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Clonotyping for precision oncology

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Abstract

Advances in identifying subpopulations of cancer cells and reconstructing the clonal evolution of tumors greatly enhance our understanding of the molecular events within a patient and their context relative to one another. In the rapidly unfolding era of personalized medicine, the ability to monitor clonal evolution throughout patient care has significant clinical implications for the appropriate development or application of targeted therapies as well as understanding the potential mechanisms driving resistance. In this review, we discuss advances in biotechnology and bioinformatics that improve precision treatment by dissecting clonal evolution, focusing first on the initial discoveries in lymphomas and leukemias followed by the more recent applications to advance our understanding of prostate cancer (PCa).

Keywords

prostate cancer; leukemia; lymphoma; precision medicine; clonal evolution; personalized medicine; subclonal architecture; metastasis

Introduction

Advances in next-generation sequencing (NGS) have facilitated our ability to detect somatic mutations and subsequently identify subpopulations of cells, or clones, in tumors. This in turn can provide valuable insight into potential morphologically or functionally distinct

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Teaser: The ability to monitor clonal evolution throughout patient care has significant clinical implications for the development and application of personalized therapy.

subclones contributing to progression of disease or treatment resistance. Pioneering work in leukemia and lymphoma has demonstrated that monitoring clonal evolution has major implications for understanding the development and application of targeted therapies in the rapidly unfolding era of personalized medicine. To date, multiple studies have focused on characterizing the molecular landscape of PCa, revealing a spectrum of mutations associated with variable clinical outcomes. Placing these molecular events in the context of the clonal evolution has enormous potential to elucidate prostate tumor biology and ultimately lead to precision medicine. This can be exemplified by recent studies exploring clonal evolution throughout the progression of this lethal disease. Here, we discuss the genomics and informatics approaches towards understanding clonal evolution, highlight seminal studies in hematological malignancies, and recent discoveries for improving PCa treatment.

Defining the subclonal architecture

Tumor heterogeneity serves as the basis for clonal evolution through the ongoing selection of clones with advantageous ‘driver’ mutations. However, this heterogeneity presents a significant challenge in accurately reconstructing the subclonal architecture. Fortunately, by leveraging the advances in NGS technology, it is now possible to identify and quantify the prevalence or cancer cell fraction (CCF) of subclonal populations. This is done by monitoring the variant allele frequency, which is the ratio of the number of reads supporting a somatic variant relative to the total reads at the locus. Currently, single nucleotide variants (SNVs) are the most commonly monitored mutational class for clonality analyses. However, placing additional classes of somatic mutation, such as insertions, deletions, and translocations, in the context of clonal evolution is also important. Furthermore, the bioinformatics methods for accurately detecting these mutational classes coupled with the more challenging validation methods required to obtain a high depth of coverage has resulted in a slower assessment of their heterogeneity. However, once estimated, a CCF serves as a molecular clock indicating when a mutation was acquired during clonal evolution. For instance, mutations presented in all cancer cells within a pure sample would have a CCF of ~100%, whereas subclonal mutations, acquired later, would be present in fewer cells and, therefore, have lower CCFs. Changes in CCF between samples collected before and after treatment can be used as an indicator of treatment sensitivity or resistance (Figure 1). Furthermore, mutations specific to a single sample suggest that they arose since the samples evolved from one another (i.e., a metastatic sample acquires additional mutations from the original primary tumor). Although seemingly straightforward, it is imperative that the bioinformatics tools used are able to reliably detect mutations. This is crucial for the more difficult to detect subclonal variants presented at low cellular fraction because they are precisely the events needed to identify the existence of a subclonal population.

The resolution for accurately reconstructing the subclonal architecture improves as the number of mutations used increases, thereby necessitating the use of both driver and passenger mutations. Therefore, the preferred strategy for comprehensively reconstructing the clonal architecture is provided by first discovering variants using whole-genome sequencing (WGS), to establish the most comprehensive mutation list, and subsequently quantifying CCFs using deep, targeted sequencing to improve the sensitivity for detecting

low-frequency clones [1–5]. Despite some existing studies that have used exome sequencing [6–8], or even limited candidate gene resequencing [9], to elucidate the subclonal architecture, the limited number of mutational events compared with WGS results in an underestimation of clonal heterogeneity. Therefore, in the context of hematological malignancies with relatively few protein coding mutations, such as acute myeloid leukemia (AML), it is beneficial to use WGS, whereas it might suffice to use exome sequencing in cancers with greater mutation burden, such as PCa or chronic lymphocytic leukemia (CLL) [8].

