Genetic and epigenetic determinants of B-cell lymphoma evolution

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Purpose of review
The success of targeted therapies fostered the development of increasingly specific and effective therapeutics for B-cell malignancies. However, cancer plasticity facilitates disease relapse, whereby intratumoral heterogeneity fuels tumor evolution into a more aggressive and resistant form. Understanding cancer heterogeneity and the evolutionary processes underlying disease relapse is key for overcoming this limitation of current treatment strategies. In the present review, we delineate the current understanding of cancer evolution and the advances in both genetic and epigenetic fields, with a focus on non-Hodgkin B-cell lymphomas.

Recent findings
The use of massively parallel sequencing has provided insights into tumor heterogeneity, allowing determination of intratumoral genetic and epigenetic variability and identification of cancer driver mutations and (epi-)mutations. Increased heterogeneity prior to treatment results in faster disease relapse, and in many cases studying pretreatment clonal admixtures predicts the future evolutionary trajectory of relapsed disease.

Summary
Understanding the mechanisms underlying tumor heterogeneity and evolution provides valuable tools for the design of therapy within an evolutionary framework. This framework will ultimately aid in accurately predicting the evolutionary paths of B-cell malignancies, thereby guiding therapeutic strategies geared at directly anticipating and addressing cancer evolution.

Keywords
cancer evolution, chronic lymphocytic leukemia, epigenetic heterogeneity, genetic heterogeneity, lymphoma

INTRODUCTION

Despite the development of effective chemotherapeutic and targeted therapies for B-cell malignancies [1,2], many patients will still experience disease relapse. This therapeutic challenge stems from the fact that within each patient's cancer, we are dealing not with a single disease entity, but rather with a collection of many distinct cell populations. Thus, our therapies are faced with the exceedingly difficult task of eliminating all of these diverse malignant subpopulations.

The intratumoral diversity results from an active evolutionary process [3]. In this process, the malignancy arises from a single transformed cell, which initiates clonal expansion. Once this process is set in motion, the growing population continues to diversify with additional somatic mutations subject to positive selection, and resulting in a highly diverse tumor population [4–7].

Recently, massively parallel sequencing (MPS) has accelerated our ability to study tumor evolution by providing base-pair resolution mapping of hundreds of tumors. In particular, the broad use of whole exome sequencing (WES) has allowed the study of large patient cohorts at high-read depth, facilitating the detection of subclonal mutations present in cancer cell populations [8▪,9▪,10,11].

An inherent characteristic of MPS is that it generates billons of independent sequencing reads [12], and provides an informative random sample of individual DNA molecules present within the tumor [13]. This allows the determination of the variant allele fraction (VAF), which is the number of reads

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KEY POINTS

- The use of massive parallel sequencing has provided new insight into B-cell lymphoma, revealing extensive intratumoral genetic and epigenetic heterogeneity.
- Therapy frequently accelerates tumor evolution, by selecting resistant and more aggressive clones of the cancer cell population.
- Increased pretreatment genetic and/or epigenetic heterogeneity is associated with shorter time until disease relapse.
- Tumor progression can be considered as an example of 'evolutionary regress', by which cancer cells explore superior evolutionary trajectories toward unicellular fitness at the expense of multicellular fitness.

harboring a determined mutation over the total reads obtained for the genomic locus (Fig. 1a). Of note, VAF is affected by the proportion of nonmalignant cells in the sample, as well as the local copy number. To account for these elements, VAF values are transformed to cancer cell fractions, which are the fraction of cells that harbor a determined genetic lesion, among all malignant cells in the sample. Measuring the cancer cell fractions of multiple mutations allows inferring the clonal composition of each individual sample (Fig. 1a).

In this context, it is important to note that we are only observing the 'tip of the heterogeneity iceberg' as our current methods rarely detect clones that are less than 1–5% in frequency, thereby delineating only the more frequent and fittest clones. Nevertheless, these studies have shown that each B-cell neoplasm is composed of multiple subpopulations, with admixtures that change over space and time. Importantly, MPS studies have provided insights as to how therapy shapes the course of evolution [13], showing that clonal evolution with therapy is the rule rather than the exception [14▪▪].

