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Long-lived Min Mice Develop Advanced Intestinal Cancers through a Genetically Conservative Pathway

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Abstract

C57BL/6J mice carrying the *Min* allele of *Adenomatous polyposis coli* (*Apc*) develop numerous adenomas along the entire length of the intestine and consequently die at an early age. This short lifespan would prevent the accumulation of somatic genetic mutations or epigenetic alterations necessary for tumor progression. To overcome this limitation, we generated F1 *Apc*^{Min/+} hybrids by crossing C57BR/cdcJ and SWR/J females to B6 *Apc*^{Min/+} males. These hybrids developed few intestinal tumors and often lived longer than 1 year. Many of the tumors (24–87%) were invasive adenocarcinomas, in which neoplastic tissue penetrated through the muscle wall into the mesentery. In a few cases (3%), lesions metastasized by extension to regional lymph nodes. The development of these familial cancers does not require chromosomal gains or losses, a high level of microsatellite instability, or the presence of *Helicobacter*. To test whether genetic instability might accelerate tumor progression, we generated *Apc*^{Min/+} mice homozygous for the hypomorphic allele of the Nijmegen breakage syndrome gene (*Nbs1*^{ΔB}) and also treated *Apc*^{Min/+} mice with a strong somatic mutagen. These imposed genetic instabilities did not reduce the time required for cancers to form, nor increase the percentage of cancers, nor drive progression to the point of distant metastasis. In summary, we have found that the *Apc*^{Min/+} mouse model for familial intestinal cancer can develop frequent invasive cancers in the absence of overt genomic instability. Possible factors that promote invasion include age-dependent epigenetic changes, conservative somatic recombination, or direct effects of alleles in the F1 hybrid genetic background.

Keywords

Intestinal neoplasia; invasive adenocarcinomas; local metastasis; microsatellite instability; chromosomal instability

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Introduction

Human colorectal cancer is a leading cause of cancer death in the world. In the United States alone, 148,810 new cases and 49,960 deaths were expected during 2008 (1). Once a colorectal tumor has metastasized to other sites, the prognosis is quite poor, since no effective treatment of disseminated colon cancer has yet been identified.

Three models have been proposed to explain the acquisition of metastatic potential (reviewed in 2). The predisposition model states that the genetic composition of the host determines whether a primary tumor metastasizes. By contrast, the initiation model states that neoplastic cells within the primary tumor have acquired mutations or other stable changes during the early stages of tumorigenesis that eventually lead to metastasis. Finally, the progression model states that a subpopulation of neoplastic cells within a primary tumor sequentially acquires genetic mutations, epigenetic alterations or both that drive progression, culminating in metastasis. Note that these models are not mutually exclusive, e.g., a combination of constitutional allelic variants and acquired somatic mutations might be required for metastasis. Also, each scenario likely requires time to unfold.

Mouse models of hereditary human colorectal cancer have not yet fully addressed the issue of metastatic potential because the existing models do not consistently develop advanced cancers. C57BL6/J (B6) mice carrying one of several different *Apc* mutations (*Apc*^{Min}, *Apc*^{580S} and *Apc*^{Δ716}) develop numerous adenomatous polyps (reviewed in 3).

Plausibly, these tumors do not progress because the mice have a shortened lifespan, owing to the heavy tumor load especially in the small intestine. This barrier can be partially overcome by introducing additional mutations. B6 *Apc*^{Min/+} mice lacking EphB2, EphB3, or p53 activity as well as *cis*-compound *Apc*^{Δ716} *Smad4*^{-/-} heterozygotes develop invasive adenocarcinomas (reviewed in 4). The frequency of invasive tumors in these models ranged from 5 to 55% when reported. Other models with low tumor multiplicity, including *Apc*^{1638N/+}, *Muc2*^{-/-}, *Apc*^{loxP/+}; *CDX2P-NLS Cre*, *Smad3*^{-/-}, and *Tgfb*^{-/-}; *Rag2*^{-/-} mice, also develop advanced cancers (5–9). *Apc*^{loxP/+}; *CDX2P-NLS Cre* mice carry a *loxP*-targeted *Apc* allele and the *CDX2P-NLS Cre* recombinase transgene; expression of Cre recombinase in the colon results in the loss of *Apc* activity and consequently the formation of tumors (5). Again, the frequency of invasive tumors varies widely, ranging from 3 to 50% when reported. In *Apc*^{1638N/+} and *Smad3*^{-/-} mice, the cancers were reported to have metastasized, although the penetrance of this phenotype in both cases was quite low (8,10). Here, we report a high frequency of invasive adenocarcinomas and local metastases in long-lived *Apc*^{Min/+} mice. In contrast to the multiply mutated mouse models, we have investigated the state of the genome in adenocarcinomas that arise in the familial Min setting. Finding no evidence for spontaneous genomic instability, we have gone on to explore whether imposed genetic instability or *Helicobacter* infection can accelerate the age-dependent tumor progression in this model.