Clonal heterogeneity can sometimes be observed from a single tumor sample [10–12]. However, comparing various samples representing spatial or temporal heterogeneity within a patient can disambiguate subclones of similar prevalence or characterize additional subclones not observed in a single sample and ultimately reconstruct more comprehensive tumor histories [1–3,6–8]. This can have profound impact on understanding various clinical scenarios, such as how subclones evolve following treatment, during the progression to metastasis, or at relapse. In addition to comparing biopsies taken at the same or varying time points from a patient, this strategy is applicable to tumor cells passaged in culture or patient-derived xenografts (PDX) to identify a subpopulation of cells that confer a specific phenotype [13]. Interestingly, such a strategy has revealed that a subset of PDX models can acquire additional mutations, thereby changing their molecular landscape such that they no longer recapitulate the original tumor [13]. Overall, the combination of deep coverage for all mutations across samples will provide a comprehensive view of spatial and temporal clonal evolution. This in turn will improve our understanding rates of tumor evolution, expansion, and tendencies to relapse, metastasize, and remetastasize that could ultimately impact patient care.

Clonal evolution in leukemia

Multiple seminal studies in hematological malignancies have improved our understanding of subclonal architecture and its utility for precision treatment. This can be exemplified by the analysis of patients with M3-AML with a known initiating event (PML-RARA), patients with normal karyotype M1-AML, and hematopoietic stem and/or progenitor cells (HSPCs) from healthy people. Interestingly, Welch *et al.* found that HSPCs from healthy donors accumulate random mutations during aging and that most somatic mutations in AML randomly occurred before the acquisition of the initiating mutation. Therefore, if a HSPC transforms to a leukemic blast, the mutational history, comprising the initiating mutation and passenger mutations, is ‘captured’ in subsequent progeny. Typically, the founding clone only requires one or two additional cooperating mutations to generate the malignant founding clone [6]. Therefore, only a tiny fraction of the total mutations in each AML genome is likely to be relevant for pathogenesis, disease classification, and targeted therapy.

To understand clonal evolution during relapse, a recent study conducted whole-genome analysis of eight primary tumors and relapse pairs to reveal patterns of clonal evolution. Ding *et al.* found that, during AML relapse, either the founding clone in the primary tumor acquired additional mutations and evolved into the relapse clone or a subclone, derived from the founding clone, escaped therapy and subsequently acquired additional mutations to

expand into the relapse clone [1]. Interestingly, chemotherapy failed to eradicate the founding clone in any of the patients. The notion that AML relapse is associated with the acquisition of new mutations has also been validated by isolating and sequencing preleukemic HSPCs, found to have a subset of mutations of their leukemic progeny [7], as well as in serial studies tracking evolution from severe congenital neutropenia [14] or myelodysplastic syndromes (MDS) [2] to AML. Through targeted and whole-genome analysis of a cohort of secondary AML (sAML) samples, Walter *et al.* found that recurrently mutated genes display a range of CCFs, demonstrating that none of the mutations are consistently associated with the founding clone and that the disease progresses through a variety of acquired mutations across patients [3]. Welch *et al.* also demonstrated that the founding clone can acquire subsequent cooperating mutations that might result in the evolution of subclones contributing to disease progression and/or relapse.

Interestingly, a follow-up study on an AML case (AML31), previously characterized in Ding *et al.* [1], by ultra-deep sequencing of samples collected at different times during the course of chemotherapy treatment, identified an additional independent subclone carrying a Tumor Protein P53 (*TP53*) mutation that was present at extremely low cellular fraction in the primary tumor. This subclone escaped chemotherapy and expanded in the relapse, and was subsequently confirmed by single cell sequencing [4]. Without temporal data, this clinically important clone would have been merged with another subclone arising from the founding clone that also resisted to chemotherapy treatment. Therefore, this result necessitates the use of ultra-deep sequencing and temporal monitoring of tumors to accurately reconstruct clonal evolution.

