Cancers Exhibit a Mutator Phenotype: Clinical Implications

Lawrence A. Loeb,[1] Jason H. Bielas,[1] and Robert A. Beckman[2,3]

[1]Department of Pathology, University of Washington School of Medicine, Seattle, Washington; [2]Oncology Clinical Research, Merck Research Laboratories, North Wales, Pennsylvania; and [3]Simons Center for Systems Biology, Institute for Advanced Study, Princeton, New Jersey

Abstract

Malignancies are characterized by mutations. We have hypothesized that the thousands of mutations in most human cancers do not result from the low mutation rates exhibited by normal human cells. Instead, cancer cells express a mutator phenotype (i.e., the mutation rate in the cancer cells is much greater than that in normal cells). We consider the following points: (*a*) Mutations in genes that govern genetic stability could be the cause of a mutator phenotype exhibited by human cancers. (*b*) A mutator phenotype increases the efficiency of acquiring mutations including those associated with cancer. (*c*) Recent experimental evidence indicates that human tumors contain a vast array of both clonal mutations and nonexpanded (random) mutations. (*d*) The presence of nonexpanded mutations in tumors has fundamental clinical implications for cancer risk assessment, grading, and prognosis including the rapid emergence of resistance to chemotherapeutic agents. Lastly, (*e*) if a mutator phenotype drives carcinogenesis, drugs that target mutator pathways might prevent cancer by delay. [Cancer Res 2008;68(10):3551–7]

Introduction

The concept that tumors have large numbers of mutations can be traced to Boveri (1), who showed that abnormal development in sea urchin embryogenesis is associated with chromosomal alterations (1). He surmised that these changes are analogous to the multiple defects in intercellular cooperation seen in cancers. Foulds' (2) experiments showed the acquisition of a malignant phenotype occurs in a stepwise fashion. With the knowledge that DNA is the genetic material and the powerful technologies now at hand, we can approach these concepts at the level of single molecules and determine which and how many mutations are required for tumorigenesis. We can now establish if Foulds' steps represent the acquisition of new mutants that underlie the phenotype of cancer cells and if these mutations are clonal and are selected from thousands of random mutations that are present in each cancer cell. Clonal mutations derive from repetitive rounds of selection for phenotypes that delineate progressive steps in carcinogenesis whereas random mutations reflect changes in DNA that have occurred primarily after the last round of clonal selection. Mutations are important in cancer, yet the recent discovery of even greater numbers of unexpanded random unexpanded mutations in cancer cells (3) could be key to quantitating the extent of heterogeneity within tumors and to stratifying patients at risk for developing drug resistance.

The concept of a mutator phenotype in cancer was initially formulated based on mutations in DNA polymerases that render them error-prone and mutations in enzymes involved in DNA repair that decrease the ability of cells to remove potentially mutagenic DNA lesions (4). As a result, there is increased genomic instability with the acquisition of mutations in oncogenes and tumor suppressor genes and in additional genes necessary for maintaining genomic integrity. With the increased number of new sets of genes that function in guaranteeing genetic fidelity (5), the mutator phenotype hypothesis has been broadened to include genes involved in microsatellite instability, chromosomal instability, checkpoint instability (6), and maintenance of the epigenome.

One can envision at least three overlapping scenarios in which mutations accumulate during carcinogenesis (Fig. 1). First, it has been proposed that there is a sequential and uniform order of mutations in cancer-associated genes, each mutation conferring a selective proliferative advantage (7). In melanomas, a series of chromosomal changes was shown to correlate with tumor grade (8). In adenocarcinomas, a timetable for mutations in oncogenes correlates with carcinogenesis (7). By this model, high-grade tumors would be expected to have accumulated mutations sequentially in each of the required cancer genes. However, fewer than 7% of colon cancers contain mutations in the three most frequently mutated genes in that tumor type (9). A second model is characterized by successive waves of clonal selection (10). This model also predicts that random mutations would be rare; given that the model does not involve an increase in the rate of mutagenesis, random mutations would not be expected to have a large influence on cell heterogeneity or the emergence of tumor resistance. A mutator phenotype entails a third model (4), where carcinogenesis is driven more efficiently via multiple pathways. By this model, the genotypes of most cells within a tumor would not be identical, but would share at least one mutation in any number of the genes that ensure DNA fidelity. The tumor would evolve as a heterogeneous collection of cancer cells, all sharing the common feature of genetic instability and all having different, but frequently overlapping, patterns of oncogenic mutations. We note that in this model, waves of clonal selection and expansion would still occur. The mutator phenotype and clonal selection and expansion are not mutually exclusive. The conceptual difference is that a linear progression model, and even one based on selection, implies that there are a very limited number of target genes or pathways that would be mutated in the vast majority of cancer cells within a tumor of a specific lineage. In contrast, cancers that evolved by a mutator phenotype would contain a large number of different oncogenic mutations and would have the ability to develop selectively advantageous ones more rapidly.

Chromosomal Instability in Human Cancers

Although chromosomal alterations are diagnostic of only a few types of cancer, they are observed in most tumors. Multiple chromosomal aberrations in cancer cells have been documented by microscopic examination using hybridization with specific probes. These changes occur in both benign and malignant tumors and include deletions, additions, amplifications, and translocations,

Requests for reprints: Lawrence A. Loeb, Department of Pathology, University of Washington School of Medicine, Seattle, WA 98195-7470. Phone: 206-543-6015; Fax: 206-543-3967; E-mail: larryloeb@earthlink.net.

