

## MECHANISMS OF DISEASE

# The Implications of Clonal Genome Evolution for Cancer Medicine

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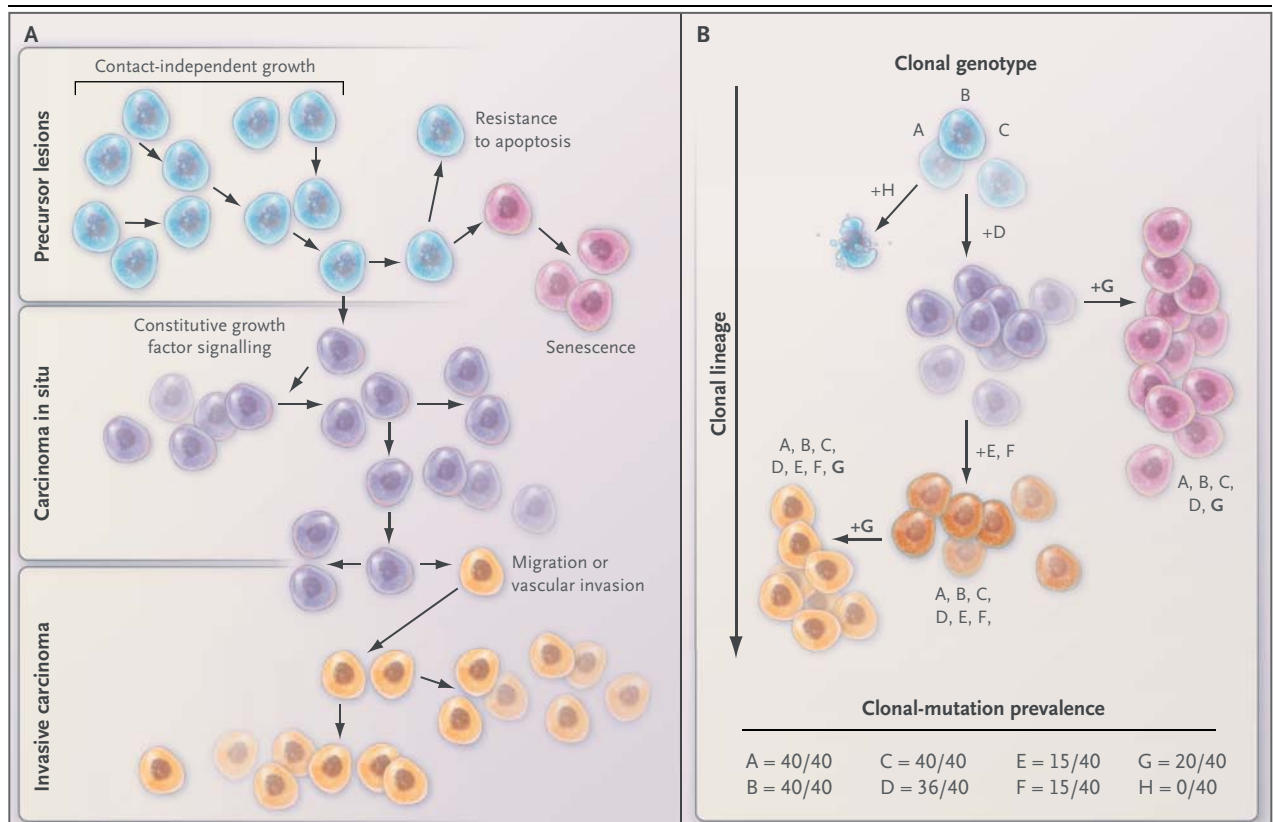
WITHIN THE NEXT 5 YEARS, INTERNATIONAL EFFORTS MAY CHARACTERIZE the distribution of clonally dominant somatic mutations (those present in the majority of cells within a cancer) in more than 21,000 cancers of diverse types.<sup>1</sup> A reduction in costs and improvements in technology have placed the sequencing of patients' tumors within practical reach. Preliminary results suggest that full characterization of cancer genomes can be accomplished in a clinically useful time frame.<sup>2,3</sup> Cancer genomics has been the subject of several recent reviews,<sup>4-7</sup> but these have not focused on the implications and opportunities afforded by the realization that cancers are composed of cellular clones. The notion that most cancers are ecosystems of evolving clones has implications for clinical application; we review these, with particular focus on epithelial cancers.

## DARWINIAN EVOLUTION AND CANCER

Darwin's theory of evolution was originally developed in the context of speciation. It has proved to be a fundamental property of biologic systems, including human cancers. Although tumor evolution has been a foundational concept in cancer biology for decades, a new focus has arrived with the advent of methods that make comprehensive sequencing of cancer genomes tractable for the first time.