In another study, Klco *et al.* used targeted sequencing to monitor the evolution of AML subclones and their potential functional heterogeneity. Whereas almost all of the AML subclones trafficked from the marrow to the peripheral blood, some subclones were enriched in specific cell populations. Single cell sequence analysis verified the identity of subclonal genotypes before undergoing various biological and experimental conditions. In some cases, morphologic or phenotypic features, as well as *in vitro* growth properties, correlated with distinct subclones, suggesting functional variation in differentiation potential that is genetically determined. Interestingly, subclones displayed variable engraftment potential in immunodeficient mice and the xenografts generated predominantly comprised a single genetically defined subclone as opposed to recapitulating the clonal architecture of the transplanted AML. Taken together, this study demonstrates that the complete spectrum of mutations within a subclone defines the morphological and phenotypic properties that correlate with distinct functional characteristics, such as *in vitro* growth, engraftment in immune-deficient mice, or relapse in patients [15]. Furthermore, this work demonstrates the potential constraints of using xenograft models for predicting clinical response, a common preclinical step for developing US Food and Drug Administration (FDA)-approved therapies.

Although DNA sequencing has enabled the reconstruction of clonal evolution and the discovery of clinically relevant mutations in leukemia, when used alone it only provides a partial molecular picture of a tumor. Combination of WGS, exome, and RNA sequencing has been successfully used in guiding personalized treatment. The best example is probably

the identification a novel target, the Fms-like tyrosine kinase 3 (*FLT3*) gene, which was overexpressed in the tumor of Lucas Wartman, who presented with a rare adult acute lymphoblastic leukemia (ALL) that was thought as incurable at his second relapse in 2011. Given that all chemotherapy failed and no actionable mutation was found via WGS and exome sequencing, using a *FLT3* inhibitor became the only option; fortunately, this resulted in complete remission and enabled a subsequent stem cell transplant [16].

Taken together, pioneering work in leukemia research has demonstrated that subpopulations of cells in an individual tumor or across tumors can be morphologically or functionally distinct and have a profound role in progression, treatment, and relapse. Moving forward, the integration of DNA and RNA sequencing data can further improve our understanding of tumor evolution and the identifying of druggable targets for precision medicine.

Molecular landscape of PCa

PCa is the most common malignancy affecting men and the second leading cause of cancer-specific deaths in the USA. To understand the molecular underpinnings of PCa, there have been multiple transcriptome, exome, and WGS analysis efforts that have focused on identifying the recurrent gene fusions and mutated genes, respectively [5,17–26]. Interestingly, approximately 85% of patients with PCa can be stratified by mutually exclusive gene fusions and outlier genes [27]. However, >80% of patients with PCa patients are multifocal and might have distinct molecular profiles [25,28,29]. Using WGS, Boutros *et al.* characterized 23 distinct regions of the primary tumors from five patients with multifocal, localized PCa. They observed a high level of spatial heterogeneity between foci (in both point mutations and large genomic rearrangements) and, in some cases, different foci did not share mutations, indicating they had independent origins. This has significant implications for assessing and prioritizing tumor foci harboring clinically relevant alterations that might have a biological role in disease progression.

Several studies have focused on understanding of how mutations arise and accumulate throughout prostate tumor progression, metastasis, and treatment resistance. Initial analysis of PCa whole genomes revealed complex rearrangements that are linked together, like a chain, which likely arose when broken DNA ends are shuffled and religated to one another [30]. Subsequent whole-genome analysis of 57 tumors revealed that many closed loops chains are likely contributing to the genesis of gene fusions. More recently, Baca *et al.* observed a phenomenon by which translocations and deletions arise in a highly interdependent manner, referred to as ‘chromoplexy’ [31]. The authors suggest that this creates a punctuated evolution where periods of sequential mutation accumulation are interspersed with dramatic changes in the mutational landscape by a few events simultaneously altering multiple oncogenes and tumor suppressors. This reiterates the need to reconstruct the subclonal architecture to elucidate subclones that might have undergone chromothripsis or chromoplexy. Given the plethora of mutations that occur during these processes, placing molecular events in the context of the clonal architecture could provide valuable insight into potentially relevant events for therapeutic intervention. For instance, recent exome and whole-genome sequencing studies revealed genes that were altered in both primary tumors and castration-resistant PCa (CRPC), such as Forkhead Box A1 (*FOXA1*),

