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Mini-review

Generation of mutator mutants during carcinogenesis

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ABSTRACT

Mutations are rare in normal cells. In contrast, multiple mutations are characteristic in most tumors. Previously we proposed a "mutator phenotype" hypothesis to explain how pre-cancer cells may acquire large number of mutations during carcinogenesis. Here we extend the "mutator phenotype" hypothesis considering recently discovered biochemical activities whose aberrant expression may result in genome-wide random mutations. The scope of this article is to emphasize that simple random point mutations can drive carcinogenesis and highlight new emerging pathways that generate these mutations. We focus specifically on random point mutations generated by replication errors, oxidative base damage, covalent base modifications by enzymes, and spontaneously generated abasic sites as a source of mutator mutants.

Contents

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1. Introduction

Carcinogenesis is a multi-step process in which cancer cells evolve from a single, normal diploid stem cell by successive rounds of clonal selection. Normal stem cells can be transformed to impending cancer cells spontaneously or by environmental carcinogens. These pre-cancerous cells typically do not have a diagnostic phenotype. In the case of solid tumors, it may take more than 20 years from the time cancer progenitor cells are formed by carcinogen exposure to the clinical appearance of a tumor. During this time there occurs sequential rounds of clonal evolution, in which a single stem cell is expanded by stepwise selection to populate a tumor. Successive waves of clonal expansion and selection of pre-cancerous cells expressing key mutations (within oncogenes and tumor-suppressor genes) drive tumor progression. It is has been postulated that in laboratory models at least six altered cellular phenotypes must be expressed [1] implying that at least six different metabolic pathways are altered. Analysis of cancer deaths as a function of age shows that between two and ten events, presumably mutations, are necessary to produce most adult human cancers. Since mutations are random events. We desire to know the source of the large number of mutations that are required to generate the key mutations that determine tumor phenotypes.

In most tumors, cancer cells are phenotypically heterogeneous and exhibit alterations in the nucleotide sequence of genomic DNA and multiple chromosomal abnormalities; these continue to accumulate spontaneously towards more and more malignant phenotypes. Given, however, that normal human cells replicate their DNA with exceptional accuracy, incorporating approximately one random error during DNA replication, it would be expected that each cell could amass only 1–2 mutant genes during its life span [2]. Thus, only if the mutations occurred exclusively on specific oncogenes and/or tumor-suppressor genes could one account for the 2–10 rate limiting changes that occur during tumorigenesis. Instead, we observe thousands of mutations in cancer cells, and we hypothesize that only a few of these occur at specific sites. In order to account for the large number of mutations present in tumors, we propose that early during tumorigenesis; pre-cancer cells must exhibit a mutator phenotype. By elevating the mutation rate, the probability of generating mutations in oncogenes and tumor-suppressor genes increases, and this can lead to clonal proliferation and selection of sequential lineages resulting in malignancy. However, an elevated rate of mutation not only leads to cancer but can also increase the incidence of apoptosis and senescence or affect other key pathways and thus the acquisition of a mutator phenotype may not always advance cells towards malignancy (Fig. 1).

In this minireview we emphasize simple point mutations (random mutations) as the source for the induction of mutator phenotype. We consider recent studies that unveil new mechanisms for the generation of genetic instability, and studies that extensively evaluate tumors for the presence of clonal mutations. Lastly, we consider the implication of multiple random mutations in tumors.