Materials and Method

Mice

All mice were investigated in the McArdle Laboratory for Cancer Research under protocols approved by the IACUC at the University of Wisconsin, following AAALAC guidelines. Two types of F1 *Apc*^{Min/+} hybrids were produced by breeding C57BR/cdcJ (BR) and SWR/J (SWR) females to B6 *Apc*^{Min/+} males. To test whether increased genetic instability affected tumor progression, females carrying the *Nbs1*^{ΔB} allele were bred to B6 *Apc*^{Min/+} males and the resulting progeny with a mixed genetic background of 129 and B6 alleles were intercrossed to generate *Apc*^{Min/+} mice with 0, 1 or 2 copies of the *Nbs1*^{ΔB} allele. Mice were genotyped at *Apc* and *Nbs1* using a PCR assay as described previously (11,12).

ENU treatment

Mice between 45 and 75 days of age were given either a single intraperitoneal injection of ethylnitrosourea (ENU) or two injections separated by 14 days. Each injection of ENU was 50 µg/g bodyweight.

Helicobacter testing

Fecal pellets were collected and DNA was isolated with spin columns using the Blood and Body Fluid protocol provided by Qiagen (Valencia, CA). Samples were analyzed using the following PCR assay. The reaction included 1 µM each of *Helicobacter* genus-specific primers 5'-TATGACGGGTATCCGGC-3' and 5'-ATTCCACCTACCTCTCCCA-3', designed from conserved regions of the 16S rRNA gene (13), 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 1.5 mM MgCl₂, template DNA, 1.25 U of *Taq* polymerase, and distilled water in a total volume of 25 µl. Reactions were heated to 94°C for 30 s once, followed by 45 cycles of denaturation at 94°C for 2 s, primer annealing at 53°C for 2 s, and extension at 72° for 30 s in a thermocycler (MJ Research PTC-100, Waltham, MA). Each PCR reaction was analyzed by electrophoresis on an agarose gel containing ethidium bromide and photographed under UV light. The following controls were included with each PCR run: no sample, a sample from a *Helicobacter*-infected mouse, and a sample from a *Helicobacter*-free mouse. Positive samples contained a 375bp band, as confirmed with DNA molecular weight standards.

Tumor scoring

All mice were sacrificed when moribund. The number of desmoid fibromas on the abdominal wall was scored and the intestinal tract was removed, opened longitudinally and laid out as described previously (14). Samples were fixed in 10% buffered formalin for 16 hours and then placed in 70% ethanol for long-term storage. The number of intestinal tumors was scored with a dissecting microscope by a single observer (RBH) blind to the genotype of the mice.

Histological Analysis

Intestinal tumors were embedded in paraffin, sectioned, and stained with either hematoxylin and eosin (H&E) or β-catenin as described previously (15). Sections were analyzed by three pathologists (HCP, MKW and RS), each blind to the genotype of the mice.

Genetic Integrity

To determine the *Apc* status of intestinal tumors, DNA was isolated from samples scraped from 20–40 paraffin sections using neighboring H&E-stained sections as a guide. The *Apc* status was ascertained using the *Apc* heterozygosity index as described previously (16).

To test for microsatellite instability, intestinal tumors and adjacent normal tissue were microdissected during necropsy. Each tumor was cut in half; one piece was snap frozen in liquid nitrogen, whereas the other piece was fixed overnight in 10% buffered formalin for histological analysis. DNA was isolated from each frozen sample using the Magnesil Genomic Fixed Tissue System from Promega (Madison, WI) and analyzed for microsatellite instability as described previously (17).

Intestinal tumors, adjacent normal tissue, and kidneys were collected for comparative genomic hybridization (CGH). Again, each tumor was divided so that histological analysis could be performed. DNA from tissue that had been snap frozen in liquid nitrogen was analyzed using FVB DNA as the reference as described previously (18). Although tumors contain both neoplastic and normal cells, our analysis of the *Apc* status in tumors by quantitative PCR indicates that the normal cell admixture is less than 30%, so the CGH platform should permit any significant change in copy number to be detected.