Cyclin-Dependent Kinase Inhibitor 1B (*CDKN1B*), and leukemia protein 2 (*MLL2*), whereas Androgen Receptor (AR), *TP53*, and Guanine Nucleotide-binding Protein, Alpha Stimulating (*GNAS*) alterations were implicated in metastasis and treatment resistance [5,17–19,26,32,33].

Indolent and aggressive disease

A current controversy exists regarding the overtreatment of patients with PCa, thereby emphasizing the need to identify markers that can distinguish indolent versus aggressive disease. Prostate tumors are heterogeneous and multifocal [28,29]. Early studies demonstrated genetic similarities across independent metastatic sites, suggesting a monoclonal origin resulting in lethal metastatic cells [29,34]. In contrast to monoclonal metastasis observation, a recent large-scale WGS of lethal PCa demonstrated the possibility of polyclonal cascades of metastasis seeding, via observation of subclones shared by multiple metastases [5,33]. Many metastatic sites were likely seeded by multiple subclones from the primary or other metastatic tumors at different times. A model of metastatic reseeding of the surgical bed was also proposed [33]. Additionally, Haffner *et al.* conducted a whole-genome analysis of primary and metastatic tumor specimens from a 47-year-old man who had succumbed to CRPC 17 years after initial presentation [35]. By monitoring the evolution of the lethal cell clone throughout the progression from the primary cancer to metastases, the authors discovered that it arose from small, low-grade cancer foci in the primary tumor instead of the higher-grade primary cancer or lymph node metastasis. This represents a first step towards understanding the mutational context throughout PCa progression that could improve precision medicine. Overall, it is still challenging to comprehensively characterize the intratumoral heterogeneity of primary tumors to rule out the possibility that metastatic clones originated from the primary tumor. Regardless, these studies challenged our current efforts in accurately reconstructing clonal histories of patients exhibiting highly intra- and intertumoral heterogeneity.

Understanding resistance to targeted therapies

Analogous to leukemia studies, understanding the clonal evolution of patients throughout progression and treatment can offer profound insights into how they become resistant as well as the development and application of targeted therapies. As an initial step towards this endeavor, the Prostate Cancer Foundation (PCF) and Stand Up 2 Cancer (SU2C)/American Association for Cancer Research (AACR) have funded a multi-institutional study that incorporates NGS to systematically evaluate patients enrolling in four clinical trials for novel drugs for CRPC. Discriminating responders and nonresponders to treatment will potentially lead to predictors of response as well as advance our understanding of how tumor cells become resistant through the acquisition of drug-resistance mutations. Initial results on a cohort of 150 patients observed common pathways altered in metastatic CRPC, including AR signaling, Phosphoinositide-3-Kinase (*PI3K*), and especially the *WNT* signaling pathway, that had not previously been seen in PCa [26]. Ideally, such studies will reveal common mechanisms that would serve as the basis for developing novel targeted therapies. However, it is plausible that we will need to understand the clonal evolution in a patient-specific manner because even uncommon genomic alterations might directly affect a

response to therapy and ultimately patient outcome [36]. Therefore, this multi-institutional study will lay the foundation for precision-based treatment.

Given tumor heterogeneity, tracking mutations in tumor biopsies and circulating tumor DNAs as tumors progress can significantly contribute to high-resolution construction of clonal evolution. A recent study that monitored commonly mutated loci in primary, metastatic, and circulating tumor DNAs in blood plasma revealed that resistant clones often have complex spatial and temporal heterogeneity that was sometimes not seen in tumor biopsies but observed in plasma samples [32]. Clinically relevant mutations, such as ones involving *AR*, emerged or regressed depending on specific treatments used. For example, several functionally relevant mutations in *AR* emerged when patients were treated with glucocorticoid and, therefore, monitoring those mutations is important in determining whether to withdraw or eliminate glucocorticoid in targeted therapy. Taken together, this study exemplified the need for continuous monitoring of both tumor biopsies and circulating tumor DNAs over time in guiding personalized treatments.

