Random mutations, selected mutations: A PIN opens the door to new genetic landscapes

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Approximately 10 years ago, at a conference meeting, a student asked Lawrence Loeb how many mutations he would expect within a tumor that consists of 10^9 tumor cells. His answer, 10^9 mutations, provoked incredulous laughter. Now, according to Bielas *et al.* (1) in this issue of PNAS, this figure may be even higher and in the range of 10^{12} per tumor. More than a number, the study is a milestone in a 30-year-old project when Loeb suggested for the first time that infidelity of DNA replication may be responsible for tumor oncogenesis (2). Most importantly, however, Loeb's study, together with a recent report from Vogelstein's group (3), makes one reconsider some popular views in cancer research and therapy development.

In 1974, Loeb *et al*. (2) hypothesized that cancers display an increased rate of errors in DNA synthesis and that this trait is causally related to malignancy. The authors foresaw that additional mechanisms might be involved to increase the mutation rate, such as deficiencies in DNA repair. However, they deemed the latter to be more difficult to verify experimentally at that time. They also speculated that errors in DNA synthesis may lead to point mutations in genes regulating the segregation of chromosomes and thus may account for chromosomal aberrations typical for solid tumors. Ironically, during the 1990s, mutations in mismatch repair (MMR) genes constituted the first intrinsic mechanism defined that causes sequence abnormalities in tumors. Loss of function of MMR genes, such as MSH2 or MLH1, leads to changes not only within repetitive noncoding sequences, also called microsatellite instability (MIN), but also within the coding regions of some tumor suppressor genes. MIN is the hallmark of distinct cancer syndromes, such as hereditary nonpolyposis colon cancer, but is also found in various sporadic cancers. In the same decade, various mechanisms initiating chromosomal instability (CIN) were identified, including point mutations in genes that regulate chromosome segregation, as predicted by Loeb. However, for a long time, it was not clear whether the original concept of a "mutator phenotype" postulating an excessive accumulation of point mutations in nonrepetitive sequences could be maintained. As the final missing link, the Loeb laboratory now provides firm evidence that point mutations occur at a 200-fold higher rate in cancers than in normal cells. Thus, after MIN and CIN, point mutation instability (PIN) has finally been identified.

This breakthrough was accomplished by an elegant experimental approach. The task was to identify random point mutations that were subjected neither to selection nor to clonal expansion. Without clonal expansion, however, point mutations in a tumor necessarily escape detection by sequencing of pooled DNA, because they must be present in at least 10% of tumor cells (i.e., in 10^8 tumor cells of a clinically detectable tumor of 10^9 cells). Not knowing the topographical location of randomly mutated cells, one needs a method for bulk DNA, and one cannot use single cells isolated from a tumor. Additionally, the method should enable the discovery of mutations indifferent to the advantage or disadvantage of the cell and should not (at least not obviously) be generated by a known mutational mechanism such as MIN. Furthermore, the assay should likewise discover nucleotide transitions and transversions at a single-molecule sensitivity. Bielas and Loeb (4) invented a strategy that fulfills all of these requirements. They chose a sequence within intron VI of *TP53*, which was enriched by magnetic beads, and after using a limiting dilution approach, they selected those target sequences by PCR that harbored a mutation that destroyed the TCGA motif of the restriction enzyme TaqI. Thereby, the authors (4) were able to screen several hundred megabases of their target sequence (see figure 1 in ref. 1).

There is substantial evidence that genetic instability promotes cancer growth.