Results

B6 *Apc*^{Min/+} mice fail to develop invasive intestinal cancers consistently, presumably owing to their short lifespan of about 100 days. This limited time likely restricts the execution of each conceivable pathway to metastasis. To test this possibility, we generated Min mice with an extended lifespan. C57BR/cdcJ (BR) females were crossed with B6 *Apc*^{Min/+} males and the resulting (BR × B6)F1 *Apc*^{Min/+} hybrids were held until moribund. The average lifespan of these mice was 232 ± 66 days, considerably longer than the average lifespan of B6 *Apc*^{Min/+} controls (Figure 1 and Table 1A). The increase in longevity was related to a dramatic reduction in tumor multiplicity: (BR × B6)F1 *Apc*^{Min/+} hybrids developed on average 19 ± 10 intestinal tumors, whereas B6 *Apc*^{Min/+} controls developed on average 122 ± 45 (Table 1A). Suppression of tumorigenesis was evident along the entire length of the intestinal tract from the duodenum to the descending colon (Figure 2). This observation indicates that the BR strain carries at least one dominant modifying allele that suppresses tumorigenesis. The majority of intestinal tumors (56%) from (BR × B6)F1 *Apc*^{Min/+} hybrids were invasive adenocarcinomas (Table 1A; Figure 3A). Longevity also affects extracolonic lesions: (BR × B6)F1 *Apc*^{Min/+} hybrids developed significantly more desmoid fibromas than B6 *Apc*^{Min/+} controls (data not shown).

The predisposition model states that metastatic potential is a heritable trait influenced by genetic polymorphisms of the host. To test whether the development of intestinal cancers was peculiar to (BR × B6)F1 *Apc*^{Min/+} hybrids, we generated another group of F1 hybrids by crossing SWR/J (SWR) females to B6 *Apc*^{Min/+} males. The resulting (SWR × B6)F1 *Apc*^{Min/+} hybrids had an average lifespan of 370 ± 99 and developed on average 15 ± 10 tumors along the entire length of the intestinal tract (Figure 1 and Table 1A). This reduction in tumor multiplicity was expected because SWR carries the resistance allele of the *Mom1* locus, which encodes the secretory phospholipase A₂ (19). Here too, the majority of tumors in (SWR × B6)F1 *Apc*^{Min/+} hybrids were invasive adenocarcinomas (Table 1A; Figure 3A). Moreover, 2 tumors out of 67 metastasized by direct extension to regional lymph nodes (Figure 3B). This frequency is likely an underestimate since the metastatic lesions were identified owing to the greatly enlarged lymph nodes which were harvested at necropsy. Thus, the development of invasive cancers in Min mice is not peculiar to a particular strain. Rather, it appears to depend upon either the elapsed time of tumor development or the age of the host – two intertwined variables in these experiments.

We analyzed the status of the *Apc* wildtype allele in 10 advanced tumors from F1 *Apc*^{Min/+} hybrids. The majority (8/10) exhibited loss, most likely by somatic recombination as in B6 *Apc*^{Min/+} mice. We did not analyze the status of *Mom1*. BR is closely related to B6 and carries the sensitive allele of *Mom1*. Therefore, the reduction in tumor multiplicity and increase in longevity exhibited by (BR × B6)F1 *Apc*^{Min/+} hybrids cannot be explained by *Mom1* status.

In the progression model, tumors grow and metastasize as a consequence of accumulated genetic mutation, epigenetic alterations, or both throughout the genome. Tumors from F1 *Apc*^{Min/+} hybrids ranging in age from 232–609 days were tested for microsatellite instability (MSI) using a multiplex assay described previously (17). One F1 adenocarcinoma was MSI-high exhibiting mutations in 2 out of 8 markers, while thirteen F1 adenocarcinomas were MSI-low exhibiting mutations only in one out of eight markers (Table 2). The majority of the mutations were found in mBAT-67, a very long mononucleotide repeat marker that is likely to be less stable than shorter mononucleotide repeat markers. Notably, the low level of MSI observed in invasive adenocarcinomas from long-lived F1 *Apc*^{Min/+} animals does exceed that observed in short-lived B6 *Apc*^{Min/+} controls. Only 1 out of 20 adenomas in B6 animals ranging in age from 90–100 days was MSI-low. Thus, a low level of MSI may participate in familial tumorigenesis, but it is not measurably enhanced during progression to the adenocarcinoma.

