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# Colorectal Pretumor Progression Before and After Loss of DNA Mismatch Repair

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**A pretumor progression model predicts many oncogenic cancer mutations may first accumulate in normal appearing colon. Although direct observations of early pretumor mutations are impractical, it may be possible to retrospectively reconstruct tumor histories from contemporary cancer mutations. To infer when and in what order mutations occur during occult pretumor progression, we examined 14 cancers from individuals with heterozygous germline mutations in DNA mismatch repair (MMR) genes or hereditary nonpolyposis colorectal cancer (HNPCC). Somatic inactivation of the normal allele occurs sometime during a lifetime and results in loss of MMR, elevated mutation rates, and subsequent widespread somatic microsatellite mutations in HNPCC cancers. Patient ages at MMR loss can be estimated because intervals between MMR loss and cancer removal can be inferred from numbers of microsatellite tumor mutations. The relative order of MMR loss during pretumor progression may also be inferred from its collective ages of occurrence. Somatic MMR loss preceded cancer removal by an average of 6.1 years, occurred relatively late in life (average of 41.6 *versus* 47.7 years at cancer removal), and was a surprisingly late (fifth or sixth) step. Calculations indicate five or six oncogenic mutations could accumulate with relatively normal replication fidelity in normal appearing colon. HNPCC pretumor progression essentially begins from birth and ends with MMR loss, implying elevated mutation rates and tumorigenesis may be unnecessary for most progression. *(Am J Pathol 2004, 164:1447–1453)***

Multiple mutations are present in colorectal cancers. Although it is uncertain when mutations occur, a pretumor progression model[1] predicts some cancer mutations are first acquired in normal appearing colons. Hereditary cancer syndromes identify mutations that may accumulate during pretumor progression. For example, individuals with familial adenomatous polyposis (FAP) or hereditary nonpolyposis colorectal cancer (HNPCC) inherit heterozygous mutations, respectively, in APC and DNA mismatch repair (MMR) genes, but are born with normal appearing colons.[2]

Although germline mutations precede tumor progression, the timing, number, and order of somatic mutations during pretumor progression are poorly characterized (Figure 1). Ideally the entire mutational history of a tumor could be documented from birth to removal. In HNPCC, there is a sequence of mutations leading to MMR-deficient cancers. Germline MMR mutations (usually MLH1 or MSH2) are followed by somatic inactivation of the normal allele, leading to functional MMR loss and elevated mutation rates, with microsatellite (MS) loci rates increased 100- to 1000-fold.[3,4] MS mutations subsequently accumulate every day, and HNPCC colorectal tumors characteristically contain ubiquitous somatic MS mutations.[2]

Recent studies illustrate that intervals between somatic MMR loss and tumor removal can be estimated retrospectively from numbers of MS tumor mutations.[5,6] Essentially the longer the intervals between somatic MMR loss and tumor removal, the greater the numbers of accumulated MS tumor mutations. A patient's age at somatic MMR loss can be estimated by subtracting the interval between MMR loss and tumor removal from age at tumor removal. Somatic MMR loss may occur during pretumor progression because congenital MMR deficiencies are compatible with normal appearing colons.[7–10] In addition, for HNPCC tumors that appear shortly after negative clinical examinations, somatic MMR loss appears to precede normal examinations by many years.[5,6,11]

Here we use methodologies of previous studies[1,5,6] to illustrate how the timing, number, and order of occult

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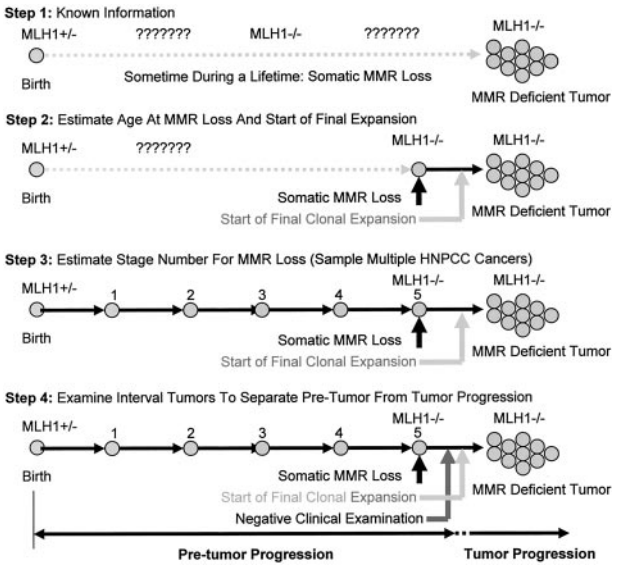


