Application of Single-Cell Sequencing to Immunotherapy

Kristin G. Beaumont, PhD*, Michael A. Beaumont, PhD, Robert Sebra, PhD

KEYWORDS

• Immunotherapy • Cancer • Single-cell analysis • Prostate cancer • Urologic cancer
• Tumor heterogeneity

KEY POINTS

- Heterogeneity of a tumor and its microenvironment (including the immune compartment) likely contributes to differences in disease development and patient response to treatment.
- Next Generation Sequencing (NGS) of bulk tissue does not always capture heterogeneity.
- Single cell sequencing of cells from tissue allows for better resolution of minor cell populations.
- Understanding these populations can help identify tumor targets and cells that can be leveraged for immunotherapy as well as to understand the effects of therapy on tumor development.

INTRODUCTION

Cancer is a complex disease rooted in heterogeneity, which is the phenomenon of individual cells, tissues, or patients having distinct phenotypic and/or genetic characteristics. This is particularly true for cancers such as prostate cancer, in which the 200,000 new cases diagnosed each year are split between men diagnosed with clinically indolent disease that does not require immediate treatment and aggressive disease that requires intervention.[1] Similar heterogeneity is observed in therapeutic response, in which some patients relapse soon after treatment, but others remain disease free for years before recurring. Such divergent disease etiology is thought to be a result of tumor heterogeneity, and therefore, better understanding the complexity of this disease may inform better diagnosis and treatment.

Tumors evolve as cancerous cells grow within an intricate network of noncancerous cell types and extracellular matrix proteins, which taken together, comprise the tumor microenvironment (TME). It is only recently that the complexity of the TME has been appreciated for its role in cancer evolution, metastasis, and treatment outcomes, and that the varied subpopulations of cells (immune, stromal, cancer, and others) each uniquely contribute to the progression of disease. The classification of distinct and important subpopulations of cells within the tumor and its associated microenvironment has remained a technical challenge. Next generation sequencing (NGS) of bulk tumor tissue has yielded some exciting results, including the discovery of certain driver mutations and variants of unknown significance. In this approach, genetic material is extracted from bulk tissue and sequenced, which provides an overall average genetic profile of the sample, but masks contributions from individual cells and minor populations of cells, particularly in heterogeneous samples. Only with the advent of single-cell sequencing technologies has it become possible to characterize the key contributions of cell subpopulations to identify new therapeutic targets and disease biomarkers, and understand modes of treatment for disease, in which single-cell sequencing characterizes and reports the genome or transcriptome of each individual cell in a population, allowing the identification of subpopulations of

[a] Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, 1425 Madison Avenue, Box 1498, New York, NY 10029, USA
* Corresponding author.
E-mail address: kgbeaumont@gmail.com

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cells that are genetically similar or distinct. This advantage is particularly important in characterizing and understanding tumor response to immunotherapy, as this therapeutic approach involves highly heterogeneous immune cell populations and tumor targets and bulk NGS approaches do not necessarily capture the complexity of such systems. In particular, single-cell analysis is uniquely suited to (1) identifying known, rare, or novel tumor targets; (2) characterizing patient-specific populations of immune cells that can be leveraged for immunotherapy; and (3) assessing the effects of immunotherapy on subpopulations of cells within a tumor to understand sensitivity or resistance. In this review, we view cancer immunotherapy through a single-cell lens and discuss the state-of-the art technologies that enable advances in this field.

HETEROGENEITY IN UROLOGIC CANCERS

Overview

Recognizing cancer as a highly heterogeneous disease is essential to studying its causes and evaluating therapeutic approaches. The extent of heterogeneity, which is the phenomenon of individual cells having distinct phenotypic and/or genomic/transcriptomic characteristics, is known to vary both as a function of disease and between patients with the same disease. For a given disease, heterogeneity may be defined as "interpatient" (mutational/transcriptional differences between tumors from different patients), "intrapatient" (mutational/transcriptional differences between tumors from the same patient, but derived from different sites), or "intratumor" (mutational/transcriptional differences between subpopulations within one tumor and its associated TME). Single-cell genomic and transcriptomic analyses facilitate a more comprehensive understanding of each level of heterogeneity, particularly intratumor heterogeneity (ITH), by permitting the identification and characterization of rare subpopulations of cells that were not previously detectable by bulk NGS methods. Moreover, single-cell analysis has been essential in identifying susceptible subpopulations of tumor cells that can serve as targets for immunotherapy, or identifying subsets of immune cells within the TME that can be leveraged for immunotherapy treatment (Fig. 1).

