Tracing Cell Fates in Human Colorectal Tumors from Somatic Microsatellite Mutations

Evidence of Adenomas with Stem Cell Architecture

Jen-Lan Tsao,* Jingsong Zhang,*
Reijo Salovaara,^{†‡} Zhi-Hua Li,*
Heikki J. Järvinen,[§] Jukka-Pekka Mecklin,^{||}
Lauri A. Aaltonen,[‡] and Darryl Shibata*

From the Department of Pathology, Norris Cancer Center, University of Southern California School of Medicine, Los Angeles, California; the Departments of Pathology[†] and Medical Genetics,[‡] Haartman Institute, University of Helsinki, Helsinki, the Second Department of Surgery,[§] Helsinki University Central Hospital, Helsinki, and the Jyväskylä Central Hospital,^{||} Jyväskylä, Finland*

Occult aspects of tumor proliferation are likely recorded genetically as their microsatellite (MS) loci become polymorphic. However, MS mutations generated by division may also be eliminated with death as noncoding MS loci lack selective value. Therefore, highly polymorphic MS loci cannot exist unless mutation rates are high, or unless mutation losses are inherently minimized. Mutations accumulate differently when cell fates are determined intrinsically before or extrinsically after division. Stem cell (asymmetrical division as in intestinal crypts) and random (asymmetrical and symmetrical division) proliferation, respectively, represent simulated cell fates determined before or after division. Whereas mutations regardless of selection systematically persist once inherited with stem cell proliferation, mutations are eliminated by the symmetrical losses of both daughter cells with random proliferation. Therefore, greater genetic diversity or MS variance accumulate with stem cell compared with random proliferation. MS loci in normal murine intestinal mucosa and xenografts of cancer cell lines accumulated mutations, respectively, consistent with stem cell and random proliferation. Tumors from patients with hereditary nonpolyposis colorectal cancer (HNPCC) demonstrated polymorphic MS loci. Overall, three of five adenomas and one of six cancers exhibited high MS variances. Assuming mutation rates are not significantly greater in adenomas than in cancers, these studies suggest the stem cell proliferation and hierarchy of normal intestines persists in many HNPCC adenomas and some cancers. An adenoma stem cell

architecture can explain the complex polymorphic MS loci observed in HNPCC adenomas and account for many adenoma features. In contrast, cancers may lose intrinsic control of cell fate. These studies illustrate a feasible phylogenetic approach to unravel and describe occult aspects of human tumor proliferation. The switch from predominantly stem cell to random proliferation may be a critical and defining characteristic of malignancy. (*Am J Pathol* 1998, 153:1189–1200)

Abnormal proliferation due to alterations of oncogenes and tumor suppressor genes is a hallmark of cancer. Cell cycle regulatory defects appear to be present in most cancers.¹ However, despite the increasingly detailed understanding of the molecular genetic pathways altered within individual tumor cells, little is known on how individual cells construct tumors.

Proliferation represents a balance between cell division and death. Normal intestinal proliferation is characterized by cell division equal to cell loss. Neoplastic proliferation is grossly evident by a net increase in cells. However, the multitude of possible division and death combinations consistent with observed changes in cell number hinder description or classification. For example, the multistep accumulation of mutations in colorectal tumorigenesis is apparently matched by the histological progression from adenomas to cancers.² Presumably, adenomas have fewer mutations and therefore fewer proliferation abnormalities compared with cancers. Adenomas may persist unchanged in size for years, and only a minority progress to cancer.^{3–5} Proliferation presumably becomes more “abnormal” in cancers.

A comprehensive and possible alternative description of occult human tumor proliferation are phylogenetic trees that describe historical relationships between individuals and populations. The clonal evolution model of cancer implies that every tumor cell traces its origin to a common precursor.⁶ In essence, a tumor represents the physical manifestation of a large phylogenetic tree re-

Supported by Public Health Service Grants CA58704 and CA70858 from the National Cancer Institute.

Accepted for publication July 18, 1998.

Address reprint requests to Dr. Darryl Shibata, 1200 N. State Street, Box 736, Los Angeles, CA 90033. E-mail: dshibata@hsc.usc.edu.

Figure 1. A: Schematic representation of a phylogenetic tree underlying tumor construction. Branching depends on relative numbers of divisions and types of deaths. Current cells originate from common precursors and adjacent cells likely have more recent common ancestors compared with cells from opposite tumor sides. B: The experimental approach examines multiple small, 200- to 400-cell tissue dots for polymorphic MS loci by DNA extraction followed by dilution and PCR. Stochastic processes superimposed on stereotypic behavior will yield random outcomes. Therefore, even if all cells proliferate equally, each dot may accumulate different MS mutations due to the independent and stochastic nature of mutation. The MS frequency distributions determined by PCR are summarized by calculating their variances.

flecting numerous divisions, deaths, and mutations. Adjacent cells within a tumor likely share more immediate common ancestors compared with cells from opposite sides (Figure 1). Branch lengths compare genetic differences between individuals. Greater genetic differences imply greater numbers of intervening divisions. Mutation rates in most tumors are too low to allow the accumulation of many mutations.⁷ However, tumors lacking DNA mismatch repair (MMR) have elevated mutation rates,⁸⁻¹⁰ most notably in microsatellites (MSs), which allow mutations to accumulate after fewer divisions. Therefore, polymorphic MS loci are informative on the number of divisions since the last common ancestor.¹¹

Tumor proliferation may be random or chaotic. Individual tumors likely progress along unique phylogenetic pathways and precise trees accounting for every division, and death may be nearly impossible to recreate. However, long-term observations of random events may reveal patterns characteristic of specific proliferation types. Here we sample MS mutations in colorectal adenomas and carcinomas and recreate these measurements with computer simulations and experimental models. These studies illustrate complex mutation patterns and suggest the stem cell architecture of normal intestinal mucosa persists in colorectal adenomas.