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frequently involving millions of nucleotides (11). Studies on adenocarcinomas of the breast (12) and ovary (13) and on leiomyosarcoma (14) have documented tumors harboring more than 20 different chromosomal alterations. Loss of heterozygosity using PCR-amplified gene fragments reveals an even greater number of alterations (15) as does competitive genomic hybridization. Klein (16) used competitive genomic hybridization to delineate chromosomal changes on isolated single tumor cells. Before the detection of clinical metastasis, they isolated single breast cancer cells from bone marrow and these cells did not exhibit the same spectrum of chromosomal abnormalities as the primary breast cancers. Their results suggest that chromosomal abnormalities in metastasis accumulate after the clinical appearance of a tumor (17). Moreover, most methods for detecting chromosomal changes score predominantly for clonal alterations and exclude changes that could be present in only a small fraction of cancer cells within a tumor.

Relative Efficiency of Mutator Pathways in Human Tumors

Mathematical models of carcinogenesis have focused on delineating the number of steps required for the expression of a malignant phenotype. The hypothesized number of mutations required to generate a cancer has been derived from the observed age-dependent cancer incidence in the population (18–21). These models fail to take into account the large number of malignant lineages that are likely eliminated during carcinogenesis as well as the likelihood that many tumors are dormant and do not cause clinical manifestations.

Our approach has been to calculate the relative efficiencies of cancer arising by pathways involving a mutator phenotype compared with nonmutator pathways as a function of key parameters that contribute to carcinogenesis (Table 1; ref. 22). Efficiency is defined as the number of malignant lineages expected from a given cell population in the amount of time required for cancer onset. We assume, as is axiomatic both in statistical mechanics and evolutionary theory, which all possible mechanisms (mutator and nonmutator) are randomly in play, with their relative contributions proportional to their relative efficiencies. By focusing on the ratio of efficiencies, we can determine the importance of mutator pathways independent of absolute mutation rates.

For example, let us consider two situations, cancers that arise as a result of 170 cell divisions (23), which mimics the situation in predominantly nondividing tissues such as liver, and cancers that