Applying this assay, the authors (4) found the frequency of random mutations in various normal tissues to be very low, $<10^{-8}$ per base pair. This is an important figure, because much of the past debate about genetic instability and the number of mutations in human cancers used mathematical models that sometimes predicted much higher frequencies in nonmalignant tissue (in general, the premises of the various authors differ widely; for a comparison, see refs. 5 and 6). The paucity of mutations in highly proliferative colon mucosa, as determined now, indicates that colonic stem cells replicate their DNA with extraordinary precision and are quite invulnerable to carcinogens. In contrast, the colonic adenocarcinoma sample displayed an almost 500-fold-higher mutation frequency. Although the frequency of point mutations in the different tumor samples differed by up to 7-fold, the only normal tissue (renal cortex) for which the assay detected random mutations still displayed 70-fold fewer mutations than the matched tumor. This control sample was infiltrated by inflammatory cells, which may increase random mutations in epithelial kidney cells or which by themselves may have different mutation rates. On average, tumors contain 2.2×10^{-6} mutations per base pair, and therefore an individual cancer cell displays ≈1,000–3,000 random mutations, which sums up to 10^{12} mutations in a tumor comprising 10^9 cells.

Embrace Complexity!

At the time the Loeb group concentrated on random mutations, the team of Vogelstein, Kinzler, Velculescu, and colleagues (3) engaged in a heroic effort to explore the mutation spectra of colon and breast cancers for all available consensus-coding sequences. Using a sequencing approach, they determined the number and identity of clonally expanded and selected mutations. It is therefore particularly informative to contrast their data with Loeb's random

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mutations (1). Of 13,023 genes evaluated, 1,149 were mutated in colon or breast cancer cells lines; 242 of these were confirmed by using microdissected primary breast cancers or colon cancer cells that were transiently propagated *in vitro* or *in vivo* as xenografts. Using an algorithm that accounted for background mutation rates, the authors (3) then determined that 191 of these 242 genes were selected mutations. Interestingly, the 191 mutations in candidate cancer genes (CAN-genes) were extracted from 816,966 putative mutations. An initial reduction of this number was achieved by subtracting 260,000 silent mutations, i.e., mutations not selected. Although the vast majority of these silent mutations may be artifacts or polymorphisms and comprise pooled data of 22 cell lines, each tumor apparently displays thousands of unselected yet expanded mutations shared by at least 10% of tumor cells. Thus, the huge number of 10^{12} random less-abundant mutations suggested by Loeb's team (1) does not seem too unrealistic.

Although the numbers are impressive, we should ask what they mean for our understanding of oncogenesis and cancer progression. Perhaps the most striking result is the finding that each cancer specimen of a given tumor type carried its own distinct combination of the 191 CAN-genes, because no cancer had more than six mutations of CAN-genes in common with any other cancer. This is true even for colon cancers, where the *APC* gene is mutated in 90% of cases, and its inactivation often is a very early event in colon oncogenesis. One could have thought that such a predominant mutation reduces the space of possibilities and hardwires the pathways for subsequent mutations. Surprisingly, this is not case, at least not in an obvious way. Various combinations of mutations apparently confer advantage to cancer cells, and it is certainly time to abandon the oversimplified linear "Vogelgram," in which APC mutations are followed by *KRAS*, *SMAD4*, and *TP53* mutations before the cancer becomes fully malignant (7). It may help to convince Vogelstein followers that this time the (previously often unheard) call for a paradigm shift comes from inside the school itself, where the talk is now of genetic "landscapes" in which cancer cells evolve. But if there are so many possible combinations of mutated genes that are selected, what does this tell us about the role of point mutations in cancer progression?

The PIN-Mutator Phenotype: Early or Late in Tumor Progression?