Tumors from F1 *Apc*^{Min/+} hybrids were also tested for chromosomal deletions and gains by CGH. We prepared genomic DNA from invasive adenocarcinomas, normal intestinal epithelium, and kidney. A few clones on the arrays consistently exhibited a change in copy number among all samples including normal tissue, indicating that they were polymorphic between B6 and FVB (Figure 4). But, no tumor-associated aberrations were detected in the DNA from these tumors. Thus, large chromosomal deletions or duplications are not necessary for the progression of invasive adenocarcinomas in this particular model of familial cancer.

Though apparently not necessary, chromosomal instability might be sufficient to drive tumor progression in Min mice. Cells homozygous for a hypomorphic allele of *Nbs1*, *Nbs1*^{ΔB}, exhibit defects in the intraS and G₂/M checkpoints and marked chromosomal instability (20). *Nbs1*^{ΔB/ΔB} *p53*^{-/-} mice develop lymphomas much earlier than *Nbs1*^{+/+} *p53*^{-/-} mice (12). We generated Min mice carrying 0, 1, and 2 copies of the mutant *Nbs1*^{ΔB} allele and held them until moribund. The significant reduction in Nbs1 activity did not affect tumor progression: 21% of the tumors in *Apc*^{Min/+} *Nbs1*^{ΔB/ΔB} mice and 12% in *Apc*^{Min/+} *Nbs1*^{+/+} mice were invasive adenocarcinomas (Table 1B). Further, no difference was observed in lifespan, tumor multiplicity (Table 1B), or tumor distribution (data not shown). Thus, a reduction in Nbs1 activity does not strongly affect intestinal tumorigenesis in the Min mouse. One explanation is that reduced Nbs1 activity might not cause genetic instability in the intestinal epithelium; this possibility has not been specifically tested.

Is genetic mutation sufficient to drive tumor progression in Min mice? We treated F1 *Apc*^{Min/+} hybrids with one or two doses of a strong somatic point mutagen. Mice received 50 μg of ethylnitrosourea (ENU) per gram bodyweight per intraperitoneal injection between 45 and 75 days of age and were held until moribund. We choose a low dose of ENU in young adults rather than a high dose because we wanted to avoid a dramatic increase in tumor multiplicity and consequently reduced lifespan. Surprisingly, treatment of (BR × B6)F1 *Apc*^{Min/+} hybrids appeared to impede tumor progression with only 9% of the tumors being invasive adenocarcinomas (Table 1C). This effect might simply reflect the significant decrease in lifespan owing to the increase in adenoma multiplicity. Treated mice developed on average 50 ± 22 tumors, whereas contemporaneous controls developed 19 ± 10 tumors. ENU had its strongest effect in the proximal half of the small intestine (Figure 2). In addition, treatment of (SWR × B6)F1 *Apc*^{Min/+} hybrids with ENU did not increase tumor progression. Thus, this situation, designed to increase the levels of somatic mutation during tumorigenesis, clearly did not enhance tumor progression in the Min mouse.

The risk of developing intestinal cancer is clearly influenced by environmental factors, including diet and bacterial flora. Several epidemiological studies indicate that risk of colon cancer is higher for individuals infected with *Helicobacter pylori*. This link is also apparent in some animal models: the development of intestinal cancers in *Smad3*-deficient mice depends on the presence of *Helicobacter*. To test whether age-dependent tumor progression in Min mice was affected by *Helicobacter* status, we analyzed tumors from F1 *Apc*^{Min/+} hybrids for which the status of *Helicobacter* was known. No effect on the frequency of invasive adenocarcinomas was observed between *Helicobacter* negative mice and contemporaneous *Helicobacter* positive controls (Table 1D).¹

¹The frequency observed in this experiment was noticeably lower than that in the initial experiment (Table 1D versus Table 1A). During the six years that elapsed between these two experiments, our colony underwent significant environmental changes including a switch in diet and the implementation of a microisolator system.

Discussion

We have identified a homogenous genetic and environmental context in which *Apc*^{Min/+} mice develop intestinal tumors that spontaneously progress and metastasize locally. A key barrier to the development of advanced cancers in the Min mouse has been the short lifespan on the sensitive B6 genetic background. F1 *Apc*^{Min/+} hybrids were generated by crossing BR or SWR females to B6 *Apc*^{Min/+} males. These mice developed one tenth the number of tumors observed in controls and often lived longer than one year. Many of the tumors in these hybrids were invasive adenocarcinomas and a few even metastasized by extension into regional lymph nodes (Table 1; Figure 3). The development of these familial cancers did not require a high level of microsatellite instability, chromosomal gains or losses, or the presence of *Helicobacter*. Several classes of genomically conservative processes can be considered to drive progression in this setting. Prominent candidates can be suggested from the known epigenetic changes observed by the analysis of the tumor transcriptome. We have previously shown that advanced cancers from long-lived Min mice exhibit core molecular signatures of the epithelial – mesenchymal transition. In particular, vimentin expression is elevated not only in neoplastic cells of the primary tumor but also in those invading the submucosa and musculature (21). A full characterization of the epigenome during tumor progression in the Min model may identify other candidate genes whose deregulation mediates progression.