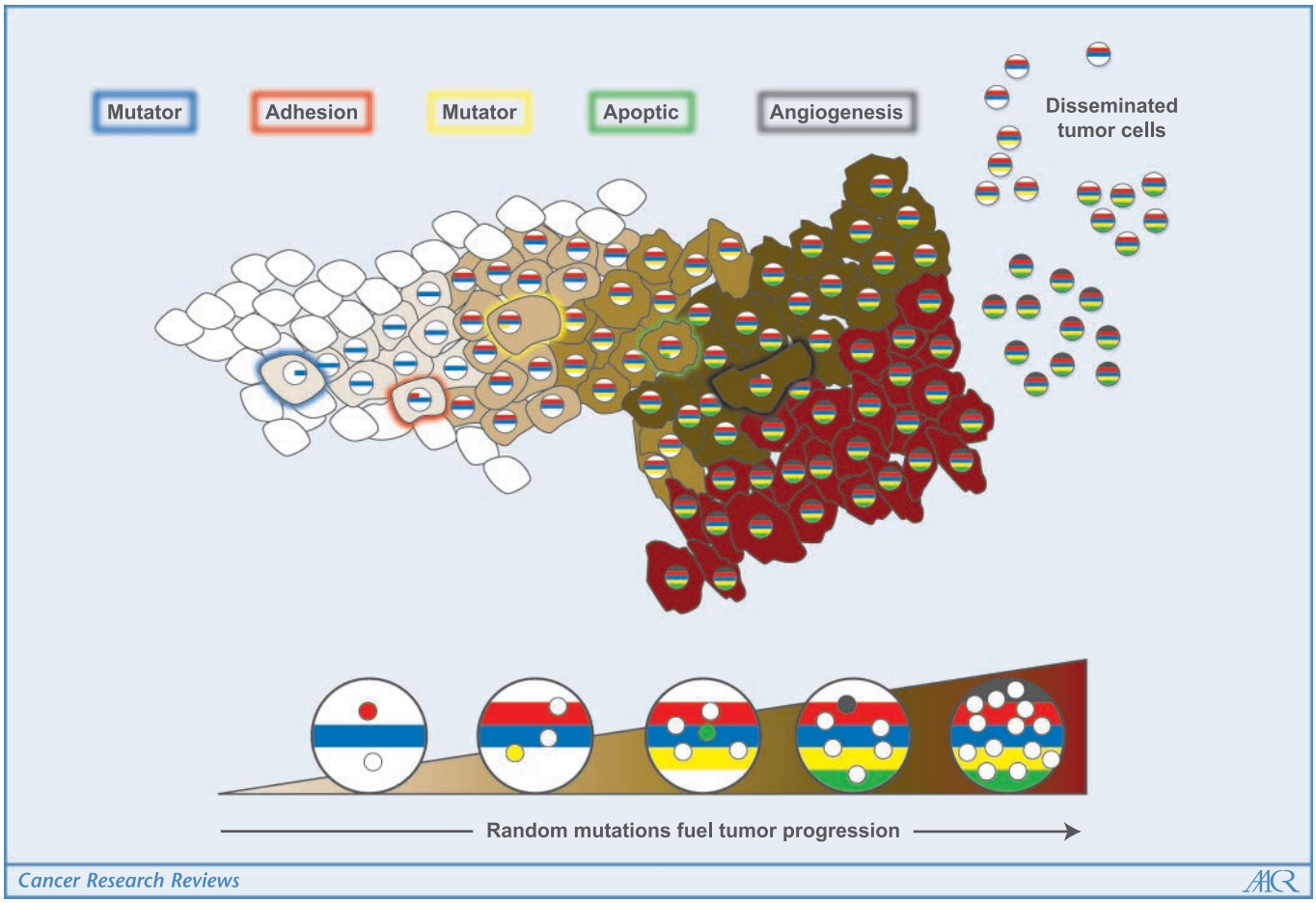

Figure 1. Mutation accumulation during tumor progression. Depicted chronologically are selective pressure (top), cells (middle) and nuclei (bottom) throughout tumor progression. Random mutations result when environmental and endogenous DNA damage exceed the DNA repair capacity of a cell. An early mutation within a mutator gene (blue) increases the rate of mutagenesis, and thus the number of random mutations (white circles) per cell generation available for clonal expansion. In this example, cells that harbor a mutation in a mutator (blue and yellow), cell adhesion (red), anti-apoptotic (green) and later an angiogenic promoting (black) gene are selected and expanded during carcinogenesis. Other than an early mutator mutation, tumor-specific chronologically ordered mutations are not consistent with the mutator phenotype hypothesis, rather random and clonal mutations accumulate stochastically. Additionally, hitchhiker random mutations that exist at other sites throughout the genome get clonally expanded alongside selected causative mutations when present in the same cells. Early- and late- mutator cells can be disseminated from the tumor and form gross metastasis.

Table 1. Relative efficiency of cancer arising via a mutator/nonmutator pathway

Increase in mutation rate (α)	Cell divisions (T)	Mutations in cancer-causing genes (C)		
		12	6	2
10×	170	2×10^{6}	0.3	4×10^{-4}
20×	170	6×10^{9}	160	2×10^{-3}
10×	5,000	4×10^{7}	80	2×10^{-2}
20×	5,000	2×10^{11}	5×10^{3}	7×10^{-2}

NOTE: Relative efficiency of a mutator/nonmutator = $\alpha^{C} k_{mut} T N_{ML} / [C + 1]$, where α, fold increase in mutation rate; C, number of mutated cancer genes; k_{mut}, mutation rate per nucleotide per cell generation (10^{-9}); N_{ML}, number of dominant mutator loci (100); T, number of cell divisions. Percentage of cancers arising via mutator pathways = [relative efficiency / (1 + relative efficiency)] × 100.

arise in cells that have undergone 5,000 cell divisions (24), which mimics the situation in colonic epithelium. We assume in Table 1 a modest 10- or 20-fold enhancement in the rate of mutation, that there are only 100 dominant mutator loci (5, 25), and that the spontaneous frequency of mutations is 10^{-9}/base per round of cell division (26). A larger variety of parameter values has been investigated. The results indicate that mutator pathways do not accelerate carcinogenesis in cases where only one or two oncogenic mutations are required, such as embryonal carcinomas (27). The relative efficiency of cancer arising via mutator pathways compared with nonmutator pathways is 10^{-3} to 10^{-4} (Table 1). This is intuitively correct because the acquisition of a mutator mutation is an extra step in carcinogenesis. When only two oncogenic mutations are required for carcinogenesis, it is unlikely that a mutator phenotype can accelerate the accumulation of oncogenic mutations sufficiently to offset the time needed to acquire the mutator mutation itself. Thus, the need for an additional mutation to generate a mutator phenotype would be critically disadvantageous. Retinoblastoma is an exception in that the critical oncogenic mutation also causes genetic instability; therefore, no extra step is required to acquire the mutator mutation (28).

In contrast, if six mutagenic events are required (18, 29, 30), it is more likely that cancers will arise via mutator pathways. If a greater number of mutations are required, such as suggested in prostate cancer (31), then the efficiency of cancer arising via mutator pathways is 10^{6}- to 10^{9}-fold greater than arising from nonmutator pathways. As discussed above, in the case of embryonal carcinomas, where mutations of both copies of a single recessive oncogene are the only two events required (27), mutator mutations are not likely to play a significant role. Thus, in hereditary nonpolyposis colon cancer, for which there is a single-copy mutation of a mutator gene and a second mutation is required to lead to a mutator phenotype, there is no increased risk of embryonal cancers despite an increased risk of colon cancer (27). This is consistent with the prediction that mutator pathways will have a greater impact on carcinogenic efficiency for cancers that involve more required oncogenic mutations in their pathogenesis. Further analysis indicates that the involvement of mutator mutants in carcinogenesis is favored by a greater increase in mutation rates, by the early onset of a mutator phenotype, by an increase in the number of required steps for carcinogenesis (more than three steps clearly favors mutator pathways, and loss of both alleles of a recessive oncogene counts as two steps), and by an increase in the number of cell generations before the development of a malignancy. It is notable that the effect of increased cell generations in favoring the mutator phenotype is still valid when the time of occurrence of the mutator mutation is allowed to vary (22). However, the greatest contribution to increased carcinogenic efficiency occurs in association with mutator mutations as a first step in carcinogenesis. If mutator pathways are more efficient routes to carcinogenesis, they will appear in the majority of cancers.

