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**REVIEW**

# Clonal evolution in hematological malignancies and therapeutic implications

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The ability of cancer to evolve and adapt is a principal challenge to therapy in general and to the paradigm of targeted therapy in particular. This ability is fueled by the co-existence of multiple, genetically heterogeneous subpopulations within the cancer cell population. Increasing evidence has supported the idea that these subpopulations are selected in a Darwinian fashion, by which the genetic landscape of the tumor is continuously reshaped. Massively parallel sequencing has enabled a recent surge in our ability to study this process, adding to previous efforts using cytogenetic methods and targeted sequencing. Altogether, these studies reveal the complex evolutionary trajectories occurring across individual hematological malignancies. They also suggest that while clonal evolution may contribute to resistance to therapy, treatment may also hasten the evolutionary process. New insights into this process challenge us to understand the impact of treatment on clonal evolution and inspire the development of novel prognostic and therapeutic strategies.

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## INTRODUCTION

The past decade has been a remarkable period of progress in the treatment of cancer in general and hematological malignancies in particular. Much of this progress has been based on exploiting knowledge of the genetic vulnerabilities of particular cancers so that they can be effectively targeted. For example, the impressive efficacy of tyrosine kinase inhibition (abrogating constitutive Abl kinase activity) for chronic myelogenous leukemia (CML) has unequivocally established the paradigm of targeted therapy for the treatment of malignant disease.[1] Likewise, understanding the role of *APML-RARA* in acute promyelocytic leukemia has led to a highly effective regimen with minimal toxicity that overcomes the effects of this gene fusion and that does not include conventional chemotherapy.[2] Collectively, these examples suggest that the promise of precision medicine is finally coming to fruition in the treatment of blood malignancies.

At the same time, this revolution has also taught us important humbling lessons. Targeted cancer therapy, even when achieving highly effective responses, typically provides only short-lived relief. The malignant process often finds alternate routes to circumvent the roadblocks imposed on it by targeted monotherapy.[3–5] An instructive example is the case of Philadelphia chromosome-positive B-cell acute lymphoblastic leukemia ($Ph^+$ B-ALL). The *BCR-ABL1* oncogene is critical for the generation of $Ph^+$ B-ALL, as shown by the high frequency of this lesion in ALL, its adverse prognostic impact,[6] and the strong *in vitro* transformative capacity of this driver.[7] The success of imatinib in the treatment of CML encouraged clinicians to attempt to inhibit the *BCR-ABL1* oncogene in $Ph^+$ B-ALL. Although a high response rate was observed (70% of patients),[8] including in patients with refractory or relapsed disease,[9] the responses were uniformly short-lived with disease progression occurring within weeks. High failure rates were also seen with more potent, second-generation, tyrosine kinase inhibitors such as dasatinib,[10] with the emergence of drug-resistant clones.

Thus, even while the genomic revolution is rapidly expanding the list of potentially targetable genetic lesions,[11] the ability of cancer to adapt poses significant limitations to the therapeutic potential of both standard chemotherapy as well as targeted therapies. As reviewed herein, several lines of evidence lead to an increasing appreciation of the plasticity of cancer—its ability to adapt both to host defenses and to therapy—as an additional facet to consider in the selection and timing of cancer therapeutics.

## CLONAL HETEROGENEITY, THE ENGINE OF CANCER PLASTICITY

Genetic plasticity is defined as one of the enabling characteristics of cancer, in which the acquisition of the multiple cancer hallmarks depends on a succession of alterations in the genomes of neoplastic cells.[12] This plasticity results from ongoing accumulation of additional somatic mutations that are then positively selected. Cases of convergent evolution have been observed in which the same genetic target may sustain several different somatic mutations within the same tumor, yet affecting different subclones (for example, the case of deletion *BTG1* in ALL[13]). These findings strongly suggest that the lesions we detect at the level of large populations of cancer cells are the products of an astonishing amount of genetic 'trial and error' that occurs in every cancerous process at the single-cell level. This high degree of genetic variability provides a ready substrate for an

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evolutionary optimization process, as subclones compete over resources and adapt to external pressures, such as cancer therapy. Cancer progression, therefore, is fundamentally a process of mutational diversification and clonal selection.[14]

The first experimental evidence supporting the idea that tumors are composed of heterogeneous subpopulations was obtained from mouse models of solid malignancies. These experiments showed that individual subclones possessed different phenotypic characteristics, including varying metastatic potential.[15] Importantly, the link between heterogeneity and resistance to therapy was apparent even in other early experiments. For example, cell lines that exhibited a higher degree of phenotypic heterogeneity also acquired resistance to chemotherapy (methotrexate) at a higher rate compared with cell lines with lower phenotypic variability.[16]