In the early 1950s, the chromosomal theory of cancer began to feed into the notion of "stem lines" related by chromosomal variation.<sup>8,9</sup> The concept of clonal structure and evolution in cancers was elegantly synthesized<sup>10</sup> in 1976 by Peter Nowell, crystallizing many prior observations of chromosomal heterogeneity during tumor evolution. Central to these ideas is the notion of a clone, a group of cells related to each other by descent from a unitary origin (Fig. 1). Clonal relationships among cells arise when selection operates on individual dividing cells to confer a survival advantage or disadvantage (Fig. 1A). Selection operates on phenotypes, which may be stable or transient. Stable phenotypes tend to be the consequence of fixed mutations, the most obvious of which are oncogenic mutations<sup>11</sup> or drug-sensitivity mutations. It is likely that tumor-specific epigenetic clonal relationships will be increasingly observed.<sup>12</sup> Clonal relationships can also emerge from the consequences of mutation over time (genetic drift) without selection (Fig. 1B), wherein the genomic differences are probably neutral or passive with respect to phenotypic consequences. In fact, neutral genetic "marking" of cells has been used experimentally for several decades to track cell lineages during development. Finally, selection can operate on transient or unstable cellular phenotypes<sup>13,14</sup> and may not be evident as lineage relationships in the genome or epigenome.

The conceptual framework of tumor genome clonality is supported by many observations in epithelial and hematologic cancers, showing that tumors are composed of clones and that clonal evolution of human cancers underpins the successive ac-



**Figure 1. Clonal Evolution and Clonal Relationships.**

As shown in Panel A, selection operates on the phenotypes termed the “hallmarks” of cancer, giving rise to clones of cells with different properties. Not all properties lead to expansions — for example, increased senescence may lead to eventual stasis or loss of a clone. The acquisition of somatic mutations and alterations in the epigenome (indicated by arrows) can be phenotypically neutral or lead to the acquisition of new phenotypes (cells shaded with distinct colors). As shown in Panel B, progressive accumulation of mutations or other heritable properties leads to clonal relationships. Letters denote a mutation or heritable mark. Colors represent distinct groupings of cells related by descent (clones). Many relationships are possible — for example, mutation G co-occurs independently, leading to two distinct clonal genotypes containing G. The presence of mutations does not necessarily imply selection; the tree depicted could arise owing to random genetic drift. Normal or supporting cells are omitted from this schematic.

quisition of the “hallmarks of cancer.”<sup>15</sup> The idea that human tumors are composed of evolving clones predicts certain features: the existence of clonal genotypes (i.e., not all mutations occur in the same cells), the expansion and decline of clonal populations over time, the existence of internal spatial variation in tumor composition, partial tumor responses to therapy and the emergence of drug-resistant malignant cells, the seeding of metastatic cells from subclones (which may be rare or common in the originating population), the absence of an observable clonal structure based on genome aberrations in some cancers, and the existence of neutral clonal relationships (e.g., arising from random genetic drift) without discernible phenotypic consequences.

#### THE DECIPHERMENT OF CLONAL EVOLUTION

Fixed somatic mutations can be used to infer lineage relationships between cells. Large-scale chromosomal aberrations have been used for decades to elucidate clonal structure in cancers. Until very recently, the ability to systematically enumerate single-nucleotide mutations within tumors was limited. However, the advent of next-generation sequencing devices<sup>16-19</sup> (see Glossary) has dramatically reduced the cost and increased the scale of genome sequencing. Moreover, most next-generation sequencing devices can provide a measure of allele prevalence for almost any aberration found in a genome (Fig. 2).

## Glossary

**Copy-number aberration:** An increase or decrease in the number of copies of DNA segments of tens to hundreds of base pairs in size (normally two copies per cell).

**Epigenetic:** Related to heritable phenotypes or modifications not based in the DNA sequence; DNA methylation and histone methylation are examples.

**Loss-of-function mutation:** A mutation that reduces or eliminates the function of the protein encoded by the gene in which the mutation lies.

**Microarray-based comparative genome hybridization:** A method for detecting copy-number aberrations in DNA.

**Next-generation sequencing (also called second-generation sequencing):** Methods that have in common the generation of a DNA sequence from single molecules of DNA and the simultaneous sequencing of hundreds of millions of DNA fragments at the same time on a single platform (massively parallel sequencing).