**Figure 1.** Inferring cancer histories. Direct measurements are possible of germline and tumor genotypes. In HNPCC, there is a germline MMR gene mutation (MLH1+/−) and the cancer is MMR-deficient (MLH1−/−). Somatic loss of the normal MLH1 allele must occur in between. In step 2, the age at MMR loss can be inferred from the number of somatic MS cancer mutations. The greater the number of cancer MS mutations, the earlier MMR loss occurred. The age at the final clonal expansion can also be inferred from the pattern of cancer MS mutations. In step 3, numbers of oncogenic mutations preceding MMR loss can be inferred from ages at MMR loss from multiple HNPCC cancers. In step 4, the transition between pretumor and tumor progression can be inferred when interval cancers are analyzed. The age at a negative clinical examination indicates persistence of pretumor progression. By our model, HNPCC progression starts from birth and a number of oncogenic mutations usually precede somatic MMR loss. Most oncogenic HNPCC mutations occur during pretumor progression.

pretumor somatic mutations may be inferred (Figure 1). Another group's modeling work suggests chromosomal instability may precede tumorigenesis;[12] we present experimental evidence that MS instability and the majority of oncogenic cancer mutations also precede HNPCC tumorigenesis.

## Materials and Methods

DNA was isolated from 14 formalin-fixed, paraffin-embedded colorectal cancers[5] from HNPCC patients with germline MLH1 or MSH2 mutations confirmed by sequencing. Somatic MS mutations were detected after dilution of the DNA to essentially single molecules, followed by polymerase chain reaction and acrylamide gel analysis.[6] Intervals between loss of MMR and cancer removal, and since the start of final clonal expansion were estimated based on MS mutation patterns or the drift of MS alleles from germline sizes.[6] Histories of 10 cancers were previously reported with an analysis that assumes one division per day and stepwise MS mutation with a rate of 0.005 per division.[5,6] Patient age at MMR loss was estimated by subtracting the time between MMR loss and tumor removal from the age of the patient at surgery. Clinical data were obtained from patient charts.

In a previous study,[1] we have presented a pretumor progression model. Given the ages of patients when they were diagnosed with cancer, we developed a methodology to infer the number of mutations necessary for cancer and their mutation rates. In the present study we use the same methodology to infer the number of mutations necessary for MMR loss and their mutation rates, given the ages of patients at MMR loss (Table 1). Our approach is Bayesian: we assume a uniform prior on the number of mutations, and we calculate the posterior probabilities of the number of mutations (Table 2). In contrast to our previous study,[1] we consider a much smaller data set; as a consequence several of these probabilities are nonzero. These probabilities can be used to form a credibility interval for the numbers of mutations.

## Results

### MMR Loss Occurs Late in Life

Mutations at 21 to 30 different CA-repeat MS loci were analyzed from 14 HNPCC cancers (Table 1). Intervals between MMR loss and tumor removal (Figure 1) were estimated from repeat unit differences between germline and tumor sizes.[6] Cancer histories were variable but MMR loss always preceded final clonal expansions (Figure 2). The average interval between MMR loss and cancer removal was 6.1 years, with an average final clonal expansion age of 1 year. Ages at MMR loss can be estimated by subtracting intervals between tumor removal and MMR loss from the age at cancer removal for each patient. MMR loss occurred relatively late during life at an average age of 41.6 *versus* 47.7 years at cancer removal.

