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Do mutator mutations fuel tumorigenesis?

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Abstract

The mutator phenotype hypothesis proposes that the mutation rate of normal cells is insufficient to account for the large number of mutations found in human cancers. Consequently, human tumors exhibit an elevated mutation rate that increases the likelihood of a tumor acquiring advantageous mutations. The hypothesis predicts that tumors are composed of cells harboring hundreds of thousands of mutations, as opposed to a small number of specific driver mutations, and that malignant cells within a tumor therefore constitute a highly heterogeneous population. As a result, drugs targeting specific mutated driver genes or even pathways of mutated driver genes will have only limited anticancer potential. In addition, because the tumor is composed of such a diverse cell population, tumor cells harboring drug-resistant mutations will exist prior to the administration of any chemotherapeutic agent. We present recent evidence in support of the mutator phenotype hypothesis, major arguments against this concept, and discuss the clinical consequences of tumor evolution fueled by an elevated mutation rate. We also consider the therapeutic possibility of altering the rate of mutation accumulation. Most significantly, we contend that there is a need to fundamentally reconsider current approaches to personalized cancer therapy. We propose that targeting cellular pathways that alter the rate of mutation accumulation in tumors will ultimately prove more effective than attempting to identify and target mutant driver genes or driver pathways.

Keywords

Mutator phenotype; Mutation; Heterogeneity; Cancer genome sequencing; Tumor evolution

1 Introduction

1.1 Do mutator mutations drive tumor evolution?

The concept of a mutator phenotype in human cancers was formulated eons ago [1]. It was put forward to explain the discrepancy between the large number of somatic changes associated with tumorigenesis and the extraordinary accuracy with which human cells normally replicate their DNA. While the genome of each human cell undergoes large numbers of DNA-damaging events per day [2], only a minute fraction escape DNA repair and are present when that genome is replicated. Many of these DNA lesions base pair with non-complementary nucleotides at a high frequency and result in mutant DNAs which, when transcribed, can subsequently alter protein function. Since these mutations are predominantly random, some of them will inevitably occur in genes encoding proteins that function in DNA replication and repair. The resulting amino acid substitutions in these enzymes would therefore result in a further reduction in the accuracy of DNA synthetic processes.

Studies in bacteria [3] and yeast [4, 5] indicate that many mutator mutations confer an initial growth advantage compared to wild type. With each successive cell generation, there is a progressive accumulation of random mutations, including those encoding additional mutators. Furthermore, with every bottleneck in tumor evolution, such as inadequate blood supply or insufficient nutrition [6], the probability of an advantageous mutation arising will be greatest in cells with an elevated mutation rate. Therefore, cells from mutator lineages will be more likely to repopulate the tumor during each successive selective sweep [7]. As a result, cancers progressively express an elevated mutation rate and accumulate mutations throughout their genomes. Considering the number of successive selective sweeps likely required for the evolution of a tumor [8], we predicted that most tumors would contain tens of thousands to hundreds of thousands of mutations [1, 9]. We recognize that epigenetic changes will also occur, contributing to the phenotypic and mutational heterogeneity of cancer cells within human tumors [10, 11]. However, epigenetic changes frequently involve multiple coordinately regulated pathways and currently are not readily subjected to detailed molecular analysis.

The concept of a mutator phenotype in human cancer has been largely ignored because it predicts (1) that many tumors are composed of cells harboring hundreds of thousands of mutations, as opposed to a small number of specific driver mutations [12]; (2) that malignant cells within a specific tumor constitute a highly heterogeneous population [13, 14]; (3) that, as a result, drugs targeting specific mutated genes or even pathways of mutated genes will have only limited anticancer potential [15]; and (4) that within such a diverse tumor cell population, cells harboring drug-resistant mutations will exist prior to the administration of chemotherapy [16], and that that preexisting resistant population will have a selective growth advantage and will rapidly repopulate the tumor.

