Genomic instability in cancer

Garth R. Anderson

Departments of Cancer Genetics and Surgical Oncology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA

An unstable genome is a hallmark feature of nearly all solid tumors and adult-onset leukemias; this instability first appears early in tumor progression, and can take several forms. While the source of instability has been established for many human family cancer syndromes to reside in inherited defects in genes relating to DNA repair, the genes generating genomic instability in sporadic cancers remain largely unknown. A clear pattern has emerged of cancer as a disease of genomic instability within a finite window, leading through accelerated somatic evolution to a genomically heterogeneous population of cells naturally selected for their abilities to proliferate and invade, while simultaneously evading host defenses.

HOW do we know cancer cells are genomically unstable? The hypothesis originated ninety years ago with Boveri's observation of aneuploidy in tumor cells; the concept has greatly expanded as new means to characterize genomes have developed and corresponding supporting data have been generated[1]. The evidence for genomic instability takes two fundamental forms, namely the extensive, progressive genomic damage observed within tumor cell genomes, and the ongoing genomic instability measurable in tumor-derived tissue culture cell lines[2,3]. But like the three blind men characterizing an elephant, current hypotheses all too often tend to attribute the observed genomic instability to whatever single area the model originator has been investigating, obscuring the broad diversity of processes destabilizing tumor cell genomes. At an extreme end of the spectrum, some dispute that genomic destabilization even exists at all in tumor cells, and argue that normal mutation rates occurring during somatic differentiation, development and ongoing proliferation combine to stochastically create a cell with the requisite mutations for malignancy; by such a model this cell then simply proliferates to create the cancer[4]. Much current thinking on the nature of tumor progression parallels the classical Vogelstein colorectal progression pathway, seeing cancer as a well-defined linear process akin to normal, stepwise biological differentiation, but accelerated in its steps due to acquired genomic instability[5]. While each model has a degree of validity, I will present evidence that solid tumors represent a far more chaotic process, with diverse routes to genomic destabilization beginning very early in tumor progression, and with a multitude of predominantly irrelevant genomic events combining with much smaller numbers of significant events to generate enormous heterogeneity within each tumor. Genomic destabilization, Darwinian evolution, and natural selection for invasive, proliferating populations of cells become the essence of cancer. Cancer cells are not intelligent beasts which cleverly learn to escape immunological defenses and therapeutic agents, but instead represent the simple but diverse evolutionary complexity of life itself evolving within the host. And while the processes facilitating which particular somatic evolutionary process predominates may influence the preferred course of events and the corresponding clinical behavior of the tumor, the ultimate outcome for the patient will be largely determined by the most aggressive but not necessarily the initially predominant branches of the evolutionary tree.

Root sources of genomic instability: Damage and repair

Intrachromosomal genomic instability in cancer reflects an increased rate of appearance of DNA alterations in tumor cells, which may arise either from increased rates of damage overwhelming the ability of normal repair systems to restore genomic integrity, or defective repair systems being unable to cope with normal rates of damage being generated through normal cellular and environmental mechanisms. This instability underlies the vast majority of genomic events. Chromosomal instability at the whole chromosome level arises from inappropriate segregation, recombination and the like, and generates relatively few events.

Increased rates of damage may arise from either external or internal sources. Thoroughly studied forms involve exogenous factors such as radiation damage and damage arising from chemical agents; while highly relevant to therapy-induced secondary cancers, exogenous damage cannot directly underlie the ongoing heritable genomic instability seen in cultured tumor cells. Endogenous factors actively and directly increasing genomic damage can include overexpression or improper nuclease sequestration, which has been demonstrated in model systems, or telomere deficiencies generating bridge–breakage–fusion events[6,7]. Telomere shortening is a natural consequence of somatic cell proliferation, and will continue up to that

e-mail: garth.anderson@roswellpark.org

point where apoptosis is activated unless a mutational event activating telomerase occurs.