As cancer is a disease that results from the accumulation of genetic alterations,[17] a natural corollary of the above studies is that phenotypic evolution must stem from underlying genotypic evolution. This concept has been indeed confirmed over the past several decades with increasing technological sophistication, using approaches based on cytogenetics[18,19] and Sanger sequencing[20] ('first-generation sequencing'). Mullighan *et al.*,[11] for example, in an elegant single-nucleotide polymorphism array analyses of pediatric pre-B cell ALL, demonstrated complex branched evolutionary growth associated with disease relapse. This landmark study further showed how relapsed disease is genetically altered compared with disease at diagnosis. In chronic lymphocytic leukemia (CLL), clonal evolution was identified in up to 43% of patients using fluorescent *in situ* hybridization or cytogenetic techniques, with frequent acquisition of the poor prognostic markers del(11q) and del(17p),[21] and occurring at a higher rate in the poor prognosis group of *IGHV* unmutated cases.[22]

Together, these experimental observations have demonstrated that the genetic makeup of hematological malignancies is constantly reshaped during disease progression. Overall, they support the prescient ideas theorized by Nowell,[23] who postulated that genetic instability would be expected to lead to enhanced heterogeneity with cancer progression, resulting in diverse, genetically distinct, subpopulations within a neoplasm.[24] Thus, the selection process would be expected to promote the outgrowth of increasingly fit subclones, thereby continuously remodeling the fitness of the overall population.

If cancer plasticity is driven by clonal heterogeneity, it is important to consider the features that fuel the generation of clonal heterogeneity.[12] Genetic instability undoubtedly has a key role in this process. The rate of acquisition of novel somatic mutations is probably closely tied to the diversification of the cancer population and therefore for enhancing its evolutionary potential, together with other features, such as the population size[25] (comprehensively reviewed elsewhere[26]). A permissive genetic context that either inhibits DNA repair (for example, *BRCA* mutations) or increases tolerance to novel mutations by removing critical checkpoints (for example, *TP53* or *ATM* mutations, enabling tolerance towards massive genomic damage[27])[28] is likely to increase the overall diversity of the tumor population. Adding to the complexity, different areas of the genome may have different rates of mutations acquisition,[29,30] which would need to be taken into account when inferring past rates of mutations from genomic information. A potentially provocative notion that arises from these data is whether genetic instability may be targeted as a measure to inhibit cancer evolution. For example, for hematological malignancies, the documented ongoing[31] mutagenic[32] activity of enzymes responsible for B-and T-cell receptor genetic modifications may be of particular interest.

## UNRAVELING CLONAL COMPLEXITY WITH MASSIVELY PARALLEL SEQUENCING (MPS)

Although confirming the basic tenet of cancer as an evolutionary disease, the above described studies are inherently limited in their ability to decipher the true extent of genomic heterogeneity, given the limited amount of genetic lesions studied at any experiment and the limited sensitivity of experimental techniques that were then available to detect smaller subclones. Both of these limitations have been largely overcome with the advent of MPS. MPS of tumors has afforded an exponential increase in the ability to characterize the genetic landscape of cancer.[33] It has revealed a very high degree of intertumoral heterogeneity (that is, different genetic lesion affecting different tumors), with hundreds of different mutations affecting different tumors with a probable effect on fitness.[34,35] Moreover, this technology has also revealed a high level of intratumoral genetic heterogeneity (that is, different genetic lesions affecting different subclones within an individual tumor), which also affects putative driver events.[36] In particular, the advent of MPS has allowed researchers to identify both subclonal somatic copy number alterations (SCNA) and subclonal somatic single-nucleotide variations (SSNV),[37,38] which can be tracked over time to study tumor evolution.[39,40] This ability to reconstruct the clonal structure is derived from an inherent property of MPS. It involves generating billions of independent sequencing reads, each derived from a single DNA molecule.[33] Thus, MPS data represent an informative random sample of individual DNA molecules contained within a tumor. At SSNV sites, the number of sequencing reads supporting the alternate and reference bases can be used to calculate a quantitative measurement of the variant allelic fraction (VAF).

In samples derived from diploid cancers such as acute myeloid leukemia (AML) that essentially lack SCNAs and are not contaminated with non-malignant cells, allelic fractions can be used to estimate SSNV clonality directly (in which any clonal SSNV should have a VAF of 0.5; while SSNVs in subclones will have lower VAFs). However, the vast majority of human cancers contain frequent SCNAs,[41] with many of them having undergone whole genome doubling during their evolution.[37] In addition, most tumor specimens contain a substantial fraction of normal cells.[37] Thus, in order to accurately infer the fraction of tumor cells that contain an SSNV from MPS data, it is necessary to account for both the copy number at the SSNV site and the overall tumor purity in the sample.

Recently, inference methods have been developed which attempt to account for these factors in order to estimate the actual cancer cell fraction harboring a specific mutation[38,42] (Figure 1). Although moderate sequencing depth may result in considerable uncertainty in the cancer cell fraction estimates of individual mutations, the fact that subclonal mutations are expected to co-occur in discrete subclonal cell populations has formed the basis of using clustering techniques to better resolve the subclonal structure of bulk tumor samples.[38,42]

Because DNA from all cells present in the bulk sample is mixed together before sequencing, information regarding which mutations co-occur in specific subclones is not readily accessible from analysis of a single sample. In general, analysis of bulk DNA from a single cancer sample cannot rule out the possibility that all subclones are nested inside one another in a linear phylogeny. One exception to this was reported in a study that applied deep whole genome sequencing (188X) of a single primary breast cancer sample and could infer branched evolution based on co-occurrence of informative alleles on sequencing reads.[38] Although this approach represents an elegant solution to the phylogenetic inference problem, the deep coverage needed over the whole genome renders it impractical for large studies using existing technology. We note that sequencing platforms capable of reliably producing longer reads will make this approach far more powerful.