When considering somatic tumor evolution, we need to address the entirety of heritable information. In addition to the genetic information, strata of epigenetic information are propagated from parent to progeny, contributing to the cell phenotype [15]. Perhaps the best-studied epigenetic modification is DNA methylation (DNAme), with ample evidence supporting its heritability, as well as its contribution to the pathobiology of B-cell malignancies [16,17]. These properties, as well as the ability to provide quantitative measurements of intratumoral heterogeneity with MPS methods, have made DNAme a prime candidate to lead the way into understanding intratumoral epigenetic diversity. Indeed, increased intrasample heterogeneity of DNAme patterns has been observed in chronic lymphocytic leukemia (CLL) compared with normal B cells [18▪], and increased intratumoral variability of DNAme patterns correlated with adverse clinical outcome [18▪,19▪,20].

In this review, we focus on non-Hodgkin B-cell malignancies, delineating MPS-driven advances that explore lymphoma intratumoral heterogeneity and evolution. Collectively, these MPS studies have highlighted several central themes of B-cell malignancy evolution, including the ability to infer the sequence of driver acquisition along the history of the disease, B-cell-specific mechanisms of diversification, vast genetic and epigenetic intratumoral heterogeneity, and the importance of tumor evolution in therapeutic failure. Thus, MPS methodologies and an advanced evolutionary perspective come together to reshape our understanding of lymphoma progression and relapse, as well as pose new exciting challenges and therapeutic opportunities.

DECIPHERING THE CHAIN OF MUTATIONAL EVENTS IN LYMPHOMA DEVELOPMENT

Genomic investigations of lymphomas revealed multiple 'drivers' (fitness enhancing somatic mutations) detected in each individual cancer. This finding prompts a fundamental question regarding driver acquisition – does it follow a particular order or sequence? In other words, does the likelihood of obtaining a particular driver event change along the evolutionary history of the disease?

Unlike the tree of life, cancer evolution repeats itself over and over. Each patient's disease represents an independent instance of the evolutionary process. This allows defining general patterns by studying many parallel and independent instances of evolution across many patients. A particular sequence of events that is overrepresented in the patient population, likely reflects an associated fitness advantage that allows many cancers to independently 'discover' this superior trajectory. We and others have capitalized on this feature of cancer evolution to infer the sequences along which drivers are acquired in lymphoma progression [8▪,14▪▪,21▪,22].

At first approximation, we can ask what proportion of cases harbors any specific driver at a clonal vs. subclonal frequency [8▪]. Although a clonal mutation involves all cells in the population, a subclonal mutation is found in only a subset of the cells (Fig. 1a), and therefore likely represents an event acquired at a later time point. Using this