The vast numbers of mutations found in human tumors and the lack of a substantial reduction in cancer death rates following treatment with most targeted chemotherapeutic agents have refocused our thoughts about the importance of cellular heterogeneity within tumors. We can no longer avoid considering the underlying mutator phenotype in devising

treatments for human cancers [17]. In this article, we present the concept of a mutator phenotype in human cancers, recent evidence that supports this concept, major arguments against the hypothesis, and the consequences of tumor evolution fueled by mutation accumulation. We then consider the therapeutic potential of altering the rate of mutation accumulation to subvert tumor evolution [18–20]. Most importantly, we argue that we need to fundamentally reconsider our approaches to cancer therapy and to take into account the possibility of targeting cellular pathways that alter the rate of accumulation of mutations, instead of focusing on targeting individual driver genes or even pathways of driver genes.

2 Mutator phenotype hypothesis—origins and evidence

The origins of a mutator phenotype in human cancer stem from observations on the multiple chromosomal abnormalities in human cancers and the striking morphological heterogeneity of cells often apparent within individual tumors [21]. This genetic and morphological diversity contrasts sharply with the highly orchestrated and controlled processes usual during normal tissue development and homeostasis. To ensure the remarkable accuracy with which a mammalian cell replicates its diploid genome, comprised of 6×10^9 nucleotides, a multistep process has evolved, catalyzed by multiple components with a variety of mechanisms for removing mistakes [22, 23]. Many gene products are required to maintain genetic stability in normal cells, and mutations in these genes can enhance mutagenesis. The mutation rate of nonmalignant cells, we argued, is insufficient to generate the large number of mutations that are present in human tumors. Instead, we hypothesized that cancers express a mutator phenotype and, as a result, more frequently accumulate mutations [1].

For many years, we and others have further developed the hypothesis that cancer cells exhibit a mutator phenotype (Fig. 1) [24, 25]. The finding of an association between inherited defects in DNA repair and an increased predisposition to cancer was a milestone in establishing the importance of ensuring DNA fidelity as a mechanism of suppressing tumor occurrence. This was first established by the finding that cells from an inherited human disease, Xeroderma pigmentosa, were sensitive to UV irradiation [26]. This single finding canonized the study of DNA repair as relevant to human disease. Subsequently, it has been shown that individuals with this disease carry defects in genes which encode proteins involved in nucleotide excision repair [27, 28] and that engineered mutations in the murine homologs of these genes result in an increase in cancer incidence upon exposure to DNA-damaging agents (reviewed in [29]).

This association of enhanced mutagenesis with development of tumors has been repeatedly modeled in mice that are either defective in DNA repair pathways or express mutator DNA polymerases [5]. In the pioneering studies from the Preston laboratory, the genes encoding the major replicative DNA polymerases, Pol- δ and Pol- ϵ , were replaced by variants harboring substitutions that abrogate their ability to excise mis-incorporated non-complementary nucleotides from the primer terminus [30]. Fibroblasts from these proofreading-deficient mice exhibit a 15- to 100-fold increase in mutation frequency and the mutant mice exhibit a reduced survival that is associated with the appearance of different cancers. In other studies on Pol- δ , mice have been generated with a leucine to lysine substitution at position 604 in the protein domain responsible for nucleotide incorporation,

rendering them error prone [31, 32]. Homozygotes of this genotype are embryonic lethal, and heterozygotes exhibit an increased hazard ratio for the risk of dying with tumors, a situation that is likely to mirror the exposure of humans to environmental carcinogens.