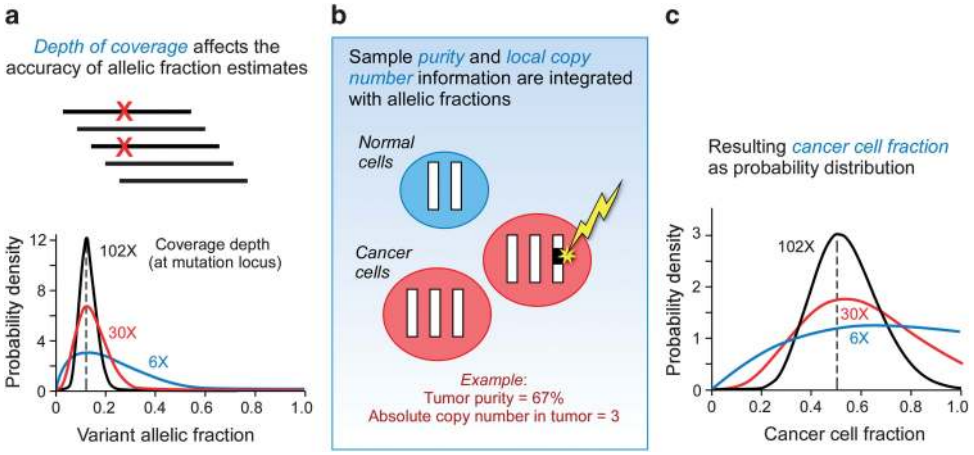


**Figure 1.** Inferring the size of a subpopulation affected by somatic mutations from genomic data. MPS provides an estimate of VAF, which is calculated by counting the number of reads with the variant alleles and dividing it by the total number of reading from the specific location. The certainty of the estimate is a function of the depth of coverage, using the Beta distribution (**a**). Subsequently, the VAF estimates are integrated with the purity and local copy number information (**b**) to yield cancer cell fractions (**c**). In the example provided, a somatic mutation with a VAF of 0.125, a local copy number of 3 and a purity of 67% yields cancer cell fraction estimates of 0.5.

An attractive approach to inferring phylogenetic structures from analysis of bulk tumor DNA is to sequence multiple specimens from the same individual's cancer. Branched evolutionary relationships can be detected as clusters in which one subclone may increase in frequency while another sibling subclone may exhibit a concomitant decrease in frequency. This approach has been used to identify branched evolution in leukemia samples taken before and after treatment[39,40] as well as in solid tumors sampled at multiple anatomical locations.[36]

## THE SURPRISING ASPECTS OF CLONAL COMPLEXITY OF HEMATOLOGICAL MALIGNANCIES

One of the first key lessons gleaned from genome-wide studies of hematological cancers is that clonal evolution which follows a complex branched path (where multiple subpopulation co-exist in the same tumor and compete for ascendency) is at least as common as a more linear trajectory (in which progeny clones replace parent clones in full selective sweeps). A traditional linear model of successive clonal expansions[43,44] could have been expected of hematological malignancies, by virtue of the mobile nature of their cellular normal counterparts (compared with solid tissues that are often embedded in fixed tissue architecture). In theory, this feature could theoretically have led to a decreased level of clonal complexity as cancer cells can readily move across tissues and hence undergo more homogenous cellular mixing. This scenario is unlike solid tumor malignancies, in which the spatial compartments are formed. To the contrary, however, whole genome/exome investigation of clonal evolution in AML,[39,45] myelodysplastic syndrome,[46] multiple myeloma[47,48] and CLL[40] have all consistently demonstrated not only a high degree of clonal heterogeneity and marked changes in the genetic makeup of the disease upon relapse but also branching rather than linear as the predominant pattern of evolution (Table 1). A major implication of these findings is that the evolutionary process is expected to result from complex interactions among multiple highly diverse populations rather than a clear succession of selective sweeps. Clonal competition among co-existing subpopulations that harbor driver lesions[49] thus shapes the eventual composition of the tumor such that multiple clonal variants are present at the same time.[50,51]

It is important to note that the published analyses to date have been limited to the detection of macroscopic clonal heterogeneity (clone size $>$1–10% of the entire cell population). This is because only clones that either represent a substantial proportion of the cancer cell mass or clones that become dominant at some point during the studied period are trackable using current methodologies. Emerging technologies capable of achieving deeper sequencing depth of bulk DNA[52,53] or single-cell genomic sequencing methods[54] may enable the study of smaller subpopulations. Delineating the full extent of cancer heterogeneity down to the single-cell level will enable us to understand how the seemingly stochastic process of 'trial and error' at the single-cell level is integrated through selection to shape the genetic makeup of the tumor. It carries the potential to refine the dichotomy of driver vs passenger mutations, by quantifying the fitness contribution of each individual mutation to selection (manifested in varying clone sizes).