Arguments against the Mutator Phenotype Hypothesis

It is appropriate and instructive in this article to consider arguments against the concept of a mutator phenotype in cancer before presenting the supporting data. First, it is argued that increasing mutagenesis generates reduced fitness, and thus a mutator phenotype would be detrimental. However, a direct mathematical analysis indicates that the generation of reduced fitness mutants during tumor proliferation would not be quantitatively significant (23). Furthermore, serial passage experiments with prokaryotes invariably result in the emergence of mutators (32). A comprehensive example is provided by the cocultivation for 320 generations of 69 *E. coli* mutants with fidelities varying by more than 6 orders of magnitude (33). At the end of 31 days, all surviving strains were moderate mutators with 10 to 47 lower fidelity than the wild type, whereas antimutators and extreme mutators had been outcompeted.[4]

Second, aneuploidy, the loss or gain of chromosomes, has been proposed as the initiating event in the conversion of a normal cell to a tumor cell (34). Indeed, aneuploidy is frequently found in human tumors; however, some tumors are not aneuploid and in others aneuploidy occurs primarily subsequent to other mutations (35).

Third, it is argued that in rapidly dividing tissues, repetitive rounds of clonal selection in the absence of an increase in mutation rate are adequate to account for the multiple mutations found in human cancers (36). However, such estimates of expected absolute mutation frequencies observed in cancers as a function of mutation rates are highly contingent on assumptions about the proliferation rate, the cell cycling fraction, the cell death probability, the number of premalignant cells in an epithelium that are desquamated and lost, the number of cancers eliminated by the host before clinical detection, and the number of early cancers or premalignant lesions that are undiagnosed in people who die of other causes. A more robust analysis involves the *relative* carcinogenic efficiency of mutator to nonmutator pathways, clearly indicating superior efficiency of mutator compared with nonmutator pathways (22), even in the presence of clonal selection.[5] Importantly, the absolute efficiency is also further increased by clonal selection as expected.

Last, the initial studies on sequencing of cancer cell genomes only identified a few mutations and were interpreted as an argument against a mutator phenotype (37). However, subsequent studies have shown the presence of large numbers of mutations in tumor cells, and even these are surely an underestimate because sequencing only

[4] E. Loh and L.A. Loeb, unpublished results.
[5] R.A. Beckman, unpublished results.

detects mutations that are present in most cells in a tumor cell population. Sequencing cannot detect changes in DNA sequence at any position if it is present in less than 10% of the cells examined (11).

Changes in Nucleotide Sequences in Cancer Cells

Three recent lines of evidence provide strong support for a mutator phenotype in cancer cells: expansion of repetitive sequences, the multiplicity of mutations in sequencing the cancer genome, and the high frequency of random substitutions in DNA from human tumors.

Microsatellite instability. The instability of the cancer genome was heralded by studies on lengths of repetitive sequences in inherited human colon cancers (38–41). In hereditary colon cancers involving mutations in mismatch repair genes, there is a striking change in the lengths of repetitive nucleotide sequences in microsatellite and other repetitive sequences throughout the genome. Changes in lengths of repetitive sequences within genes are also frequently observed and can result in gene inactivation by frameshift mutations. Based on frequency of mutations in a limited number of microsatellites examined, it has been estimated that thousands of loci are mutated within each tumor cell (42). In addition to hereditary nonpolyposis coli, microsatellite instability has been documented in a variety of nonhereditary human cancers (43). We consider changes in the lengths of repetitive DNA sequences as mutation "hotspots" in which slippage by DNA polymerase exceeds the capacity for correction by mismatch repair and results in elongation of the repeats that can cause frameshift mutations or affect spacing within DNA regulatory elements. Whereas this was the first demonstration of a mutator phenotype at the level of nucleotide sequence, it is unlikely to be unique.

DNA sequencing. A major goal of The Cancer Genome Atlas has been to identify new oncogenes that are commonly mutated in human cancers and that could serve as targets for therapy or signals for early detection. Sjoholm et al. sequenced 456 Mb from 11 breast and 11 colon cancer cell lines and identified 1,307 tumor specific somatic mutations. This may be a minimal estimate because they excluded mutations in known single nucleotide polymorphisms; such loci would be mutated at higher frequency than other loci in cancer cells. Furthermore, most of the mutated genes were not previously known to be associated with cancers. An analysis of 189 of the mutated genes in 24 breast and 24 colon cancers indicated that, on average, there were 12 mutated genes per tumor, that each tumor exhibited a different panel of mutated genes, and that the consensus sequence is different for breast and colon cancers. In another study (44), the mutation frequency in the kinase gene family in one breast carcinoma was 10^{-3}. More than 1,000 somatic mutations were identified in 274 Mb of DNA in the coding exons of 518 kinase genes in diverse tumors, most of which were not known to be associated with cancers (45). Some tumors contained mutations in as many as 79 of the kinase genes. In each of these studies, mutations were identified by DNA sequencing. Because sequencing involves large populations of molecules, isolated random changes in nucleotide sequence would not be detected unless that change was present in 10% of the tumor. We interpret these results as confirmation of the diversity of mutations in human cancer and consistent with the mutator phenotype hypothesis.

Random unexpanded mutations. To detect random mutagenic events, it is necessary to interrogate single DNA molecules. We have developed and validated an assay to capture specific segments of DNA and identify mutations in single molecules that render the DNA noncleavable by restriction enzymes (46). Mutations are quantitated by amplification of single molecules from primers flanking the intact cut site using real-time PCR and confirmed by DNA sequencing. The random mutation capture (RMC) assay offers unprecedented sensitivity for mutation detection, allowing detection of one single-base substitution in 10^9 copies of human genomic DNA. As an initial target, we selected a TCGA sequence in intron VI of p53 and showed that mutations at this locus were neutral, conferring neither a positive nor a negative selection pressure on cells that harbor such mutations. The RMC assay allows us to calculate the random mutation frequency of the cells that compose various tumor and normal tissues (Table 2).