The relationship between somatic mutations and cancer has also been approached using different mathematical models [33–35]. One approach that is limited to oncogenic mutations addresses the question: What is the likelihood of a tumor arising by a non-mutagenic *versus* a mutagenic pathway [17]? If less than three mutations are required, mutator mutations are not favored in the development of cancer. However, if more than three mutations are required, then it is necessary to postulate a mutator pathway to account for the number of mutations. For example: if 12 mutations are required and the mutation frequency is increased 20-fold, then in 170 cell generations, the likelihood of a tumor evolving by a mutator pathway compared to a non-mutator pathway is 5.6×10^7 . Importantly, this result is true over a wide range of values for all relevant parameters. These studies indicate that an increase in mutation rate is more likely for cancers that require large numbers of mutations to be clinically manifested. In addition, these studies indicate that (1) mutator mutations have a greater effect if expressed early in carcinogenesis, (2) the more mutations in a tumor, the more likely the tumor is associated with the expression of a mutator phenotype, and (3) a mutator phenotype markedly increases the efficiency by which a tumor will acquire the multiple genetic changes required during tumorigenesis.

It is somewhat ironic that the strongest evidence for a mutator phenotype in cancer comes from the results of The Genome Cancer Atlas [14, 15]. The major goal of this project was to identify specific cancer-associated mutations common to large numbers of human cancers in the hope of discovering potential targets for chemotherapy; indeed, some new potential targets have been identified as “targetable,” for example IDH1 and IDH2 in gliomas [36]; however, the number of potential targets that are repeatedly mutated has been disappointingly low, particularly in common cancer types. Significantly, as more and more tumor genomes have been analyzed in greater detail, more and more mutations per tumor have been identified [37]. Furthermore, no mutations are invariant in any specific type of histologically designated cancer, and there is very little evidence for a consensus of mutations in any histological type of tumor. Sequencing, even next-generation sequencing, detects only mutations that are present in the majority of cells in a sample and thus is limited predominantly to identification of clonal mutations [15]. In addition, mutations occurring at regions with very high GC content such as telomeres and repetitive sequences, which constitute a substantial portion of the genome, are excluded from analysis due to difficulties in copying these stretches of DNA during PCR amplification and/or due to alignment difficulties [38]. Even with these exclusions, most tumors contain on average 50–100 mutations in exons and >10,000 mutations. More than 200,000 mutations have been identified in glioblastomas and melanomas, implying that the number of mutations required to result in malignancy may be much larger than previously assumed [39, 40]. As a general hypothesis, the malignant state of a tumor is positively correlated with the number of mutations.

The accumulation of mutations within the cancer genome is not necessarily uniform with regard either to timing or spatial distribution. Multiple point mutations, for example, can be

acquired in one-off bursts [41]. Clustering of TpC to TpT mutations occurring on the same strand in breast cancer genomes has also been described [42]. While the mechanism of this phenomenon remains unclear, Roberts *et al.*, by examining the distribution of over 200,000 mutations in 32 cancer genomes, have identified clusters of coordinated C>T mutations that occurred at motifs specific for a subfamily of APOBEC cytosine deaminases [43]. It has been proposed, therefore, that these mutation clusters likely arise in single stranded DNA, formed after replication fork stalling and strand resection at the resulting double-strand breaks, which is subsequently targeted by the specific APOBECs.

Whole genome sequencing has also uncovered a process of genome shattering and reassembly that has been termed chromothripsis [44]. This results in a distinctive pattern of as many as several hundred genomic rearrangements localized to one or a few chromosomes. Again, the exact mechanisms behind chromothripsis remain to be elucidated, although it has been proposed to involve micronucleus formation [45]. Other examples of variable rates of mutation across the genome include the increased rate of heterozygous and homozygous deletion at chromosomal fragile sites [46], the increased rate of end-to-end chromosome fusion caused by telomere attrition and the ensuing cycles of repeated breakage–fusion–bridge cycles [47], and clusters of mutations created by bypass polymerases [48].