## EPIGENETIC CLONAL HETEROGENEITY

Although genetic alteration has been the main focus of evolutionary dynamics in cancer thus far, epigenetic modifications are probably responsible for a large part of phenotypic differences[55] that ultimately affect fitness. Similar to genetic alterations, epigenetic modifications are heritable and therefore subject to natural selection. The contribution of epigenetic modification to selection in cancer is probably substantial, as epigenetic alterations accumulate as the cell population evolves and diversifies at rates estimated to be orders of magnitude higher compared with somatic genetic alterations.[56] Indeed, a large degree of intratumor epigenetic heterogeneity was recently described in lymphoma using DNA methylation arrays.[57]

Genetic and epigenetic changes probably have complex bidirectional interactions and co-operate to mold the evolutionary landscape. This complex and bidirectional interplay between genetic and epigenetic features in cancer has been perhaps most deeply explored in the area of cancer stem cells. Specifically, early xenograft studies of ALL revealed leukemic repopulation that recapitulated the genetic heterogeneity of the patients' original leukemia.[58,59] Similar findings were also demonstrated in solid tumor malignancies.[60] Anderson *et al.*[58] concluded that cancer stem cells—an epigenetically uniform population—are genetically diverse. On the other hand, even genetically uniform cell subpopulations have been reported to reveal profound epigenetic differences leading to differences in the phenotypes of survival capacity and pluripotency potential.[61,62]

**Table 1.** Next-generation sequencing studies of clonal evolution in hematological malignancies

| *Disease* | *Methodology* | *Number of cases* | *Insights* |
|---|---|---|---|
| AML[39] | WGS, followed by targeted deep sequencing | 8 | Relapse after chemotherapy is associated with clonal evolution and acquisition of new mutations |
| Secondary AML[46] | WGS, followed by targeted deep sequencing | 7 | Secondary AML clones are often evolved progeny of MDS clones |
| Multiple myeloma[47] | WES | 1 | Clonal shifts occur along the history of the disease |
| Multiple myeloma[118] | WES | 1 | Clonal shifts occur along the history of the disease |
| CLL[76] | WGS, followed by targeted deep sequencing | 3 | Different patterns of evolution evident through cycles of therapy |
| CLL[40] | WES | 149 (18 longitudinal samples) | Subclonal drivers can anticipate clonal evolution and impact outcome |
| Essential thrombocytosis[115] | Single-cell WES | 1 | ET is monoclonal in origin |
| Follicular lymphoma[119] | WES | 8 | Early and late drivers identified |

Abbreviations: AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; ET, essential thrombocytosis; WES, whole exome sequencing; WGS, whole gemone sequencing.

Together, these observations prompt a model of cancer evolution in which epigenetic and genetic heterogeneity are integrated, thereby accounting for epigenetic heterogeneity of genetically uniform populations and genetic heterogeneity of epigenetically uniform populations. Such a model would designate cells with high self-renewal capacity (an attribute encoded in the epigenetic state) as the crucial units subjected to selection forces in genetic evolution. Hence, the appearance of a new somatic mutation within these specialized cells could lead to their clonal propagation. At the same time, such a model would also acknowledge the existence of a far less homogenous cancer stem cell population then previously considered with regard to various features, including drug sensitivity.[63]

It is important to consider that in cancer the movement between different epigenetic states (along the spectrum of pluripotency to differentiation, for example) may be altered as well. In multicellular organisms, epigenetic transitions are tightly controlled through numerous regulatory mechanisms.[64] Neoplastic transformation can unhinge those mechanisms, reverting to a state more closely resembling unicellular organisms,[65] in which the fluid movement across diverse states can achieve high adaptivity by 'trial and error.' Epigenetic heterogeneity, thus, can be a hedging strategy for enhanced survival.[65] Cancer progression, therefore, may be viewed as a scenario in which both genetic and epigenetic population structures become increasingly malleable, such that the lines between populations with different 'stemness' potential become more blurred. Within this framework, 'stemness' may exist as a functional phenotype, which can be manifested by any member of a malignant population given the appropriate endogenous and exogenous factors.[66] Thus, a high degree of interclonal competition would probably select for cells with the highest self-renewing capacity at the expense of more differentiated cells, as has been demonstrated in CML.[67] Therapy may also accelerate this process by providing a strong selection for cancer stem cell survival and proliferation.[67,68] Acquired genetic alterations probably have an important role in this scenario as well. For example, the loss of *TP53*, often seen with disease progression,[21] provokes stem-cell-like transcriptional programs.[69,70] Other oncogenes may also afford leukemogenic potential to committed myeloid progenitors, again demonstrating that genetic lesions may enlarge the available pool of cells with stem-like features.[71]

In summary, integrating the stem cell hierarchy and the genetic phylogenetic tree yields a complex evolutionary picture that has only begun to be unraveled. In concert with genetic diversification and fitness optimization, a similar process very likely occurs at the epigenetic level. Cancer stem cells constitute a growing proportion within the cancer cell mass[72,73] and have a greater plasticity in terms of bidirectional conversion from stem cells to more differentiated cells. Together, this leads to enhancement of the cellular substrate available for selection, with large, treatment-resistant and genetically heterogeneous cancer stem cell population.