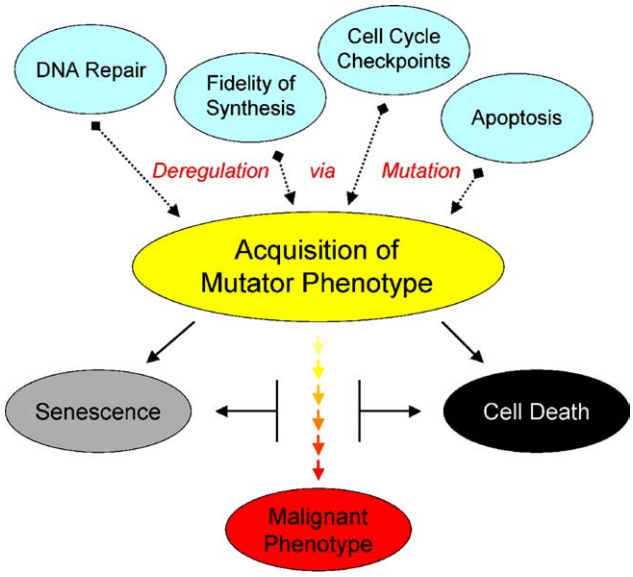

Fig. 1 – The maintenance of the genome is ensured by high fidelity DNA synthesis, DNA repair systems, cell cycle checkpoints, and apoptosis. Deregulation of any of these pathways can be achieved via induced or spontaneous gene mutation and results in the acquisition of a mutator phenotype, and thus an increase in the rate of mutagenesis. The increased mutational load enhances the probability of generating mutations in proto-oncogenes and tumor-suppressor genes. This can lead to the clonal proliferation and selection of a series of lineages resulting in malignancy. In principle an elevated rate of mutation, need not always result in malignancy. Rather, intact genomic integrity pathways that were activated via an increase in mutational load can halt carcinogenesis by triggering apoptosis or cellular senescence.

2. Misincorporation and unrepaired DNA lesions may drive origin of cancer cell lineages

On undamaged DNA templates, the accuracy of replicative DNA polymerases is approximately one misincorporation event per 10^5–10^6 nucleotides polymerized [3]. Errors in DNA synthesis, coupled to excision events by proof reading exonuclease activity and mismatch repair (estimated to contribute further about 100–1000-fold toward accuracy of DNA synthesis), are infrequent enough to account for the low mutation rates in normal human somatic cells [3]. Thus, in principle one can account for the accuracy of DNA replication by studies with purified components. A reduction in the accuracy of DNA replication can be a major source of spontaneous mutagenesis.

Endogenous and environmental agents constantly damage the human genome and this damage needs to be repaired prior to cell division. Normal human cells are very proficient at the repair of DNA damage and additional processes prevent the accumulation of mutations during development by regeneration and replacement of tissues. One can envision that the overall mutation rate in cells is governed by equilibrium

between mechanisms that generate changes in DNA sequence (DNA damage and errors in DNA synthesis), counterbalanced by the efficiency of DNA repair. In normal human cells, the protective shield provided by DNA repair pathways is extensive, and consequently the majority of lesions are excised during DNA replication and therefore mutations in normal cells are exceedingly rare event. Mutations that inactivate DNA repair enzymes have been demonstrated in rare recessive diseases, the classical prototype is xeroderma pigmentosum (XP), in which there is a 1000-fold increase in skin cancer. Such inherited rare diseases have provided definitive evidence for the association of deficits in DNA repair with increased incidence of human cancers. On the other hand, the evidence for the involvement of mechanisms that generate mutations, i.e. mutator mutants is less well established.

3. Cellular origin of human cancers

It has been hypothesized that many cancers arise from stem cells and evidence toward cancer-stem cell hypothesis has continued to accumulate [4,5]. First, stem cells isolated from acute myelogenous leukemia (AML) and breast cancers form tumors when introduced into NOD/SCID mice that are immunologically defective [6,7]. Second, Kielman et al. reported that embryonic stem cells (ES cells) with mutations in *APC* (found in human colorectal cancer) could no longer differentiate into various cellular lineages. They hypothesize that *APC* mutations prevent embryonic signaling cues for differentiation into different cellular lineages and confer a stem cell-like phenotype [8]. Third, Hemmati et al. investigated the properties of different pediatric brain tumors (medulloblastoma, astrocytoma and glioma) and observed that tumor-progenitor cells were multi-potent. As xenografts in neonatal rat brains they differentiated into neurons and glial cells [9]. Furthermore, Singh et al. isolated from human medulloblastomas and glioblastomas, tumor-initiating $CD133^+$ cells which after injection into brain of SCID mice formed tumors [10]. These empirical observations indicate that some human cancers may arise from totipotent cells. Until recently mutation rates and DNA repair capacity of stem cells have not been delineated. Cervantes et al. now report that mutation rate of mouse embryonic stem cell is 10–100-fold less than that in mouse embryonic fibroblasts [11]. This important study needs to be verified with other markers and extended to human stem cells. These results, if extrapolated to human stem cells that are the progenitors of cancer, indicates that it is even more unlikely that normal mutation rates can generate the large numbers of mutations required to form tumors. It can be argued that long-lived stem cells would accumulate DNA damage and special mechanisms would be required to guarantee that this damage is not passed on to daughter cells. Cairns has postulated that stem cells keep the same parental DNA strands through successive divisions [12].

