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REVIEW

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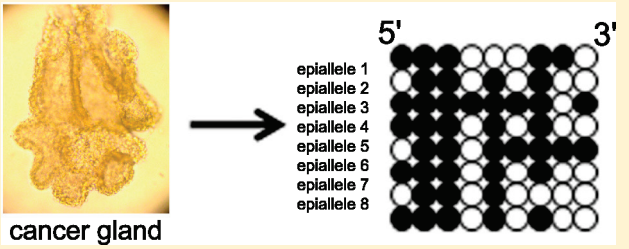
Molecular Tumor Clocks To Study the Evolution of Drug Resistance

Darryl Shibata*

Norris Comprehensive Cancer Center, University of Southern California Keck School of Medicine, 1441 Eastlake Avenue, NOR 2424, Los Angeles, California 90033, United States

ABSTRACT: It is likely that drug resistance evolves after transformation. Exactly how these resistant cells arise is uncertain. This review outlines how the evolution of individual human cancers may be inferred by comparing genomic variation from different parts of the same tumor. The past of a tumor may help predict its responses to chemotherapy.

KEYWORDS: DNA methylation, pairwise distance, cancer stem cells, colorectal cancer, selection



INTRODUCTION

Resistance to chemotherapy is commonly observed, but the mechanisms underlying drug resistance are uncertain and likely to be diverse. One mechanism is tumor heterogeneity, by which subsets of pre-existing resistant cells are already present before chemotherapy is administered. The Codie−Goldman model outlines how cell variants spontaneously arise or evolve during the many cell divisions necessary to form a visible tumor.[1,2] Instead of a homogeneous population of cells with the same susceptibilities, tumors are more likely to be composed of heterogeneous populations of cells with different susceptibilities. The surviving, more resistant variants are responsible for tumor regrowth after chemotherapy.

Another postulated mechanism of chemoresistance is secondary to "cancer stem cells" (CSCs). In this model, a tumor is composed of a hierarchy of self-renewing CSCs and their more numerous non-CSC progeny that have more limited cell division potential.[3,4] Chemotherapy may initially eliminate the more numerous non-CSC progeny, but regrowth occurs because CSCs are intrinsically more resistant and not eliminated, and subsequently produce more non-CSC progeny.

There is evidence to support both mechanisms of chemoresistance. Tumor heterogeneity is a well-known phenomenon,[5] and the purpose of this review is to outline a new approach to address how one can characterize CSCs and tumor heterogeneity in primary human tumors. This approach translates molecular phylogeny techniques to reconstruct ancestral trees of individual human cancers from passenger DNA methylation changes.

HUMAN CANCER ANCESTRAL TREES

Ancestral trees help illustrate the evolutionary relationships between CSCs and tumor heterogeneity (Figure 1). An ancestral tree has four basic parts: a start or common ancestor, present day individuals, and no longer present individuals, which are either ancestors or dead ends (Figure 1A). Translated to an individual human cancer, the start is the first transformed cell, and the tumor contains present day cells. In between transformation and the present day tumor cells are no longer present cancer cells, which are either ancestors or dead ends.[6] All cancer cells (past and present) must fit somewhere on this tree. Conveniently, CSCs fit the definition of ancestors (with present day progeny) whereas dead ends are non-CSCs, including "CSCs" that failed to express their potential. Because all trees have ancestors, all cancers must contain CSCs. CSCs retrospectively defined by ancestry may differ from CSCs or tumor initiating cells experimentally defined by the prospective ability to form xenografts in immunocompromised mice. Nevertheless, ancestors share the tumor propagating properties of CSCs because they were able to undergo self-renewal ever since transformation.

Tumor heterogeneity naturally arises along an ancestral tree because replication errors accumulate within a genome with each division. Starting from the first transformed cell and its genome, subsequent progeny (including CSCs) and their genomes will become increasingly more different from replication errors. The amount of heterogeneity in a present day tumor depends both on the mitotic age of the tumor (numbers of divisions since transformation or the last clonal expansion) and the numbers of long-lived CSC lineages. Tumor heterogeneity is therefore intrinsically linked to CSC frequencies. A relatively homogeneous tumor would have a short mitotic age (i.e., a recent clonal expansion) and few CSCs, whereas a more heterogeneous tumor would have a greater mitotic age and many CSCs.