## HOW DOES CLONAL EVOLUTION CONTRIBUTE TO RESISTANCE TO THERAPY?

Relapsed malignancy shows an almost universal phenotypic evolution, resulting in a more aggressive and treatment-refractory phenotype.[74] We and the others have shown that frequent genetic evolution underlies the phenotypic evolution.[11,40] Therefore, a central question in cancer treatment is what is the precise nature of the interaction of clonal evolution with cancer therapy. Initial studies highlighted the potential role of chemotherapy to induce novel mutagenesis[75] and thereby to enhance the process of genetic diversification (Figure 2a). Although studies of WGS (whole gemone sequencing) are inherently limited by the power to detect minute subclones within a sample, studies in acute myeloid malignancies have nonetheless suggested that the novel mutagenesis may result from the genotoxic effects of chemotherapy, supported also by a changing spectrum of somatic single-nucleotide alterations.[39,76] In contrast, in case of indolent blood malignancies such as CLL, evidence for the contribution of the chemotherapy's mutagenizing effect is limited. Previous purine analog-based therapy was not associated with an increased total number of mutations in CLL[77] and also was not associated with an altered mutational pattern.[40] Therefore, although chemotherapy-induced mutagenesis has the potential to contribute to further clonal diversification, other sources for generating evolutionary shifts appear to be at play, and probably involve pre-existing genetic variants or subclones.[78]

How then does therapy induce evolution from pre-treatment genetic variation? Two explanations are considered, depending on the tumor kinetics, the efficacy of cell kill with treatment and other factors related to both the tumor type and the specific treatment strategy. The first is that resistant clones may be actively selected by therapy (Figure 2b). Examples for this model are numerous,[79–81] including *MSH6* mismatch repair gene mutations in recurrent glioblastoma multiforme after treatment with temozolomide[82,83] and the *BCR-ABL T315I* mutations in CML.[84,85] Indeed, this model of clonal evolution induced by the selective pressure of therapy maybe particularly relevant in the context of targeted therapy, as