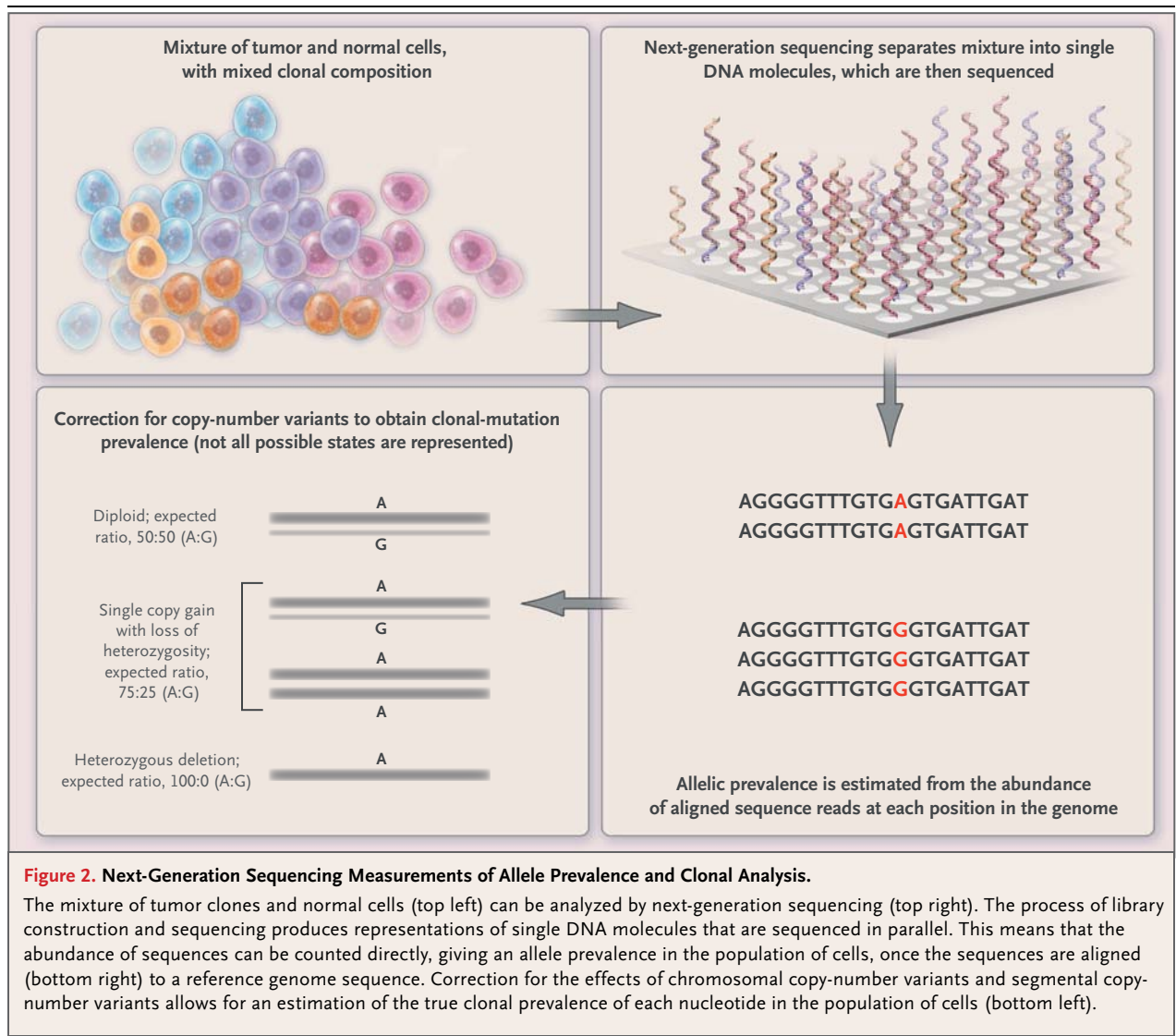
In a mixed population of tumor clones, three key concepts relate the prevalence of fixed aberrations measured in the whole population (allele prevalence) to that of the underlying clones (Fig. 1B). First, clonal-mutation prevalence is a compound measure of the population abundance of the mutation in question and is a function of the size of each clonal population bearing the mutation. Second, a clonal genotype refers to the set of common fixed mutations that define a clone. Third, clonal lineage defines the relationship between clones as they evolve over time. Clonal relationships are usually conceptualized as branched, treelike structures. However, many complex topologies may exist<sup>20,21</sup> with the potential for extinction events, the independent occurrence of secondary mutations (including identical secondary mutations), and mutations occurring at different scales (from chromosome aberrations to single-nucleotide variants) in the genome.