### Intervals Between MMR Loss and Cancer Correlate with Patient Age but Not Clinical Tumor Stage

Mutations are associated with incremental changes in phenotype during tumor progression.[2] Therefore, clinically more advanced cancers might have longer intervals between MMR loss and cancer removal, and greater numbers of MS mutations. However, there was no correlation between tumor age and clinical stage (Figure 3A). Higher stage cancers tended to have older clonal expansions (Figure 3B), but overall final clonal expansion intervals were short (average of 1 year). Of interest, intervals between MMR loss and cancer removal correlated with patient age at cancer removal (Figure 3C). Average tumor age was significantly greater for patients older than 50 years old at the time of surgery (8.0 *versus* 5.2 years, $P = 0.022$, two-tailed *t*-test).

### Older Cancers in Older Patients

A relationship between tumor and patient ages is consistent with multistep models[13–15] that postulate that cancers have the same number of oncogenic mutations regardless of patient age at cancer. For example, cancers arising in a 42-year-old patient and a 60-year-old patient would have the same number of oncogenic mutations.

**Table 1.** Tumor Histories

| Tumor | Stage | Age at removal | Age at MMR loss* | No. loci tested | Tumor age since loss of MMR (CI†) | Age of clonal expansion |
|---|---|---|---|---|---|---|
| H1 | D | 47 | 44 | 24 | 3.4 (1.0 to 4.8) | 0.82 |
| H2 | D | 35 | 31 | 28 | 4.1 (1.6 to 5.7) | 0.77 |
| H3 | C | 43 | 38 | 29 | 4.6 (2.0 to 6.6) | 0.52 |
| H4 | A | 58 | 53 | 23 | 5.1 (2.0 to 7.6) | 0.27 |
| H5 | D | 40 | 34 | 21 | 5.5 (2.0 to 7.7) | 2.0 |
| H6 | B | 46 | 40 | 20 | 5.6 (1.8 to 8.5) | 0.93 |
| H7 | B | 44 | 38 | 30 | 5.7 (2.2 to 7.9) | 0.96 |
| H8 | B | 43 | 37 | 29 | 5.8 (2.5 to 8.2) | 0.25 |
| H9 | B | 42 | 36 | 28 | 5.9 (2.4 to 8.3) | 0.71 |
| H10 | A | 54 | 48 | 22 | 6.0 (2.1 to 7.7) | 1.2 |
| H11 | D | 38 | 32 | 21 | 6.1 (2.2 to 8.6) | 1.8 |
| H12 | B | 57 | 51 | 24 | 6.3 (2.7 to 9.2) | 0.52 |
| H13 | B | 64 | 55 | 26 | 8.8 (4.1 to 13) | 0.77 |
| H14 | B | 57 | 44 | 24 | 13 (5.3 to 19) | 0.96 |
| Average | | 47.7 | 41.6 | 24.9 | 6.1 | 0.9 |

*Age (in years) at cancer removal minus tumor age.
†95% confidence intervals.

Simplistically, the difference between these cancers is the time to acquire their mutations. If cancers require six oncogenic mutations, then on average each mutation requires ~7 years in the 42-year-old patient and 10 years in the 60-year-old patient because mutations accumulate sequentially (Figure 4). The process starts at birth and the first mutations stochastically accumulate at early ages. The older ages at MMR loss for cancers removed from older patients generally fit a multistep model (Figure 4).

The late ages at MMR loss relative to cancer removal allow for earlier mutations. Numbers of mutations can be inferred from ages of occurrence of any measurable event. For example, cancer frequencies increase exponentially with age and this age at cancer information can be used to infer total numbers of oncogenic cancer mutations. Similarly, numbers of oncogenic mutations preceding MMR loss can be estimated if ages at MMR loss are known. The most likely number of mutations can be estimated with a Bayesian approach[1] for small samples. From the ages at MMR loss (Table 1), MMR loss most likely represented the fifth or sixth oncogenic mutation (Table 2). A similar analysis using ages at cancer removal (Table 1) estimated HNPCC cancers most likely require five or six oncogenic mutations (Table 2). Both estimates are consistent with HNPCC cancers requiring multiple oncogenic mutations for transformation, with MMR loss representing a late oncogenic mutation.