INFERRING CANCER ANCESTRAL TREES

A goal of molecular phylogeny has been the development of methods and measurements to distinguish between candidate ancestral trees. Molecular clocks (homologous sequences that differ between individuals) are commonly used to infer ancestry.[7] Essentially, the greater the time since a common ancestor, on average the greater the numbers of differences between their

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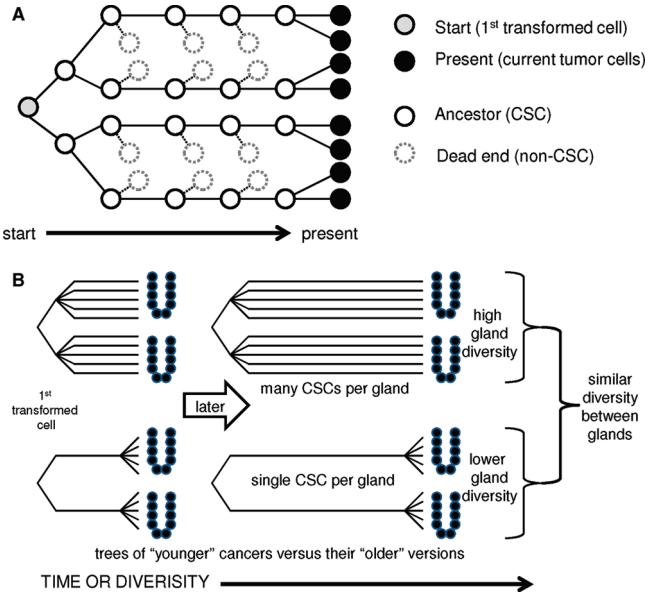

Figure 1. Human somatic cell ancestral trees. (A) A tree has four basic cell types. (B) Two possible cancer trees illustrating the ultimate cancer common ancestor (first transformed cell) and more recent common gland ancestors. Branch lengths and therefore diversity increase with time after transformation. The upper tree illustrates cancer glands maintained by multiple long-lived CSC lineages whereas the lower tree has only a single long-lived CSC lineage per gland. Although the times since the first transformed cell are the same when comparing cells between the glands, the glands from the upper tree will be much more diverse because random replication errors can independently accumulate in the multiple CSC lineages.