Clonal-mutation prevalence gives a compound measure of the underlying clonal complexity of a tumor (Fig. 1B and 2) but does not allow for direct inference of clonal genotypes. The latter requires single-cell methods,<sup>22,23</sup> which are still far from being routine. Measurements of the prevalence of single-nucleotide clonal mutations can, however, give an indication of how populations of clones evolve over time and space (Fig. 3), provided that chromosomal copy-number aberrations are also accounted for. The first study to show clonal evolution with allele-prevalence measurements from whole-genome sequencing compared a lobular breast-cancer metastasis with the primary tumor from the same patient over a period of 9 years.<sup>24</sup> Measurements of allele

prevalence in diploid or copy number invariant regions in this tumor pair showed that a substantial number of subdominant somatic mutations in the primary cancer evolved to become dominant in the tumor-cell population. With the use of similar approaches, the shift in somatic allele prevalence in a xenotransplanted human basal breast cancer<sup>25</sup> suggested that selection during xenografting rendered the clonal composition more similar to that of a metastasis from the same patient than to the primary cancer of origin.

An important consequence of the clonal nature of cancers is spatial and temporal variation in the composition of primary cancers. This has now been observed in several epithelial cancers with next-generation sequencing methods. The sequencing of genomes of renal cancers,<sup>26</sup> for example, has shown an extensive degree of spatial variation in the clonal composition within single cancers, implying that subregional diagnostic assessment will be fraught with sampling issues. Extensive spatial heterogeneity has also been shown in breast,<sup>27,28</sup> prostate,<sup>29,30</sup> and pancreatic<sup>31,32</sup> cancers. One of these studies showed how different metastatic clones arise from spatially separated regions of the primary pancreatic cancer and subsequent clonal evolution in the metastatic cancers. Extreme variability in the extent of clonal evolution in primary breast cancers has also recently been shown for triple-negative breast cancers<sup>33</sup> and estrogen-receptor–positive breast cancers.<sup>34</sup> In the former study, methods for measuring the prevalence of clonal mutations showed that primary triple-negative breast cancers considered to be similar by current diagnostic assessment methods at the time of diagnosis were at very different stages of genomic and clonal evolution.

Common to the most recent studies are observations of subdominant mutations in known tumor suppressors and oncogenes.<sup>33,35,36</sup> It will be critical to determine a means for gauging which clones in any given cancer are most biologically relevant to disease, such as those with the genotypes that confer risk of progression or drug resistance. When sequential genome analysis has been undertaken, minor subclones in a primary tumor have been shown to eventually become the lethal drug-resistant clones.<sup>29,36-40</sup> The assessment of primary cancers for clones that bear determinants of progression or metastasis is limited by current protocols, which involve biopsy of subregions of tumor tissue.



Clonal relationships may be observed through any heritable mark of the genome.<sup>12</sup> Recent single-cell studies of breast cancers have shown extensive clonal structure as revealed by copy-number aberrations,<sup>27,28</sup> and similar relationships have been inferred at the subchromosomal scale for pancreatic and renal cancers. Not all tumors are necessarily clonally complex at the outset. Recent single-cell sequencing studies — one of a renal cancer<sup>41</sup> and another of a myeloproliferative disease sample<sup>42</sup> — suggested the absence of major clonal variation at moderate-to-high population frequencies but extensive variation of low-prevalence, dispersed mutations. Thus, most of the cancer cells were of one clonal genotype, with a minority of cells containing additional

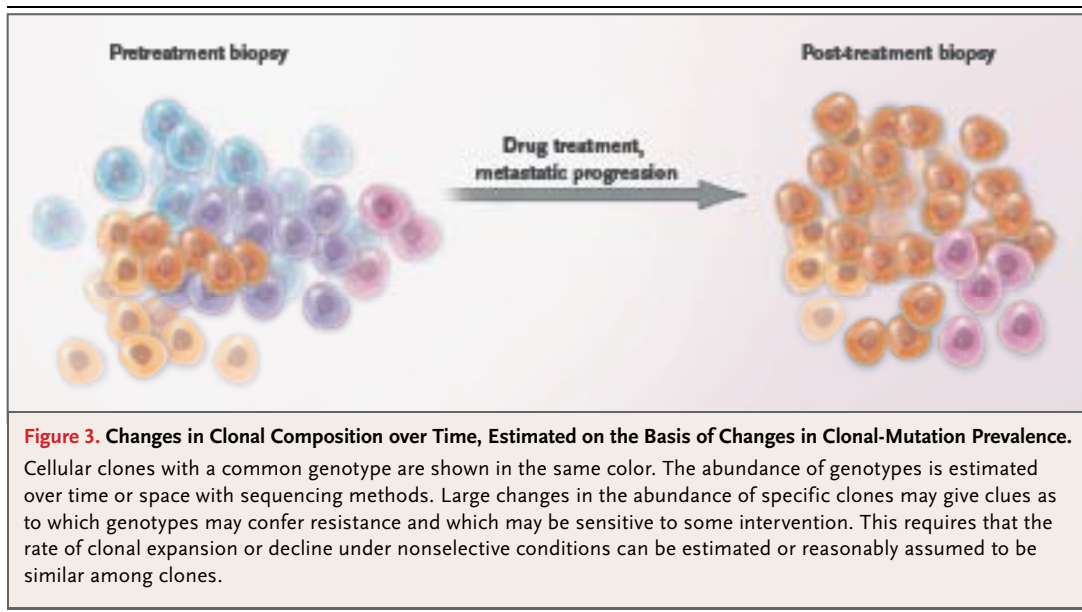
mutations at a wide variety of genomic locations. Determining whether this particular clonal structure is normative will require future studies.