Heterogeneous tumors consist of many different cellular clones, or subpopulations of cells. The types and relative numbers of these cells differ between patients and tumors: there is now evidence that the degree of heterogeneity of a solid tumor and its associated TME affects its propensity to proliferate as well as its susceptibility to treatment. The clonal distribution of tumor cells may change during therapy, in an effect known as clonal evolution. Clonal evolution occurs when therapy targets only certain subsets of tumor cells, and the overall therapeutic profile of a tumor changes over the course of treatment: previously treatment-sensitive tumors may become treatment-

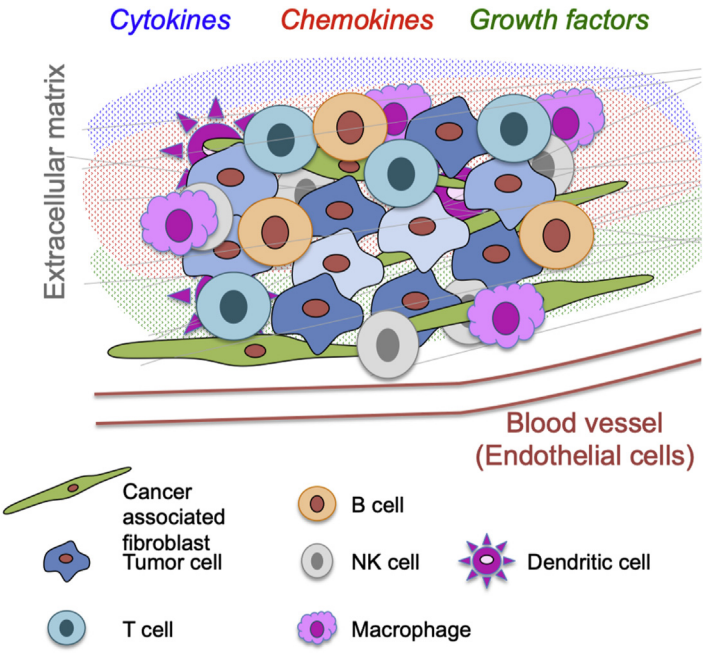

Fig. 1. Identifying subsets of immune cells within the TME that can be leveraged for immunotherapy treatment. NK cell, natural killer cell.

resistant following therapy, where one possible consequence of therapy is preferential selection for survival of treatment-insensitive clones (which may be potentially rare and difficult to detect).[2] Leveraging single-cell data analysis to characterize clonal evolution following therapy can help inform clinicians as to the success of the approach or suggest whether a combined approach is necessary (Fig. 2).

As one particularly striking example, muscle invasive bladder cancer is known to have one of the highest mutation rates of any cancer, which results in extensive ITH at both the genomic and transcriptomic levels.[3] Although TP53 mutations were recognized early on as one of the key drivers of bladder cancer, targeting these tumors therapeutically yielded limited results, likely due at least in part to their highly heterogeneous nature. Cote and coworkers[4] authored an early report suggesting that patients bearing tumors with TP53 mutations responded better to chemotherapy, but follow-up clinical trials failed to support this observation. In one TP53-targeted phase III trial, which assessed treatment response to adjuvant cisplatin-based chemotherapy (vs surveillance) based on TP53 status, yielded no difference in 5-year recurrence-free or overall survival based on this marker.[5] The investigators noted that immunohistochemistry, which was the primary method for assessing p53 dysregulation, likely failed to accurately reflect the range of patients' TP53 statuses, given that some mutations in TP53 result in p53 protein that is not reactive to the immunohistochemistry antibody.[6] This contradictory result was one of the first cautionary indicators that more detailed understanding of the characteristics of subpopulations of cells within these tumors was necessary to be able to predict therapeutic outcome.