Inefficient or defective repair unable to cope with normally occurring damage is well documented and provides indirect means of generating genomic instability[8]. This can arise from deficiencies in the repair enzymes themselves, or from checkpoint defects which fail to halt the cell cycle until repair can be effected. The reader is referred to an excellent recent article by Wood *et al*. for a comprehensive review of the genes involved in DNA repair[9].

DNA repair is essential to preserve the fidelity of genomic information, removing damage generated by naturally occurring environmental insults as well as from the inevitable errors arising during a cell's manipulating its genome. The genome is particularly vulnerable during its replication, and segregating chromosomes to daughter cells provides a further opportunity for large losses of genetic information. Damage repair deals with single events through base excision repair, nucleotide excision repair, or mismatch repair. Specialized genes exist for various forms of repair optimized for the particular damage, such as large or small chemical adducts, replication fork errors, or UV-generated cytobutane pyrimidine dimers. Xeroderma pigementosum and hereditary nonpolyposis colorectal cancer provide clear examples of how defects in these genes can contribute to genomic instability and malignancy[8,10].

DNA double strand breaks are normally repaired at high fidelity through homologous recombination repair, which utilizes the sister chromosome as the framework to ensure proper repair. With the human breast cancer genes *BRCA1* and *BRCA2* both involved in this pathway, genomic instability arising from defects in this pathway also can clearly contribute to solid tumor development[11]. Nonhomologous end joining produces an emergency repair of broken chromosomes, although at the cost of not preserving fidelity. Since break originating processes such as deletions, insertions, inversions, amplifications and translocations are abundant in cancers, this pathway is evidently often involved. The Nijmigen breakage syndrome with its defective *nibrin* gene illustrates how defective repair at this level can also contribute to genomic instability and the development of cancer[12].

Where is the genomic damage occurring?

At the largest scale size of events, genomic instability can arise through inappropriate chromosomal segregation, generating gains or losses of entire chromosomes. Losses become particularly consequential if intrachromosomal instability has already generated a number of small events, which can no longer be complemented once diploidy is lost. Trisomies generate consequences due to extra gene copies spanning an entire chromosome, producing in essence chromosome-wide gene amplification. While aneuploidy clearly contributes to genomic instability, the rampant intrachromosomal damage also present in most tumor cell genomes (with the exception of acute leukemias), shows aneuploidy is nowhere near the whole picture.

At a slightly smaller level, chromosomal translocations are widely seen in cancers; these have been invaluable in understanding the genomically relatively stable acute leukemias[13]. By cloning translocation points several activating oncogene systems have been identified such as *bcr–abl*, *myc* and *PML–RARa*. In leukemias the genes altered at the translocation points are providing valuable and effective therapeutic targets, as well as clinically important diagnostic tools. But at this point the picture for chromosomal translocations in solid tumors is much less well characterized; with tumor heterogeneity there appear to be multitudes of secondary events as well as a few key recurring events such as are seen with gliomas[14]. The advent of SKY chromosome painting is permitting rapid progress in this area, although the need to use metaphase spreads generated in cultured cells and its minimal resolving power combine to limit the use of this technology[15]. Higher resolution array-based approaches are not yet suitable to detect translocations at the genome-wide level.

For intrachromosomal instability, comparative genomic hybridization has had an enormous impact by providing a visual image of the larger genomic damage events occurring within tumor chromosomes. This methodology utilizing competitive painting of normal cell metaphase spreads using parallel fluorescent-tagged PCR copies of tumor and normal cell DNA was developed by Kallioniemis and others nearly a decade ago[16]. This approach has been limited by its resolving power of about 10 Mb, revealing allelic imbalances in the form of amplifications and deletions, but not revealing if these events are within intact chromosomes or in alternative forms such as small, multi-copy double minute chromosomes. A further inherent limitation of this methodology is its inability to reveal translocations, insertions, inversions and the like. But its applicability to small biopsy specimens, combined with its widespread application to numerous tumor types, has provided highly valuable insights as to locations of recurrent amplifications and deletions, in turn pointing to naturally selected events that are likely to harbor genes playing important roles in tumor progression. With the advent of comparative genomic hybridization, the number of genomic events believed to be required to produce cancer quickly went up from the 5 or so of the White–Vogelstein pathway, to more than 20.