3 Arguments against a mutator phenotype

Several arguments have been raised against the concept of a mutator phenotype in human cancers [49]. First, it is argued that most mutations decrease fitness of gene products. This is likely true, however when one considers Darwinian evolution in the context of tumors overcoming environmental bottlenecks that restrict growth, it is apparent that only a few cells harboring a favorable mutation are required for escape. A direct mathematical analysis indicates that the generation of mutations that reduce fitness during tumor proliferation would not be quantitatively significant [50] while studies in prokaryotes have demonstrated that sequential rounds of clonal selection almost invariably result in the emergence of mutators [3]. Comprehensive co-cultivation studies have been performed with *Escherichia coli* strains differing by more than one million fold in replication fidelity. After 320 generations, all winning strains were moderate mutators with 10- to 47-fold lower fidelities than that of wild type [51]. Whether human mutators exhibit a similar competitive advantage either in culture or *in vivo* remains to be established.

Second, aneuploidy, the loss or gain of whole chromosomes, has been proposed to be the primary event in the initiation of tumorigenesis [52]. It is not clear whether a change in ploidy can also be classified as a mutation, in the absence of other alterations and it should be noted that normal human hepatocytes are frequently polyploid. Changes in chromosome number in the absence of genetic instability are, in fact, rare in cancer [53, 54], and as such may be considered a particular form of chromosomal instability.

Third, it has been proposed that the rate of mutation of normal cells is sufficient to account for the multiple mutations in human tumors [49, 55]. This assumes that most of the cells within a tumor are dividing most of the time. It is contingent upon assumptions about cell

proliferation rates prior to the last clonal expansion, cell cycling, cell death probabilities, and the number of premalignant cells. Also, the arguments presented for the adequacy of a normal mutation rate to account for the multiplicity of mutations in tumors assume a mutation rate of 10^{-8} mutations/bp/cell division [55]. In contrast, using data derived from cultured human fibroblasts, we estimate a mutation rate of 10^{-10} [56]. The strongest argument for the adequacy of a normal mutation rate can be invoked for rapidly proliferating epithelial cells such as those that line the crypts of the colon. However, this assertion becomes problematic for tumors that do not rapidly divide. There are many tumors in which the rate of cell division is similar to or less than that of corresponding normal cells. Also, there is considerable evidence for tumor dormancy [57], a situation in which malignant cells are found in isolated nondividing colonies. Furthermore, it is generally assumed that some cancers arise from mutated stem cells; however, we currently lack extensive information on mutation frequency or rates in human stem cells and in cancer stem cells [58]. There is one important report that the mutation frequency in normal stem cells is 100-fold lower than in somatic fibroblasts [59]. If these results also pertain to cancer stem cells, then the somatic mutation rate of the progenitor stem cells would likely be lower than that calculated from human fibroblasts, further favoring the requirement for a mutator phenotype in tumorigenesis.

Lastly, it can be argued that only a limited number of mutations have been discovered in human tumors in genes that encode proteins required for genetic stability. The most direct expectation would be that mutations in these genes would be found more frequently than in others throughout the genome. For example, dysfunction of mismatch repair is common in certain sporadic cancers, frequent mutations in DNA polymerase β have been reported in human gastric tumors, and Pol- ϵ mutations in colon cancers have been reported [60, 61]. There are two important circumstances that could account for the apparent underrepresentation of tumor-associated mutations in genetic stability genes. First, many and possibly the majority of mutations in these genes are likely to be lethal or to be disadvantageous for cell growth. Second, mutations in more than 100 genes in yeast have been shown to suppress genetic instability [62]; thus, it may be difficult to identify potential mutator variants. An extreme example of this is whether mutations of TP53, the most frequently mutated gene in human cancers, can be considered mutator alleles. Dysfunction of TP53 undoubtedly enables the incipient cancer cells to circumvent the normal pro-apoptotic signals and bypass certain cell cycle checkpoints [63]; however, because of its central role in ensuring genome integrity [64, 65], its dysfunction almost certainly decreases the fidelity with which cancer cells can replicate their DNA. In either case, subclonal mutations in this class of genes should be detectable through advancements in DNA sequencing, such as duplex sequencing [66].