The genomically conservative pathway to invasive intestinal adenocarcinomas that we have observed in the familial Min model does not rule out a role for genomic instability in other contexts such as sporadic colon cancer. Indeed, mutations in a number of genes have been shown to enhance the tumor phenotype of Min and other mouse models. We have investigated whether a generalized increase in somatic mutation can increase the number of invasive adenocarcinomas and metastatic lesions as well as decrease the time required for these types of tumors to form in the Min model. We treated adult F1 *Apc*^{Min/+} hybrids with a low dose of ENU and found that this treatment shortened the lifespan, increased tumor multiplicity, and reduced progression.² Thus, any molecular changes that occur spontaneously during tumor progression cannot be fully replaced by treating mice with a strong point mutagen. Perhaps, the salient changes are not point mutations. Other possibilities will be discussed below.

Our results do not yet support a particular model of metastatic potential. Consider the predisposition model. Both (BR × B6)F1 and (SWR × B6)F1 *Apc*^{Min/+} hybrids develop a significant number of invasive adenocarcinomas. BR and SWR are quite distinct and were derived from separate stocks in the 1930s and 1940s (23). Nonetheless, these strains could share one or more allelic variants that affect metastatic potential. The propensity for metastasis must be mapped to particular host loci and tested by transplantation for autonomy *versus* non-autonomy to investigate this hypothesis. Lifsted and colleagues recently demonstrated that the development of mammary cancers and extensive metastasis in mice carrying the (*MMTV*-*PyMT*)634*Mul* transgene was profoundly affected by genetic background (24). In addition, Park and colleagues demonstrated that resistant and susceptible strains can be stratified based on the expression of 17 genes in normal mammary tissue (25). Together, these findings have lent support to the host predisposition model.

Constitutional polymorphic variants are known to affect other aspects of intestinal tumorigenesis in the *Apc*^{Min/+} mouse model. B6 *Apc*^{Min/+} mice develop on average 100 tumors, whereas AKR *Apc*^{Min/+} mice develop on average less than 1 tumor (16). This difference is partially due to *Mom1*, encoding *Pla2g2a* and at least one other modifier of intestinal

²The increase in tumor multiplicity was unexpected, since our previous study had found that treatment of young B6 *Apc*^{Min/+} adults with ENU failed to affect tumor multiplicity (22). Key differences between this study and that of Shoemaker and colleagues are genetic background (F1 vs B6) and the age at which the treated mice were sacrificed (≤ 100 days *versus* 188 ± 32).

tumorigenesis (19). The reduction in tumor multiplicity does not necessarily lead to increased longevity because AKR mice frequently develop lymphomas. Other polymorphic modifiers of the Min phenotype have been identified including *Mom2*, *Mom3*, *Mom6* and *Mom7* (4). *Mom2* is a spontaneous mutation that maps to distal Chromosome 18 and disrupts *Atp5a1*, encoding the α subunit of the ATP synthase (26). *Mom7* maps to the pericentromeric region of Chromosome 18 and may directly regulate the loss of heterozygosity of distal elements (27). The genome-wide identification of polymorphic modifier loci for the Min phenotype is just beginning.

Somatic mutations also can affect intestinal tumorigenesis. Armitage and Doll estimated that 5–6 changes are necessary for a cancer to form (28). Analyses of human tumors at different stages have revealed that the activation of KRAS promotes growth and the loss of p53 favors metastasis. These alterations appear not to occur spontaneously during the development of intestinal adenomas and invasive adenocarcinomas in studies of two mouse models (29,30). Possibly, the context, e.g. sporadic versus familial disease or even the specific mutation in *Apc*, dictates which pathway is favored. The possible role of somatic mutations in progression in mouse models is suggested only by studies of these models carrying germline mutations in loci other than *Apc*. Janssen and colleagues demonstrated that constitutional expression of an activated form of KRAS does affect tumor multiplicity and progression in *Apc*^{1638N/+} mice (31). Nearly half of the tumors from *KRAS*^{V12G}; *Apc*^{1638N/+} mice progressed to invasive adenocarcinomas by 105 days of age (16/34), unlike *Apc*^{1638N/+} controls (1/8). Similarly, we have demonstrated that a lack of p53 activity leads to tumor progression in the intestines of young Min mice (14). B6 *Apc*^{Min/+} *p53*^{-/-} mice developed a small proportion of invasive adenocarcinomas by 90 days of age (2/42), in contrast to B6 *Apc*^{Min/+} *p53*^{+/+} controls (0/78). Tumor progression in mouse models is also affected by mutations in several other genes including *Atp5a1* (*Mom2*), *Ephrin-A1*, *EphB2*, *EphB3*, *ER α* , *Fen1*, *Netrin-1*, and *Tsp1* (reviewed in 4). The genome-wide identification of mutant alleles that modify the Min phenotype is also just beginning.