## *HNPCC Pretumor Progression*

The past appearance of a tumor progenitor may be inferred when a negative clinical examination precedes tumor removal. Unless a tumor was missed, a negative clinical examination suggests the absence of a visible tumor phenotype at that age. Negative examinations (surgery or colonoscopy) preceded removal of four HNPCC cancers (Figure 2). Loss of MMR was estimated to precede these negative examinations, indicating MMR loss and earlier oncogenic mutations occur during pretumor progression.

To test the feasibility of this conclusion, mutation rates required to accumulate five or six oncogenic mutations by the estimated ages of MMR loss were calculated as in Calabrese and colleagues.[1] For our baseline model (15 million crypts, 64 stem cells per crypt, one stem cell division per day) and neutral mutations, rates of $\sim 8.8 \times 10^{-7}$ to $4.4 \times 10^{-6}$ per locus are needed to collect five or six oncogenic mutations in a single HNPCC cancer progenitor stem cell by the times of MMR loss indicated in Table 1. If every mutation is followed by a sweep that results in very rapid fixation, lower rates ($3.1 \times 10^{-8}$ to $6.8 \times 10^{-8}$) are needed to collect five or six mutations. Rates would be between the two scenarios if early progression combines selective and neutral mutations. These estimated mutation rates are within the realm of normal replication fidelity,[16,17] indicating it is feasible to accumulate multiple oncogenic somatic mutations before MMR loss during pretumor progression.

## *Discussion*

Ideally one could identify all oncogenic cancer mutations, their relative order, and when during a lifetime they oc-

**Table 2.** Oncogenic Mutations to MMR Loss or Cancer

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| MMR loss | 2 | 3 | 4* | 5* | 6* | 7* | 8* | 9 | 10 |
| Probability | 0.00 | 0.02 | 0.11 | 0.29 | 0.32 | 0.19 | 0.07 | 0.02 | 0.00 |
| Cancer | 2 | 3 | 4* | 5* | 6* | 7* | 8* | 9 | 10 |
| Probability | 0.00 | 0.02 | 0.10 | 0.26 | 0.31 | 0.20 | 0.08 | 0.02 | 0.00 |

*Results from a Bayesian analysis[1] using the data in Table 1. We assumed a uniform prior on the number of mutations being between 2 and 10. The 95% credible interval is 4 to 8, with 5 or 6 the most likely numbers of oncogenic mutations to MMR loss or cancer.

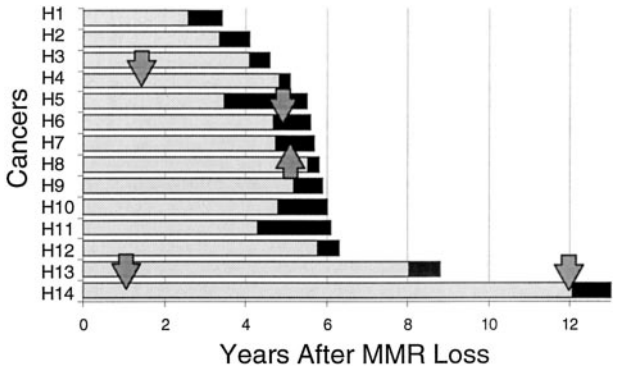


**Figure 2.** Histories of 14 HNPCC cancers. Times since MMR loss (**gray** plus **black bars**) or since starts of final clonal expansion (**black bars**) and tumor removals were estimated from MS mutation patterns. Times of negative clinical examinations (ie, no visible tumors) are indicated with **arrows** for four cancers.

curred. Here we outline an approach (Figure 1) that can characterize progression from birth to tumor removal based on a pretumor progression model.[1] In HNPCC, somatic loss of MMR occurs between birth and tumor removal because normal cells are MMR proficient (MLH1+/−) but cancers are MMR-deficient (MLH1−/−). MMR loss results in greatly elevated mutation rates and large numbers of somatic MS mutations are present in HNPCC cancers.[2] The longer the intervals after MMR loss, the greater the numbers of MS tumor mutations. A quantitative analysis of MS tumor mutation patterns[5,6] can estimate time since MMR loss and time since the start of the final clonal expansion (Figure 2). With this information, patient age at the time of somatic MMR loss can be estimated (Figure 1).