Materials and Methods

Specimens

Adenomas and cancers removed from hereditary nonpolyposis colorectal cancer (HNPCC) patients were fixed in formalin and paraffin embedded. Clinical information was obtained from medical records. Screening intervals are the periods between colonoscopy or surgery.

MMR-deficient colorectal cell lines HCT116 and LoVo (American Type Tissue Collection, Rockville, MD) were diluted to single cells and grown in culture for 2 to 4 weeks. Approximately 5×10^6 cells were injected subcutaneously into both flanks of nude mice (BALB/c nu/nu). At various times, the mice were sacrificed and the xenografts were fixed in formalin and paraffin embedded. Data for each time point were obtained from both xenografts present in the same mouse. The initiation of single-cell clones in tissue culture defined day 0.

MS Analysis

The same approach was used for all tissues. Multiple small tissue regions or dots of approximately 200 to 400 cells were isolated by selective ultraviolet radiation fractionation (SURF)¹¹ from microscopic sections (Figure 1B). For human tumors, SURF dots were estimated to contain at least 70% tumor cells. To be included for analysis, at least 35 alleles were amplified from a dot. The DNA in these dots were diluted to essentially single alleles with approximately 20 to 80% of reactions yielding polymerase chain reaction (PCR) products, which were analyzed on 6% denaturing polyacrylamide sequencing gels and a phosphorimager (Molecular Dynamics, Sunnyvale, CA). PCR products were labeled with [³³P]dCTP (NEN Research Products, Boston, MA) incorporated during 38 to 43 PCR cycles. Each tissue was examined with at least two different MS loci and with at least two independent microdissections. The CA-dinucleotide repeat MS loci were DXS556, DXS1060, DXS418, DXS453, MIT129, and MIT38 (Research Genetics, Huntsville, AL).

Tumor-specific MS alleles were distinguished from germline alleles originating from contaminating normal cells by two methods. A MS distribution separate from the germline allele was assumed to arise by somatic mutation in the tumor cells. In this case, the germline alleles were eliminated by truncation. When MS distributions included

germline-sized alleles, some germline alleles must originate from contaminating normal cells. In these cases, up to 30% of all alleles were considered to arise from normal cells, and germline allele frequencies were reduced as far as possible by this number. The remaining alleles were considered tumor-specific MS distributions. Bimodal tumor distributions were observed in a few dots of tumors 3 and 11. The two distributions were considered as two different tumor populations, and variances were calculated for each peak. No corrections were necessary for the xenografts as the human MS primers did not amplify murine DNA.

Computer Simulations and Calculations

Simulations were performed as previously described.¹² Statistical analysis was performed with Excel 7.0 (Microsoft, Bellevue, WA). Composite variances were calculated by combining the MS distributions of the individual HNPCC tumor dots or by sampling DNA isolated from entire xenograft sections.

Results

Definitions, Logic, and Computer Simulations

The stochastic nature of mutation can mask underlying stereotypic proliferation patterns. For example, every branch of Figure 1 may independently accumulate different numbers of MS mutations even if division and death histories were identical. Therefore, the general strategy first simulates two simple but fundamentally distinct proliferation patterns. The simulations are then compared with the MS polymorphisms in experimental models and human tumors. Comparisons between tissues are difficult as tumors vary in size and may be heterogeneous with respect to morphology, age, or cell of origin. To facilitate comparisons between physically disparate tissues, the current approach focuses on similar sized groups of 200 to 400 cells or dots isolated by microdissection and examined genetically by PCR (Figure 1B). Another simplification is the use of X-chromosome MS loci and tumors from male patients. Every MS allele should represent a single cell as MMR-deficient tumors are typically near diploid.^{13,14} The focus on small tissue dots requires experimental examination of multiple dots because stochastic variation is increased. However, as noted below, some behaviors become evident when small populations are examined.

Somatic mutations that increase the fitness of their cells ensure prevalence due to selection. Noncoding MS mutations must be considered differently as they lack selective value and may be eliminated by chance through death. However, mutations can attain tenure regardless of selection if they occur in cells with systematic renewal. Therefore, MS mutations accumulate differently depending on their cell fates.

This analysis concentrates on two fundamentally different proliferation patterns: stem cell or random proliferation (Figure 2). These two models were selected for their

Figure 2. Two distinct proliferation patterns. Mutations in noncoding MS loci lack selective value and therefore are "passively" dependent on lineage survival. Cell fate may be determined before or after division. With stem cell proliferation, fate is determined intrinsically before asymmetrical division to reproduce one stem cell and one mortal cell. Every lineage will persist, and inherited mutations accumulate regardless of selection. With random proliferation, fate is determined extrinsically after division. Therefore, both asymmetrical and symmetrical divisions are possible. Nonselective mutations never attain tenure as they are at risk for elimination after every division due to symmetrical losses in both daughter cells. Consequently, fewer MS mutations accumulate with random compared with stem cell proliferation.