The recent introduction of array-based approaches to comparative genomic hybridization has substantially increased its resolving power, limited only by the size of the array probes[17]. Chromosomal regions which had been believed to represent single amplicons are now found to represent complex events with numerous small internal

amplifications and deletions. The revealed number of genomic events having occurred per cell correspondingly rises sharply. While arrayed BAC clones have increased the resolution to around 150 kb, in theory there seems no technical reason why the much higher resolving power of SNP arrays cannot be soon brought to bear. Still, this approach remains limited to allelic imbalances, and cannot reveal the full extent of genomic damage.

Anomalous laddering events in allelotyping reactions in 1993 led Ionov and Perucho to identify a new type of genomic instability in cancer, named microsatellite instability for its visible expansions and contractions in microsatellite repeat sequences[18]. This form of instability predominated in the syndrome hereditary nonpolyposis colorectal cancer, but was also occasionally observed in sporadic colorectal cancers. Astute observations by Kolodner and Fishel permitted their recognition that DNA mismatch repair was being affected; subsequent sequencing studies showed most such tumors arose from defects in the *hMSH2* or *hMLH1* genes, or from DNA methylation silencing of *hMLH1* (ref. 19). By measuring the number of events in a sampled microsatellite population, Perucho estimated about 600,000 events were occurring within each tumor cell genome where microsatellite instability was present[20]. Most of these events must be in noncoding repeat sequences, but Markowitz elegantly showed that repeat sequences within the *TGFbRII* gene were the first known functional gene target of this process[21].

The six hundred thousand events for hereditary nonpolyposis colorectal cancer was far, far greater than generally had been hypothesized for sporadic cancers, largely based on CGH data. But how might we learn the number for sporadic cancers, and in particular for sporadic colorectal cancer? We approached this by sampling the genome, testing for genomic events within these samples, and then based on the sample size compared to the size of the entire genome, a simple extrapolation can be used to estimate the total number of events. The problem becomes how best to representatively sample the genome. Working with Daniel Stoler and Mark Basik, we selected the technique inter-(simple sequence repeat) PCR which exploits the several hundred thousand repeat sequences scattered throughout the genome. Some of these will inevitably be in inverted orientation and within a few kilobases of one another, thus enabling a single PCR primer to generate a series of products which may be electrophoretically resolved. This technique had already been used to study primate and plant evolution, so why not apply it to the much more rapid evolutionary processes of cancer? Our studies generated an estimate that about eleven thousand events had occurred within each sporadic colorectal cancer cell, calculated by conservatively excluding amplifications and deletions of more than about two kilobases in size[22]. This precaution was taken since a single large amplification might alter more than one PCR product with a single event, if multiple primer-binding sites should exist within that amplicon. Similarly, large deletion events could affect more than one PCR product by simultaneously removing many primer-binding sites. Without eliminating amplifications and deletions, the estimated number of events rises to about one hundred thousand per cell. Why does not this genomic damage kill the cell? Eukaryotic cells evidently need only about 3,500 essential genes to survive and proliferate, based on yeast. Additional mutations might well prevent normal development, but this is not a concern of the cancer cell.

Rubin and colleagues have argued somatic variation occurring at normal rates can generate these same large numbers of genomic events, with cancer simply the natural outgrowth of a heavily altered but still genomically stable cell[4]. By their model, individual normal, clonal colonic crypts should show a degree of variation similar to that seen when colorectal carcinomas are compared to normal colonic mucosa. A direct testing of this was recently carried out by Bruce Brenner in our laboratory using inter-(simple sequence repeat) PCR as well as loss of heterozygosity assays. In clear contrast to studies comparing tumor and normal tissue, genomic variation between normal crypts was not seen at all with inter-SSR PCR, and loss of heterozygosity differences were minimal (Anderson *et al.*, *Cancer Res.*, submitted). For sporadic colorectal cancer at least, the Rubin model cannot be valid.