If cancer is a multistep process (one mutation after another), ages at cancer and ages at intermediate mutations should also follow a multistep pattern. Cancer ages of occurrence are consistent with five to seven oncogenic mutations or stages.[13–15] Ages at cancer from the current patients were consistent with HNPCC colorectal cancers requiring five or six oncogenic mutations for transformation. Similarly, ages at MMR loss from multiple HNPCC cancers (Table 1) can be used to estimate numbers of oncogenic mutations preceding MMR loss. Potentially

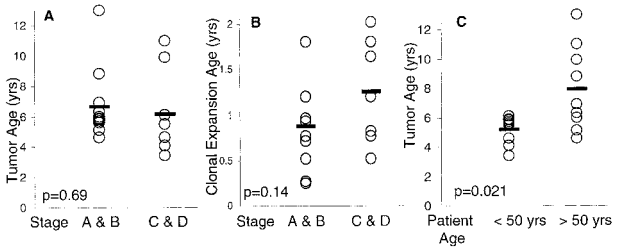


**Figure 3.** Relationships between tumor ages (years between MMR loss and cancer removal) and clinical stages. **A:** Time after MMR loss did not significantly affect cancer phenotypes. Cancers with higher clinical stages had shorter intervals between MMR and removal compared to lower stage cancers. **B:** Cancers with higher clinical stages had older final clonal expansions, indicating greater time may be required for invasion and metastasis. **C:** Time after MMR loss was significantly greater ($P = 0.022$) for cancers removed from older patients. This relationship between cancer age and patient age at removal is consistent with a multistep pretumor progression model (see Figure 4).

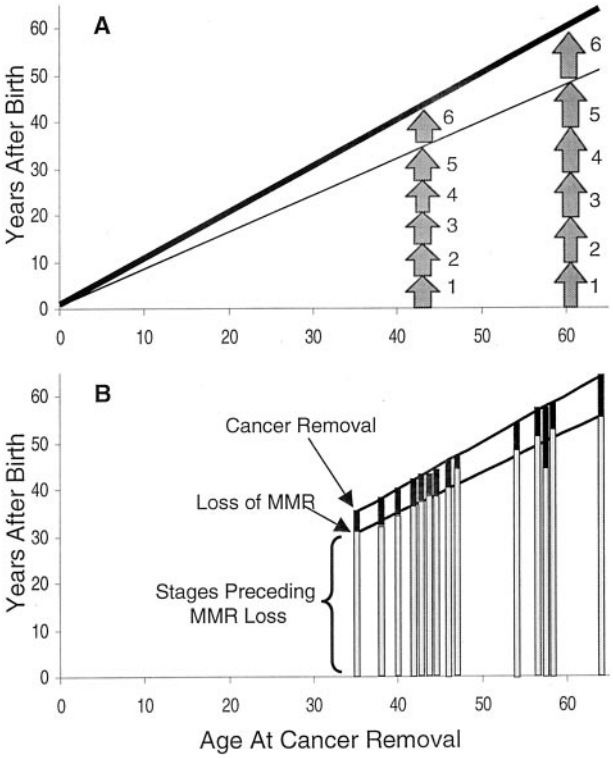


**Figure 4. A:** Multistep models postulate cancers have the same number of oncogenic mutations. A cancer that appears in an older individual takes on average longer to acquire each mutation. For cancers requiring six oncogenic mutations, on average each mutation takes 7 years or 10 years if their cancers, respectively, appear at 42 or 60 years of age. Mutations occur stochastically and rates may differ between loci, so some mutations may involve more or less time than the average mutation. **B:** Consistent with a multistep process, trend lines for ages at cancer removal and ages at MMR loss indicate all intervals are generally longer for cancers removed from older individuals. MMR loss occurs late during life allowing time for earlier oncogenic mutations.