In contrast to the paucity of mutations in normal human tissues, adjacent tumor tissues were found to exhibit a high frequency of single-base substitutions. In a blinded study with six tumor samples, the mean mutation frequency was 2.2×10^{-6} (47). Mutation frequencies ranged from 0.6 to 4.8×10^{-6}, an average increase of

Table 2. Random mutation frequency in human tissue and cells

Normal	Nucleotides analyzed, $\times 10^{-6}$	Mutation frequency,* $\times 10^{8}$	Neoplastic	Nucleotides analyzed, $\times 10^{-6}$	Mutation frequency,* $\times 10^{8}$
Tissues [†]					
Squamous epithelium	115	<1	Ovarian carcinoma	18	75
Renal cortex	108	<1	Perirenal liposarcoma	24	65
Colonic mucosa	115	<1	Colonic adenocarcinoma	10	475
Inflamed renal cortex	55	4	Renal carcinoma	15	270
Skeletal muscle	110	<1	Pleomorphic sarcoma	15	141
			Non-Hodgkin's lymphoma	27	300
Cultured fibroblasts [‡]					
Untreated	218	2	ENU treated[§]	24	175

*Measured by the random mutation capture assay.
[†]Normal and neoplastic tissues listed in the same row are paired samples from the same individual.
[‡]Data for cultured normal dermal fibroblasts.
[§]Treated with 1 mg/mL *N*-ethyl-*N*-nitrosourea for 1 h.

>220-fold compared with paired normal tissues. The diversity of nucleotide substitutions in the tumor DNA indicates that each mutation is present in only one or a few cells and arose after the last round of clonal selection. These results suggest that a mutator phenotype is expressed throughout the course of carcinogenesis and persists late in the process or occurs as a late event. We note that the observed mutation frequency at a given nucleotide locus is approximately equal to the average mutation rate per locus per cell generation multiplied by the number of cell generations. However, it is unlikely that a >200-fold increase in mutation frequency can be explained by an increase in cell generations alone because that would require a >200-fold increase in proliferation rate and/or cycling fraction. Thus, it is likely that much of this >200-fold increase in mutation frequency relative to wild type reflects inherent genetic instability. The results suggest that each tumor cell contains more than 10^4 random point mutations, when the single-base mutation frequency is multiplied by the number of bases in the genome. A dramatic increase in the frequency of random mutations has also been demonstrated in tumors from mice with the RMC assay (48).

Changes in Nucleotide Sequences in Normal Tissue

The mutation frequency in a variety of normal adult human tissues was determined to be $<5 \times 10^{-8}$ mutations/bp (Table 1). Based on the total numbers of nucleotides interrogated, we estimate the mutation frequency in normal human tissues to be less than 10^{-9}; this is an upper estimate and is comparable to measurements of frequencies as the number of mutations per base pair observed in normal human T-lymphocytes via the use of the hprt mutational assay (26). Thus, based on a minimum of 45 cell divisions required to generate an adult containing 5×10^{13} cells, we estimate that the average mutation rate is $<10^{-10}$ mutations/base per cell division. We lack information about the mutation frequency in human stem cells or in putative cancer stem cells (49). However, the mutation frequency in mouse embryonic fibroblasts is reported to be 100-fold lower than that in somatic fibroblasts (50). If these cells can be equated with cancer stem cells, then the spontaneous mutation frequency in tumor progenitor cells before a mutator mutation is likely to be even lower than that in somatic cells.

Consequences of Random Mutations in Tumors

Tumor cell heterogeneity. Irrespective of mechanism, genetic heterogeneity has important consequences with respect to stratification of tumors. Variability of random mutations within different grades might provide an additional criterion to guide therapy. Tumors with marked elevation in random mutations would be more likely to harbor a greater variety of functionally important mutations in key genes that determine malignant potential. It can be hypothesized that individuals harboring tumors with fewer random mutations should be treated more conservatively, whereas individuals harboring tumors with a higher frequency of random mutations should be treated more aggressively.

Development of resistance. The presence of 10^4 different point mutations within each cancer cell means that within a tumor of 10^9 cells (at the time it is detected clinically), there could be as many as 10^{13} different mutations. The majority of these mutations are single-base substitutions, many of which could fail to inactivate proteins (51) and yet could alter substrate specificity. As a result, tumors are likely to contain mutant genes that encode proteins that can render the cells resistant to any therapy directed against the tumor. It should be noted that, even with this high mutation burden, few cells would contain mutations that render them resistant to multiple non–cross-resistant agents. Thus, the presence of large numbers of random mutations in tumors provides an additional argument for the simultaneous administration of multiple therapies (52). However, the effective doses of some combined therapies may be limited by toxicity.

The emergence of resistance to imatinib (Gleevec) in patients with chronic myelogenous leukemia (CML) provides clear evidence for the involvement of random mutations in drug resistance. Imatinib specifically targets the BRC-ABL protein kinase, and based on successful responses it has become the primary treatment for CML (53). Unfortunately, resistance develops in 30% to 90% of patients (54) and is mediated by point mutations in the ATP binding site (55). Resistance can be overcome by administration of a second drug that targets a different site on the BRC-ABL protein kinase; yet further resistance is mediated by the emergence of additional point mutations, which, when present in the same molecule, confer resistance to both drugs (56). Evidence suggests that many of these mutations that yielded resistant genes were present in bone marrow before the onset of therapy.[6] A mathematical model of resistance in CML suggests that most of the imatinib resistance occurring within 2 years of therapy can be accounted for by preexisting resistance if one assumes a mutation rate of 10^{-8}/base per cell division (57, 58). This mutation rate is quite high compared with that which has been observed in other normal tissues, but is consistent with that observed in solid tumors (3).