Genomic instability in tumors is not limited to the nuclear genome. Vogelstein and collaborators have shown that mitochondrial DNA, at least in colorectal tumors, is also mutated[23]. Events tend to cluster near the DNA replication origin, suggesting a defect in replication fidelity may be generating the instability. Natural selection may then be selecting the most rapidly replicating tumor mitochondria, although not necessarily the most functional.

When does instability begin in tumor progression?

Our findings with inter-(simple sequence repeat) PCR produced an ancillary important finding, as to when in tumor progression does genomic instability begin. The Loeb model of genomic destabilization facilitating the evolutionary process of tumor progression had pointed out the need for genomic instability to begin early. For several years the finding that p53 was mutated only late in progression, combined with the dogma that p53 was the 'guardian of the genome' led many to view genomic instability as perhaps only a consequence of malignancy.

Our studies examining early sporadic premalignant adenomatous polyps of the colon revealed nearly as much genomic damage as had occurred in them as in fully progressed carcinomas themselves[22]. This was initially difficult to understand, in that if tumor progression is an evolutionary process mediated by genomic destabiliza-

tion, then many more events should be present in carcinomas. There was a simple explanation; we had been extracting DNA from million cell tissue specimens, and each sample represented around twenty cell generations. Late occurring evolutionary events would average each other out in the PCR reactions, meaning we were detecting only early events common to most of the evolutionary descendents in the relatively large cell population we were assaying. When laser capture microdissection was brought to bear, allowing analysis of thousand cell specimens, about five times as many more events were revealed in the carcinomas as in the adenomas.

Genome-wide allelotyping examining loss of heterozygosity patterns has independently confirmed that instability is already present at the early adenomatous polyp stage[24]. Similar results are being seen with breast and thyroid cancer, establishing that genomic instability begins early in tumor progression just as Loeb had long predicted. Genomic instability is not a consequence of malignancy; is it really its cause?

The genes behind genomic instability

It is time to momentarily leave the world of experimental evidence and go to a more conceptual level. For only then can we see how the diverse experimental findings fit a coherent pattern.

Our genomes represent the core of our temporary individual existence, reflecting three billion years of Darwinian evolution. While cell proliferation and metabolism were the appropriate primary foci for our primitive single-cell ancestors, the development of more and more intricate multicellular organisms gave rise to correspondingly intricate patterns of cellular regulation, differentiation and coordination. These capabilities have been carefully preserved and perpetuated within our genomes, subject only to the exceedingly slow process of Darwinian evolution itself.

Preserving genomic integrity is of obvious immense value, as manifested by our genome's investment of some 250 genes for purposes of DNA damage repair, more than 230 genes for high-fidelity DNA replication, and perhaps 500 more for chromosome segregation, cell cycle checkpoints, telomeres, centromeres, damage sensing and the like (Table 1)[25]. What happens if any one of these genes should mutate within the host, or be inherited in defective form? Our diploid nature initially will minimize the impact of such events, except for rare dominant cases. But once the remaining normal allele is lost, the genome becomes vulnerable and unless redundant safeguards exist, genomic damage begins. If genomic damage is widespread or an essential gene target is hit, cell death will follow either directly or through activation of the apoptotic response. But what happens when genomic damage is more subtle, accelerating somatic evolution without having severely adverse consequences on cellular survival (Figure 1)? Genes arising during our long heritage of Darwinian evolution will mutate, along with non-coding regulatory sequences as well as marginally significant intervening, non-coding sequences. Most of these mutations will have no discernible significance. But those which promote proliferation, particularly by activating processes in the pathways of signal responsive cell proliferation or eliminating removal of inappropriate proliferative cell populations, will give rise to colonies of expanding cell populations harboring the mutant regulatory gene. This may be seen as a small initially insignificant mass of cells, perhaps as a nevus on the skin or perhaps as an adenomatous polyp in the colon. And from this expanding cell population, out of the sea of expanding genomic damage, new advantageous mutations will be selected for. If an angiogenic factor such as VEGF or bFGF becomes overexpressed, neovascularization will proceed. Lose a proliferation inhibiting gene, and proliferation is further facilitated. Mutate genes controlling protease secretion, and the opportunity arises for such cells to slip between their neighbors. Continue the process, let cells enter and exit the vasculature, and new colonies are seeded. Metastatic cancer, flowing out of genomic destabilization and natural selection, thus becomes a simple concept. But where is the evidence proving the principles of this concept? Do mutations in genes giving rise to loss of genomic integrity truly give rise to cancer? And are there other routes to malignancy?