#### IMPLICATIONS FOR CLINICAL ONCOLOGY

##### GENOMIC STRATIFICATION

Many clinical observations of different tumor types (e.g., colorectal,<sup>43,44</sup> breast,<sup>45</sup> and cervical<sup>46</sup> cancer) have documented the variability in responses to therapy that can occur between metastases and even within a single tumor mass. In part, this variability may be explained by the emergence of genomically distinct clones of malignant cells. Yet, genomic stratification of can-





cers<sup>47,48</sup> has to date relied on bulk tumor profiling and hence reflects mutations present only in the majority of cancer cells. Minor subclones can be clinically relevant, as shown in colorectal cancer, in which *KRAS* mutations present in minor subclones identify patients with resistance to anti-epidermal growth factor receptor (EGFR) antibodies.<sup>49</sup> Moreover, intratumoral heterogeneity of *KRAS*, *BRAF*, and *PIK3CA* mutations was noted among at least 1 to 8% of colorectal cancers,<sup>44</sup> a finding that suggests that even among early, “driver” mutations, subclonality occurs sufficiently often to demand explicit assessment. In some cases, primary tumors will need to be subject to multiple sampling, requiring a revised approach to the pathologists’ evaluation of surgical specimens and to initial biopsy assessments. Whether functional imaging of tumors or related techniques can be used to guide sampling remains to be shown, but it is conceivable that discordant results of functional imaging, especially in patients with metastatic disease, may be a consequence of underlying clonal differences.

Clonal heterogeneity itself constitutes a “molecular phenotype.” For example, breast cancers have been characterized with the use of copy-number profiling with microarray-based comparative genome hybridization as monogenomic (cancers that have a dominant clone and no heterogeneity) or polygenomic (cancers that have several clones).<sup>27,28</sup> These patterns of genomic heterogeneity may prove to be prognostic.<sup>50</sup>

Another important issue is stratification of targeted therapies in patients with metastatic disease. At present, this is done mostly with the use of archived primary-tumor material. There is evidence for discordance of mutations between primary and corresponding metastatic disease — for example, mutations in *KRAS*, *BRAF*, and *PIK3CA* in colorectal cancer<sup>44</sup> and in other genes in breast,<sup>24,51</sup> pancreatic,<sup>31,32</sup> and renal<sup>26</sup> cancers and in medulloblastoma.<sup>39</sup> These examples suggest that characterizing and measuring clonal heterogeneity is key to understanding the biology of metastasis at different sites and the evolution of different clones under the selective pressure of therapy (see below). They also underscore the need to rebiopsy tumors in the management of metastatic disease. The implementation of methods for sequencing and clonal analysis of free circulating tumor DNA and fine-needle aspirates may facilitate this in practice.

#### TUMOR MONITORING

How could the monitoring of clonal evolution be made less invasive? The complement of mutations in any clone within the tumor effectively constitutes a “barcode” that uniquely identifies that clone. Whenever cells from the clone die in situ or enter the circulation as part of the dissemination cascade, tumor DNA from these cells may be released into the bloodstream as plasma DNA. If circulating tumor DNA can be detected and sequenced, it can be used to assess tumor dynamics (Fig. 4).

This approach has already been used in metastatic cancers of the colon and breast and in osteosarcoma.<sup>52-54</sup> In all these examples, the detection and measurement of circulating mutant DNA required prior knowledge of the mutations by the sequencing of a tumor sample. A similar approach has been extensively used in leukemia (e.g., testing for *BCR-ABL* translocation in chronic myeloid leukemia) to track leukemic burden after the initiation of therapy or to detect minimal residual disease.<sup>55</sup>

Even more exciting is the ability to use direct sequencing of plasma DNA to identify the mutations in circulating tumor DNA (ctDNA), effectively transforming a blood sample into a “liquid biopsy.”<sup>56</sup> This approach has the potential to be used, for example, in patients with multiple or inaccessible metastases to characterize the mutational complement in some or all of the metastatic lesions. This has been reported for one gene, *PIK3CA*, in which mutations were identified in the plasma of 28 to 29% of patients with metastatic breast cancer. The presence of the mutation in *PIK3CA* in plasma DNA correlated with the mutation status of *PIK3CA* in a metastatic-tumor specimen collected synchronously with the blood sample.<sup>57</sup> The development of ctDNA genotyping for liquid biopsy will depend on the demonstration that mutations are sufficiently sensitive and specific to monitor tumor burden.