Two pathways lead to genetic instability in colorectal cancer (32): microsatellite instability (MSI) and chromosomal instability (CIN). Alberici and colleagues reported that adenomas in *Apc*^{1638N/+} mice display numerous chromosomal aberrations (33). Further, several investigators have found that sporadic human colorectal tumors display numerous chromosomal aberrations. For example, Martin and colleagues reported that those chromosomal regions that had undergone amplification and deletion carried known cancer genes including *EGFR* and *Myc* and many of affected regions were also altered in lung cancers, glioblastomas, or multiple myelomas (34). However, it remains a challenge to determine whether the genetic and genomic instabilities observed in these advanced cancers are triggers rather than bystanders acquired during growth under the relaxed constraints of the autonomous neoplasm. Thus, a chromosomal region that is commonly altered may either carry a critical gene affecting progression or else simply be inherently unstable in its structure.

Selection for genetic instability may be much stronger in sporadic disease than familial disease. Some intestinal adenomas from *Apc*^{Min/+} mice and FAP patients have been reported to form with relatively stable karyotypes (15,35). Conservative somatic recombination at the *Apc*/*APC* locus plays a role in the formation of these tumors. Two independent studies have demonstrated that a reduction in activity of the Bloom's Syndrome gene (*Blm*) enhances the Min phenotype (36,37). In both cases, the analysis of markers on Chromosome 18 revealed that the wildtype allele of *Apc* was lost by somatic recombination in several tumors. Haigis and his colleagues demonstrated that the wildtype allele of *Apc* is almost always lost by somatic recombination to initiate tumorigenesis in B6 *Apc*^{Min/+} mice (38). They found the Robertsonian translocation Rb(7.18)9Lub (Rb9) reduces the multiplicity of intestinal adenomas in this mouse model by disrupting the pairing of the Chromosome 18 homologs during mitosis. The extent

of somatic recombination throughout the entire genome is unknown. Interestingly, McMurray and Gottschling demonstrated that wildtype yeast cells switch to a hyper-recombinational state as they age (39). Loss of heterozygosity occurs by somatic recombination in young cells and by break-induced replication in old cells. These forms of genetic instability would not be detected by CGH since copy number is unaltered. Thus, somatic recombination in tumors of F1 *Apc*^{Min/+} hybrids could generate homozygosity for allelic variants that drive progression, culminating in metastasis. We did find loss of heterozygosity at the *Apc* locus in the majority of advanced tumors from F1 *Apc*^{Min/+} hybrids.

By contrast, epigenetic silencing may play a role in the formation or progression of these genetically stable neoplasms. Silencing may be reflected by the CpG island methylator phenotype, CIMP (reviewed in 40). Intriguingly, DNA hypermethylation is an age-dependent process in the intestinal epithelium (41,42).

In summary, we have tested invasive adenocarcinomas from long-lived F1 *Apc*^{Min/+} hybrids for CIN and MSI. Overall, the tumors in this particular model of familial disease display a stable karyotype and only a low level of MSI. These invasive tumors can metastasize by extension into regional lymph nodes. Bodmer and Tomlinson have argued that genetic instability is not required for tumor development because normal mutation rates coupled with selection are sufficient to account for the molecular changes observed (43,44). Thus, though genetic instability may be able to drive progression in some biological contexts, it is not the only route to the locally invasive adenocarcinoma. Rusan and Peifer have reviewed the possible positive and negative impacts of genomic instability on cancer (45). Indeed, genetic instability can inhibit tumorigenesis in certain other biological contexts by triggering apoptosis (46). Rao and colleagues have reported that the lack of mitotic aberrations in Min mice reduces tumor multiplicity in the small intestine but increases it in the colon (47).