Prostate cancer is also known to be particularly heterogeneous, starting even at the level of gross morphology. Classified as a "pluriform" neoplasm, it can consist of glandular, cribriform, trabecular, solid, and single-cell tumor patterns.[7] Moreover, prostate cancer is often multifocal, where some reports estimate that 50% to 90% of all radical prostatectomy specimens have more than one disease foci, and this characteristic is associated with higher grade, stage, and recurrence rates.[8] The origin of multifocal disease in prostate cancer is

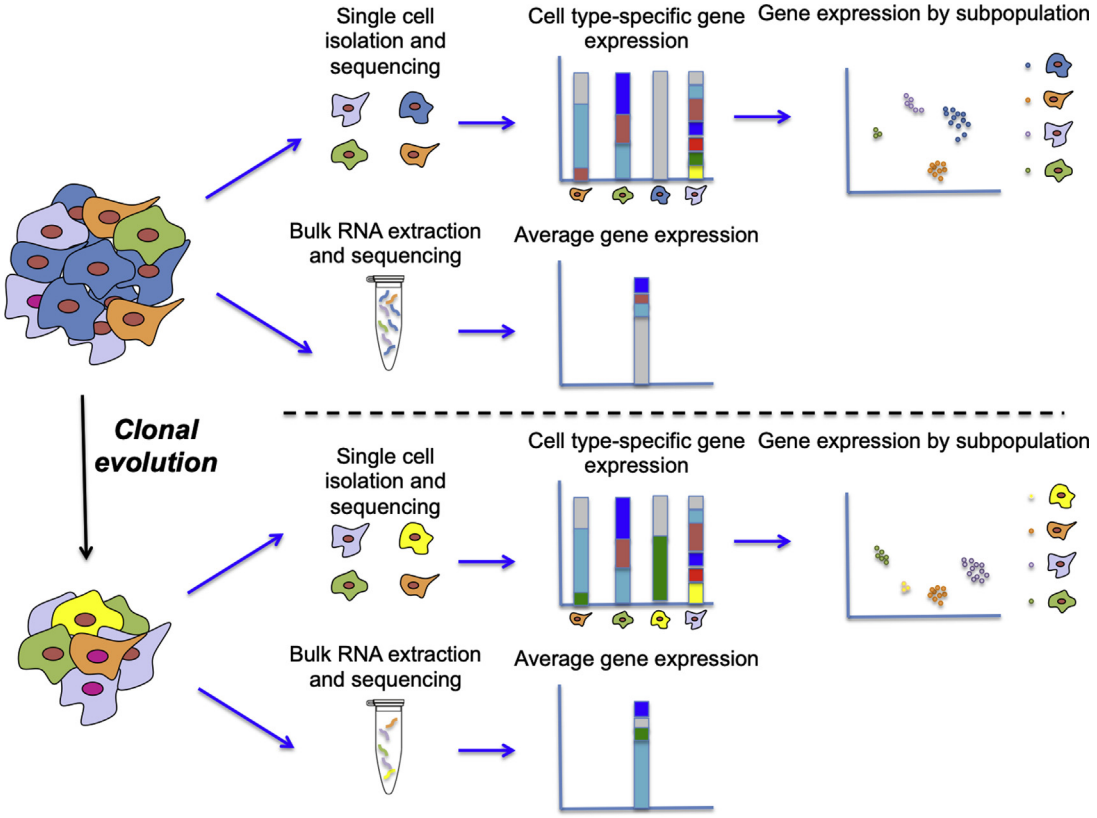

Fig. 2. Leveraging single-cell data analysis to characterize clonal evolution following therapy can help inform clinicians as to the success of the approach or suggest whether a combined approach is necessary.

not clear: one school of thought is that different genetic and epigenetic alterations occur in a single clone, which evolve through generations of daughter cells, resulting in different disease foci ("monoclonal origin"),[9] whereas an alternative view is that genetic abnormalities arise independently in otherwise healthy cells ("polyclonal origin," known as the cancer field effect).[10] Bulk sequencing of foci provides evidence supporting both views and clearly illustrates the genomic and transcriptomic heterogeneity in this disease.[11–14]

Heterogeneity in Immune Response

By definition, immunotherapy leverages the patient's own immune system to target tumor cells. The complexity of human immune response relies on the choreography of many different subpopulations of cells that are not only defined by cell type (eg, T cell, B cell, natural killer cell), but by state (activated, exhausted, cytotoxic). The necessity of single-cell analysis in characterizing immunotherapy mechanism and effectiveness becomes even more clear when heterogeneity in the immune response is considered alongside heterogeneity in the tumor and tumor-associated cells that will serve as targets for the immune campaign. Moreover, both sides of the immunotherapy coin (immune cells and their tumor targets) undergo phenotypic, genomic, and transcriptomic changes that vary spatially and temporally as well as in response to specific stimuli, further motivating the need for single-cell analysis of these effects.