functional simplicity and represent fates determined intrinsically before or extrinsically after division. Stem cell proliferation resembles the programmed hierarchy of normal intestinal mucosa,^{12,15} and random proliferation models an unbiased or random elimination of potentially immortal cells in cancers. Stem cell proliferation is asymmetrical division intrinsically programmed to reproduce one stem cell daughter and one mortal daughter. The mortal daughter may continue to divide (three to six times

Figure 3. Computer simulations of random and stem cell proliferation. **A:** Simulated output of 200 trials with a mutation rate of 0.0025 per division and stem cell (20 immortal stem cells) or random proliferation (200 immortal cells). The observed variations in MS variance result from the stochastic nature of mutation (and death for random proliferation), and outcomes are summarized by median (50%) and confidence (95%, 75%) intervals. Larger populations decrease confidence intervals.¹² Median variances increase linearly with division and stem cell proliferation but are relatively limited with small populations and random proliferation. **B:** Summary of random proliferation simulations (100 to 200 trials for each point). Median MS variances are limited in small populations and low mutation rates and fail to exceed 1.0 when mutation rates are less than 0.005 per division and "immortal" cell numbers are less than 200.

in a normal intestinal crypt¹⁵), but her progeny all eventually die. Random proliferation is when asymmetrical and symmetrical (both daughters survive or die) division are equally likely. None of these cells are formally "mortal" because fate is unknown at the time of division and death is determined by extrinsic factors. Whereas every lineage persists with stem cell proliferation, symmetrical loss of both daughter cells can occur with random proliferation. Consequently, MS mutations are sporadically lost with random proliferation but become "fixed" within a stem cell lineage once inherited by a stem cell daughter. With identical mutation and division rates, greater numbers of MS mutations accumulate with stem cell proliferation.

These models were simulated assuming constant mutation rates, stepwise mutation, constant cell number, and equal numbers of divisions for all cells. Variables are mutation rates, numbers of divisions, and numbers of immortal cells. Constant mutation rates are generally assumed for phylogenetic analysis. A complex of proteins are required for DNA MMR, and homozygous loss of MLH1 or MSH2 appears to substantially diminish MMR.^{2,6,16} Cells with heterozygous MLH1 or MSH2 deficiencies are repair proficient, suggesting that loss of the normal allele is primarily responsible for the high mutation frequencies observed in MMR-deficient tumors. Stepwise (single unit additions or deletions) mutation is assumed

as most mutations observed in MMR-deficient cell lines are single repeat unit frameshifts.^{9,10}

Cell numbers remain constant in normal mucosa, but growth must occur with neoplasia. However, kinetic studies suggest that most tumor cells die (>90%) as mitotic rates generally exceed growth rates.^{17,18} Therefore, as an approximation, cell division and death are considered equal between small microdissected tumor dots. Finally, tumors are characterized by defects in cell cycle regulation.¹ Assuming a monoclonal origin, it is likely that adjacent cells will initially have identical MS alleles and divide approximately equally. MS frameshift mutations appear to arise secondary to slippage during replication.^{19,20} Therefore, division and MS mutation should be tightly linked and MS loci become polymorphic with division.

The accumulation of mutations were simulated for different numbers of divisions, cells, or mutation rates (Figure 3 and Figure 3 in Ref. 12). MS diversity is summarized mathematically by the variance of each MS frequency distribution. More polymorphic MS loci have larger variances. Each single trial may yield a different variance, illustrating that small populations with identical proliferation histories stochastically accumulate different numbers of mutations. Therefore, median variance is used to summarize the spread of the variances from multiple trials. As previously noted for stem cell proliferation,¹² median MS variance increases linearly with division, independent of

Figure 4. A: MS variance increases with age in small populations of normal intestines of mice deficient in MMR (Pms2^{-/-}). The graph represents previously published data¹² with additional data points. The bold marks represent median variances (× for MIT129 and + for MIT38). The scatter of the variances calculated from the individual intestinal dots are expected (see stem cell proliferation in Figure 3A) if small numbers of stem cells are responsible for mucosal renewal.¹² B: MS variances (squares for DXS556 and circles for DXS1060) of small xenograft populations (LoVo and HCT116) of different ages. After initial increases, median variances (filled symbols) do not appear to increase with time, consistent with random proliferation. Day 0 is the initiation of the single-cell clones in tissue culture. One DXS556 point with a variance of 4.7 in the 275-day HCT116 xenograft is omitted in the graph for clarity.

the number of simulated cells, and with the variation expected of stochastic mutation. In contrast, MS variance with random proliferation is limited except with high mutation rates or larger populations. With smaller populations or low mutation rates, random losses of mutations limit increases in variance. Theoretically, high MS diversity in small tissue dots is more consistent with stem cell rather than random proliferation.

Proliferation in Normal Mucosa and Xenografts

The simulations were compared with two experimental models. The first examines the accumulation of somatic MS mutations with age in normal intestinal mucosa of mice with germline deficiencies (Pms2) in MMR. These studies (with additional data points) have been previously published¹² and are summarized in Figure 4A. The increases in median variance with age are consistent with simulated stem cell proliferation. The variation of the multiple intestinal dot variances mimics the simulated variation with small numbers of stem cells (<20).