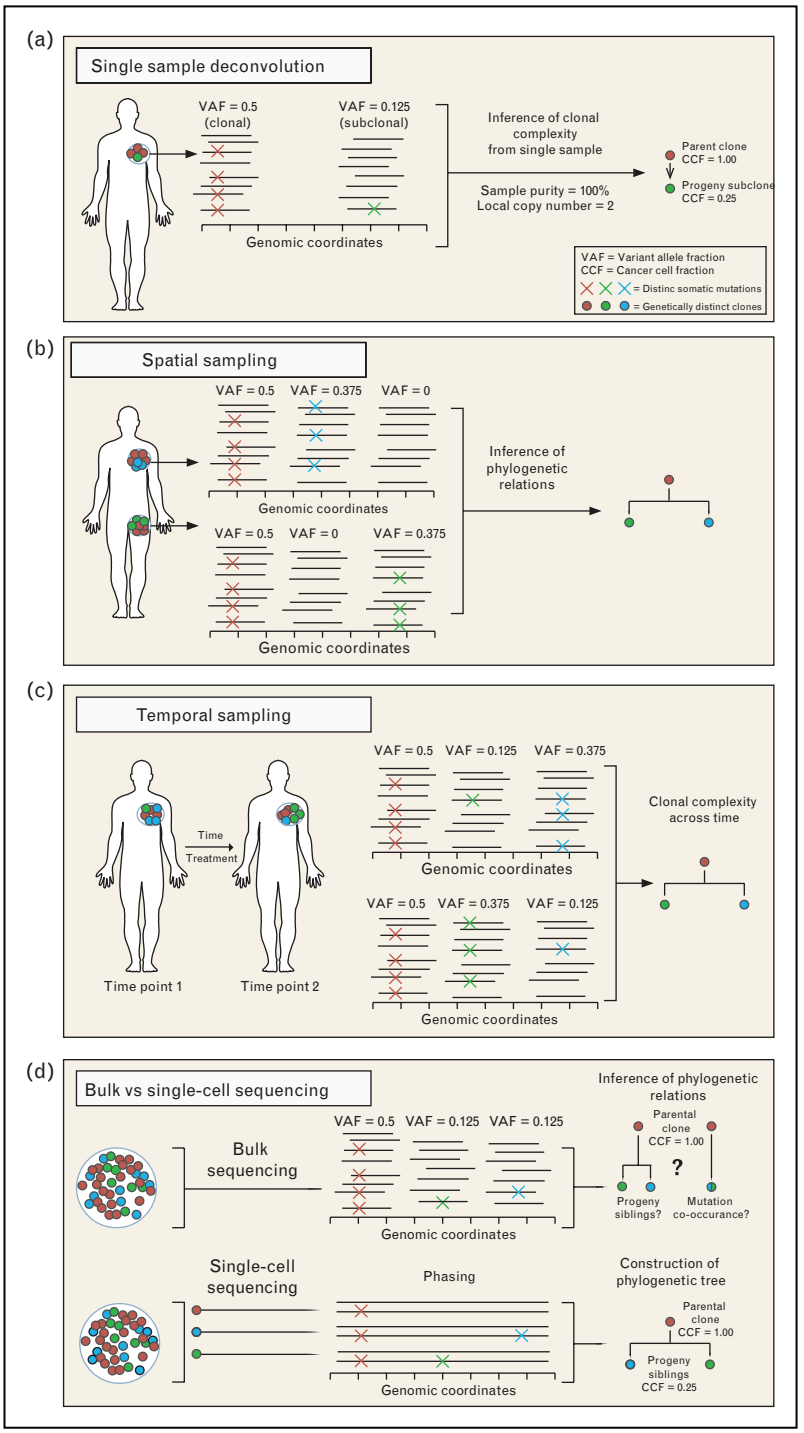

FIGURE 1. Sampling methods for heterogeneity analysis and clonal complexity reconstruction. (a) Massively parallel sequencing allows inference of clonal evolution from VAF estimation, as the number of reads carrying the mutation over the total reads obtained for a given region. Correction by the sample purity (in all cases we assume 100% purity, as all cells in the sample being cancer cells) and a somatic copy number alteration of two allows determination of CCF carrying a given mutation. (b) Sampling from different locations allows inference of more complex phylogenetic relations and clonal divergence between different tumor sites. (c) Longitudinal sampling at different times allows determination of the evolutionary trajectory followed by the tumor population, by determining the temporal sequence of lesion acquisition. (d) Although in whole exome sequencing studies clonal phylogeny has to be inferred from relative VAF values, single-cell sequencing allows complete phasing of the lesions, assigning each mutation to a single corresponding cell. CCF, cancer cell fraction; MPS, massively parallel sequencing; VAF, variant allelic fraction.

methodology, we showed that distinct lesions appear early [e.g., *MYD88*, trisomy 12, and hemizygous *del*(13q)] or late (e.g., *TP53*, *ATM*, and *SF3B1*) in the history of CLL [8▪]. This method has also been applied to follicular lymphoma, showing mutations in chromatin modifiers (e.g., *EZH2*, *MLL2*, and *CREBBP*) as early driver events [23▪▪]. This suggests that the fitness associated with each driver genotype is also a function of the particular moment it appears along the evolutionary time course of the disease.