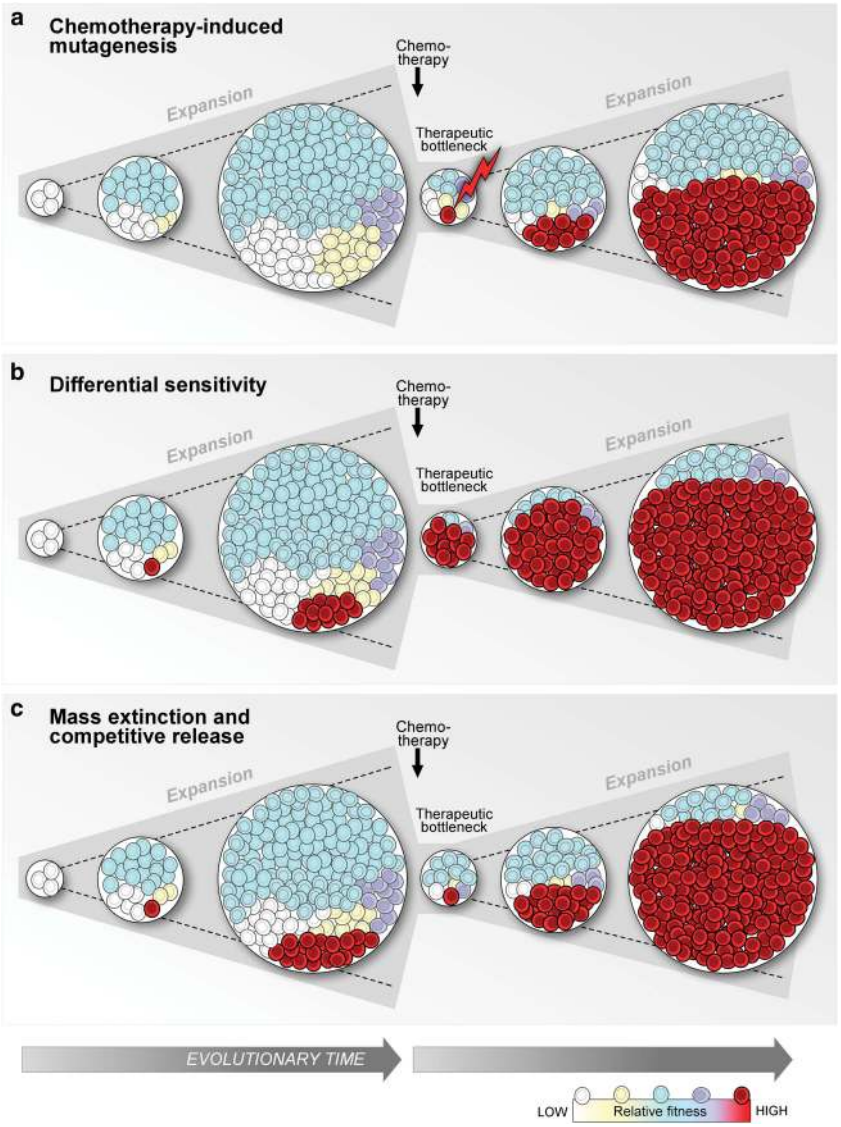


**Figure 2.** Three models of how cancer therapy may accelerate clonal evolution. First, cancer therapy, particularly containing genotoxic agents, can induce novel mutagenesis (**a**). Second, therapy can accelerate clonal evolution by selecting a clone (here illustrated in red) containing a mutation that confers resistance to the therapeutic agent used (**b**). The resistance of the selected clone is reflected in the depiction of the cell population after cytoreduction, composed almost entirely of the resistant clone (in red). A third model postulates similar sensitivity to treatment of the different subpopulations, reflected in similar proportions before and after cytoreduction (**c**). The clearing niche alters the dynamic evolutionary landscape allowing a faster rise of a fitter clone.

the therapy is often directed at a particular genetic context which may not be shared by all subclones. This relationship between therapy and genetic adaptation is likely to result in convergent evolution, in which a mutation that confers resistance will become highly prevalent in relapsed disease. Indeed, this process has been reported in relapsed T-cell ALL after treatment with nucleoside-analog chemotherapy drugs.[86]

An alternative process contributing to the emergence of continuously more aggressive clones may be entirely independent of differential sensitivity to therapy (Figure 2c). We recently observed a higher number of large subclones (>10% of cancer cells) in 149 CLL cases that were exposed to treatment before sampling compared with patients who received therapy after the sample was obtained. This finding of increased clonal diversity with treatment held true even after accounting for potential confounders, such as longer follow-up time.[40] We interpret this observation to result, at least in part, from the outgrowth of many diverse pre-existing minor but fit subclones.[76,87] This latter interpretation is further supported by our observation of an increased frequency of subclonal-driver events (presumably fitter) in treated relative to untreated patients. Overall, our data support the idea that CLL therapy, by markedly reducing disease bulk, may act as a classic evolutionary restriction point and reset interclonal dynamics.[88]

Within this conceptual framework, when subclones with high fitness already exist within a tumor population, treatment could favor the development of more aggressive clones, potentially reducing post-relapse survival.[40] In this context, cytotoxic therapy would effectively remove the incumbent clone[89]—acting like a 'mass extinction' event[89]—and thereby shift the evolutionary landscape[90,91] in favor of one or more aggressive subclones.[92] Thus, highly fit subclones probably benefit from treatment and exhibit rapid outgrowth.[78] These data provide mechanistic support to the observation that the 'watch and wait' strategy for

CLL leads to superior clinical results,[93] as the earlier administration of chemotherapy may accelerate clonal evolution and the emergence of fitter clones with more aggressive disease phenotypes. This form of relationship between therapy and evolution maybe particularly important to CLL, as this cancer type is highly dependent on growth and survival signals provided by the local microenvironment.[94] This dependency may augment the importance of the role of interclonal competition in the evolutionary dynamics of CLL. Future in-depth studies would assist in confirming this model as well as whether it is generalizable to malignancies other than CLL, and in particular in other more indolent cancers.

## TRANSLATING CLONAL EVOLUTION TO THE CLINIC

A major priority of precision cancer genomics is to use information on genetic lesions to define patient prognosis. In an illustrative example, Patel *et al*. could demonstrate that lesions such as internal tandem duplication of *FLT3* (*FLT3*-ITD), partial tandem duplication in *MLL* (*MLL*-PTD), as well as mutations in *ASXL1* and *PHF6* associated with reduced overall survival in AML, while *CEBPA* and *IDH2* mutations associated with improved overall survival. These associations were independent of established risk factors.[95] Similar efforts have been carried out in other hematological malignancies, including CLL,[77,96,97] and multiple myeloma.[98]

Across the blood malignancies, patients with apparently poor prognostic markers can nonetheless exhibit good survival, and vice versa.[88] The several studies reviewed above (Table 1) suggest that intratumoral clonal heterogeneity may be an important contributor to this complex picture. In aggregate, studies of clonal evolution have revealed cancers to be genetically heterogeneous in space and time.[99] Hence, simply labeling an individual cancer as harboring a genetic lesion or not is not fully precise. From a practical standpoint, for a solid tumor mass, or even leukemia cells that are present in different tissue compartments (that is, blood vs marrow vs lymph node), multiple samplings may be required to correctly assert the genetic landscape of an individual case (Figure 3).

Finally, clonal heterogeneity in and of itself may impact clinical outcome. Our studies have shown that the presence of a strong subclonal-driver event, but not a clonal driver, negatively impacts clinical outcome in CLL.[40] The link between clonal heterogeneity and specifically the presence of a subclonal driver to adverse clinical outcome adds an additional dimension to the current efforts of linking discrete somatic mutations to outcome. In other words, it is not only the presence or absence of a mutation that should be considered but also the size of the subpopulation it affects.