Loeb (5) originally suggested that the mutator phenotype is required for oncogenesis. There is substantial evidence from hereditary cancers and genetic mouse models that genetic instability promotes cancer growth, specifically for CIN and MIN. For PIN, a mouse model lacking the 3′-5′ exonuclease and thus proofreading activity of DNA polymerase δ (8) is characterized by rapid development of several types of malignancies in the animals. In contrast, inheritable mutations in human polymerases seem to be incompatible with embryogenesis; however, they could occur as somatic mutations and promote cancer growth. For a better understanding of the mutator phenotype, an important application of the Bielas protocol will be to address the question of at what stage of tumor progression the mutation rate increases and in which cells (stem cells or further differentiated cancer cells). Does it rise gradually or suddenly, and is the increase associated with particular tumor types, phenotypes, or genotypes, or any clinical parameter? It is tempting to speculate that such a PIN-mutator phenotype prepares the mutational space from which specific combinations of mutations are subsequently selected. However, there are reasons to think the PIN phenotype is expressed relatively late in cancer progression. Evidence comes from the Sjöblom *et al*. data (3). Although not included in the paper, it can be deduced from the supporting information (3) that a major fraction of the 191 candidate oncogenes were selected after the surgeon had removed the tumors. The breast cancer cell lines displayed on average 12 mutated CAN-genes, whereas the microdissected primary breast cancers harbored on average only six mutations per tumor ($P < 0.004$). Thus, 50% of the CAN-genes were selected during the short time of adaptation to *in vitro* culture and not during the decade's lasting natural progression of the cancer. In addition, the mutated gene with the highest score for being selected in breast cancer was *TP53*. However, it is well known that *TP53* mutations are found significantly less frequently in small than in large tumors (9). Therefore, the generation and selection of TP53 mutations occur in most cases when the tumor grows beyond 1.5 cm in diameter.

Conceptual and Clinical Implications

Although the CAN-genes may be selected late in the local tumor, the mechanisms generating random mutations may be expressed earlier, because the time required for *in vivo* selection is unknown. In any case, the activation of a mutator phenotype likely produces different combinations of CAN-genes in primary tumors and the metastatic seed growing at ectopic sites, because metastatic dissemination is an extremely early event in breast cancer (10). Hopefully, critical reflections of PIN in the context of evolutionary theories of cancer, such as those recently proposed (11, 12), will stimulate approaches to therapy that differ from a thoughtless race for novel inhibitors for each CAN-gene. The papers by Bielas *et al*. (1) and Sjöblom *et al*. (3) send at least a note of caution to those investing money in all-encompassing sequencing approaches of cancer genomes. No doubt additional mutations will be discovered, but the landscape they create seems too wide and broken to strike a tumor at a specific location. Even if a specific inhibitor is available, treatment makes double-mutated clones a frequent occurrence, which is obviously a consequence of selection (13). Thus, the money may be better invested in identifying the origin of the PIN phenotype and its prevention. Even if the mutator phenotype emerges late in cancer progression, its inhibition may slow down cancer progression substantially and prolong the life of cancer patients. This is another hypothesis that Lawrence Loeb suggested years ago (14).

1. Bielas JH, Loeb KR, Rubin BP, True LD, Loeb LA (2006) *Proc Natl Acad Sci USA* 103:18238–18242.
2. Loeb LA, Springgate CF, Battula N (1974) *Cancer Res* 34:2311–2321.
3. Sjöblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, *et al.* (2006) *Science* 314:268–274.
4. Bielas JH, Loeb LA (2005) *Nat Methods* 2:285–290.
5. Loeb LA (1991) *Cancer Res* 51:3075–3079.
6. Tomlinson I, Sasieni P, Bodmer W (2002) *Am J Pathol* 160:755–758.
7. Fearon ER, Vogelstein B (1990) *Cell* 61:759–767.
8. Goldsby RE, Lawrence NA, Hays LE, Olmsted EA, Chen X, Singh M, Preston BD (2001) *Nat Med* 7:638–639.
9. Olivier M, Langerod A, Carrieri P, Bergh J, Klaar S, Eyfjord J, Theillet C, Rodriguez C, Lidereau R, Bieche I, *et al.* (2006) *Clin Cancer Res* 12:1157–1167.
10. Schardt JA, Meyer M, Hartmann CH, Schubert F, Schmidt-Kittler O, Fuhrmann C, Polzer B, Petronio M, Eils R, Klein CA (2005) *Cancer Cell* 8:227–239.
11. Breivik J (2005) *Semin Cancer Biol* 15:51–60.
12. Vineis P, Berwick M (2006) *Int J Epidemiol* 35:1151–1159.
13. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H (2005) *PLoS Med* 2:e73.
14. Loeb LA (2001) *Cancer Res* 61:3230–3239.