Immune cells residing in the tumor microenvironment, or the tumor immune microenvironment (TIME), are particularly important in dictating tumor behavior and response to treatment. These populations of cells have been the focus of a great deal of recent research and are known to be highly heterogeneous in both identity and function. T cells and tumor-associated macrophages (TAMs) have both shown promise as biomarkers for immunotherapy, in which CD4+ helper T cells and cytotoxic CD8+ T cells can prevent tumor growth, and highly plastic TAMs can repress antitumor immunity, angiogenesis, and cell migration. In many cases, the makeup of the TIME can be used for predicting prognosis and is often associated with patient outcome. As one example, significant tumor infiltration of type 1 T-helper (Th1) cells, CD8+ cytotoxic T cells, and their associated cytokines typically suggest that a patient's immune system is capable of some degree of tumor suppression. Characterizing and defining an antitumor microenvironment is challenging given that tumor-associated immune cell populations vary by patient, disease subtype, and time. Furthermore, the function and interactions of different subpopulations have not been well-characterized, but studies toward this goal have yielded promising therapeutic targets, such as PD-1 and CTLA-4, which can be targeted by antibodies to overcome T-cell exhaustion.[15,16] Prostate cancer is somewhat unique in that it is both a known immunogenic disease and, for certain molecular subtypes, driven by hormone stimulation, making this an ideal disease for targeting with combination therapy. Designing combination therapy is inherently complex, particularly when both the tumor targets and immune system compartments are heterogeneous and temporally dynamic. It is also encouraging that prostate cancer has several well-described tumor-associated antigens (including prostate-specific antigen, prostatic acid phosphatase, and prostate-specific membrane antigen) that may serve as targets for immunotherapy.[17]

In evaluating immunotherapeutic approaches, single-cell analysis is uniquely suited to identifying tumor targets (known, rare, or novel), characterizing patient-specific populations of immune cells that can be leveraged for immunotherapy and assessing the effects of immunotherapy on subpopulations of cells within a tumor to understand sensitivity or resistance. One particularly notable example of using single-cell characterization to understand potential tumor response to immunotherapy was published by Chevrier and colleagues.[18] They developed an immune atlas of clear cell renal carcinoma from 73 affected patients and 5 healthy controls using single-cell mass cytometry (a complementary technique to single-cell sequencing that relies on antibody panels). By interrogating 3.5 million single cells, they identified 17 tumor-associated macrophage phenotypes and 22 T-cell phenotypes, but most significantly, were able to identify a signature immune composition that was associated with progression-free survival. This study clearly demonstrates that single-cell analysis reveals additional layers of cellular complexity and that this additional resolution can be clinically important.

Contributions of Bulk Next Generation Sequencing to Understanding Urologic Cancer

The Cancer Genome Atlas, the International Cancer Genome Consortium, and efforts of individual groups have illuminated some of the genetic roots of cancer (including prostate cancer) using bulk NGS approaches, such as whole genome sequencing (WGS), whole exome sequencing (WES), and bulk RNA sequencing.[19–24] These

approaches rely on genetic material isolated from tissue and result in an average, overall genetic profile of the entire sample where the genetic contributions of individual cells are masked. Novel, common alterations have been identified in prostate cancer using this method, including SPOP and CHD1 mutations, and previously known targets have been validated (including PTEN and TP53 loss or mutation).[25] Using prostate cancer as a key example, though, the clinical utility of these efforts has been somewhat limited because, as one might expect, the genetics of this disease are very complex. Several genetic anomalies (fusions in ERG, ETV1/4, and FLI1 and mutations in SPOP, FOXa1, and IDH1) are common among patients with prostate cancer, and these have led to the evolution of 8 distinct molecular subtypes of the disease: 7 groups, each based on 1 conserved genetic alteration and 1 group of "other." However, samples within each of these classifications have demonstrated additional contributors to heterogeneity (for example, in epigenetic profiles and androgen-receptor activity) and these classifications have not been linked to either prognosis or therapeutic effectiveness.[7,19,26]

It is interesting that the Gleason score, which is a very strong predictor of disease progression in prostate cancer, has been demonstrated in some studies to be related to underlying genetic alterations.[7] As one example, Rubin and colleagues[27] performed WGS and WES on samples from 426 prostate cancer samples, with the goal of identifying genomic support for prognostic grade groupings, which are clinical categorizations based on Gleason number. They observed increasing polyploidal frequency with increases in genomic amplification, deletions, and nonsynonymous point mutations with increasing grade.[27] This observation, taken together with the knowledge that single-cell analysis can better characterize the genetic complexity of disease, leads to the hope that single-cell sequencing and characterization may contribute significantly toward clinically actionable information, particularly in highly heterogeneous treatment scenarios, such as immunotherapy of urologic cancers.

