determining the specific tissue distribution of MMR-deficient tumors. In Msh2<sup>−/−</sup> mice, however, the time of onset, tissue distribution, and number of tumors were not significantly affected by the absence of a CTL-mediated immune response. Therefore, immune surveillance is likely not responsible for determining the HNPCC tumor spectrum.

Our analyses show that (aside from lymphomagenesis) the tumor spectrum in both immunocompetent and immunocompromised homozygous Msh2 knockout mice is highly similar to that found in humans with a hemizygous defect in MMR genes. Thus far, no HNPCC cases have been found with germ-line defects in both copies of a MMR gene. However, several patients were described with a ubiquitous MMR defect, possibly due to inheritance of dominant negatively acting versions of the MMR genes MLH1 and PMS2 (60). In these patients, only colorectal cancer developed, albeit at an early age. Together, these observations indicate that the tissue distribution of tumorigenesis in HNPCC patients is not a consequence of a tissue-specific selective pressure on the accumulation of MMR-deficient cells, e.g., by methylating agents. We rather envisage that in some tissues, MMR-deficient cells have a higher mutation rate than in others. Thus, the accumulation of oncogenic mutations will certainly depend on the rate of tissue turnover and may be further enhanced to a critical level by exposure to mutagens. In this respect, it is noteworthy that prenatal administration of ENU accelerated lymphomagenesis in Msh2-deficient mice far more strongly than it did in wild-type animals, showing that the combination of mutagenesis by MMR deficiency and by exposure to carcinogens is highly carcinogenic. We speculate that additional mutagenesis by exogeneous carcinogens in spontaneously arising MMR-deficient cells in the intestine and the endometrium of HNPCC patients plays an important role in directing carcinogenesis to these organs.

**Tumorigenesis in Msh2-hemizygous Mice.** Considering the 100% tumor incidence in Msh2<sup>−/−</sup> mice, it is remarkable that hemizygosity of Msh2 did not affect survival. Nonetheless, in Msh2<sup>−/−</sup> animals, more tumors were found than in wild-type mice. With one exception, none of these tumors displayed the RER<sup>+</sup> phenotype or LOH at Msh2. The increased tumor incidence may therefore be a consequence of an increased mutation rate in cells that are hemizygous for Msh2. It is surprising that Msh2<sup>−/−</sup> animals hardly developed lymphomas and developed no tumors at all of the small intestine and sebaceous glands, which are characteristic for complete Msh2 deficiency. The increased tumor incidence in Msh2<sup>−/−</sup> mice included mammary gland or skin tumors, uterine tumors, hemangiosarcomas, teratocarcinomas, and bladder carcinomas, which were less abundant or absent in the wild-type control group. We therefore speculate that MMR was compromised by hemizygosity of Msh2 only in cell types from which these tumors originate, leading to an enhanced risk of oncogenic transformation. This hypothesis is currently under investigation.
Intestinal Tumorigenesis in Apcc<sup>+/Min</sup>;Msh2 Mutant Mice. The vast majority of intestinal tumors that developed rapidly in Apcc<sup>+/Min</sup>;Msh2<sup>−/−</sup> mice did not show LOH of Apc that is normally seen in MMR-proficient Apcc<sup>+/Min</sup>;Msh2<sup>−/−</sup> mice. This may indicate that the Apc<sup>−/−</sup> allele was lost by mutational inactivation, e.g., by framshifts at one of the many small mono- or dinucleotide repeats in the murine Apc coding sequence. Thus far, however, we have not obtained direct evidence for genetic inactivation of the Apc<sup>−/−</sup> allele. Because Apc is expressed only in the villi and not in the crypts, the absence of Apc coding sequence. Thus far, however, we have not obtained direct evidence of the many small mono- or dinucleotide repeats in the murine Apc gene in MMR-proficient Apc<sup>+/Min</sup>;Msh2<sup>−/−</sup> mice. This may indicate that the Apc<sup>+/−</sup> allele in 8—18% of the intestinal tumors that developed in Apcc<sup>+/Min</sup>;Msh2<sup>−/−</sup> mice. In the completely Msh2-negative tumor, which showed no LOH of Apc, this was certainly an early event preceding or obviating inactivation of Apc; in the tumors in which a part showed loss of the Msh2<sup>−/−</sup> allele, this might have been a late event, occurring after the loss of Apc. Remarkably, however, some of these tumors were evidently biconal, consisting of two adenomas that originated from different ancestor cells; one of these retained the Msh2<sup>−/−</sup> allele but lost the Apc<sup>−/−</sup> allele, whereas the other displayed the inverse genotype, indicative of an early loss of MMR preceding or obviating inactivation of the Apc<sup>−/−</sup> allele.

Biclonal intestinal tumors were observed previously in chimeric mice (62) and more recently in a familial adenomatous polyposis coli patient (63). Biclonality may have resulted from collision between different ancestor cells; one of these retained the Msh2<sup>−/−</sup> allele and the other one of the two parental Msh2 alleles. The Apc<sup>+/Min</sup>;Msh2<sup>−/−</sup> tumor model may provide new insights on the factors and mechanisms that contribute to the development of MMR-deficient intestinal tumors in HNPCC patients.

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