Xenografts are considered experimental models of cancer. Cell line daughters are both potentially immortal. However, xenografts do not grow exponentially but rather exhibit Gompertzian kinetics¹⁷ characterized by relatively stable tumor sizes after an initial growth spurt. Therefore, cell division is likely balanced by death in macroscopic xenografts. Serial xenografts composed of MMR-deficient colorectal cancer cell lines HCT116 (hMLH1 deficient) and Lovo (hMSH2 deficient) were examined for MS mutations (Figure 4B). There was variation in the MS

variances measured from multiple small xenograft dots, consistent with the simulated process of stochastic mutation and cell death. Median variance initially increased but did not further increase with xenograft age (up to 275 days), consistent with simulations of random proliferation with small numbers of cells and mutation rates less than 0.005 (Figure 3B).

The numbers of cells destined to further divide ("immortal") within xenograft dots are less than the total numbers of microdissected cells, and depend on the length of mortal cell survival after division. Assuming equal periods between cell division and death, the number of immortal cells is half of the microdissected cells. Therefore, with one division per day, random proliferation, and 100 to 200 immortal cells per tumor dot, xenograft MS mutation rates are estimated at less than 0.005 per division. This mutation rate is similar to the rate (0.002 to 0.0025) estimated in MMR-deficient murine intestines.¹²

Mitotic Cell Populations in Xenografts

Scenarios other than random proliferation may also explain the xenograft data. For example, division may decrease in older xenografts leading to fewer than expected mutations. This possibility seems unlikely as the cell-cycle-associated antigen Ki-67²¹ was detected in the majority (greater than 75%) of cells regardless of xenograft age (data not shown). In addition, simulations of random proliferation predict variance will progressively increase if larger populations are sampled. When MS alleles were sampled from entire xenograft sections, "composite" variance increased with xenograft age (Figure 5A). Therefore, decreased division does not appear to account for the limited median MS variances observed in older xenograft dots.

A succession of clones with increasingly greater selective advantages could also limit genetic diversity as each expansion would homogenize the xenograft. However, the progressive increase of composite MS xenograft variance with age suggests that clonal succession either did not occur or was limited in extent.

Stem Cell versus Random Proliferation

Small 200- to 400-cell dots accumulate different numbers of mutations with random *versus* stem cell proliferation. Although MS variances will be initially low after clonal expansion with both types of proliferation, differences arise with division. Based on the experimental and simulated data, median variances over 1.0 appear to distinguish between random and stem cell proliferation when mutation rates are less than 0.005. Specifically, if rates are 0.0025 mutations per division or less, median MS variance will exceed 1.0 after 400 or more stem cell divisions whereas median variance will not exceed 1.0 even after 1000 random divisions. Therefore, high median MS variances (>1.0) in small tumor dots imply either stem cell proliferation and 400 or more divisions or random proliferation with mutation rates greater than 0.0025.

Figure 5. Comparisons of median variances (filled bars) from small tumor dots (small populations) and composite variances (immediately adjacent to right) sampled from entire tumor sections (large populations). **A:** Median variances are relatively constant in the xenografts whereas composite variances increase with age. These differences with age are predicted by simulations of random death and different population sizes (Figure 3B). **B:** Median variances are generally less than or equal to composite variances in the HNPCC adenomas and cancers (see Discussion).

Tumors from Patients with HNPCC

With this theoretical and experimental background, the MS mutations in small dots from human mutator phenotype tumors were sampled. Although serial analysis of primary isogenic human tumors is impractical, synchronous and metachronous (asynchronous) tumors in HNPCC patients provide opportunities to minimize confounding factors and observe different tumors arising within the same genetic and similar environmental backgrounds. Primary differences between these tumors should be relative ages (numbers of divisions since the last clonal expansion) and proliferation patterns.

Metachronous tumors (a cancer and three adenomas) from a HNPCC patient demonstrated different amounts of MS diversity, as expected from independent tumors of different ages (Table 1 and Figure 6). The adenomas were removed during biennial surveillance colonoscopy.

Some of the highly polymorphic and complex topographical MS distributions are illustrated in Figure 7. The different patterns between loci within the same tumor likely reflect independent stochastic mutation- and locus-specific differences in mutation rates. Median variances exceeded 1.0 for the smallest (adenoma 1, <0.5 cm) and largest (adenoma 3, 1.0 cm) adenomas. Median variances were less than 1.0 for adenoma 2 (0.5 cm) and the large carcinoma (number 4).

A second HNPCC patient with multiple tumors was also examined. Median variance exceeded 1.0 for a synchronous adenoma (number 5) adjacent to a cancer (number 6), whereas two metachronous cancers (numbers 6 and 7) exhibited median variances less than 1.0. The synchronous adenoma (number 8) and cancer (number 9) from another patient (C) with the same germline hMLH1 mutation also exhibited median variances less

Table 1. Summary of HNPCC Data

Tumor	Patient	Screening interval	Median variance				Proliferation
			DXS556	1060	418	453	
Adenoma 1, <0.5 CM	A (MSH2)	24 M	1.48	1.65	2.24	0.91	S
Adenoma 2, 0.5 CM		28 M	0.47	0.63	0.66	ND	S or R
Adenoma 3, 1.0 CM		23 M	2.58	1.31	0.96	ND	S
Cancer 4, PD, Dukes' B		ND	0.62	0.55	0.60	ND	S or R
Adenoma 5,* 1.0 CM	B (MLH1-A)	ND	ND	1.26	2.71	ND	S
Cancer 6,* PD, Dukes' C		ND	ND	0.34	0.74	ND	S or R
Cancer 7, MD, Dukes' B		6 M	0.51	0.33	0.82	ND	S or R
Adenoma 8,* 0.8 CM	C (MLH1-A)	46 M	0.46	0.13	0.40	0.33	S or R
Cancer 9,* PD, Dukes' A		46 M	0.42	0.43	0.16	ND	S or R
Cancer 10, MD, Dukes' B	D (MLH1-B)	37 M	0.35	0.51	0.58	ND	S or R
Cancer 11, MD, Dukes' B	E (MLH1-B)	60 M	2.00	1.14	1.72	ND	S

MS variances of 11 mutator phenotype tumors from five HNPCC patients with germline mutations in hMSH2 (codon 619–620 insertion⁴¹ for patient A) and hMLH1 (exon 6 splice site⁴² for patients B and C (MLH1-A), exon 16 deletion for patients D and E (MLH1-B)).