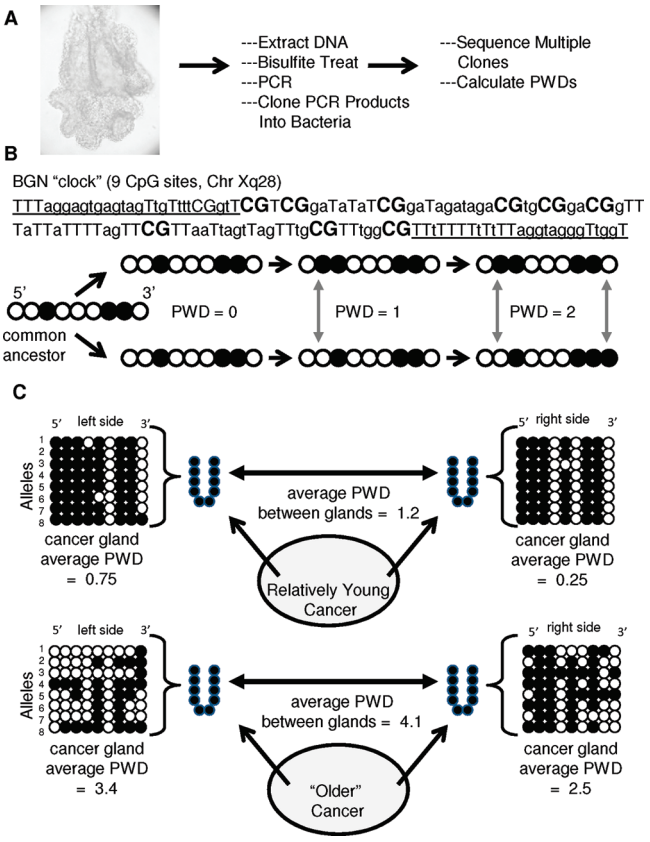

Figure 2. A strategy to measure and analyze colorectal adenocarcinoma genomic diversity. (A) Individual adenocarcinoma glands can be isolated from fresh CRC specimens. The relative diversities of these neighboring cells can be measured by sampling multiple epialleles. (B) Example of an epigenetic molecular clock with 9 CpG sites on the X chromosome. PCR primer sites are underlined. In theory, a single 5′ to 3′ pattern (circles represent CpG sites with filled circles being methylated) in the first transformed cell will initially be similar in all cancer cells, but will subsequently randomly drift with time from independent replication errors. The amount of drift can be quantified with a pairwise distance (PWD) that counts the numbers of differences between homologous sites. (C) Sample data[13] illustrating comparisons within and between glands from opposite sides of the same cancer. Eight epialleles are sampled from each cancer gland, and usually multiple glands are sampled (not illustrated). Average PWDs within glands infer both numbers of CSCs and times since the last common gland ancestor. Average PWDs between glands infer times since transformation since these cells probably last shared common ancestors around the time of transformation. The bottom cancer is more diverse and therefore probably removed later after transformation than the upper cancer.

genomes ("molecular clock hypothesis"). For example, there are more differences between chimpanzees and humans (about 1 base per 100) than between humans (about 1 base per 1,000) corresponding to a divergence of ~5 million years ago between chimps and humans, and the more recent emergence of modern humans out of Africa ~50,000 years ago. Therefore, it is possible to infer something about the past of a population by measuring and comparing the genomes of present day cells or individuals.

It should be possible to distinguish between the human cancer ancestral trees in Figure 1B because a bushy tree with many CSCs should accumulate more genomic diversity compared to a tumor with very rare ancestors or CSCs. Variation that arises in non-CSCs cannot accumulate and is lost in these dead ends. A diverse population contains many long-lived lineages, favoring the evolution of variants (CSCs and their progeny) with different drug susceptibilities.

MEASURING SOMATIC CELL GENOMIC VARIATION

The time frame for somatic cell evolution is relatively short compared to species evolution, and it is difficult to use DNA sequences as molecular clocks because somatic mutations are relatively rare. For example, the frequencies of clonal cancer mutations (present in all cells) are typically lower than one per 100,000 bases,[8,9] and frequencies of new variant mutations that arise in individual cancer cells after transformation are likely to be even lower. To overcome this problem, it is also possible to examine epigenetic genomic variation such as DNA methylation at CpG sites because the 5′ to 3′ order of methylation is usually copied after DNA replication. Methylation patterns are quite diverse in human cancers at certain CpG rich loci,[10] and the 5′ to 3′ order of DNA methylation can be read with conventional DNA sequencing after bisulfite treatment.[11]

DNA methylation has a number of regulatory roles, and patterns in certain genes may reflect functional selection. However, many genes are not expressed in a tissue of interest, and DNA methylation in these regions may reflect passenger of neutral variation. Certain of these CpG rich regions also appear to accumulate methylation with aging in mitotic tissues like the colon, and therefore this methylation may represent neutral replication errors. The variation of these passenger methylation patterns can potentially be used as "epigenetic molecular clocks", with the numbers of CpG site differences a function of the numbers of

divisions since a common ancestor.[12] A pairwise distance (PWD) can be used to count the number of CpG methylation differences between any two alleles, with average PWDs summarizing the diversity or heterogeneity of larger tumor populations (Figure 2). PWD can be used as a surrogate for mitotic age or the numbers of divisions since two genomes shared a common ancestor.

AN EXPERIMENTAL STRATEGY TO UNRAVEL HUMAN TUMOR EVOLUTION

There are a number of barriers to reconstructing human cancer ancestral trees because little is known about the progression of individual human cancers. Genetic and epigenetic variation may arise by a number of mechanisms, and error rates may be variable and depend on the microenvironment. How does one adequately sample the variation in a billion cell tumor and then use this information to reconstruct its tree?