To answer the last question first, non-genetic routes to genomic destabilization are presumed to exist where long-term exposure to carcinogens occurs, as occurs with tobacco use, environmental radiation exposure and the like. But do we know that processes of ongoing genomic destabilization have not also been activated within the tumors of such individuals? A recent report by Li *et al*.[26] showed that a variety of DNA damaging and non-damaging stress exposures generate heritable genomic instability in a minority but significant fraction of cells surviving the stress; this genomic instability persists more than 30 generations. The likeliest explanation is that

Table 1. Gene families likely or known to contain members contributing to genomic instability in human cancers

Family	Number of known human genes*
DNA repair	247
DNA replication	233
Chromosome segregation	423
DNA damage	228
Cell cycle checkpoint	49
DNAse	26
Recombinase	26

*Data are from the OMIM database[25]. Some overlap exists between families, although the potential number of genes involved in genomic instability remains relatively large.

mechanisms preserving genomic integrity become irreversibly altered at the genetic or epigenetic level during the initial exposure to the stressing agent.

Heritable family cancer syndromes provide conclusive proof of principle evidence that gene-driven genomic destabilization can produce cancer. Hereditary nonpolyposis colorectal cancer, with its origins in defective DNA mismatch repair genes[8], familial breast or ovarian cancer driven by defective BRCA-1 or BRCA-2 producing defective homologous recombinatorial repair[11], ataxia telangiectasia with defective ATM causing defective DNA damage sensing, and the Li–Fraumeni syndrome arising from a defective p53 checkpoint system all prove the eventual oncogenic consequences of genomic destabilization.

What about sporadic, non-familial cancers, which constitute the vast majority of cancer cases? For colorectal cancer, about ten per cent of sporadic cases arise through mutations in hMSH2, hMLH1, or epigenetically through methylation silencing of hMLH1; any of these routes generates microsatellite instability. But for the rest of colorectal cancers, the source of the genomic destabilization remains largely unknown, although microsatellite instability is not seen.

Two routes have predominated in searching for the genes behind genomic instability. Richard Kolodner's group, in particular, has systematically examined yeast mutants containing a reporter plasmid, and several genes have been found which generate genomic instabilities resembling those of human cancers[27]. Studies with knockout mice losing functional DNA repair genes have additionally demonstrated genomic instability and an ensuing propensity to develop malignancies[28].

Man or mouse

Those genes that have the capability of generating genomic instability in lower organism model systems have only rarely been found to be defective in human cancers, and those cases are generally familial cancer syndromes which are themselves rare. And why might such model systems not reflect the genes most often rendered defective in human tumor genomic destabilization? A potential reason is again that the window for the degree of genomic destabilization generally producing cancer is likely to be relatively narrow, and this will differ between species largely as a function of their life spans. That degree of genomic destabilization ideal to complete tumor progression in three to six months or less in the short-lived mouse is very unlikely to be appropriate for the degree of instability needed for human tumor progression occurring over a ten- or twenty-year period, as illustrated in Figure 2. Mice can tolerate natural mutation rates which would theoretically produce cancer in ten years, if mice only lived that long. For all species, evolution presumably has selected apoptotic and other defenses against rapid mutation producing malignancy before reproductive success is assured. Humans must also have selected effective genomic defenses to ensure successful procreation, requiring a much higher degree of genomic stabilization than occurs in mice. And correspondingly, the genetic systems underlying the genomic destabilization and evolution to malignancy occurring in humans must differ from those seen in mice.