Screening intervals were periods between biopsy and the last previous examination. S, stem cell proliferation; R, random proliferation; ND, no data.

*Synchronous tumors.

Figure 6. MS variances (squares for DXS556, circles for DXS1060, triangles for DXS418, and inverted triangles for DXS453) of the HNPCC adenomas and carcinomas arising from three different germline mutations. Median variances (filled symbols) exceed 1.0 for adenomas 1, 3, and 5 and cancer 11, suggesting stem cell proliferation. The lower median variances in the other tumors are consistent with random proliferation or young tumors with stem cell proliferation.

than 1.0. Another germline hMLH1 mutation was present in two other patients (D and E). Median variances were less than 1.0 for cancer 10 but exceeded 1.0 for cancer 11.

Overall, three of five adenomas and one of six cancers exhibited median variances over 1.0. These observations suggest that many adenomas but only rare cancers accumulate evidence of stem cell proliferation. Adenomas with stem cell proliferation and low median variances are expected as they may be removed soon after clonal expansion. As noted above, median variances less than 1.0 are uninformative because they are compatible with either random proliferation or recent expansions with stem cell proliferation.

Cancer 11 was the exception to the low variances in cancers. This moderately differentiated, sessile tumor with crypt-like structures partially invaded the underlying muscularis and was classified as an invasive (Dukes' B) adenocarcinoma (Figure 8). The apparent stem cell behavior of this cancer was not evident by histological examination as three of the cancers were moderately differentiated and three were poorly differentiated. This exception highlights potential conflicts between morphological phenotype and the dynamic phenotype²² inferred with molecular clocks. The resolution of such conflicts are limited as morphology is dogmatic, and the current MS model is consistent with but not definitive evidence of stem cell proliferation. However, this patient is still alive 8 years after resection. Persistent stem cell proliferation could account for the relatively infrequent metastases and better prognosis of some HNPCC patients with colorectal carcinomas.^{23,24}

The Number of Stem Cells Appears Small

The variance ranges of the small dots provide information on the numbers of stem cells.¹² Tumors with apparent stem cell behavior (1, 3, 5, and 11) exhibited large variance ranges (Figure 6) consistent with the stochastic variation expected with small proportions (estimated at less than 20 cells) of adenoma stem cells (Figure 3A). Differences in ages between adenoma regions can also

account for the variation. This possibility seems unlikely as consistently high or low variances at different MS loci were not observed from specific tumor regions (Figure 7 and data not shown). Note that adenoma stem cells do not have to be directly isolated and examined because their differentiated cells will represent stem cell genotypes if limited numbers of divisions intervene.¹²

Adenomas with Multiple Precursors?

A further analysis (Figure 5B) compares median variances from individual dots (small populations) and "composite" variances of the MS alleles sampled from entire tumors (large populations). For random proliferation, composite variances should equal (for recent expansions) or exceed median variances. This expectation was generally met for the HNPCC cancers, although some loci exhibited higher composite variances compared with other loci. The etiologies for these deviations are unknown and may reflect technical problems measuring MS alleles or limitations of the simulations.

Composite and median variances should be equivalent for stem cell proliferation. The adenomas met this general expectation, although in some cases composite variances were slightly greater than median variances. Greater composite variances could arise if some of the adenoma divisions reflected random rather than stem cell proliferation. However, composite variances greatly exceeded median variances for adenomas 3 and 8 and therefore are inconsistent with stem cell proliferation. One possibility for the high composite variances is that these two adenomas were older than the other tumors. However, their screening intervals of 24 and 46 months were similar to the other tumors (6 to 60 months) and suggest they were not excessively old.

These two adenomas not only are inconsistent with stem cell proliferation but also represent a paradox for the general hypothesis that mutation rates increase with tumor progression.^{6,7} Regardless of proliferation type, the high composite MS variances of these adenomas imply mutation or division rates much greater than other adenomas or in the cancers.

The topographical distributions of MS mutations in these two adenomas suggest an etiology for their apparent high mutation rates (Figure 7). Instead of the relatively uniform distributions of the other tumors (Figure 7B and data not shown), these two adenomas (Figure 7, C and D) had heterogeneous regional subpopulations. The MS distributions were often different between dots. Bimodal distributions consistent with completely distinct subpopulations were also evident in some dots. These complex polymorphisms are not easily explained by a simple phylogenetic tree (Figure 1) as neighboring cells were often genetically different. A possibility consistent with stem cell proliferation and modest MS mutation rates is that these two adenomas originate from more than one immediate precursor. Tumor dots from each precursor would become polymorphic after clonal expansion. However, as each precursor may initiate expansion with a different genotype, the overall genetic diversity will be greater

Figure 7.