With larger cohort sizes [14▪▪], we could probe for potential temporal relationship in acquisition of 'pairs' of drivers. By studying all the instances in which a pair of drivers is found within the same leukemia, one in clonal and the other in subclonal frequency, we found enrichments of distinct 'sequences' in CLL evolution. This observation adds important additional context specificity to the definition of driver lesions – the fitness associated with each genotype is also a function of its somatic background.

This framework can be supplemented by large cross-sectional sequencing of different clinical phases, as has been implemented in CLL, where a premalignant entity exists – monoclonal B-cell lymphocytosis (MBL). For example, Klinger *et al.* [24] explored MBL by performing deep sequencing of the immunoglobulin heavy chain variable locus, finding clonal complexity to be present even at this early stage [24]. Also, CLL-associated driver lesions were found in MBL months before disease progression [25], and *NOTCH1* mutations, shown to be an earlier event in CLL [8▪], were already detected as small subclones in MBL [26].

Other studies have utilized longitudinal sequencing to delineate the evolutionary sequence of driver acquisition, such as the emergence of clones bearing new mutations, as in progression from follicular lymphoma to transformed-follicular lymphoma (tFL) [27]. Indeed, extensive clonal diversity [23▪▪,28▪,29▪] in follicular lymphoma was shown to lead to tFL [23▪▪,29▪].

In summary, evolutionary inference based on a mixture of large cross-sectional studies and longitudinal sampling provided a novel understanding of B-cell neoplasm's drivers. By uncovering distinct sequences of events, we learn that the fitness associated with driver lesions is highly context specific, and depends on its particular position within a probabilistically defined chain of events.

The determination of the sequence of events within B-cell malignancies provides valuable information upon which parameterized mathematical models can be built [30▪]. This quantitative approach has been previously addressed with the development of stochastic models [31], as well as deterministic models [32] to follow tumor evolution dynamics. Recently, attention has been centered on modeling fitness landscapes, which may present particular topologies as the result of epistatic interactions [33]. Consequently, certain evolutionary trajectories would become inaccessible, a feature that has given rise to the possibility of using 'evolutionary traps' [34] and 'temporal collateral sensitivity' [35,36] to drive cancer cell populations toward extinction [37▪]. Therefore, empirical information regarding the sequence of events in B-cell malignancies can empower these quantitative approaches to provide evolutionary-driven design of rational therapeutic interventions [30▪].

A MOTLEY CREW – THE REMARKABLE INTRALYMPHOMA GENETIC HETEROGENEITY

The ability of MPS to capture numerous somatic mutations at high throughput has provided insight into intratumoral genetic heterogeneity in B-cell malignancies. This has been done with two complementary approaches: deconvoluting clonal heterogeneity within each sample as described above, based on the different allele frequencies of different mutations (Fig. 1a), and sequencing spatially or temporally distinct samples from the same patient (Fig. 1b and c).

With the these methods, widespread intratumoral genetic heterogeneity has emerged as a common theme across B-cell neoplasms, with evidence of branched rather than linear evolution being the more common evolutionary trajectory [8▪,9▪,14▪▪,38]. Vast intratumoral genetic heterogeneity was found to involve recurrent driver events across many B-cell malignancies, including CLL [14▪▪,39], diffuse large B-cell lymphoma (DLBCL) [10,38,40], mantle cell lymphoma (MCL) [41], and follicular lymphoma [42].

The study of cancer heterogeneity and evolution has been particularly enhanced by sequencing of multiple sites within a single patient or primary tumor [43–45] (Fig. 1b). These studies have shown that much of the heterogeneity we observe may result from spatial constraints, as tumor cells are unlikely to undergo ideal mixing [46▪▪]. As the evolution of cancer populations is dependent on the selective pressures of the environment, greater intratumoral heterogeneity is expected if cancer cell populations reside in different ecological niches (e.g., different tissues). For example, studies comparing clonality in lymph nodes and peripheral blood revealed differences in clonal distribution between these two compartments [47▪]. Further studies applying spatial sequencing in lymphomas are

crucial to define the magnitude of such process that resembles allopatric speciation, and to quantitatively delineate the dispersion of clones within the entire malignant population [46▪▪].