Single-Cell Sequencing: Current State of the Art

Single-cell sequencing has grown extensively in the past several years, both in the characterization of single-cell genomics (DNA) and single-cell transcriptomics (RNA). Single-cell genomic analysis has been particularly useful in understanding the evolution of somatic mutations in disease. As one example, Zhang and coworkers[28] used single-cell WGS to characterize the somatic mutational landscape in B lymphocytes as a function of age and found that cells from newborns contained fewer than 500 mutations per cell, whereas cells from centenarians contained more than 3000 mutations per cell. They were also able to use this approach to identify mutational hotspot regions, some of which were located at genes associated with somatic hypermutations, similar to mutational signatures observed in B-cell tumors.[28]

Single-cell transcriptomic analysis reflects the functional diversity of cells and can capture the functional consequences of tumor/TME interactions as well as the effects of treatment. As one cogent example, Horning and coworkers[29] used single-cell RNA sequencing to characterize prostate cancer cell response to androgen stimulation, given that there is evidence that small androgen-independent subclones of cells are preferentially selected during androgen deprivation therapy and drive post-therapy disease progression. In this work, they profiled the transcriptomes of 144 single LNCaP prostate cancer cells, following cell-cycle synchronization and androgen treatment, which revealed previously unappreciated heterogeneity in the cellular response of 8 potential subpopulations. In particular, they identified one subpopulation of cells with enhanced expression of 10 cell-cycle genes, and decreased dependence on androgen-receptor signaling, which resulted in advanced growth and sphere formation capability of these cells. This work clearly highlights the need to understand the heterogeneity in this disease to better understand disease progression and therapeutic mechanism.

Single-cell immune profiling approaches also have proven useful in understanding immune response at the single-cell level. In brief, the V(D)J sequences of T cells and B cells are characterized such that T-cell and B-cell clonotype diversity profiles and antigen specificity can be evaluated. This is invaluable not only as an additional dimension for understanding the TIME, but also in evaluating personalized medicine approaches.

OVERVIEW OF SINGLE-CELL SEQUENCING APPROACHES

Single-Cell DNA Sequencing

Although much of the discussion in this review has been focused on the value of single-cell transcriptomics (via single-cell RNA sequencing), it is important to note that single-cell characterization of the genome has also been evolving toward novel and impactful data (**Fig. 3**). Single-cell copy number variation analysis has proven useful

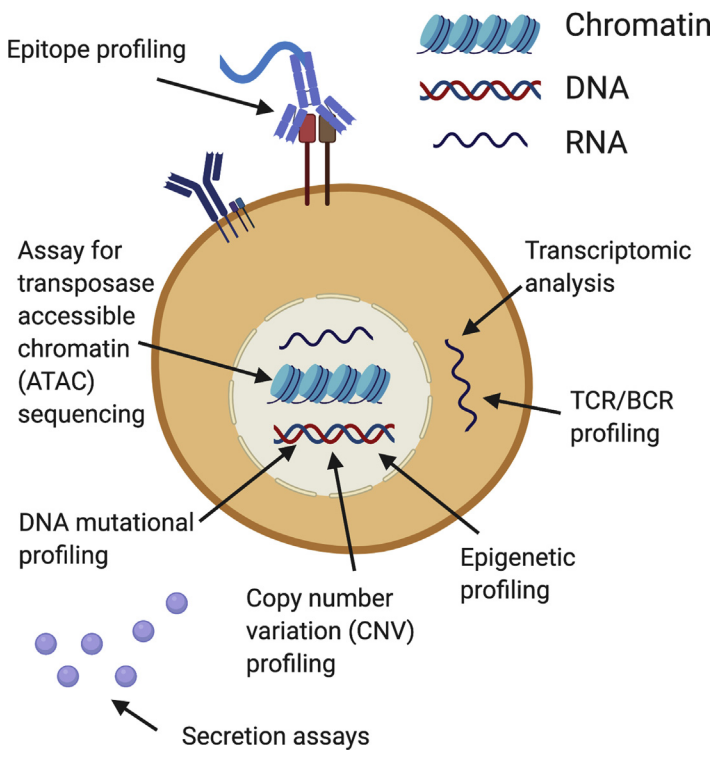

Fig. 3. Single-cell characterization of the genome has also been evolving toward novel and impactful data.

in characterizing genomic heterogeneity and clonal evolution, particularly in diseases driven by such aberrations (such as multiple myeloma).[30] Single-cell approaches to understanding epigenetic heterogeneity have also been developed, particularly those aimed at characterizing chromatin accessibility (single-cell assay for transposase-accessible chromatin sequencing)[31,32] and methylation,[33] where these data are useful in understanding accessibility and activity of transcriptional promoters.