Does the Accumulation of Random Mutations Drive Carcinogenesis?

Although the concept that cancer results from a mutator phenotype introduces many impediments for cancer therapy, it also presents a new opportunity for cancer prevention. For most adult tumors, it takes some 20 years from the time an individual is exposed to a carcinogen to the time a tumor is clinically detected, and for many tumors an additional 0.5 to 15 years before death of the host. If mutations, generated via a mutator phenotype, accelerate carcinogenesis, there is the opportunity to prevent clinical manifestation of many cancers by interfering with mutation accumulation. Even a 2-fold reduction in the efficiency of carcinogenesis would significantly lower cancer mortality (11). For a cancer that requires six oncogenic mutations, a 2-fold reduction in carcinogenic efficiency corresponds to only an 11% reduction in mutation rate (22). If invasiveness and metastasis are late mutational manifestations of a tumor, one might also reduce cancer morbidity by retarding the underlying evolutionary forces that govern their development. Compounds that interfere with mutation accumulation might be designed *de novo* based on known targets or identified by high-throughput screening.

Antimutagenic prevention strategies could include reduction in environmental exposure or (more easily) administration of agents that decrease endogenous reactive DNA-damaging species in high-risk populations. In addition, human cells contain at least five error-prone DNA polymerases that are believed to function by enabling

[6] J.P. Badich, unpublished results.

the DNA synthetic apparatus to copy past potentially blocking template lesions. In doing so, they frequently incorporate noncomplementary nucleotides. Pol κ mutations are reported to be increased in small-cell lung cancer (59), and Pol β is mutated in gastric cancers (60). Thus, DNA polymerases represent one of the targets for prevention by antimutagenesis. Based on our theoretical results, prevention by antimutagenesis is expected to be particularly effective for tumors in which a larger number of oncogenic steps are required for malignant transformation, such as prostate cancer.

The use of antimutagenic therapies for established cancers may slow the development of resistance to accompanying standard therapies, if such resistance was not already preexisting. For example, some tumors may harbor mutations that render replicative DNA polymerases error-prone by distorting the substrate binding pocket, inactivating the 3′→5′ proof-reading exonuclease, or interfering with transport between the polymerase and exonuclease sites. These mutant polymerases would also be more likely to misincorporate nucleotide analogues that terminate or slow DNA replication. Lastly, the error rates of DNA polymerases are dependent on the relative concentrations of the four deoxynucleoside triphosphate precursors, which, if altered in specific tumors, may potentially be corrected by the administration of nucleosides, antimetabolites, or other pharmacologic interventions.

Sequential passage of bacteria and yeast has invariably showed that mutators have a selective advantage in culture. It may be possible to screen libraries of small molecules for those that reduce mutation frequencies and therefore shift the curve of mutation frequency versus survival in culture. One would predict that strains with an initial mutation frequency higher than the optimum would, in the presence of an antimutator compound, be shifted to mutation frequencies nearer the optimum, and thus acquire a selective advantage in culture. The similarities of mutator pathways in prokaryotes and eukaryotes suggest that the powerful genetics that has been elaborated in bacteria and yeast might serve to initially identify candidate antimutator compounds.

Disclosure of Potential Conflicts of Interest

R.A. Beckman: stockholder in Merck and Co., Inc. The other authors disclosed no potential conflicts of interest.