Figure 7. A: Autoradiographs of the different sized DXS1060 alleles present within small tumor dots. The arrows mark the allele sizes and the solid circles represent the germline alleles. The left is a sample with low variance (final value was 0.34) from cancer 4, and the right is a sample with high variance (final value was 4.08) from adenoma 3. B: Locations of tissue dots and their MS frequency distributions and variances for adenoma 2. The composite graph summarizes the data from all dots. The x axis indicates repeat unit additions or deletions compared with the germline allele (marked with an arrowhead). The scale is the same for all tumors. C: Locations of tissue dots and their MS frequency distributions and variances for adenoma 3. D: Locations of tissue dots and their MS frequency distributions and variances for adenoma 8.

than expected for a single precursor, leading to very high composite tumor variances.

Discussion

Oncogenic mutations accumulate during progression due to selection. In the absence of selection, the essential premise is that highly polymorphic MS loci cannot exist in small populations unless mutation rates are high, or unless mutation losses are inherently minimized. Although the complex HNPCC MS mutations are difficult to fully interpret, they are consistent with two relatively simple proliferation patterns. Assuming mutation rates are not significantly greater in adenomas than in cancers, the current studies suggest a model that may account for some of the occult structural features of HNPCC tumors (Figure 9). Adenomas retain the basic stem cell architecture of normal mucosa. Only a small proportion of adenoma stem cells are responsible for maintaining the larger number of mortal "differentiated" cells. In contrast, most cancers lack this stem cell hierarchy. Instead of an inherent determination of cell fate, external factors determine life or death after every division.

These two general but distinct architectural designs simplify robust HNPCC tumor construction by individual cells. The polymorphic MS loci observed within tumor dots imply relatively stable populations as clonal expansions initially homogenize genotypes. Cell fates can be predetermined by asymmetrical segregation of intrinsic factors.²⁵ A stem cell proliferation program likely pre-exists in the crypt stem cell precursors of intestinal tu-

Figure 8. A: The moderately differentiated sessile carcinoma (number 11) with MS distributions consistent with stem cell proliferation. The tumor partially invades but does not penetrate through the muscularis. H&E; magnification, X20. B: Higher-power view (X200) of its crypt-like structures. Note that the MS analysis cannot identify the locations or the morphology of tumor stem cells.

Figure 9. Architectural models of HNPCC adenomas with stem cell proliferation and cancers with random proliferation. Cells with different fates are required of only the first asymmetric adenoma division as subsequent adenoma cells are terminally differentiated and destined to die. This intrinsic stem cell adenoma hierarchy is consistent with many adenoma features (see Discussion). Cell fates are indeterminate with random proliferation, and decisions occur after every division. Cells are equivalent, and nonselective mutations are lost at random as no mechanism exists to ensure their tenure.

mors. Modifications or temporary perturbations of this programmed hierarchy in adenomas could allow neoplasia without eliminating asymmetrical division as only the first adenoma stem cell division produces cells with different fates. Alterations of subsequent symmetrical divisions or survival could increase numbers of mortal adenoma cells.

Similarly, random proliferation is a likely consequence of the loss of programmed asymmetrical division. When every daughter cell is potentially immortal, external rather than internal factors mediate mortality. For example, cell lines exhibit exponential growth under optimal tissue culture conditions. In contrast, the same cell lines show limited growth and random proliferation as macroscopic xenografts. Although control of cell number depends on specific selective criteria, the process may effectively mimic random selection if there are few differences between clonally related tumor cells. Intermediate or varying degrees of symmetrical and asymmetrical division are possible and likely occur but would require more complex intrinsic programs or external modulation. Random or stem cell proliferation can operate indefinitely between periods of selection (clonal expansion) and therefore may be the proliferation most often recorded as MS loci become polymorphic.

The polymorphic MS loci observed in the HNPCC tumors are consistent with but cannot completely verify this model as other types of proliferation may also generate similar mutations. However, as these architectural foundations broadly organize cell behavior, they should also explain other properties of adenomas and cancers. Most adenomas do not progress to cancer,³ they express low levels of telomerase,²⁶ and serial radiological observations demonstrate relative size stability.^{4,5} The failure of most adenomas to progress to cancer is puzzling as the risk is increased by the number of adenoma cells. A stem cell adenoma architecture reduces the number of cells at risk for further progression because mutations acquired in nonstem cells will be lost. Expression of oncogenic transgenes in differentiated but not stem cells can induce hyperplasia or dysplasia but not adenomas or cancers in murine intestines.²⁷ A stem cell adenoma architecture may also explain adenoma persistence despite very low

to absent telomerase expression²⁶ because, similar to normal mucosa, only a small number of stem cells need to maintain their telomeres.

Many adenomas grow slowly or even disappear.^{4,5} The ability of normal mucosa to resist adenoma expansion seems limited as most intestinal cells are terminally differentiated and destined to die. However, an equilibrium between adenoma and normal mucosa could be stabilized indefinitely if their junctions were maintained by terminally differentiated adenoma and normal cells.²² Whatever advantage conferred by an adenoma genotype, further expansion would be inherently limited. Adenoma stem cell locations cannot be determined with the current analysis, and the disorganized division patterns observed in adenomas^{28,29} may reflect abnormalities of topography but not in an underlying stem cell hierarchy.