Implications of clonotyping for treating PCa

Although various mechanisms have been studied to understand PCa progression, including androgen signaling and gene fusions, we are faced with the clinical challenge of treating a heterogeneous population with differing molecular backgrounds. Therefore, it is crucial to speciate patients with indolent or aggressive disease and, subsequently, their progression and resistance to therapy. Thus, it is conceivable to establish longitudinal monitoring of the care of patients with PCa to improve clinical management through precision medicine. Under such a strategy, patients with newly diagnosed PCa would have their biopsies assayed with NGS technologies and stratified as either being low risk and remaining on surveillance, or high risk, in which case they would undergo local therapy. Although hormonal therapy would serve as the standard of care, it could be supplemented with targeted therapy, when applicable, identified through sequencing. Additional biopsies could be sequenced upon progression or metastasis to identify molecular alterations that could guide subsequent targeted therapies.

To implement longitudinal patient management, there currently exist numerous commercial high-throughput strategies. Although WGS is an attractive diagnostic platform, the decreasing costs have not yet reached a level for routine use in the clinic. Furthermore, it still requires a fair amount of analysis time and the moderate–low coverage is not sensitive to detect crucial low-frequency subclonal mutations. To circumvent these issues, several targeted NGS assays exist with the intent of providing deep coverage across a panel of candidate genes typically selected from previous large-scale sequencing efforts (i.e., The Cancer Genome Atlas) that have characterized prevalent somatic cancer mutations. Furthermore, a single hybrid-capture NGS platform improves efficiency and scalability over a variety of disparate methods otherwise required to discover the full spectrum of mutations (i.e., gene fusions, SNVs, and indels), while providing deep read counts to improve the likelihood of detecting residual disease. However, such strategies are reliant on capturing the full spectrum of relevant mutation *a priori* and, therefore, could miss an uncommon molecular event responsible for treatment response or progression. In addition to optimizing

the appropriate NGS assay for patient monitoring, additional efforts are being placed on sequencing from formalin-fixed, paraffin-embedded tissue, requiring lower genomic input, as well as scaling such approaches to WGS [37,38]. Furthermore, noninvasive monitoring using circulating tumor cells, plasma DNA, exosomes, and urine are also being explored for diagnostic and predictive biomarker detection [32,39–42].

Concluding remarks

In summary, advances in bioinformatics and genomics technologies have enabled a more accurate reconstruction of the subclonal architecture of tumors, which offers profound insights into their molecular evolution throughout progression and in response to treatment. We are still witnessing improvements in the analytical development and genomic assays to comprehensively monitor crucial events within rare and emerging subclones, assaying using noninvasive specimens and single cells, and the application to more diverse clinical scenarios in PCa. Although tumor heterogeneity presents a crucial challenge, taken together, these advances will improve understanding of PCa progression and serve as a guide for treatment strategies aimed at the elimination of all, rather than just some, of the cancer cells within a patient. Taken together, improved longitudinal and temporal monitoring of clonal architecture throughout patient care will enhance the successfulness of precision medicine.

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Highlights

- Subpopulations of cancer cells can be morphologically and/or functionally distinct
- Clonal evolution is complex and clinically relevant to disease progression, metastasis, and treatment resistance
- Intra- and inter-tumoral heterogeneity presents a significant challenge in tracking clonal evolution to guide precision medicine
- Longitudinal and temporal monitoring of clonal populations in tumor biopsies and blood plasma can improve personalized treatments

Figure 1.

Reconstructing subclonal evolution. **(A)** Following the detection of somatic mutations in samples collected before and after treatment, the corresponding cancer cell fraction (CCF) of the mutations in each sample can be estimated. Subsequent clustering of variants can be used to identify somatic mutations with similar CCF that might correspond to a clone and/or subclone. This in turn can be used to infer the model of clonal evolution **(B)** that can reveal clinically relevant patterns, such as a subclone that regresses (subclone 2), expands (subclone 3), or arises (subclone 4) following treatment.