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References

1. Boveri T. Uber mehrpolige Mitosen als Mittel zur Analyse des Zellkerns. Veh. Dtsch. Zool. Ges. Wurzburg; 1902.
2. Foulds L. The experimental study of tumor progression: a review. Cancer Res 1954;14:327–39.
3. Bielas JH, Loeb KR, Rubin BP, True LD, Loeb LA. Human cancers express a mutator phenotype. Proc Natl Acad Sci U S A 2006;103:18238–42.
4. Loeb LA, Springgate CF, Battula N. Errors in DNA replication as a basis of malignant change. Cancer Res 1974;34:2311–21.
5. Myung K, Kolodner RD. Suppression of genome instability by redundant S-phase checkpoint pathways in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 2002;99:4500–7.
6. Paulovich AG, Toczyski DP, Hartwell LH. When checkpoints fail. Cell 1997;88:315–21.
7. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759–67.
8. Balaban GB, Herlyn M, Clark WH, Jr., Nowell PC. Karyotypic evolution in human malignant melanoma. Cancer Genet Cytogenet 1986;19:113–22.
9. Smith G, Carey FA, Beattie J, et al. Mutations in APC, Kirsten-ras, and p53—alternative genetic pathways to colorectal cancer. Proc Natl Acad Sci U S A 2002;99:9433–8.
10. Nowell PC. The clonal evolution of tumor cell populations. Science 1976;194:23–8.
11. Loeb LA, Loeb KR, Anderson JP. Multiple mutations and cancer. Proc Natl Acad Sci U S A 2003;100:776–81.
12. Kallioniemi A, Kallioniemi O-P, Piper J, et al. Detecton and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. Proc Natl Acad Sci U S A 1994;91:2156–60.
13. Riopel MA, Spellerberg A, Griffin CA, Perlman EJ. Genetic analysis of ovarian germ cell tumors by comparative genomic hybridization. Cancer Res 1998; 58:3105–10.
14. El-Rifai W, Sarlomo-Rikala M, Knuutila S, Miettinen M. DNA copy number changes in development and progression in leiomyosarcomas of soft tissues. Am J Pathol 1998;153:985–90.
15. Kerangueven F, Noguchi T, Coulier F, et al. Genome-wide search for loss of heterozygosity shows extensive genetic diversity of human breast carnomas. Cancer Res 1997;57:5469–74.
16. Klein CA. Single cell amplification methods for the study of cancer and cllular ageing. Mech Ageing Dev 2005;126:147–51.
17. Klein CA, Holzel D. Systemic cancer progression and tumor dormancy: mathematical models meet single cell genomics. Cell Cycle 2006;5:1788–98.
18. Armitage P, Doll R. The age distribution of cancer and a multi-stage theory of carcinogenesis. Br J Cancer 1954;8:1–12.
19. Beckman RA, Loeb LA. Genetic instability in cancer: theory and experiment. Semin Cancer Biol 2005a;15: 423–35.
20. Cairns J. The origin of human cancers. Nature 1981; 289:353–7.
21. Mendelsohn ML, Pierce DA. A multi-mutational model for cancer based on age-time patterns of radiation effects: 2. biological aspects. In: Proceedings of International Conference on Low Doses of Ionizing Radiation, Biological Effects and Regulatory Control. Seville (Spain): International Atomic Energy Agency; 1997.
22. Beckman RA, Loeb LA. Efficiency of carcinogenesis with and without a mutator mutation. Proc Natl Acad Sci U S A 2006;103:14140–5.
23. Beckman RA, Loeb LA. Negative clonal selection in tumor evolution. Genetics 2005b;171:2123–31.
24. Tomlinson IP, Sasieni P, Bodmer W. How many mutations in cancer? Am J Pathol 2002;100:755–8.
25. Kolodner RD, Putnam CD, Myung K. Maintenance of genome stability n *Saccharomyces ceerevisiae*. Science 2002;297:552–7.
26. Albertini RJ, Nicklas JA, O'Neill JP, Robison SH. *In vivo* somatic mutations in humans: measurement and analysis. Annu Rev Genet 1990;24:305–26.
27. Hethcote HW, Knudson AGJ. Model for the incidence of embryonal cancers: application to retinoblastoma. Proc Natl Acad Sci U S A 1978;75:2453–7.
28. Knudson AGJ. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 1971;68:820–3.
29. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
30. Weinberg RA. The biology of cancer. New York (NY): Garland Science; 2007. 796 pp.
31. Renan MJ. How many mutations are required for tumorigenesis? Implications from human cancer data. Mol Carcinog 1993;7:139–46.
32. Sniegowski PD, Gerrish PJ, Lenski RE. Evolution of high mutaton rates in experimental populations of *E. coli*. Nature 1997;387:703–5.
33. Loh E, Choe J, Loeb LA. Highly tolerated amino acid substitutions increase the fidelity of *E. coli* DNA polymerase I. J Biol Chem 2007;282:12201–9.
34. Duesberg P, Rausch C, Rasnick D, Hehlmann R. Genetic instability of cancer cells is proportional to their degree of aneuploidy. Proc Natl Acad Sci U S A 1998;95: 13692–7.
35. Mitelman F. Catalog of chromosome aberrations in cancer. New York (NY): Wiley-Liss; 1994.
36. Tomlinson I, Bodmer W. Selection, the mutation rate and cancer: ensuring that the tail does not wag the dog. Nat Med 1999;5:11–2.
37. Wang TL, Rago C, Silliman N, et al. Prevalence of somatic alterations in the colorectal cancer cell genome. Proc Natl Acad Sci U S A 2002;99:3076–80.
38. Fishel R, Lescoe MK, Rao MRS, et al. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell 1993;75: 1027–38.
39. Leach FS, Nicolaides NC, Papadopoulos N, et al. Mutations of a *mutS* homolog in hereditary non-polyposis colorectal cancer. Cell 1993;75:1215–25.
40. Paillard F, Sterkers G, Vaquero C. Transcriptional and posttranscriptional regulation of TcR, CD4 and CD8 gene expression during activation of normal human T lymphocytes. EMBO J 1990;9:1867–72.
41. Peinado MA, Malkhosyan S, Velazquez A, Perucho M. Isolation and characterization of allelic loss and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. Proc Natl Acad Sci U S A 1992;89: 10065–9.
42. Perucho M. Cancer of the microsatellite mutator phenotype. Biol Chem 1996;377:675–84.
43. Eshleman JR, Lang EZ, Bowerfind GK, et al. Increased mutation rate at the *hprt* locus accompanies microsatellite instability in colon cancer. Oncogene 1995;10:33–7.
44. Stephens P, Edkins S, Davies H, et al. A screen of the complete protein kinase gene family identifies diverse patterns of somatic mutations in human breast cancer. Nat Genet 2005;37:590–2.
45. Greenman C, Stephens P, Smith R, et al. Patterns of somatic mutation in human cancer genomes. Nature 2007;446:153–8.
46. Bielas JH, Loeb LA. Quantification of random genomic mutations. Nat Methods 2005;2:285–90.
47. Bielas JH, Venkatesan RN, Loeb LA. LOH proficient