The overwhelming evidence for genomic instability facilitating the somatic evolution process of tumor progression consists of the ongoing genomic instability of tumor cells, the multitude of genomic events seen in tumor genomes, the greater number of loss of heterozygosity events seen in colorectal carcinomas compared to premalignant adenomatous polyps, the propensity of DNA repair gene mouse knockouts to develop malignancies, and that the finding that most familial cancer syndromes have their origins in genes known to generate genomic instability. Could there be any alternative explanations? The Loeb argument is very strong for enhanced

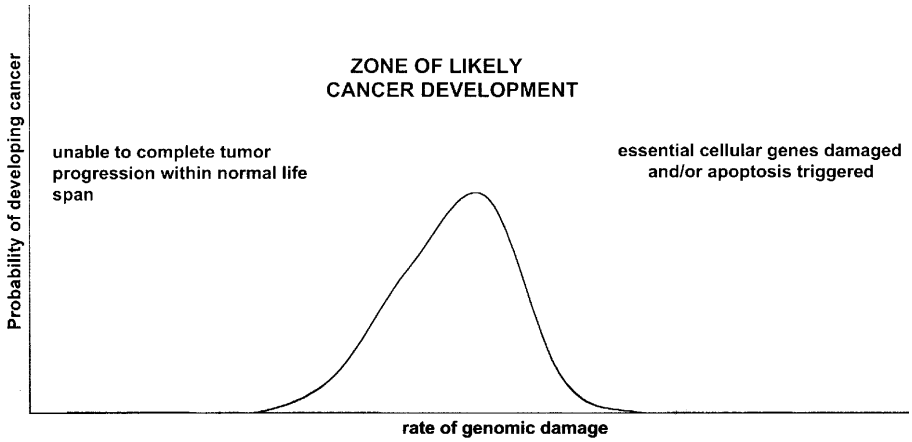

Figure 1. Cancer will develop within only a finite window of genomic instability. Not all genes involved in maintaining genomic integrity will be involved in the progression to malignancy.

mutation rates being necessary to produce even five or six essential mutations in tumor progression. But are the numerous other genomic events truly relevant, or do they conceivably reflect that (i) tumors contain one relatively stable lineage which constitutes most of the tumor, containing few genomic events beyond those essential to generate malignancy, and (ii) other lineages with rampant instability exist transiently but are evolutionary dead ends. Fluorescent *in situ* hybridiation studies show considerable cell-to-cell variability when many markers are examined within a tumor, while other approaches such as comparative genomic hybridiation have suggested far fewer events occur. This has been considered by some to support the transient lineage hypothesis. But these data become reconcilable with the simple Darwinian model as one considers first that evolution within a tumor is an ongoing process, and that events occurring later in progression within a tumor population will average each other out in any assay which pools cells.

But is there direct evidence for tumor progression representing somatic accelerated Darwinian evolution? Characterization of Barrett's esophagus provides one model, where ongoing biopsies of tissue left *in situ* allows reconstruction of the evolutionary pathways occurring over several years. A particularly elegant recent study of bladder cancer by van Tilborg *et al*. examined individual patients over periods as long as fourteen years[29]. With as many as fifteen tumors recurring in a single patient, it was again possible to deduce the direct evolutionary trees. And with forty-eight markers analysed for allelic imbalances on each specimen, it was possible to independently calculate the total number of genomic events to be in the thousands.