To simplify this process, one strategy is to sample the variation within a single cancer gland (Figure 2A). Glands are small isolated groups of 2,000 to 10,000 adjacent cells, and a relatively easier question to ask is whether neighboring cells are more related than distant cells. Human colorectal adenocarcinomas consist of many such glands, which can be isolated intact from fresh specimens with an EDTA solution.[13] DNA from each gland is extracted and bisulfite treated, PCR products at an epigenetic molecular clock are cloned into bacteria, and then multiple clones are sequenced (Figure 2A). This process is equivalent to the sampling of genomes from individual cancer cells. Typically multiple patterns are present within a gland, and this heterogeneity can be numerically summarized by its PWD, which should on average increase with greater numbers of divisions since a common ancestor (Figure 2B). A new gland formed from a recent clonal expansion would have little diversity, but its diversity should progressively increase with time if the gland is a stable population maintained by multiple long-lived lineages (Figure 1B).

The heterogeneity of a single gland is relatively uninformative, but patterns emerge when multiple different glands from the same tumor are compared (Figure 2C). Essentially we try to ask whether the evolutionary histories of multiple glands within a single tumor are similar or different. At one extreme, all of the glands within an adenocarcinoma share similar histories because they are founded by similar cells. At the other extreme, each gland may have a different history molded by the "chaos" of genomic instability and relentless cycles of stepwise selection in response to its unique microenvironment.

Consistent with the idea that human cancers are removed at different times after transformation, passenger methylation pattern diversities were different between individual human CRCs.[13] Presumably, more diverse tumors were present longer compared to less diverse tumors, but there was no consistent relationship between tumor passenger methylation pattern diversity and tumor diameter.[13] Remarkably, methylation pattern diversities in multiple glands from the same human CRC are usually similar.[13,14] This observation suggests that the ancestries of the different glands within the same CRC are also similar. Two possible scenarios can produce uniform tumor gland ages. In one scenario, selection is so frequent that none of the glands can become very diverse because the mitotic age of a gland is constantly reset back to zero by clonal evolution when a single most fit cell attains dominance over neighboring cells within the gland. In another scenario, selection is so infrequent that all of the glands within a single tumor become progressively more diverse after transformation.

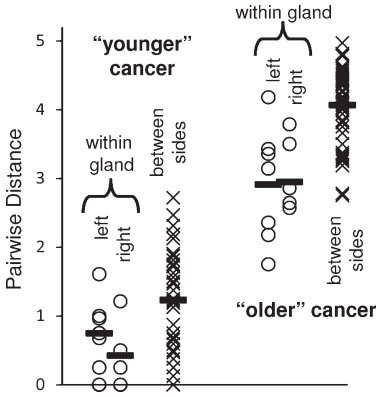

Figure 3. PWDs within glands are nearly as great as PWDs between glands from opposite cancer sides. That PWDs within the glands are smaller than between glands may reflect that, even with high frequency CSCs, many cells within a gland are closely related daughter cells. The scatter of values may be attributed to the stochastic nature of replication errors and CSC renewal.

It is possible to distinguish between these two scenarios by using tumor topography as a surrogate for ancestry. What is needed is a method to compare the mitotic age of a gland (time since a last common gland ancestor) with the mitotic age of its tumor (time since transformation). Is a gland as old as its tumor? It is possible to infer the mitotic age of a tumor by comparing genomes from cells isolated from opposite cancer sides. Although exceptions are possible, cancer cells from opposite sides of an adenocarcinoma are likely to be among the least related cells (i.e., have the greatest numbers of intratumoral genomic differences), with last common ancestors arising around the time of transformation.

This strategy can be employed by sampling tumor glands from opposite cancer sides (Figure 2C). Instead of comparing PWDs or methylation patterns within a gland, we now compare PWDs between glands from opposite cancer sides. Are cells from opposite cancer sides significantly less related than neighboring cells within a small cancer gland? If cancer glands are unstable, rapidly evolving populations, then cells within a gland should be much more related than cells from opposite tumor sides.