4 Random and subclonal mutations in human tumors

Until recently, it has not been feasible to quantify the number of random or subclonal mutations in human tumors. The error rates of conventional and next-generation DNA sequencing are from 1 in 10 to 1 in 1,000 miscalls per nucleotide sequenced [67]. As a result, even with the deepest sequencing, it is difficult to confidently detect mutations at a frequency less than 1 in 10. We have developed a method to measure random sequence

substitutions at restriction sites that render them non-cleavable by the cognate restriction enzyme [68]. Mutations are quantitated by PCR amplification of single molecules using primers flanking the uncut restriction site. This random mutation capture assay offers exceptionally high sensitivity but is limited to restriction sites which are cleaved by highly efficient restriction enzymes. Using intron VI of p53 in a blinded study, we determined that rapidly dividing tumors exhibited an average increase of up to 220-fold compared to paired normal tissues. Since most of the mutations were all found in single DNA fragments, we considered them to be random substitutions. The increase in random mutations has been confirmed using the same assay in mice [69]. Also using this methodology, tumors that harbored a mutation in the FEN1 gene have independently been shown to exhibit a 20-fold increase in random mutations relative to normal tissues [70].

A new method, referred to as Duplex Sequencing, offers the prospect of detecting and cataloging subclonal mutations in human tumors using next-generation DNA sequencing, potentially making any sequence context amenable to analysis [66]. This technique takes advantage of the inherent complementarity of DNA and scores mutants in single DNA molecules only if substitutions are present at the corresponding positions on both strands and are complementary. It eliminates errors due to mis-incorporations during PCR amplification and during copying of damaged DNA templates. As a result, we have been able to achieve a background error frequency of less than 10^{-7} using DNA from normal human tissue; the theoretical frequency of artifactual errors is less than 10^{-9} . Application of this methodology to human tumors and corresponding normal tissues is in progress.

5 Consequences of multiple mutations in human tumors

By the time a tumor is clinically detected, it is about 1 cm² and contains approximately 10^9 cells. It also contains 1,000 to 200,000 clonal mutations as detected by DNA sequencing and is likely to contain an even greater number of subclonal and random mutations (however, this assumption requires further verification) [37]. Since the random mutations are different in each of the tumor cells, a given tumor is likely to contain cells with mutations rendering them resistant to any particular chemotherapeutic agent. There is already evidence to substantiate this assertion. In chronic lymphatic leukemia, mutant cells harboring imatinib-resistant mutations have been detected prior to initiation of chemotherapy [71]. In colorectal tumors, *KRAS* mutations conferring resistance to anti-EGFR therapy have been detected in peripheral blood prior to therapy [72, 73]. The presence of preexisting resistant cells would be an important contraindication for use of specific drugs in cancer chemotherapy. In specific situations, for example in hematological malignancies, it might be feasible to take serial biopsies to detect the preferential growth of cells with resistance mutations, allowing chemotherapy to be altered in response to changes in the tumor composition [17].

The marked heterogeneity of cancers and the Darwinian evolution of a tumor suggest that mutations in tumors may be geographically delineated; that is, different regions of the tumor may have different mutations (Fig. 2). There is already evidence based on DNA sequencing of pancreatic tumors [74] and renal cell carcinoma [75] indicating that different mutations are present at distinct sites within a tumor. If this evidence is substantiated with other tumors, and the intra-tumoral heterogeneity is further magnified by the presence of different

subclonal mutations, then it might be necessary to carry out multiple biopsies on individual tumors in order to determine an appropriate personalized drug therapy regimen.

6 Targeting mutation accumulation during tumorigenesis

For some human cancers, it has been documented that there could be more than one decade between exposure to a chemical carcinogen and the clinical diagnosis of cancer. Efforts have been made to catalog chemical carcinogens in our environment to reduce exposure and thus reduce the initiation of carcinogenesis. Perhaps of equal importance would be the development of drugs to reduce the rate of mutation accumulation particularly in individuals with precancerous lesions. Even a small decrease in mutation rate could profoundly delay the manifestations of tumor, the production of drug-resistant mutations, and metastatic spread [18, 19].