Despite these advances, a central remaining challenge stems from the reliance of these methods on the short DNA fragments used in MPS. This limits the ability to 'phase' distinct somatic events, since the determination of whether two mutations are found within one or two clonal populations is only indirectly inferred by clustering methods [8▪]. This limitation may be addressed with the development of single-cell capture sequencing [48,49], which allows complete phasing, whereby all the mutations can be assigned to individual cells (Fig. 1d). Single-cell sequencing has allowed the reconstruction of evolutionary pathways in myeloma [50], childhood acute leukemia [51▪▪] and breast cancer [52] genomes [53]. Hence, these methodologies carry a significant potential to increase the resolution of genetic heterogeneity mapping, confidently assigning mutations to low-frequency subclones.

B-CELL MALIGNANCIES: A RECIPE FOR DIVERSIFICATION

The extensive intratumoral heterogeneity of B-cell malignancies prompts an important question: how is such intratumoral diversity generated? Although B-cell malignancies are subject to mutational processes observed across cancer [8▪], B cells are unique in their ability to edit their genomes as part of their normal physiology, introducing novel mutations. Although many mechanisms are in place to guide these processes to immunoglobulin loci [54–57], these physiological mutagenesis mechanisms are often hijacked in malignancy. In this regard, many B-cell malignancies harbor translocations affecting the immunoglobulin locus, which is associated with activation-induced deaminase (AID) activity in class switch recombination [58].

In addition to chromosomal breakage induced during class switch recombination, AID continues to introduce single base pair mutations through the process of somatic hyper mutation of the immunoglobulin gene [58]. In work by Alexandrov *et al.* [59▪▪], a mutational signature associated with AID was found outside the immunoglobulin locus in both CLL and B-cell lymphomas. More recently, whole genome sequencing (WGS) of CLL has shown AID-related mutational activity both in the very early phases of CLL and along the progression of the disease [60▪]. Notably, AID genomic targeting shows a tropism for highly active enhancers and promoters [61,62]. Therefore, the role of AID in lymphoma evolution will likely expand as we begin to fully characterize the noncoding 'drivers' through large-scale WGS initiatives [63▪,64].

SHAPING THE COURSE OF EVOLUTION THROUGH THERAPY

In most B-cell malignancies, we hold powerful therapeutics to eliminate the majority of the malignant population, enforcing tight therapeutic bottlenecks [13]. This strong external pressure, applied to a genetically diverse population, is likely to result in significant evolution of the genetic makeup of the disease. Indeed, WES of matched diagnosis and relapse samples demonstrates that therapy frequently induces clonal evolution, as shown by changes in the relative clonal frequencies after therapy [8▪,14▪▪,39]. Clonal evolution with therapy has been broadly observed across lymphomas such as MCL [65] and follicular lymphoma [42], showing that clonal evolution in disease relapse is an almost uniform outcome of effective therapy.

In CLL, this is exemplified by drivers, which are present at subclonal frequencies in the pretreatment sample and increase in frequency after therapy, consistent with positive selection (e.g., *TP53* mutations with fludarabine-based therapy). Notably, other drivers are equally likely to increase or decrease in frequency with therapy (e.g., *ATM, SF3B1,* and *POT1*) [14▪▪]. Thus, although these latter drivers are not likely to confer selective resistance to therapy, these clones can still show significant changes of clonal frequencies with therapy [66]. This observation suggests that other elements determine the course of evolution, in addition to selective resistance to therapy [13].

Targeted therapies have also been found to be associated with specific resistance mutations in B-cell malignancies. Some examples are the detection of a relapse-specific mutations in MCL and CLL samples from patients with acquired resistance to ibrutinib treatment [67▪,68]. These studies have shown that effective targeted therapies select for clones that harbor mutations that render the drug inactive or bypasses its inhibition.