Serial sequencing of plasma could also be used to monitor the dynamics of distinct clones and thus be used as a clonal tracking tool. Supporting this proposal were the recent findings that 10 concomitant mutations in a patient with metastatic breast cancer could be monitored by sequencing ctDNA and that the dynamic change in ctDNA bearing the individual mutations varied with the sequential administration of two different regimens of chemotherapy, a finding that suggests a differential response to therapy of the individual subclones.<sup>56</sup>

The extent to which detection and assay of ctDNA will prove useful will depend on the extent to which ctDNA reflects tumor evolution. Two recent reports describe the detection of mutant alleles of *KRAS* in the blood of anti-EGFR antibody-treated patients with metastatic colorectal cancer as early as 10 months, and on average 21 weeks, before any radiographic evidence of disease progression.<sup>58,59</sup> Although preliminary, these results suggest that the genotyping of ctDNA could be used to better understand drug resistance (see

below) and to track newly emerging clones in metastatic disease before these become evident by imaging, allowing therapeutic intervention when the tumor burden is comparatively small. The sensitivity and specificity of the method need to be established in representative patient populations and in different clinical situations.

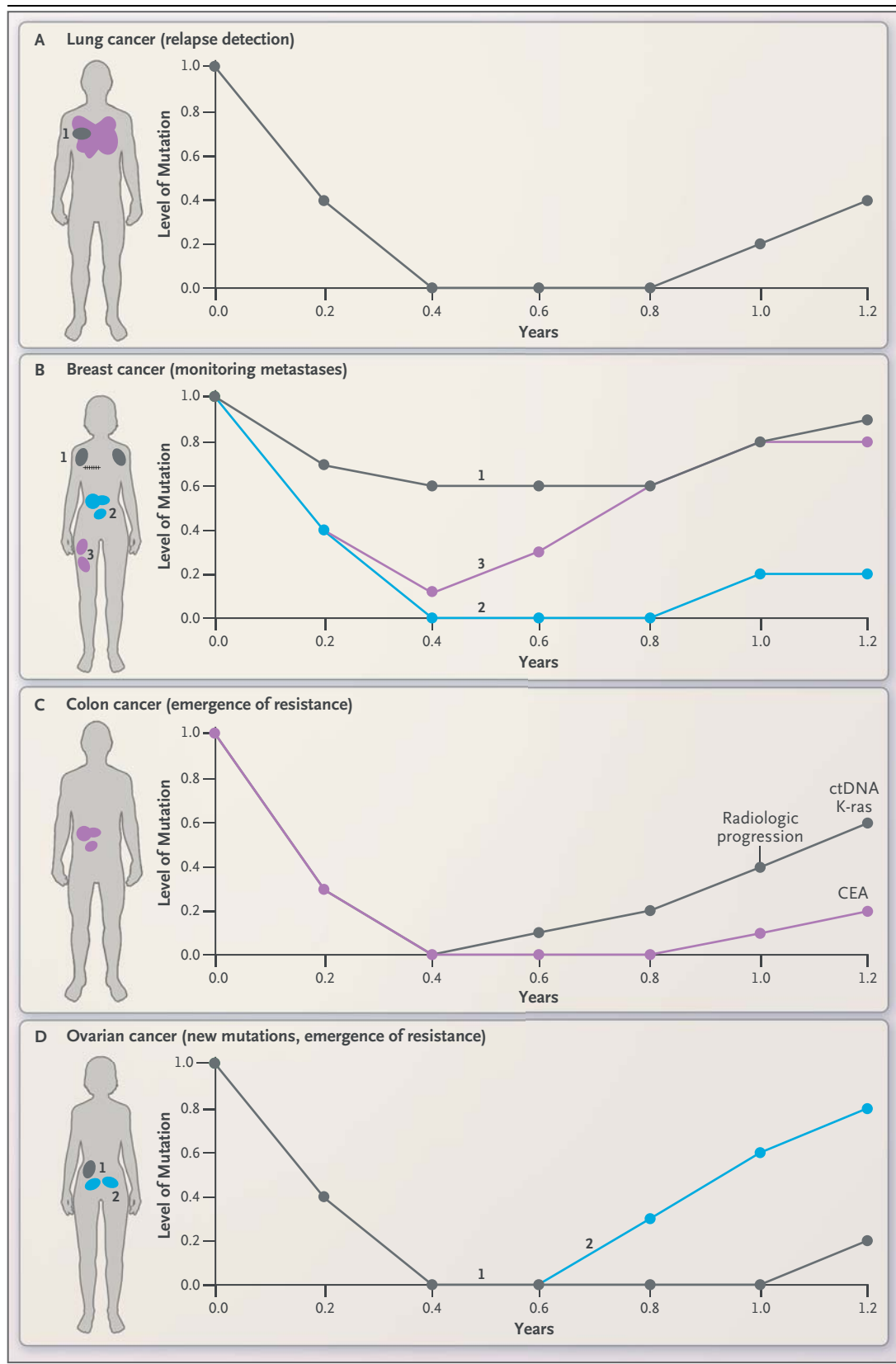
#### THE EMERGENCE OF TREATMENT RESISTANCE