The genetically conservative pathway to invasion described in this report must now be analyzed more deeply by newly emerging methods. Does the development of invasive potential reflect the elapsed time of tumor development, or the age of the host? Longitudinal studies of individual tumors arising at different times in an animal may enable one to disentangle these two factors. Does it reflect an accumulation of conservative somatic recombination events that render particular constitutional alleles homozygous in F1 *Apc*^{Min/+} animals? Does it reflect an age-dependent epigenetic silencing of one or both alleles at particular anti-progression loci? The capacity of the Min mouse model to display a high frequency of locally metastatic behavior represents another step in the development of better animal models for human colon cancer.

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Figure 1. F1 *Apc*^{Min/+} hybrids live significantly longer than B6 *Apc*^{Min/+} controls. 200 (SWR × B6)F1 *Apc*^{Min/+}, 184 (BR × B6)F1 *Apc*^{Min/+}, and 97 B6 *Apc*^{Min/+} mice were allowed to age until moribund. The percent surviving versus age is plotted.

Figure 2.

Tumors develop along the entire length of the intestinal tract in F1 *Apc^{Min/+}* hybrids. Mice were allowed to age until moribund and then sacrificed. The small intestine was divided into four equal segments with region 1 being closest to the stomach and region 4 being closest to the cecum. The number of tumors in these segments and the colon was scored and is plotted as the percentage of the total. Interestingly, the distribution of tumors skewed towards region 1 if F1 *Apc^{Min/+}* hybrids are treated with ENU ($P < 0.001$ each by Chi-square test for ENU-treated BR and SWR hybrids compared to their untreated counterparts.)

Figure 3.

Tumors in long-lived F1 $Apc^{Min/+}$ hybrids often become invasive adenocarcinomas and sometimes even metastasize by direct extension to regional lymph nodes. F1 $Apc^{Min/+}$ hybrids ranging from 224–387 days of age became moribund and were sacrificed. (Panel A) H&E-stained sections of four invasive adenocarcinomas from these mice are shown. The top two photographs are of (SWR $\times$ B6)F1 and the bottom two are of (BR $\times$ B6)F1. The tumors often exhibit ulceration of the mucosal surface and extensive invasion of neoplastic cells through the muscle wall into the serosa and mesentery. These examples are representative of tumors observed throughout the study. Size bar, 1mm. (Panel B, C, and D) In a 367-day old (SWR $\times$ B6)F1 $Apc^{Min/+}$ mouse, a tumor appeared to have spread by direct extension into a regional

lymph node. The tumor, mesentery, and enlarged lymph node were removed, fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Sections were assessed by IHC for overexpression of β -catenin (left) or stained with H&E (center; each area outlined with a black rectangle is shown enlarged at the right). In this tumor, neoplastic cells have invaded into the muscle wall of the gastrointestinal tract (panels B and C), and largely effaced and replaced normal tissue in the lymph node leaving only residual intact areas (panel D). The cytoplasm and nucleus of each tumor cell stained intensely for β -catenin (brown) unlike normal tissue. Size bar for β -catenin photographs, 1mm.

Figure 4.

Invasive adenocarcinomas from long-lived F1 $Apc^{Min/+}$ hybrids do not exhibit chromosomal instability. Two invasive adenocarcinomas (panels A and B, size bar, 2mm), adjacent normal tissue, and kidneys were removed from a (BR $\times$ B6)F1 $Apc^{Min/+}$ male that lived for 312 days. DNA was prepared and analyzed by CGH (panel C). The $\log_2$ ratio is plotted for each locus. None of the autosomal chromosomes exhibited any gains or losses when comparing results obtained with DNA from tumors (red square and red triangle) to those obtained with DNA from adjacent normal tissue (black diamond). By contrast, the X chromosome appears to be lost in all samples. This change merely reflects the fact that test DNA for this example was isolated from a F1 $Apc^{Min/+}$ male and the control DNA was isolated from a female. A total of

seven invasive adenocarcinomas from three F1 $Apc^{Min/+}$ hybrids were analyzed. Similar results were observed in all cases.