By comparing epigenetic molecular clocks within and between glands from the same human CRC,[13] it appears that individual glands are typically nearly as diverse or "old" as their tumors (Figure 3). The implication is that the glands in a single cancer were formed shortly after transformation and that very little selection or clonal evolution has occurred in the intervals between transformation and surgical removal. Most CRCs (10 of 12 analyzed tumors) appear to be relatively simple single clonal expansions.[14] Moreover, simulations based on a number of assumptions are more consistent with relatively frequent long-lived lineages or CSCs within individual cancer glands. Simplistically, if CSCs were extremely rare (fewer than 1 per 100,000 cells), then individual small glands would be relatively homogeneous populations because nearly all of the cells within the gland would be short-lived non-CSCs and related to a very recent common gland ancestor. Simulations infer that the high diversities present in small cancer glands were consistent with between 4 and 1,000 CSCs per 8,000 cell glands.[13]

Much is known about tumor evolution, but very little is known about how any given individual cancer evolved. Therefore, tumor specific ancestries encoded by methylation patterns are uncertain. Furthermore, this analysis of tumor methylation patterns does not reveal information about clonal expansions prior to transformation. Modeling and simulations[13−15] suggest that the PWD variations within and between glands (Figure 3) can be attributed to the stochastic nature of replication errors and CSC renewal. Additional data and better algorithms are needed to better infer human cancer ancestries. Along these lines, an initial analysis of partial DNA sequencing of multiple individual cancer genomes from the same breast tumor also favors a relatively rapid clonal expansion rather than gradual stepwise progression.[16]

SELECTION AND SURVIVAL OF THE FITTEST

Selection is a mysterious parameter that is difficult to measure. It is commonly assumed that selection can exploit even small difference between cells, resulting in relentless cycles of clonal dominance and further progression by increasingly more fit cells. If selection efficiently optimized fitness, it would be difficult for heterogeneity to accumulate within small glands because less fit variants would be quickly replaced by newer, fitter variants. Therefore, the high passenger methylation diversity of individual CRC glands is somewhat surprising because it implies that selection and clonal evolution does not frequently recur after transformation. Cells with different genotypes can also be found intermixed in breast cancers,[17] illustrating the potential weakness of selection to confer even local clonal dominance. Of note, frequent selection would mimic infrequent CSCs because selective sweeps cause bottlenecks that reduce diversity and numbers of long-lived lineages. The relatively high gland passenger methylation pattern diversities (nearly as diverse as their tumors) imply that single cancer cells do not commonly acquire selective advantages sufficient to even allow focal sweeps or clonal dominance over neighboring cells within small glands.

Selection is local in the sense that direct competition occurs between neighboring cells. A cell may acquire a selective advantage over another tumor cell, but such an advantage is moot unless that other cell is its immediate neighbor. A potential paradox is that, within a gland, the closest neighbors are often siblings, yet competition or selection would be least efficient among such nearly identical cells. Perhaps the physical constraints on selection imposed by a glandular architecture are responsible for the maintenance of the high diversity and infrequent selection inferred within human adenocarcinomas. Along these lines, it has been noted that a crypt architecture itself acts as a tumor suppressor mechanism.[18] However, weak selection would also facilitate the random accumulation of drug-resistant phenotypes, which may not confer any selective value until chemotherapy is administered.

EVOLUTION OF DRUG RESISTANCE

Although the evolutionary histories inferred from passenger methylation patterns are uncertain, it is easy to see how drug resistance may evolve within a tumor population after transformation. One type of progression that can produce "flat" cancers with similar ages throughout a tumor is one that starts with a very rapid initial clonal expansion.[19] Instead of stepwise evolution, the first transformed cell may already have most of the capabilities needed for tumor formation.[20] The ancestry that emerges from the sampling and analysis of passenger methylation patterns at a limited number of epigenetic molecular clocks is consistent with Gompertzian growth,[21] where tumors initially grow quickly and then slow such that the growth of clinically detectable tumors is relatively slow. A rapid expansion after transformation would tend to generate tumors with relatively uniform diversities or ages because most glands and lineages are created shortly after transformation. Initially homogeneous glands will subsequently become diverse as cell division continues, with minimal growth if cell division is largely balanced by cell death. The inferred ancestries of human CRCs (many long-lived CSC lineages, little selection) appears to maximize the potential for cancer population diversity, increasing the probability that variant cells resistant to chemotherapy will arise after transformation. That cancers are relatively uniform single clonal expansions helps validate the common practice of basing therapies on the analysis of small random biopsies from large tumors.