A broader corollary that emerges from the evolutionary perspective on cancer is that increased heterogeneity would provide greater adaptive capacity to the cancer cell population, thereby facilitating disease relapse. Indeed, recent studies showed that higher pretreatment tumor genetic heterogeneity at diagnosis is associated with shorter time until disease relapse in B-cell malignancies [8▪,14▪▪] (Fig. 2a), and adverse clinical outcome across cancer [69,70▪,71].

Collectively, these insights have three important clinical implications. First, the genetic makeup

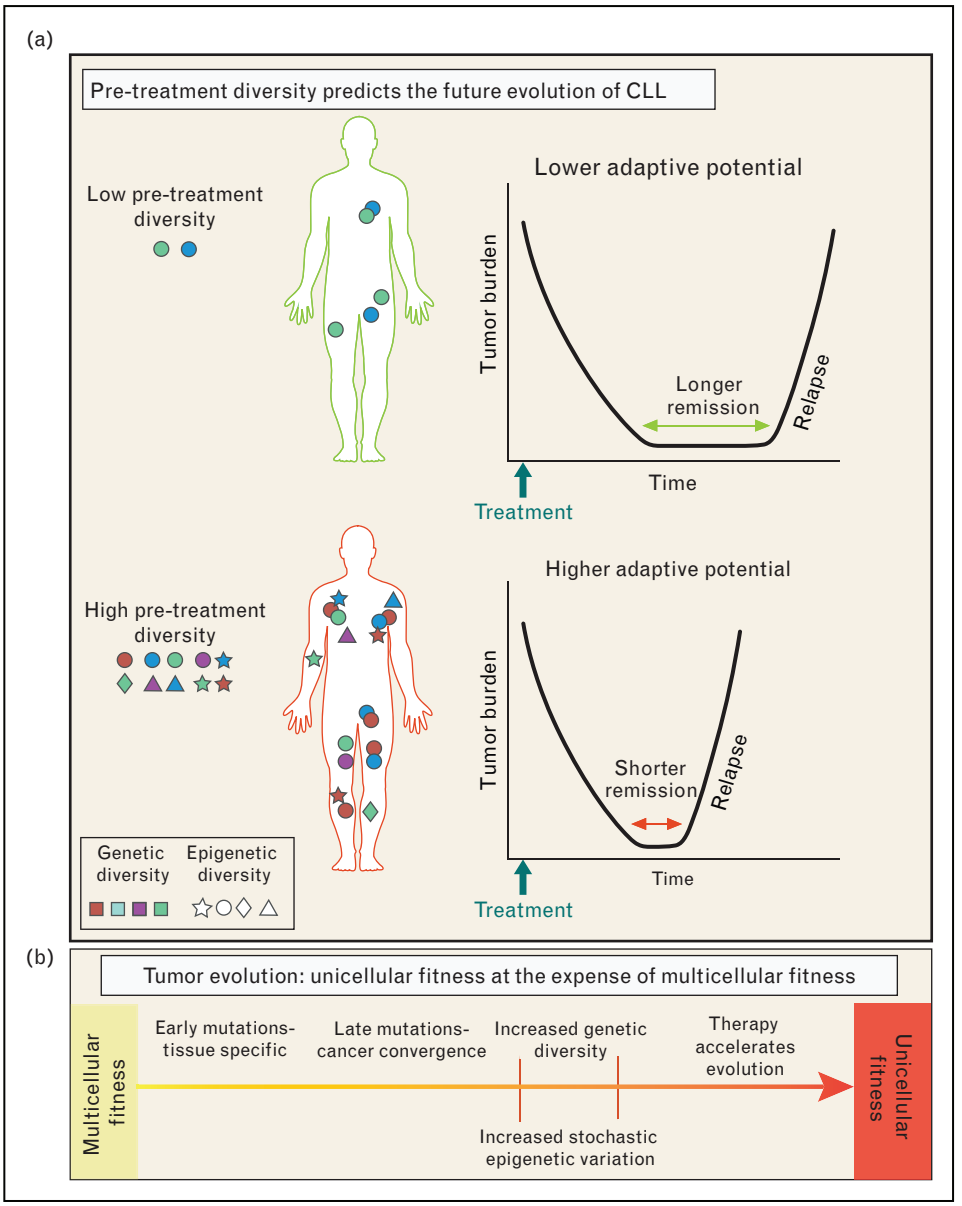

FIGURE 2. Evolution of lymphoma in progression and relapse. (a) Increased pretreatment genetic and epigenetic heterogeneity is associated with shorter time until disease relapse in B-cell malignancies, reflecting a higher adaptive potential from highly heterogeneous cancer cell populations. (b) A model for the sequence of events occurring in the path from multicellular fitness to unicellular fitness, along the progression of lymphoma evolution. CLL, chronic lymphocytic leukemia.

of relapsed disease is frequently different from that of the pretreatment disease, suggesting that a genetic risk profile assessment would need to be repeated at each treatment decision juncture. Second, the major clone present in the relapse sample can often be detected as a minor clone in the pretreatment sample. Therefore, a pretreatment assessment of clonal diversity can help anticipate the future evolutionary trajectory leading the disease relapse. Lastly, the frequent clonal evolution points to a fundamental failure to therapeutically address the collective of subpopulations. Therapies suppress some populations, while allowing others to thrive, which ultimately leads to disease relapse. This understanding challenges us to rethink our therapeutic strategies such that they will directly anticipate and address clonal evolution. An exciting opportunity emerges to integrate frequent measurements to resolve clone-specific growth patterns (either of circulating cells [66] or using liquid biopsy technology [72▪]), with ecological models to inform the therapeutic strategies required to generate an extinction event of the cancer cell population [37▪].

EPIGENETIC VARIABILITY AS SUBSTRATE FOR TUMOR EVOLUTION