Understanding and being able to pharmacologically counter the clonal evolution of tumors under the selective pressure of therapy are two of the greatest challenges in oncology, and yet determining the mechanisms of resistance and finding combinations of drugs that will suppress clonal evolution are key to treating recurrent disease. The systematic genomic sampling of tumors before treatment, at the completion of treatment if any residual disease is left, and at the time of relapse is critical to meeting these goals (Fig. 4). For every molecularly targeted therapy subjected to a clinical trial, starting with imatinib, resistance eventually develops in patients who initially responded to therapy.

Genomic profiling has consistently identified the mutations that drive resistance. These are often in the gene encoding the target protein, or they are mutations in a gene encoding a protein that bypasses the inhibition of the target.<sup>59-66</sup> Although this appears to suggest a bleak picture for targeted therapies, it also portends the knowledge for tackling the problem by developing new drugs that are active against the mutant resistant to the initial drug,<sup>60,61</sup> by using combinations that inhibit the bypass pathway<sup>62</sup> or by using combinations in efforts to stave off the emergence of resistance, similar to the approach used in controlling human immunodeficiency virus (HIV) infection. Such an approach has already succeeded by trial and error in testicular cancer and childhood leukemias, in which the underlying clonal complexity is not a barrier to successful chemotherapy. The elucidation of clonal genotypes that are responsive or resistant provides a basis for determining which combinations of drugs will be effective in preclinical and clinical studies.

#### MUTATIONAL AND CLONAL ASSESSMENT IN PRACTICE

In the hierarchical model of clonal evolution, early somatic mutations (“stem” mutations) tend to be propagated in many or all clones, whereas later events occur only in some clones (“clade”



**Figure 4 (facing page). Concept of Plasma DNA for Monitoring Circulating Tumor DNA and Clonal Evolution.**

On the basis of a tumor-resection sample subjected to next-generation sequencing, chromosomal rearrangements and point mutations could be identified as tumor “barcodes” (Panels A and B) and used to monitor recurrence or tumor burden. Alternatively, direct sequencing of plasma DNA could be used to identify new mutations in circulating tumor DNA (ctDNA) as a means to the early identification of resistance to targeted therapies (Panels C and D). Colors (and accompanying numbers) indicate different cellular clones. Panel A shows a hypothetical patient with lung cancer treated with curative intent by lobectomy, in whom a chromosomal rearrangement present in all cancer cells in the primary tumor (e.g., *ROS1* translocation) is used for early detection of relapse. Panel B shows a hypothetical patient with breast cancer; at the time of the mastectomy, the tumor was sequenced, showing three mutations (1, 2, and 3) present in different fractions of cancer cells. When the patient presents with metastatic disease, the tracking of all three mutations is used to monitor the burden of different clones within the tumor. Whereas two mutations (2 and 3) suggest an excellent early response, levels of mutation 1 continue to rise in plasma DNA, showing that the cells in the clone containing this mutation are not responsive to therapy. Panel C shows a hypothetical patient with metastatic colorectal cancer, in whom the primary tumor is *KRAS* wild-type; the patient is treated with cetuximab, and levels of carcinoembryonic antigen (CEA) show an excellent response to the anti-epidermal growth factor receptor (EGFR) therapy. Three months before CEA levels start to rise, direct sequencing of plasma DNA is already indicative of the development of resistance because of the emergence of mutant *KRAS* sequences in plasma. Panel D shows a hypothetical carrier of a *BRCA1* germline mutation with advanced-stage ovarian cancer, including multiple intraabdominal tumor masses, in whom assay of TP53 mutation in ctDNA (1) shows a very good response to platinum-based therapy. After 7 months, direct plasma sequencing identifies the emergence of a “revertant” mutation in *BRCA1* (2), providing an early indication of eventual resistance to platinum therapy.