Therapeutic implications

With solid tumors arising out of mutational or epigenetic events generating genomic destabilization, followed by many years of ongoing evolution and natural selection, the tumor as it presents clinically thus is already a genomically diverse, heterogeneous population of cells. Many events which occurred early in progression will still be present in most of the tumor cells, and many specific events such as p53 loss will be recurrently but not invariably selected. Particularly aggressive lineages will predominate, creating a degree of homogeneity, but overall the tumor still represents a highly heterogeneous population. So what is the physician to do? Surgical resection of the entire tumor cell population remains the first choice.

If that is not possible or suitable, how can we attack 'the tumor cell'? For some hematopoietic malignancies where overproliferation is the principal selected process, tumor cell genomes are relatively stable and excellent responses may be achieved with either more general antiproliferative agents or with newer, molecularly targeted agents such as STI-571. For solid tumors where genomic heterogeneity is rampant in any given tumor, a far less promising picture exists. If tumors are genomically diverse, where is 'the target'? Is it the first event in the progression pathway, hoping that event itself has never been replaced during the subsequent years of evolution? Is it the genomic instability itself, even though there may be new secondary instability pathways activated later in progression? Or do we simply try to exploit this understanding by developing improved means of early detection, diagnosis, or perhaps prevention through partial restoration of genomic stability?

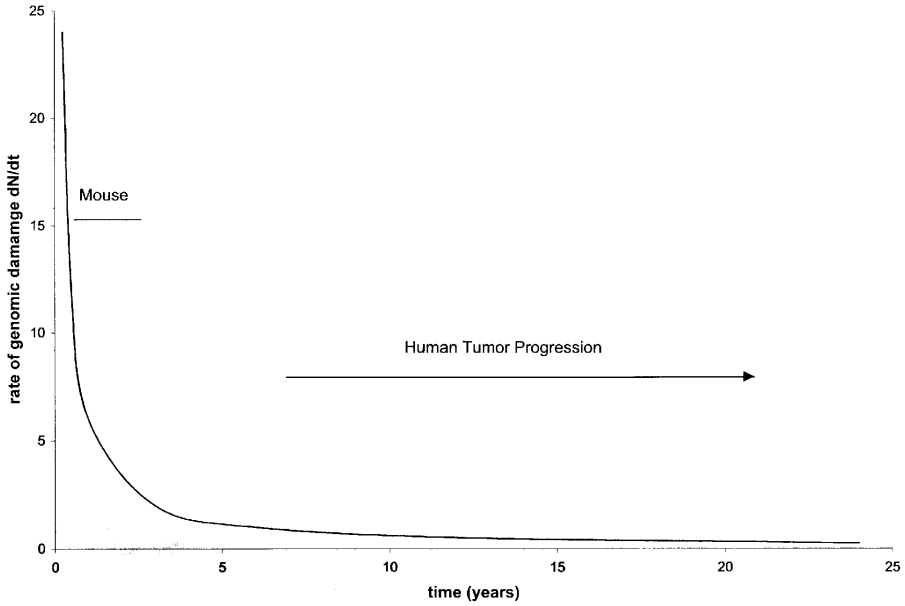

Figure 2. Different lifespans will impact tumor progression. Longer-lived species must have more effective genomic safeguard mechanisms; tumor progression is also able to take a more leisurely pace. Very rapid tumor progression which is essential in the mouse will rarely be seen in human.

There is an alternative. Instead of directly attacking the heterogeneous population of genomically unstable tumor cells, the invariant, genomically stable cells of the tumor vasculature become an especially appealing target[30]. Instead of focusing on the aberrant pathways within each individual tumor cell, or trying to target the proliferative features of tumor cells within a host where normal cell proliferation is also essential, targeting the tumor vasculature composed of relatively recently proliferated endothelial cells appears to offer a way around the problem of genomic instability. With wound remodeling providing a natural pattern for the wholesale destruction of such vasculature, and with compounds such as endostatin already proven effective in animal models, the concept becomes even more attractive. But even here genomic instability can enter the equation as individual tumor cells are likely to produce a diversity of angiogenic factors, and those particular approaches targeting a single angiogenic factor or its receptor may well soon lead to selection for tumor cells producing another alternate angiogenic factor.

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