The inherent expansion limitations of a stem cell adenoma architecture can also account for the regional genetic heterogeneity often observed in adenomas but not in cancers.^{30,31} Periodic genetic heterogeneity followed by homogeneity is expected if adenomas progress through a succession of clones with greater selective advantages. However, the ability of an adenoma clone to attain dominance over another adenoma clone seems paradoxical when normal mucosa (presumably lacking selective mutations) is resistant. Alternatively, genetic heterogeneity is a natural option of a stem cell architecture as minor advantages conferred by related or unrelated³² adenoma genotypes would not readily allow clonal dominance over each other or normal cells. The polymorphic and genetically distinct tumor subpopulations but lack of apparent age heterogeneity in adenomas 3 and 8 provide evidence for coexistence rather than succession of adenoma clones.

A stem cell adenoma architecture implies that adenoma size depends primarily on the number of terminally differentiated cells. As in normal mucosa, this hierarchy allows for differentiation into a variety of cell types. Multipotent differentiation is also observed in murine adenomas,^{33,34} and human adenomas typically demonstrate phenotypic heterogeneity.³⁵ Somatic mutations in genes not expressed in stem cells may alter downstream differentiation.²⁷ Growth could result from an increase in the number of adenoma stem cells³⁶ or the extended survival or production of differentiated adenoma cells. For example, increasing by one the number of post-stem cell divisions could double the number of adenoma cells. Conversely, regression, as seen with chemopreventive trials, may reduce the number of differentiated adenoma cells but have little influence on cancer prevention.^{37,38}

The analysis of cancers is less informative as their less polymorphic MS loci do not usually distinguish between proliferation types. Most cancers appear to rely on extrinsic control of cell fate. Although the survival of one, none, or both cancer daughter cells may be specifically and individually determined by complex environmental and genotype interactions, the net effect appears consistent with a random selection process after most divisions. Note that random proliferation may coexist with a hierarchy of differentiation if cells selected to die further differentiate or divide into terminally differentiated cells. In this

case, cell fates are not all indeterminate as, similar to stem cell proliferation, differentiation may be intrinsically programmed. However, the overall pattern is consistent with random proliferation as the initial selection of cell fate (one, none, or both daughters survive) is still by extrinsic factors.

This study represents an initial attempt to reconstruct the proliferation responsible for the complex polymorphic MS mutations in HNPCC tumors. These studies illustrate a feasible experimental approach and genetic support for the well known but occult stem cell tumor concept.^{17,39,40} Further refinements are limited by the laborious analysis of greater numbers of MS loci and the complexity of comprehensive simulations. The dynamic information recorded most frequently by MS mutations can be translated with a simple model of asymmetrical proliferation by small numbers of stem cells in normal mucosa and many adenomas, and random proliferation in most cancers, although alternative and more complex models cannot be eliminated. The switch from predominantly stem cell to random proliferation may be a critical and defining characteristic of malignancy.

Acknowledgments

We thank Atsushi Arakawa, Nathan Yum, Karim Adzhyan, and Corey B. Blake for their technical assistance.