4. Characterization of mutator phenotypes

Mutator phenotype and genetic instability are general concepts associated with an accumulation of random genetic changes. This includes simple point mutations, changes in the number of repetitive DNA sequences termed microsatellites (MIN or MSI) as well as rearrangements, losses, and gains of large segments of DNA within chromosomes termed chromosomal instability (CIN).

The MIN phenotype has been most clearly observed in individuals with hereditary non-polyposis coli (HNPCC) that harbor mutations in mismatch repair genes. This instability occurs in repetitive sequences both in exons and introns. Furthermore, smaller degrees of changes in lengths of repetitive sequences are observed in many other tumors. Some of these alterations are associated with decreased expression or aberrant methylation of mismatch repair genes. It has been implied that microsatellite instability is generated by slippage of DNA polymerases during copying of simple tandem repeats and thus repetitive sequences are hot spots for mutagenesis. So far, we lack definitive evidence for mutations in DNA polymerases in tumors that exhibit MIN phenotype. The prevalence of microsatellite instability in tumors harboring mutations in mismatch repair genes (listed in [13]) provides an important indicator of the pervasiveness of MIN in tumors.

The CIN phenotype is characterized by alterations in large segments of chromosomes including: losses, gains, translocations, inversions, deletions and amplifications [14,15]. Unlike cancer cells with MIN phenotype, which are frequently diploid, cells exhibiting the CIN phenotype are usually aneuploid. The majority of human cancers display a CIN phenotype and harbor anueploid cells [16]. Duesberg and coworkers have argued that aneuploidy is the somatic event that initiates tumorigenesis [17,18]. Recently, Rahman and coworkers have provided evidence supporting the link between aneuploidy and cancer, they reported biallelic mutations in spindle checkpoint gene *BUB1B* is associated with aneuploidy in a human disease, MVA (mosaic-variegated aneupoidy) [19]. MVA is a rare recessive disease characterized by early onset of cancer. Thus in at least one rare inherited disease, mutations in a gene that affects chromosomal segregation is associated with human cancer [19].

5. Mechanisms for acquiring random mutation in the genome

5.1. *Mutations in DNA repair genes can increase burden of point mutations and thus can initiate carcinogenesis*

Human cells encode proteins that are tailored to specifically remove different lesions in DNA, e.g. alkylated, abasic and modified bases are removed by base excision repair (BER), UV-damaged, cross-linked DNA are eliminated by nucleotide excision repair (NER) [20]. Increasingly, we are presented with the evidence that many of the DNA repair proteins are mutated or inadequately expressed in human tumors, and consequently resulting in deficits in DNA repair perhaps accounting for or contributing to mutation accumulation during tumorigenesis. In each of above repair pathways, hereditary diseases have been identified that contain mutations in DNA repair proteins. Until recently, the one exception has been BER. However,

hereditary colon cancers that lack mutations in mismatch repair proteins have been found to harbor mutations in the MutY, a DNA glycosylase in the BER pathway. MutY protects against oxidative damage; deficits in MutY result in G to T transversions throughout the genome including the *APC* gene [21,22].

5.2. *Spontaneous mutations generated by low-fidelity DNA synthesis catalyzed by family Y DNA polymerases*

Despite the proficiency of DNA repair in normal human cells, some DNA lesions escape detection and repair and are present when cells divide resulting in misincorporation of non-complementary nucleotides. The classic replicative DNA polymerases (α, δ, and ε) stall upon encountering base adducts. This presented an interesting paradox; many base alterations that block DNA synthesis by replicative DNA polymerase are present in cells and yet cells harboring these DNA lesions replicate their genome and undergo cell division. The recent discovery of a new family of DNA polymerases (family Y: η, ι, κ) known as translesion (or bypass) polymerases and an additional member of family B known as DNA polymerase ζ, which are able to bypass DNA lesions addresses this paradox. The ability to synthesize past lesions thus provides a backup mechanism for rescue of stalled replication forks [23–26]. One model for lesion bypass suggests that at stalled replication forks, family Y DNA polymerases are recruited to bypass the DNA lesion, which is followed by resumption of processive DNA synthesis by replicative DNA polymerases [27]. The bypass polymerases are perhaps one of the major constitutive sources for the generation of spontaneous random mutations — this is clearly the case in bacteria and yeast but remains to be adequately demonstrated in human cells [28]. The Y-family DNA polymerases synthesize DNA with very low-fidelity using both damaged and undamaged DNA templates. Their fidelity has been estimated to be ~1000–4000-fold lower than the replicative DNA polymerases [29]. Loss of Pol η expression in XP-V patients results in a 1000-fold increase in the incidence of skin cancers after UV exposure [30]. It is has been postulated that spontaneous skin cancers are also associated with mutation and/or diminished production of Pol η [31–33]. Synthesis past the UV-lesions is also presumed to occur through collaboration with another error-prone bypass DNA polymerase, Pol ζ. Considering the large number of bypass polymerases, the mechanism for specificity and recruitment is likely to be more complex.