MMR loss may occur anytime and in any order during HNPCC progression. However, instead of a random order for MMR loss, its ages of occurrence were consistent with MMR loss late in life as a fifth or sixth oncogenic mutation (Figure 1).

A final uncertainty is the separation between pretumor and tumor progression. Although MMR loss is compatible with normal phenotypes,[7–10] MMR loss may also occur in tumor cells. Interval tumors or cancers that appear shortly after negative clinical examinations provide insights into past phenotypes of tumor progenitors. Although false-negative examinations are possible, a negative examination indicates an age at which a tumor progenitor still retains a normal phenotype. In conjunction with an estimate for the start of final clonal expansion, a negative clinical examination marks the minimal age for the end of pretumor progression (Figure 1).

These approaches describe HNPCC progression as a stepwise series of mutations leading to a MMR-deficient cancer. Most oncogenic mutations including somatic MMR loss initially fail to confer visible changes in phenotype and accumulate in stem cells during pretumor progression. Somatic MMR loss is preceded by a number of other somatic oncogenic mutations. Visible tumor progression is restricted to the terminal few years (Figure 1).

Our model assumes mutation rates are normal until both MMR alleles are lost. We note that cells with heterozygous MMR mutations are repair proficient[18] and mice with heterozygous MMR mutations do not exhibit increased mutation frequencies.[19] However, subtle increases in mutation rates because of haploinsufficiency or dominant-negative interactions are also consistent with our analysis because inferred mutation rates during pretumor progression were slightly higher for HNPCC cancers compared to sporadic cancers ($8.8 \times 10^{-7}$ to $4.4 \times 10^{-6}$ for HNPCC cancers *versus* $3.2 \times 10^{-7}$ to $7.4 \times 10^{-7}$ per locus for sporadic cancers and the neutral model[1]). Of note, yeast with heterozygous MMR mutations exhibit a threefold increase in mutations compared to wild-type yeast.[20]

A number of assumptions underlie our pretumor progression model and its conclusions.[1] Therefore, rather than providing a final description, the current analysis demonstrates how modeling can produce a coherent and internally consistent description of progression throughout a lifetime. To test the model for consistency with HNPCC biology we consider two observations, congenital MMR deficiency and surveillance attempts to prevent HNPCC cancers.

## *Congenital MMR Deficiency*

A major uncertainty is whether MMR loss occurs early or late during progression. Progression to cancer could be rapid after MMR loss because mutation rates are elevated.[2] However, early MMR loss alone does not appear sufficient for rapid or efficient progression because colorectal cancers are infrequent in individuals with homozygous germline repair mutations,[7–10] and before 30 years of age in HNPCC families.[21] With congenital MMR deficiencies, cancers are rare even though every colon stem cell lacks MMR.

The lack of colorectal cancers with congenital MMR loss is consistent with our model, in which a number of oncogenic mutations usually precede MMR loss. Progression to cancer is efficient once MMR loss occurs, with an average interval estimated here at 6.1 years, but a number of other mutations must first precede MMR loss. Most individuals with congenital MMR deficiencies die within the first decade of life[7–10] and may fail to develop colorectal cancers because they seldom live long enough to acquire these other stages. Potentially these mutations may be difficult to acquire after MMR loss because our model suggests they are usually acquired in MMR-proficient cells (Figure 1). Although our pretumor model does not explain why a number of oncogenic mutations usually precede somatic MMR loss, it is consistent with the observed rarity of colorectal cancers with congenital MMR deficiencies.