Genetic heterogeneity cannot fully explain the outstanding adaptive capacity observed in tumors [73,74]. Another candidate mechanism for enhancing cancer's evolutionary plasticity is epigenetic variation, as epigenetic information may be stably inherited and results in phenotypic changes [75]. In particular, intratumoral DNAme heterogeneity has been studied in B-cell malignancies, given its established role in the pathobiology of these diseases [17]. Moreover, higher intrasample DNAme heterogeneity is associated with increased aggressiveness in DLBCL and follicular lymphoma, as was first demonstrated with 450K methylation arrays [76▪].

More recently, these questions have been studied using MPS coupled with bisulfite conversion, which provides genome-wide base-pair resolution of methylation patterns [77]. Each MPS read represents an individual DNA fragment, originating from a particular cell. Thus, similar to measurement of somatic mutation VAF, the study of multiple reads covering the same locus provides the frequency of distinct methylation patterns (epi-alleles) in the cellular population [78]. Furthermore, by comparing the methylation of CpGs contained within such a short sequencing read, the concordance in methylation state of closely neighboring CpGs – from the very same cell – can be determined [18▪,79].

Applying this perspective to CLL showed that leukemic cells differ from normal B cells not only in differential methylation of specific loci, but also in the amplitude of stochastic variation in methylation patterns between cells within the same sample [18▪,80]. This represents a departure from the traditional view of cancer epigenetics. Our perspective was largely adopted from developmental biology, where cell-specific genome-wide coherent epigenetic identities were identified. Now, we understand that in addition to these coherent profiles, a significant component of 'trial and error' exists, very much akin to genetic diversification in cancer.

This intraleukemic DNAme heterogeneity is likely not without consequences. High level of promoter methylation discordance between neighboring CpGs was shown to correspond to an intermediate transcriptional output, which interferes with both complete silencing and high gene expression, increasing transcriptional entropy as shown by single-cell RNA sequencing [18▪]. Thus, epigenetic variability is closely correlated with transcriptional heterogeneity and cancer cell phenotypic diversity.