mutations). Pathogenesis-associated driver mutations tend to be identified as early events, whereas the more promiscuous clade mutations may confer additional hallmarks of cancer. Examples of common stem driver mutations include the translocation between *BCR* and *ABL* (*BCR-ABL*) in chronic myeloid leukemia, activating *KRAS* mutations in pancreatic cancer, amplifications of *EGFR* in glioblastoma multiforme, and loss-of-function mutations of *KIT* in gastrointestinal stromal tumor. But clade mutations can also be driver mutations.<sup>63</sup> Most current-generation diagnostic genomic

tests (e.g., mass spectrometry-based and Sanger sequencing-based) for cancer are binary (present or absent) and take no account of the proportion of the tumor that contains the feature (mutation or amplification). Thus, most current mutation-detection tests fail to deal explicitly with clonality and in particular will tend to miss clade mutations. In principle, all somatic mutations in cancer and some clonal structures can now be enumerated by whole-genome deep sequencing. In practice, the sample requirements and analysis required do not yet make this a practical prospect outside research studies.

How might clonal analysis be used in practice in the near term? We envision that, for the foreseeable future, clinical genomics laboratories will need to provide targeted deep-sequence coverage for analysis of clonal-mutation prevalence, focusing on genes or mutations that are already clinically “actionable” and those implicated in conferring the hallmarks of cancer. Examples include *KRAS* mutations to stratify patients with colon cancer for anti-EGFR antibody therapy, driver-mutation barcodes to be tested in plasma for tumor monitoring, and *EGFR* mutations to predict benefit from erlotinib or gefitinib. At current reagent costs, by using a target-enrichment step,<sup>64,65</sup> deep sequencing of 400 to 500 genes (roughly 3 Mb of linear sequence) is in the range of a few hundred dollars, although regulatory hurdles and patent licensing may inflate these costs in some jurisdictions.

**IMPLICATIONS FOR DRUG DEVELOPMENT AND CLINICAL TESTING OF NEW AGENTS**

The ability to track clonal evolution in cancers, both experimentally and in patients, may provide new avenues and opportunities for drug development. Most drugs are still developed against targets expressed in cell lines with unknown or only partially characterized genomic backgrounds. Although efforts are under way to genomically characterize cell lines currently used for drug development,<sup>66-68</sup> the majority of these lack germline reference DNA (which makes it difficult to distinguish germline from somatic variants). The mutational diversity of somatic tumors in patients is also underrepresented for most tumor subtypes by current cell lines. The mutational landscape of cancers suggests a vast array of po-



tentially targetable pathways, yet sorting through the therapeutic hypotheses individually is a daunting task. Through the observation of evolutionary selection — namely, the clonal genotypes that undergo positive or negative selection in patients or in polyclonal human tumor xenografts — focus may be brought to bear on the most important pathways. This suggests that progress may be possible by prospectively developing sequenced polyclonal xenografted human tumors and by mapping clonal genotypes of therapeutic resistance and sensitivity in patients.

A greater issue is that most agents are developed to inhibit single targets (or a couple of closely related targets). Targeting the most common driver mutation alone will not succeed if mutations that confer resistance are already present as minor clones in the cancer (see above). The genetic variability of cancer and its capacity to evolve mean that most single-target approaches select for resistant clones, which expand and become dominant. Durable control of viral replication was achieved in HIV therapy only when triple combinations of antiretroviral agents, sufficient to suppress clonal evolution of the virus, were developed. In the context of cancer research, better knowledge of how single targets could be

combined from the outset is essential. The ability to follow which clonal genotypes are sensitive and which are resistant could be valuable in both the early stages (xenograft studies) and late stages (phase 1–2 trials) of drug development.

Molecular analysis of tumors is still in its infancy in the clinic, in part because the molecular–clinical correlates needed to inform decision making take many years to obtain. In some jurisdictions, the cost–benefit ratio of developing and certifying tests is a financial disincentive. However, mutation sequencing is gradually becoming adopted, as more therapies are linked to the presence of specific mutations or as disease-monitoring paradigms emerge. Tumor clonality adds an important new dimension. In this respect, biomarker or stratification approaches will require overhaul to incorporate clonality measures. An important opportunity may also lie in coupling functional imaging with information about clonal genotype. The clinical-trials community will need to retool to incorporate tissue biopsies and ongoing studies of tumor evolution as part of the correlative science of clinical trials.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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