Increasing the mutation load of a tumor beyond an “error threshold” has been proposed as an alternative therapeutic strategy [19]. This concept, referred to as lethal mutagenesis, has been repeatedly demonstrated in RNA viruses and in retroviruses [76, 77]. By increasing mutation frequency above the limit required for maintenance of a viral population, it has been shown that an error catastrophe can be induced which results in the loss of viral infectivity. Populations of rapidly evolving viruses are maintained as a quasi-species; vigorously replicating viruses in the population maintain the rapidity of growth and infectivity, while poorly replicating viruses provide genetic flexibility and facilitate survival [78]. A similar situation may be pertinent to specific human cancers, particularly those that have acquired large numbers of mutations as a result of a mutator phenotype. A further increase in mutation frequency therefore might also result in an error catastrophe. Noncanonical nucleotides that induce an increase in incorrect base pairing may be particularly effective in this regard, particularly if the incorporated nucleotide is poorly repaired. Each round of replication would see an increase in mutation frequency in the malignant population. While a similar increase may occur in normal cells, malignant cells will have previously accumulated large numbers of mutations, placing them close to their error threshold. Clearly, there are important concerns with respect to using mutagenesis as the basis for therapy: could, for example, this strategy result in secondary cancers or even facilitate progression of the disease? Despite these concerns, specific types of cancers, particularly those with high mutation rates, might be amenable to treatment by mutagenic nucleotide analogues.

7 Concluding remarks

The mutator phenotype hypothesis was proposed long before the possibility of conducting whole genome analyses at base pair resolution. The ongoing revolution in DNA sequencing technologies has for the first time documented the extensive mutational diversity common to all solid cancers studied to date, validating a central prediction of the hypothesis. The implications of the mutator phenotype for future development of cancer therapies are stark—the mutational diversity within solid tumors will confound the appropriate selection of targeted, personalized therapies as well as presenting a preexisting reservoir for the emergence of resistance. A fuller understanding of the mutator phenotype however offers

unique avenues for therapeutic intervention. As the rate of mutation fuels disease progression, strategies that attenuate the accumulation of mutation could dramatically slow the rate of progression of early stage cancers. Conversely, by inducing excessive levels of mutation, it might be possible to treat certain tumors by lethal mutagenesis.

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Fig. 1.

Tumor cells carrying mutator mutations will preferentially outcompete non-mutator lineages during repetitive rounds of selection. Multiple barriers exist to prevent tumor progression. The mutator phenotype hypothesis proffers that incipient tumor cells with an elevated mutation rate will preferentially accumulate driver mutations and therefore outcompete non-mutator lineages. The elevated mutation rate of the developing cancer will result in extensive genomic heterogeneity. *Blue circles* represent mutations in genes that result in an elevated mutation rate. *Red triangles* indicate driver mutations that confer a selected phenotype that allows the tumor to overcome a particular barrier to progression and *green bars* represent passenger mutations

Fig. 2.

Multiple premalignant genetic trajectories result in a convergent cancer phenotype within a single tumor. The early acquisition of mutator mutations leads to substantial genetic heterogeneity between the cell populations that will ultimately comprise a cancer. Multiple different combinations of driver mutations give rise to genetically distinct lineages with similar phenotypes but which constitute geographically distinct regions within the resulting cancer. The marked genomic heterogeneity observed between different cancers therefore also exists within individual cancers. *Blue circle* represents mutator mutations that result in an elevated mutation rate. *Red circles* indicate driver mutations that confer a phenotype that allows a given lineage to overcome a particular barrier to progression. *Green bars* represent passenger mutations accumulated as tumor progression proceeds which do not in themselves confer selected phenotypes, however do contribute significant genetic heterogeneity. *Blue and red squares* represent mutator and driver mutations that result in outcompeted lineages that do not progress to malignancy