## *HNPCC Cancers Commonly Appear During Surveillance*

Cancers should be preventable if most mutations accumulate during tumor progression. A cancer should be preceded by an adenoma, and periodic surveillance and polypectomy should prevent cancers. However, HNPCC cancers frequently appear relatively soon after screening surveillance examinations.[22–24] Potentially these interval cancers develop from missed adenomas. In addition, tumor progression may be extremely rapid because of the increased mutation rate after MMR loss.[2] However, this last possibility contradicts observations that colorectal cancers are rare with congenital MMR deficiencies.[7–10]

With our model, cancers that appear shortly after negative examinations represent neither missed adenomas nor extremely rapid tumor progression. Instead, our model predicts that it will be difficult to prevent all HNPCC cancers by surveillance because the majority of oncogenic mutations occur during pretumor progression (Figure 1). Interval and noninterval cancers contain essentially the same numbers of MS mutations,[5,6,11] indicating a sudden visible emergence of an HNPCC cancer is not unusual. Interval cancers do not arise *de novo* because pretumor progression starts from birth and an occult stem cell tumor progenitor may accumulate nearly all its required mutations at the times of negative examinations.

Although our model suggests it will be difficult to prevent all HNPCC cancers, MS tumor histories suggest surveillance may help prevent cancer death. After transformation, a single cell must physically generate sufficient progeny for invasion and metastasis. MS tumor histories indicate advanced cancers tend to represent older clonal expansions (Figure 3B), suggesting more time is needed for invasion and metastasis. Therefore, although a cancer may suddenly emerge after pretumor progression, early detection may prevent its spread. Consistent with our model, surveillance does not prevent all HNPCC cancers, but interval cancers tend to be of low clinical stage.[22–24]

## *Progression after MMR Loss*

Frameshift mutations in short mononucleotide repeats of coding loci such as TGFBR2 and BAX are common in MMR-deficient cancers.[25–27] Such frameshift mutations likely occur after MMR loss and their high frequencies suggest substantial progression after MMR loss. However, frameshift mutations may be frequent or biologically important, but not rate limiting[15,28,29] because they arise quickly after MMR loss. Some frameshifts in MMR-deficient cancers may be passenger mutations because shifts in noncoding mononucleotide repeats are also frequent[30] and some TGFBR2 mutations lack selective value in cell culture assays.[31]

Only certain mutations are likely critical for a tumor phenotype. MS mutations are neutral and in this analysis they merely serve as molecular odometers to measure time after MMR loss. If most oncogenic HNPCC tumor mutations accumulate after MMR loss during tumor progression, there could be a relationship between incremental changes in phenotype and time after MMR loss. However, there was no relationship between clinical cancer stage and the interval after MMR loss (Figure 3A).

Instead, and consistent with our multistep model, MS histories indicate cancers in older individuals generally require more divisions to achieve the same number of oncogenic mutations before and after MMR loss (Figures 3C and 4).

## *Summary*

A pretumor progression model[1] illustrates how experimental mutation and epidemiological data may be integrated into a coherent plausible lifetime tumor history (Figure 1). Many parameters of the model are uncertain,[1] and therefore its conclusions represent a framework for analysis rather than a definitive description. Instead of a quiescent prelude before tumor progression, HNPCC tumor histories suggest MMR loss and most oncogenic mutations accumulate in stem cells during pretumor progression. Consistent with a conclusion that pretumor progression to cancer does not require elevated mutation rates or tumorigenesis,[1] most oncogenic HNPCC cancer mutations appear to accumulate before MMR loss in normal appearing colon.

The identities of the mutations that precede MMR loss are unknown. However, pretumor progression predicts that mutations that confer stem cell survival advantages within niches will accumulate faster and more frequently. Interestingly, activating somatic $\beta$-catenin mutations are common in HNPCC tumors,[32] but disruption of the mutant $\beta$-catenin allele in a MMR-deficient colorectal cancer cell line enhanced or had minimal effects on tumorigenicity,[33] suggesting the mutation had a different role early in progression.[34] This $\beta$-catenin mutation (like others in HNPCC cancers[32]), likely occurred before MMR loss because it was a nonrepeat sequence deletion rather than a mononucleotide repeat frameshift. $\beta$-Catenin and other members of the WNT signaling pathway appear to influence stem cell survival,[1] and the activating $\beta$-catenin mutation may have conferred its selective advantage during pretumor progression. In the next study[35] we illustrate heterozygous APC mutations that enhance niche stem cell survival in normal appearing FAP crypts.

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