From the therapeutic standpoint, studies of cancer genomics highlight the concept that cancer is not a single disease entity but rather a collection of related disorders; hence, treatment should be targeted to the molecular subtype of disease. For example, high-dose daunorubicin, as compared with standard-dose daunorubicin, improves the rate of survival among patients with *DNMT3A*, *NPM1* mutations or *MLL* translocations in AML but not among patients with wild-type *DNMT3A*, *NPM1*, and *MLL*.[95] The potential to integrate the available high-throughput sequencing technologies (for example, DNA-seq, RNA-seq and ChIP-seq) to provide a patient-specific genomic-epigenomic map may provide crucial prognostic perspective and inform therapeutic choices.[100] Furthermore, targeted treatments that are based on the presence of specific molecular lesions may greatly improve therapeutic response, as seen in, for example, *FIP1L1-PDGFRA* eosinophilia-associated myeloproliferative disorders.[101] These 'actionable mutations' where a clinician matches a tumor mutation to a cancer drug may either be missed given genetic heterogeneity in time and space, or alternatively might involve only a small subclone, which begs the question of the clinical efficacy were it to be solely targeted. For instance, synthetic lethal approaches were found to be highly effective in situations in which all cancer cells contain the targeted variation, as witnessed by the potent efficacy of PARP (poly ADP-ribose polymerase) inhibition in tumors of *BRCA* germline carriers.[102]

These observations together raises the provocative question of whether it is preferable to target genetic variations that are found in the 'trunk' compared with those found in 'branches' of the evolutionary phylogenetic tree.[99] Intuitively, the former may be considered the superior approach. 'Trunk' events, by definition, are mutations present in all the cells of the malignant process. Targeting of this event in theory carries the potential of a complete extinction of the entire population of malignant cells. Conversely, it is unclear whether the cell remains dependent on the specific 'trunk' target after acquiring additional oncogenic events (a 'branch' target), and therefore, how well they will be impacted by therapy directed against these founder targets. In solid malignancies, such as non-small cell lung cancer, *KRAS* and *EGFR* mutations are rarely detected together; however, when they co-occur, targeting the 'trunk'-type mutation (that is, *EGFR*) is no longer effective.[103] Similarly, *BRAF* canonical mutations are discovered in benign colonic polyps and are therefore probably to be earlier, 'trunk'-type events. However, the response to *BRAF* targeting has been disappointing.[104] One may hypothesize that at least in more indolent malignancies, targeting 'branch' mutations ('pruning') may be an effective strategy, which could promote clonal equilibrium and hinder the selection of more aggressive phenotypes.

The differential effects of targeting 'branch' vs 'trunk' lesions may be determined, in part, by the complex epistatic relationship between different genetic lesions within the same clonal population. As new mutations do not occur in isolation but rather enter into an established genomic landscape, the existing gene network may have a profound effect on the fate of the cell and determine whether the novel mutation will result in cell death or clonal expansion. For example, activation of many oncogenes together, including *KRAS*, can lead to a state of 'oncogene-induced senescence'.[105] A similar relationship has been demonstrated for *c-MYC*-induced apoptosis that is relieved in the context

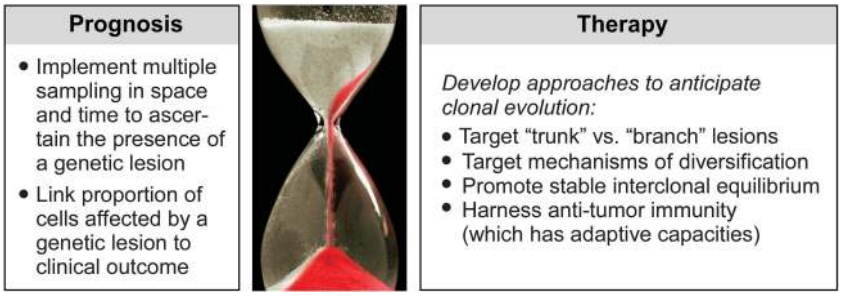


**Figure 3.** Translating clonal heterogeneity insights to the clinic. Possible prognostic and therapeutic implications of clonal heterogeneity are outlined. Image courtesy Broad Institute/Lauren Solomon; hourglass photo iStockphoto/Dominik Pabis.

of *BCL-2* overexpression.[106] Hence, further study of the epistatic relationships in model systems as well as in clinical trials will help clarify in what context optimal effects will result from targeting the 'trunk' event and when it is preferable to target the 'branches'.