These observations prompt an important question; how is such DNAme heterogeneity achieved? Although yet unexplored, it has been proposed that increased cell replication rate may underlie this extensive epigenetic heterogeneity, given that the error rate estimated for a given CpG per cell division can be up to 4% [81,82]. This process could be enhanced by somatic genetic mutations within components of the DNAme machinery [18▪].

In both CLL and DLBCL patients, high rate of pretreatment intratumor DNAme heterogeneity of gene promoters was found to be associated with adverse clinical outcome and shorter time until disease relapse [18▪,19▪,20] (Fig. 2a). Notably, the majority of the patients displayed decreased intratumor methylation heterogeneity in DLBCL upon treatment [19▪], showing that therapy may result in a more epigenetically homogeneous population, suggestive of therapeutic selection. Thus, epigenetic heterogeneity likely plays a central role in providing plasticity to cancer cell populations; as it arises in a stochastic fashion, it generates a continuum of epigenetic variability, providing a rich substrate for tumor evolution.

Ongoing studies of DNAme are geared toward utilizing the improved understanding of stochastic variation to enhance our ability to discover epi-drivers through statistical inference, as well as the ability to experimentally introduce epigenetic editing. Adaptation of the clustered regularly-interspaced short palindromic repeats/Cas9 system to carry out epigenetic modifications at specific loci [83] could provide information regarding the phenotypic consequences of these epigenetic modifications, as well as the means to validate putative epi-driver events observed during tumor evolution.

Our exploration of the epigenetic contribution to heterogeneity of cancer cell populations has only begun. Emerging technologies provide new opportunities to gain insight on other fundamental epigenetic aspects of the evolutionary unit of cancer – the single cell. Single-cell RNA sequencing has provided knowledge on the transcriptional heterogeneity with unprecedented resolution [84,85]. In addition, recent efforts aimed toward the development of single-cell ChIP-seq [86] and single-cell assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) [87] could allow the study of variations in the distribution of histone modifications and its consequences. Finally, the combined analysis of genetic and epigenetic somatic variation will likely provide an exciting perspective on tumor progression and evolution, as well as insights into the interplay between genetic and epigenetic lesions [75].

CONCLUSION

Throughout evolution, life forms became increasingly complex, transitioning from unicellular to multicellular organisms. In this context, cancer is a fascinating example of 'evolutionary regress'; whereby cells gradually rescind the multicellular contract, and propagate to more closely resemble a unicellular life form. Large-scale application of MPS technology to B-cell malignancies has improved our understanding of this 'evolutionary regress' (Fig. 2b).

Wherein early driver mutations are cell-type specific, likely enhancing the malignant cells' ability to compete with their normal counterparts, late drivers show convergence to mutations found across cancer, and likely reflect competition between different malignant subpopulations. As the lymphoma population grows, genetic and epigenetic diversification intensifies, as both of these phenomena are closely linked to cell replication and the size of the malignant population.

In particular, stochastic epigenetic variation provides a natural avenue for 'reverse engineering' of the multicellular epigenome into a unicellular one. As evolutionary graph dynamics demonstrate [21▪], the multicellular strict unidirectional differentiation structure suppresses the selection of fitter mutants. In contrast, a relaxation of the hierarchical structure through epigenetic variation may allow for enhanced selection and fitness optimization. Thus, as cancers progress and grow, greater epigenetic variation occurs, leading to blurring of epigenetic identities, enhanced evolutionary potential, and adverse outcome [18▪].

These studies have also taught us an important humbling lesson. Cancers are more complex than we have previously appreciated, and the recipe to overcoming our best therapies is typically already known to small subclones present upon treatment initiation. To overcome this complexity, we would need to develop new therapeutic approaches that cease to regard cancers as monolythic populations. Instead, we would need to study cancer diversity to devise the next facet of precision medicine – a treatment that is personalized not only to an individual patient, but also to individual clones within a single cancer. This perspective will catalyze the next generation of therapeutic algorithms, which maximize overall tumor elimination, instead of merely selecting one clone over another.

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Conflicts of interest

There are no conflicts of interest.

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