Table 1

Effect of background/age, *Nbs1* genotype, ENU treatment, and *Helicobacter* status on tumor multiplicity and progression

Mouse type	N	Lifespan (d), mean ± SD	Intestinal tumor count, mean ± SD	Invasive adenocarcinomas/total tumors [*]
<i>A. Phenotype of F1s and controls</i>				
(BR × B6)F1	72	232 ± 66	19 ± 10	104/185 (56%)
(SWR × B6)F1	79	370 ± 99	15 ± 10	39/45 (87%)
B6	97	100 ± 18	122 ± 45	0/74 (0%)
<i>B. Effect of Nbs1</i>				
B6 <i>Nbs1</i> ^{+/+}	17	144 ± 48	23 ± 23 [†]	6/53 (11%)
B6 <i>Nbs1</i> ^{+/^ΔB}	28	175 ± 79	24 ± 14 [†]	ND
B6 <i>Nbs1</i> ^{ΔB/ΔB}	22	142 ± 42	31 ± 27 [†]	10/47 (21%)
B6 <i>Apc</i> ^{+/+} <i>Nbs1</i> ^{ΔB/ΔB}	13	ND	0 ± 0	NA
<i>C. Effect of ENU treatment</i>				
(BR × B6)F1, one ENU treatment	9	202 ± 47	44 ± 22	8/94 (9%) [#]
(BR × B6)F1, two ENU treatments	6	180 ± 8	58 ± 20	
(SWR × B6)F1, one ENU treatment	5	289 ± 86	19 ± 14	13/35 (38%) [#]
(SWR × B6)F1, two ENU treatments	8	190 ± 21	40 ± 17	
<i>D. Effect of Helicobacter</i>				
(BR × B6)F1, <i>Helicobacter</i> negative	61	312 ± 82	10 ± 4	9/30 (30%)
(BR × B6)F1, <i>Helicobacter</i> positive	20	319 ± 100	12 ± 7	13/41 (32%)
(SWR × B6)F1, <i>Helicobacter</i> negative	58	396 ± 144	13 ± 10	12/38 (32%) ^{&}
(SWR × B6)F1, <i>Helicobacter</i> positive	12	498 ± 75	8 ± 4	7/29 (24%) ^{&}

Note: All mice are *Apc*^{Min/+} unless otherwise specified.

^{*} Intestinal tumors were isolated from a randomly selected subset of mice of each strain.

[†] Wilcoxon Rank Sum P-values of the three pair-wise comparisons ≥ 0.3. ND, not done; NA, not applicable.

[#] One or two ENU treatments.

[&] One tumor in this set showed metastasis by direct extension to a regional lymph node.

Table 2

MSI analysis of invasive intestinal adenocarcinomas with mononucleotide repeats

Genotype	Mouse ID	Age (d)	Sample pair	Change in allele size (bp)							
				<i>D1Mit79</i>	<i>mBat-24</i>	<i>mBat-26</i>	<i>mBat-27*</i>	<i>mBat-37</i>	<i>mBat-59</i>	<i>mBat-64</i>	<i>mBat-67</i>
<i>B6.MH1^{+/+}</i> (BR × B6)F1 <i>Apc^{Min/+}</i>	4934	269	1	-2	0	-3	-2	0	-3, -7	-7, -11	-6, -12
	1851	362	2	0	0	0	0	0	0	0	0
(SWR × B6)F1 <i>Apc^{Min/+}</i>	2012	297	3	0	0	0	0	0	0	ND	0
	2056	232	4	0	0	0	0	0	0	0	0
	2058	530	5	0	0	0	0	0	0	0	0
	2079	536	6	0	0	0	0	0	0	0	-24
	2217	520	7	0	0	0	0	0	0	0	ND
	2232	517	8	0	0	0	0	0	0	0	-25, -34, -40
			9	0	0	0	0	0	0	0	0
			10	0	0	0	0	0	0	0	ND
			11	0	0	0	0	0	0	ND	0
	1840	601	12	0	0	0	0	0	0	0	-22, -38
	1888	591	13	0	0	0	0	0	0	0	-30
	1902	595	14	0	0	0	0	0	0	0	-21
	1904	595	15	0	0	0	0	0	0	0	ND
	2000	571	16	0	0	0	0	0	0	0	-38
	2001	571	17	0	0	0	0	0	0	0	0
	2221	533	18	0	0	0	0	0	-25	0	-15, -26, -35
			19	0	0	0	0	0	ND	ND	0
	2223	533	20	0	0	0	0	0	0	0	-27, -37
	1986	579	21	0	0	0	0	0	0	0	-12, -20
	1885	604	22	0	0	0	0	0	0	0	-12, -24, -33
	1898	609	23	0	0	0	0	0	0	0	-18
	2041	578	24	0	0	0	0	0	0	0	-26
	2047	577	25	0	0	0	0	0	0	0	0
			26	0	0	0	0	0	ND	0	-21

* mBat-27 (accession #L24372) primer sequences were GGGAAAGACTGCTTAGGGAAGA and ATTGGCTTTCAAGCATCCATA (48). The other microsatellite markers were described previously (17). ND, not done.