ES cells: a model of cancer progenitor cells? Trends Genet 2007;23:154–7.
48. Zheng L, Dai H, Zhou M, et al. Fen1 mutations result in autoimmunity, chronic inflammation and cancers. Nat Med 2007;13:812–9.
49. Wicha MS, Liu S, Dontu G. Cancer stem sells: an old idea—a paradigm shift. Cancer Res 2006;66:1883–90.
50. Cervantes RB, Stringer JR, Shao C, Tischfield JA, Stambrook PJ. Embryonic stem cells and somatic cells differ in mutation frequency and type. Proc Natl Acad Sci U S A 2002;99:3586–90.
51. Guo HH, Choe J, Loeb LA. Protein tolerance to random amino acid change. Proc Natl Acad Sci U S A 2004;101:9205–10.
52. Goldie JH, Coldman AJ. Drug resistance in cancer: mechanisms and models. Cambridge UK: Cambridge University Press; 1998.
53. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 2001; 344:1031–7.
54. Soverini S, Martinelli G, Rosti G, et al. ABL mutations in late chronic phase chronic myeloid leukemia patients with up-front cytogenetic resistance to imatinib are associated with a greater likeihood of progression to blast crisis and shorter survival: a study by the GIMEMA Working Party on Chronic Myeloid Leukemia. J Clin Oncol 2005;23:4100–9.
55. Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kurivan J. Structural mechanism for STI-571 inhibition of Abelson tyrosine kinase. Science 2000;289: 1938–42.
56. Shah NP, Skaggs BJ, Branford S, et al. Sequential ABL kinase inhibitor therapy selects for compound drug-resistant BCR-ABL mutations with altered oncogenic potency. J Clin Invest 2007;117:2562–9.
57. Hughes TP, Kaeda J, Branford S, et al. Frequency of major molecular responses to imatinib or interferon α plus cytarabine in newly diagnosed chronic myeloid leukemia. N Engl J Med 2003;349: 1421–30.
58. Michor F, Hughes TP, Iwasa Y, et al. Dynamics of chronic myeloid leukemia. Nature 2005;435:1267–70.
59. O-Wang J, Kawamura K, Tada Y, Ohmori H, Kimura H, Sakiyama S, Tagawa M. DNA polymerase κ, implicated in spontaneous and DNA damage-induced mutagenesis, is overexpressed in lung cancer. Cancer Res 2001;61:5366–9.
60. Sweasy JB, Lauper JM, Eckert KA. DNA polymerases and human diseases. Radiat Res 2006;166: 693–714.

Response

The most fundamental problem with the mutator phenotype hypothesis is that it simply does not take adequate account of the power of natural selection. Loeb et al. draw the distinction between "clonal" mutations, which have populated a tumor because of the selective advantage they confer on the cells carrying the mutation, and "unexpanded random mutations" which are presumably those that do not confer any significant selective advantage and so mostly do not increase in frequency in a tumor. The primary mutational event is always, however, effectively random. The validity of the assessment of mutation rates in normal tissues is questionable and does not take into account the different clonal histories of a tumor, as compared to a mixture of normal cells. There is no clear evidence to support a difference in mutation rates between tissue stem cells and differentiated cells. However, most mutations must occur in tissue stem cells, as their progenitors only have a relatively short half-life. Germ line estimates of mutation rates are of the order of at least 10-9 per base pair per generation (see ref. 23 in Bodmer's article) and it seems most unlikely that the mutation rate in tissue stem cells would be much less than this.

On this assumption, a clone of 109 cells, representing about 1gm of wet tissue, will produce, on average, approximately one mutation in every base pair per cell generation. The vast majority of these mutations will not confer any selective advantage and are the "unexpanded random mutations" of Loeb et al. They will occur in the tumor at very low frequencies and so will be largely irrelevant for the overall biology of the tumor. However, even in such a relatively small tumor, the chance of a mutation arising that does confer a selective advantage must already be quite high without any need for an increased mutation rate. This clearly implies that it is only at the earliest stages of a cancer that the mutation rate might be limiting, and yet the evidence clearly is that is not when mutator gene mutations are found. The model proposed by Loeb et al. seems to assume that all cells in a tumor somehow acquire a mutator phenotype, and then calculates the accumulation of mutations under different assumptions of cell numbers and mutation rates. But this is done with no regard to the possibility of certain mutations conferring selective advantages on the cells that carry them. The model also assumes dominant mutator gene mutations, but these have just not been found in sporadic tumors. So far, the results of large scale sequencing of cancers have shown remarkably few genes with mutations that occur in more than one or two tumors out of more than 20. Given normal mutation rates of the order of 10-9, and the sort of population sizes a tumor reaches in late stages, namely 1011 to 1012 cells, and the number of base pairs and of genes in which mutations can occur, standard population genetics would not make it surprising to find a number of mutations in a detectable fraction of a tumor's cells even by chance. If some of these mutations confer even a slight selective advantage then the expected number would be even higher, and it would be mainly these that are seen. Mutations which occur in, 5% or less of tumors of a given type are not likely to be relevant for the overall development of targeted antitumor therapies.

Many, if not most, solid tumors, with the notable exception of those connected with smoking or sunlight exposure, are most probably not initiated by the action of specific carcinogens. It therefore seems most unlikely that "anti-mutagenic" therapies will have any effect. Not smoking and not exposing oneself to excess sunlight are clearly the most effective, already known, "anti-mutagenic therapies".

I. Walter Bodmer
Cancer and Immunogenetics Laboratory,
Weatherall Institute of Molecular Medicine,
University of Oxford,
Oxford, United Kingdom

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Lawrence A. Loeb, Jason H. Bielas and Robert A. Beckman

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