The broader evolutionary perspective allows us to view cancer as an ecology of different subpopulations in the context of their environment.[87] Intriguing data suggest that, at least in some cases, complex co-dependency relationships between subpopulations may exist,[107] in addition to competition. The understanding that disease is composed of diverse subpopulations is a challenge to our traditional schemes of clinical trials. A future in which both trunk and branch events are characterized, and in which no two cancers share the same genomic features, may be envisaged.[108] In this setting, performing large-scale clinical trials using present-day methodologies, in particular utilizing combinations of targeted agents, may prove highly challenging. The disease can no longer be defined as a single entity containing a uniform set of genetic abnormalities. Furthermore, the degree of genetic heterogeneity of a tumor is likely to be an important determinant of therapeutic outcome.[92,109]

A better understanding is needed of the impact of therapy on the evolutionary landscape, possibly through the use of the more applicable whole-exome sequencing technologies to study large cohorts on patients.[37] Researchers may consider incorporating approaches such as WES (whole exome sequencing) that identify at least larger subpopulations ($>1$–10% of cancer mass) and characterize their evolution in ancillary studies of prospective clinical trials. Such information may inform us regarding the adaptive processes responsible for treatment failure as well as eventually spark the development of novel therapeutic paradigms. For one, it has been proposed that alternative approaches could potentially maintain interclonal equilibrium at the expense of trying to maximize cell kill.[110] This approach supports preventing the elimination of therapy-sensitive clones, as they (theoretically) could continue to suppress the growth of therapy-resistant clones in a competitive manner and thereby maintain an equilibrium state. A second approach that requires further consideration is the idea of limiting the underlying diversification that serves as the substrate for clonal evolution before the full expression of the genetic or the epigenetic heterogeneity in cancer is evident. Finally, the therapeutic challenge posed by a continuously adapting and reshaping malignant process provides strong rationale to support the pursuit of immunity-based therapies, as this approach may effectively pit one complex adaptive process against another. There is already limited evidence that allogeneic hematopoietic stem cell transplant (a non-specific example of a immunity-based therapy) imposes evolutionary pressures on the tumor that are distinct from other therapeutic modalities (leading, for example, to loss of donor–recipient mismatched *HLA* alleles[111,112] or multiple cytogenetic abnormalities[113]). These alterations demonstrate that the leukemic cell population is being molded by a powerful immune response and hence to the efficacy of the immunity-based therapy. The process of co-evolution of the cancer cells and the immune response in the setting of effective immunotherapy is an area of great interest for future study.

## CONCLUSIONS AND FUTURE DIRECTIONS

Understanding the evolutionary capacity of cancer is emerging as a key element in developing improved therapeutic strategies in the era of precision medicine, as it presents one of the most formidable obstacles to the successful application of targeted therapy.

As aforementioned, the intensive application of high-throughput genomic platforms has enabled rapid progress in our understanding of the process of clonal evolution in hematological malignancies. Collectively, these studies have provided several core insights, including: first, that clonal heterogeneity is common in malignancy both at the genetic and the epigenetic level; second, that clonal evolution is frequently observed in relation to therapy, leading to emergence of more aggressive and resistant disease; and finally, that the process of clonal evolution is linked to adverse clinical outcomes.

Although these recent studies point to the key role played by clonal evolution in cancer progression, current perspectives of cancer as an evolutionary problem are fairly limited. Even as knowledge about germline and acquired genetic lesions associated with cancers has grown exponentially, we still know only little about the background rate of heterogeneity—which is the substrate of evolution—and possess only a rudimentary understanding of how the epigenetic program affects this substrate. In this respect, developing methodologies to integrate data from complementary—genetic and epigenetic—high-throughput platforms is key, both at the cell population and at the single-cell level. Moreover, the dynamics of interactions between clones—whether they compete or co-depend on each other—has not been elucidated. Additionally, the examination of key mechanistic question relating to clonal evolution using genomic tools (for example, different types of selective pressure, interaction with microenvironment niches and interactions between multiple genetic lesions within the same cell) has yet to be accomplished. Thus, the ability to foresee the evolutionary trajectory of any individual cancer is presently still in its infancy. Improving this capacity to predict how cancer will evolve with treatment carries a significant potential to allow us to anticipate and tailor treatment to the probable future trajectory (so-called 'anticipation-based chemotherapy').[114]

Ongoing technological developments are now generating tools ideally suited for the study of these questions. Recently, proof-of-principle studies of single-cell sequencing have been conducted that have catalogued the point mutations in protein-coding regions.[115,116] In the not too distant future, single-cell sequencing will allow the detailed study of genetic heterogeneity that provide the backdrop against which evolution at the subpopulation level occurs. The application of single-cell RNA-seq[117] to the study of hematological malignancies would enable the study of the heterogeneous transcriptional changes and signaling networks that stem from heterogeneous somatic genetic alterations. In addition to single-cell examination, novel methodologies are capable of deconvoluting subpopulations from bulk material[37] and may be helpful in delineating the basic underlying principles of evolution in *in vivo* and *in vitro* models, with deeper sequencing providing both higher sensitivity for smaller subclones as well as more precise estimates of their size. The ability to use WES as an alternative approach to WGS,[40] as well as the projected downtrend of sequencing costs, will enable multi-sampling in time and space of hematological malignancies, clarifying the nature of spatial heterogeneity in blood malignancies as well as questions regarding the nature repopulation of the ecological niche upon relapse. It may also allow the study of these questions in large clinical trials. These efforts can potentially answer questions of fundamental importance to the clinical application of these insights, namely what is the prognostic significance of the size of the subclone that harbors a genetic marker, when should we target branch or trunk lesions and how to integrate this knowledge in combinatorial therapies.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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