DNA polymerase ι exhibits the lowest fidelity amongst known DNA polymerases [34]. Under certain conditions Pol ι is able to bypass abasic, 8-oxo-dG, and *N*-acetyl-2-aminofluorene-dG lesions [35]. The UV-induced cyclobutane dimers (CPD) blocks Pol ι; bypass is inefficient and occurs in an error-prone manner and is dependent on the sequence flanking the lesion [36,37]. It has been suggested that Pol ι is required for somatic hypermutation in human cells but maybe dispensable in murine cells [38,39]. Because Pol ι violates "Watson–Crick" base pairing complementarity rule during replication of template thymine and is highly error-prone while synthesizing past damaged or undamaged DNA, it has been suggested that Pol ι activity may be a major factor in generating spontaneous mutations [34].

DNA polymerase κ efficiently bypasses abasic site, 2-acetylaminofluorene (AAF), oxygen-damaged thymine (thymine glycols), 1,N^6-ethenodeoxyadenosine and base adduct generated by polycyclic hydrocarbons (benzo[a]pyrene, (B[a]P) that are present in tobacco smoke and environmental pollutants [40–45]. Murine cells lacking Pol κ are highly sensitive to killing and mutagenesis by B[a]P and in contrast to wild type cells, Pol κ deficient cells fail to recover from S-phase arrest after treatment with B[a]P-dihydrodiol epoxide [46,47]. Mutagenic properties of Pol κ may arise from its proficient ability to extend from both damaged and undamaged mispaired primer termini [48,49]. Thus the benefit of ensuring continuity of DNA synthesis by copying the damaged DNA template may result in generation of spontaneous mutations.

5.3. *Unusual biochemical activities of family X DNA polymerases*

DNA polymerases λ, μ, and terminal transferase (TdT) belong to the family X DNA polymerases which includes DNA polymerase-β. Pols λ and μ have been implicated in nonhomologous end joining pathway of double-strand break repair and V(D)J recombination, respectively [50]. TdT is expressed only in lymphoid cells, incorporates deoxynucleotides on to a primer in a template-independent manner, and plays a pivotal role in diversification of variable region of immunoglobulins during V(D)J recombination [51]. The constitutive expression of TdT serves as a useful diagnostic marker for lymphoblastic lymphomas [52]. Pols λ and μ possess exceptionally low-fidelity which is biased toward generation of frameshift mutations [53–55]. They proficiently bypass abasic sites and other DNA lesions by template slippage, microhomology-mediated template misalignment or DNA looping [56–59]. These enzymes have been reported to exhibit unusual biochemical properties; they initiate DNA synthesis de novo, synthesize unusual structures especially at the site of DNA lesion or double-strand breaks and incorporate ribonucleotides in vitro onto a primer in a template-independent manner (Pol μ only) [59,60]. Based on their exceptionally low-fidelity and atypical polymerase activities, Hubscher and coworkers have proposed a model where the unusual DNA structures synthesized by Pols λ and μ at the site of DNA lesions may act as signal for recruitment of DNA damage checkpoint pathways [60]. Therefore, the proposed processing of double-strand breaks by Pols λ, μ and TdT is a highly mutagenic event leading to generation of large number of mutations.

Recently Albertella and coworkers have reported a comprehensive gene expression analysis of DNA polymerases in matched normal and malignant tissues. Family X DNA polymerases β, λ and family Y DNA polymerase ι, were frequently overexpressed, whereas family-Y DNA polymerase κ and replicative DNA polymerases α and δ were underexpressed [61]. The most compelling evidence for the involvement of family X polymerases in human cancer is detection of expression of mutant/variant forms of DNA polymerases β in ~30% (44/149) human cancer [62]. It would be interesting to determine any correlation between expression levels of different polymerases and their effect on mutagenesis.