References

1. Strauss M, Lukas J, Bartek J: Unrestricted cell cycling and cancer. *Nature Med* 1995, 1:1245-1246
2. Kinzler KW, Vogelstein B: Lessons from hereditary colorectal cancer. *Cell* 1996, 87:159-170
3. Koretz RL: Malignant polyps: are they sheep in wolves' clothing? *Ann Internal Med* 1993, 118:63-68
4. Stryker SJ, Wolff BG, Culp CE, Libbe SD, Ilstrup DM, MacCarty RL: Natural history of untreated colonic polyps. *Gastroenterology* 1987, 93:1009-1013
5. Otchy DP, Ransohoff DF, Wolff BG, Waver A, Ilstrup D, Carlson H, Rademacher D: Metachronous colon cancer in persons who have had a large adenomatous polyp. *Am J Gastroenterol* 1996, 91:448-454
6. Nowell PC: The clonal evolution of tumor cell populations. *Science* 1976, 194:23-28
7. Loeb LA: Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res* 1991, 51:3075-3079
8. Parsons R, Li GM, Longley M, Fang WH, Papadopoulos N, Jen J, de la Chapelle A, Kinzler KW, Vogelstein B, Modrich P: Hypermutability and mismatch repair deficiency in RER⁺ tumor cells. *Cell* 1993, 75:1227-1236
9. Shibata D, Peinado MA, Ionov Y, Malkhosyan S, Perucho M: Genomic instability in repeated sequences is an early somatic event in colorectal tumorigenesis that persists after transformation. *Nature Genet* 1994, 6:273-281
10. Bhattacharyya NP, Skandalis A, Ganesh A, Groden J, Meuth M: Mutator phenotypes human colorectal carcinoma cell lines. *Proc Natl Acad Sci USA* 1994, 91:6319-6323
11. Shibata D, Navidi W, Salovaara R, Li ZH, Aaltonen LA: Somatic microsatellite mutations as molecular tumor clocks. *Nature Med* 1996, 2:676-681
12. Tsao JL, Davis SD, Baker SM, Liskay RM, Shibata D: Intestinal stem cell divisions and genetic diversity: a computer and experimental analysis. *Am J Pathol* 1997, 51:573-579
13. Thibodeau SN, Bren G, Schaid D: Microsatellite instability in cancer of the proximal colon. *Science* 1993, 260:816-819
14. Lengauer C, Kinzler KW, Vogelstein B: Genetic instability in colorectal cancers. *Nature* 1997, 386:623-627
15. Potten CS, Loeffler M: Stem cells: attributes, cycles, spirals, pitfalls, and uncertainties. Lessons for and from the crypt. *Development* 1990, 110:1001-1020
16. Marsischky GT, Filosi N, Kane MF, Kolodner R: Redundancy of *Saccharomyces cerevisiae* MSH3 and MSH6 in MSH2-dependent mismatch repair. *Genes Dev* 1996, 10:407-420
17. Steel GG: *Growth Kinetics of Tumors*. Oxford, Clarendon, 1977
18. Steel GG: Cell loss as a factor in the growth rate of human tumors. *Eur J Cancer* 1967, 3:381-387
19. Streisinger G, Okada Y, Emrich J, Newton J, Tsugita A, Terzaghi E, Inouye M: Frameshift mutations and the genetic code. *Cold Spring Harbor Symp Quant Biol* 1966, 31:77-84
20. Strand M, Prolla TA, Liskay RM, Petes TD: Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* 1993, 365:274-276
21. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H: Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984, 133:1710-1715
22. Shibata D: Molecular tumor clocks and dynamic phenotype. *Am J Pathol* 1997, 151:643-646
23. Sankila R, Aaltonen LA, Järvinen HJ, Mecklin JP: Better survival rates in patients with MLH1-associated hereditary colorectal cancer. *Gastroenterology* 1996, 110:682-687
24. Lynch HT, Lanspa S, Smyrk T, Boman B, Watson P, Lynch J: Hereditary nonpolyposis colorectal cancer (Lynch syndromes I and II): genetics, pathology, natural history, and cancer control, part I. *Cancer Genet Cytogenet* 1991, 53:143-160
25. Jan YN, Jan LY: Asymmetrical cell division. *Nature* 1998, 392:775-778
26. Chadeneau C, Hay K, Hirte HW, Gallinger S, Bacchetti S: Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res* 1995, 55:2533-2536
27. Kim SH, Roth KA, Moser AR, Gordon JI: Transgenic mouse models that explore the multistep hypothesis of intestinal neoplasia. *J Cell Biol* 1993, 123:877-893
28. El-Deiry WS, Tokino T, Waldman T, Oliner JD, Velculescu VE, Burrell M, Hill DE, Healy E, Rees JL, Hamilton SR, Kinzler KW, Vogelstein B: Topological control of p21 (Waf1/Cip1) expression in normal and neoplastic tissues. *Cancer Res* 1995, 55:2910-2919
29. Polyak K, Hamilton SR, Vogelstein B, Kinzler KW: Early alteration of cell-cycle-regulated gene expression in colorectal neoplasia. *Am J Pathol* 1996, 149:381-387
30. Shibata D, Schaeffer J, Li ZH, Capella G, Perucho M: Genetic heterogeneity of the c-K-ras locus in colorectal adenomas but not adenocarcinomas. *J Natl Cancer Inst* 1993, 85:1058-1063
31. Borland CR, Sato J, Appelman HD, Bresalier RS, Feinberg AP: Microallelotyping defines the sequence and tempo of allelic losses at tumor suppressor gene loci during colorectal cancer progression. *Nature Med* 1995, 1:902-909
32. Novelli MR, Williamson JA, Tomlinson IPM, Elia G, Hodgson SV, Talbot IC, Bodmer WF, Wright NA: Polyclonal origin of colonic adenomas in an XO/XY patient with FAP. *Science* 1996, 272:1187-1190
33. Moser AR, Dove WF, Roth KA, Gordon JI: The Min (multiple intestinal neoplasia) mutation: its effect on gut epithelial differentiation and interaction with a modifier system. *J Cell Biol* 1992, 116:1517-1526
34. Gould KA, Dove WF: Localized gene action controlling intestinal neoplasia in mice. *Proc Natl Acad Sci USA* 1997, 94:5848-5853
35. Perzin KH, Fenoglio CM, Pascal RR: Neoplastic diseases of the small and large intestine. *Principles and Practice of Surgical Pathology*. Edited by Silverberg SS. New York, John Wiley and Sons, 1983, pp 915-918
36. Bjerknes M: Expansion of mutant stem cell populations in the human colon. *J Theor Biol* 1996, 178:381-385
37. Niv Y, Fraser GM: Adenocarcinoma in the rectal segment in familial polyposis coli is not prevented by sulindac therapy. *Gastroenterology* 1994, 107:854-857
38. Thorson AG, Lynch HT, Smyrk TC: Rectal cancer in FAP patient after sulindac. *Lancet* 1994, 343:180

39. Mackillop WJ, Ciampi A, Till JE, Buick RN: A stem cell model of human tumor growth: implications for tumor cell clonogenic assays. *J Natl Cancer Inst* 1983, 70:9–16
40. Cairns J: Mutation selection and the natural history of cancer. *Nature* 1975, 255:197–200
41. Nyström-Lahti M, Wu Y, Moisio AL, Hofstra RM, Osinga J, Mecklin JP, Järvinen HJ, Leisti J, Buys CHCM, de la Chapelle A, Peltomäki P: DNA mismatch repair gene mutations in 55 kindreds with verified or putative hereditary non-polyposis colorectal cancer. *Hum Mol Genet* 1996, 5:763–769
42. Nyström-Lahti M, Kristo P, Nicolaidis NC, Chang SY, Aaltonen LA, Moisio AL, Järvinen HJ, Mecklin JP, Kinzler KW, Vogelstein B, de la Chapelle A, Peltomäki P: Founding mutations and Alu-mediated recombination in hereditary colon cancer. *Nature Med* 1995, 1:1203–1206