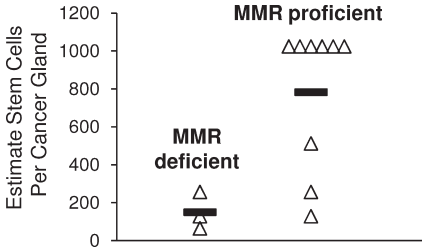

Figure 4. Estimated numbers of CSCs per gland are lower for MMR deficient CRCs. Clinical outcomes for patients with MMR deficient CRCs are generally better, suggesting fewer CSCs (and therefore lower overall cancer diversity) correlate with better survival.

Tumor heterogeneity may play an important role in determining response to therapy. Newer, targeted therapeutics are often characterized by promising responses in tumors that lack certain mutations (such as cetuximab with wild type KRAS CRCs[22]), but relapses are common. Potentially heterogeneous tumors are more prone to relapse because of greater probabilities of containing resistant cells. However, outcomes may also be better with more heterogeneous tumors because they may represent relatively older and therefore biologically more stable or less aggressive tumor populations. It may be that more homogeneous and therefore newer clonal expansions have poorer outcome because they are biologically more aggressive and come to clinical attention sooner. The relationships between tumor heterogeneity and clinical outcome are correlations that remain to be characterized.

An interesting clinical observation is that patients with CRCs with higher mutation rates due to loss of DNA mismatch repair (MMR deficient or microsatellite instability high) tend to have better outcomes.[23] It is possible that chromosomal instability (CIN[24]) more effectively generates drug resistant variants compared to MMR deficiency. Interestingly, estimated numbers of CSCs per cancer gland were fewer in MMR deficient CRCs compared to MMR proficient CRCs (Figure 4). Although the numbers of examined cancers are small,[13] potentially MMR deficient CRCs intrinsically have fewer CSCs per gland. Another possibility is that the 100- to 1000-fold higher point mutation rates in MMR deficient CRCs[25] allow for more genetic variation and selection, and therefore fewer long-lived lineages. In view of the better clinical outcomes of MMR deficient CRCs,[23] and because mutations tend to be neutral or deleterious,[26] one possibility is the lower passenger methylation pattern diversities

are a consequence of greater negative rather than positive selection as cancer cells become less fit after transformation. However the true effects of most mutations in a cancer genome are uncertain. These preliminary data suggesting that less frequent CSCs or long-lived lineages (and less tumor heterogeneity) may be associated with better clinical outcomes.

SUMMARY

This review has attempted to describe methods and a strategy to unravel the evolution of individual human tumors by measuring the genomic variation in cells from different parts of the same tumor. The approach is analogous to studies in population genetics that seek to infer the histories of present day individuals. There are many possible ways a cancer may evolve, and the development of methods to infer the past of each tumor may help guide the treatment of individual cancers. The ability to measure cancer populations before and after therapy may also help unravel exactly why therapies fail. Advances in sequencing will make it increasingly possible to sample and compare multiple genomes from the same tumor. There are multiple ways to measure tumor heterogeneity (genetic, epigenetic, chromosomal structure), and potentially any hereditable variation may encode the past. The ancestries of individual human tumors are likely encoded within their genomes, awaiting to be read with appropriate algorithms and sampling schemes.

AUTHOR INFORMATION

Corresponding Author

*University of Southern California Keck School of Medicine, Norris Comprehensive Cancer Center, 1441 Eastlake Avenue, NOR 2424, Los Angeles, California 90033, United States. E-mail: dshibata@usc.edu. Tel: 323 865 3298. Fax: 323 865 0077.

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