6. Mutagenesis by activation-induced cytosine deaminase (AID)

In at least one mammalian tissue, mutagenesis is an important physiological mechanism that generates diversity. B cell lymphocytes generate diverse panels of low affinity antibodies. Upon antigenic-stimulation, antibodies with progressively higher affinity are produced by class switching and then followed by somatic hypermutation. Recently, activation-induced cytidine deaminase (AID) has been identified as the key enzyme required for generation of high affinity antibodies. AID is a member of RNA-editing APOBEC-1 family. It is expressed specifically in the germinal center B cells but has been also been detected in low levels in other tissues [63,64]. AID deaminates cytidine residues in DNA resulting in U:G mismatches that are substrates for base excision repair in the targeted sequences [65]. Constitutive expression of AID has also been detected in B cell non-Hodgkin lymphomas and high expression levels have been reported in chronic lymphocytic leukemia [66–68]. AID activity has been reported to be essential for c-myc/IgH chromosomal translocations that are diagnostic of Burkitt's lymphoma [69].

Ectopic expression of AID and APOBEC-1 family members in *Escherichia coli*, mouse pre-B cell line (AID), human B cell and non-B cell lines (AID) result in elevation of mutation frequency of DNA targets [65,70–72]. Liver-specific expression of APOBEC-1 in rabbits and mice can result in liver dysplasia and hepatocellular carcinoma [73]. In another transgenic mice study, constitutive expression of AID in all tissues resulted in development of malignant T-cell lymphomas and micro-adenomas in lung [74]. These combined experiments suggest that deregulated expression of AID and APOBEC-1 family members could result in tumorigenesis by induction of genome-wide random somatic mutagenesis [75,76].

7. Viral induction of mutator phenotype

The possibility that infection by oncogenic viruses can destabilize cellular genome has been frequently considered but has lacked experimental evidence. Viral reverse transcriptases can copy DNA and incorporate non-complementary nucleotides that induce mutations. A mechanism of tumorigenesis by a RNA virus designated as "hit-and-run" has received experimental support by Machida et al. Infection with hepatitis C virus (HCV) both in vitro and in vivo induces mutator phenotype [77]. There is a 5–10-fold elevation in the mutation frequency at multiple loci: Ig heavy chain, BCL-6, p53 and *β-catenin*. Direct sequencing of the target genes (BCL-6, p53 and *β-catenin*) after HCV-infection revealed a wide-spectrum of mutations including single-base substitutions, deletions and insertions. There are multiple sources for these mutations. In tumors (lymphomas and hepatocellular carcinoma) associated with HCV-infection, family Y DNA polymerases ζ, and ι and AID were upregulated. HCV-infection also leads to upregulation of immunologic (type II) isoform of nitric oxide synthase (iNOS), which generates all forms of reactive nitrogen species; NO (nitric oxide), nitrites, and nitrates are mutagenic [77].

8. Mutator phenotype hypothesis versus cellular proliferation and selection hypothesis

To explain the empirical observation that tumor cells harbor large number of mutations, we have previously proposed that cancer progenitor cells must express a "mutator phenotype" which drives selection and evolution of cells to those with unlimited growth potential. The mutator phenotype hypothesis proposed that spontaneous mutations in genome-stability genes, for e.g. DNA polymerases, DNA repair enzymes, DNA damage checkpoint control and chromosome segregation, results in a state of hyper-mutagenesis that increases the probability of acquiring additional random mutations. The random mutations drive the clonal selection for cells with neoplastic properties [78]. Even if expression of mutator phenotype is transient, it is still likely that tumor cells would contain large numbers of clonal mutations that were initially generated as random mutations. It can be argued that a mutator phenotype would generate predominantly mutations that imparted a "reduced fitness" and thus cells bearing this phenotype would undergo negative clonal selection. However, mathematical modeling of elevated mutagenesis suggests that negative clonal selection is unlikely to abnegate mutation accumulation either by cell death or senescence (Fig. 1) (Beckman and Loeb, Genetics, in press). A more likely outcome is the persistence of a mutator phenotype during tumor progression.

It is instructive to consider arguments against the concept of a mutator phenotype in cancer. First, computational modeling studies of Tomlinson and Bodmer indicate that pre-cancerous colonic stem cells need not express a state of hypermutability to acquire large numbers of new mutations; the spontaneous mutation rate exhibited by somatic cells (5×10^{-9}/nucleotide/generation) might be adequate. They postulate that colonic stem cells could undergo several thousand rounds of cell division during a human life span. This model could be sufficient to account for mutations in rapidly dividing tissue that exfoliate but seems inadequate for tissues in which only a small fractions of cells divide such as in liver [79,80].

Second, Wang et al. sequenced 3.2 megabases of exonic DNA from 12 tumor cell lines and revealed only three tumor-specific coding mutations [76]. The authors conclude that because cells lining the intestinal epithelium periodically regenerate therefore the small number of substitutions observed could result from normal mutation rates. However, when this mutation frequency is extrapolated genome-wide; the tumor genome could harbor 3000 mutations. The limitation of their DNA sequencing protocol is the inability to detect random mutations that have occurred after the last few rounds of clonal selection. With DNA sequencing only the most frequent mutation at any position would be detected.

Third, Futreal et al. compiled a comprehensive catalog of genes mutated in human cancer from published literature [81]. Only 1% (291) of human genes in tumors harbor clonal mutations, predominantly within gene families that include protein kinases, transcriptional regulators, and DNA binding proteins. Mutations in genes directly involved in maintenance of DNA sequence integrity like mismatch repair, base excision repair and nucleotide excision repair only accounted for a

very minor fraction. Two points emerge from the above study which argues against the mutator phenotype hypothesis: (1) only a small number of point mutations have been reported in tumors and (2) mutations in genes responsible for maintenance of DNA sequence integrity are rarely found in human tumors. Human tumor tissue comprises of genetically heterogeneous cells and it is possible that some cells may harbor multiple mutations in genome-stability genes and only those that are present in majority of cells would be detectable by direct DNA sequencing.

9. Random mutations in non-coding sequence

We have postulated that random mutations drive the expression of a mutator phenotype and underlie mechanisms that generate tumor heterogeneity. The human genome is comprised mostly of non-coding sequence and probability of acquiring random deleterious mutations in these sequences is much lower compared to coding sequences. Thus it seems reasonable to assume that random mutations should preferentially accumulate in non-coding DNA in tumors and recent techniques have made in feasible to verify this assumption [82]. However, non-coding segments of DNA may also be subject to selection and mutations may have deleterious effects, for example: (1) mutations in enhancer and promoter regions can modify temporal and spatial control of gene expression pattern. Further, if mutations are localized in regulatory regions such as insulator or heterochromatin, which can alter chromatin structure and perhaps global gene expression pattern and may initiate unscheduled epigenetic events. A precedent for such mechanism has already been reported in some sporadic colon cancers, where both alleles of MLH1 gene promoter has been inactivated by methylation resulting in loss of expression of MLH1 gene [83,84]; (2) mutations in the intronic regions can affect splice-site selection resulting in aberrantly spliced message and non-functional protein preferentially in cancer cells [85]; (3) mutation in non-coding 5′ and 3′ untranslated regions can affect message stability and/or half-life of the proteins [86–89]; (4) mutations in the tRNA can result in global mutagenesis at the protein level that would be undetectable by direct DNA sequencing of genes. Such mutators have been described in *E. coli* and pathological consequences of tRNA mutations (nuclear and mitochondrial) in humans are unknown. Rare spontaneous mitochondrial tRNA mutations have been found in patients with cancer susceptibility and neurological dysfunction [90,91]. If the aforementioned mutations are clonal in tumors they should yield clues towards the function of non-coding sequences in cellular metabolism.

10. Consequences of a mutator phenotype in cancer

There are at least three important consequences that can be evoked when one considers the presence of large numbers of random mutations in cancer cells. First, if indeed thousands of random mutations are present within each cancer cell, then a tumor containing 10^9 cells would harbor cells that are resistant to any drug. During treatment these cells would have a selective growth advantage, which could account for the ability of tumors to evade cancer chemotherapy. Second, if a mutator phenotype is expressed and required throughout tumor progression, then agents that inhibit mutation accumulation might delay tumor progression. Third, if cancer cells contain large numbers of random mutations then an increase in mutation frequency might selectively kill cancer cells by exceeding the error threshold required for cell viability. Many cancer chemotherapeutic agents are mutagens and mutation induction may account for their selectivity.

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