Single-Cell RNA Sequencing

Early, plate-based approaches to single-cell analysis focused on single-cell isolation followed by Smart-Seq library preparation and Illumina-based sequencing. There are a variety of methods that have been useful for single-cell isolation, including laser dissection, targeted fluorescence-activated cell sorting (FACS), or more novel microfluidic approaches such as the C1 (Fluidigm, South San Francisco, CA), DEPArray (Menarini Silicon Biosystems, Bologna, Italy), or Beacon (Berkeley Lights, Emeryville, CA). Cells are directly sorted into well plates containing cell lysis buffer and reagents for reverse transcription. The resulting complementary DNA (cDNA) then undergoes amplification and tagmentation in preparation for short-read NGS sequencing, where libraries are prepared cell by cell and then barcoded before pooling. Although these methods are highly useful in that they yield full-length transcripts for each cell, throughput is limited by sorting capacity, manual effort required for library preparation, and per cell cost.

Droplet-Based Single-Cell RNA Sequencing Approaches

To address the limited throughput of plate-based approaches, several groups have developed droplet-based approaches (Drop-Seq and InDrop); these are the most recent innovative approaches that facilitate the isolation and sequencing of large numbers (thousands) of single cells in a highly efficient manner.[34,35] Droplet-based methods combine single-cell isolation (via Poisson loading of single cells into fluid droplets) with cell lysis; in this approach, single cells in suspension flow into a microfluidic network and are captured into fluid droplets that also contain all the necessary reagents for cell lysis, messenger RNA (mRNA) capture/labeling with a Unique Molecular Identifier (UMI), and reverse transcription. Then, cDNA is amplified and libraries are constructed for short-read NGS sequencing, where droplet (and usually single-cell) identity is retained

by a barcode. Sequencing is initiated from either the 3′ or 5′ end of the transcript, but current read lengths (100–150 base pairs) yield only partial information from the transcript. Research efforts demonstrating targeted, long-read/full-length, single-cell sequencing (ie, scIsoSeq) are ongoing and aim to address this current limitation.[36]

TECHNICAL CONSIDERATIONS IN SINGLE-CELL SEQUENCING

Sample Preparation and Handling

One of the major challenges associated with any approach to single-cell sequencing is in the generation and/or selection of single viable, intact cells from tissue; this is particularly true as more and more patient and patient-derived samples are being interrogated via this method.[37] Tumor or tissue dissociation requires optimization and validation to ensure that all populations are appropriately represented and that disaggregation methods do not selectively lyse sensitive cells. Moreover, enrichment for cell populations of interest is often necessary and strategies to accomplish this (eg, depletion of bulk cells, targeted FACS, bead-based enrichment of target cells) must also be optimized and validated. Dynamic changes in the tissue and isolated cells also must be considered in designing and evaluating single-cell experiments; changes in cell health, signaling, and transcription (even those induced solely from dissociation) will all be reflected in single-cell sequencing data, particularly as the sensitivity of this method increases.

Number of Cells Analyzed and Sequencing Depth

One important consideration in designing single-cell analyses is the size of the cell population of interest; this is especially true if rare cell populations are the target of a study, as many fold more total cells must be analyzed to yield information from a sufficient number of rare cells (where, for example, rare cells might be treatment-resistant clones, circulating tumor cells, or cancer stem cells) with adequate sequencing depth (historically 50,000–100,000 reads per cell for differential gene expression analysis, where deeper sequencing can reveal additional genes up to the point of sequencing saturation).[37] This is a very important clinical consideration when analyzing samples from patients who are either at an early stage of their disease or have low levels of refractory disease, as cells of interest from these patients may be present in very low numbers. In some cases, it is necessary to combine cells derived from multiple samples/patients/animals to achieve the total number of cells required; this is especially true when analyzing small tumor biopsies or highly acellular tissue (such as some bladder tumors). Although retaining the sample identity of single cells is straightforward in plate-based approaches, historically, this has been more challenging for droplet-based methods, as there is no way to track droplets through the microfluidic system. Recent advances, such as "cell hashing," enable the pooling, analysis and de-multiplexing of multiple samples such that data from individual cells can be traced back to their original sample source.[38] In this novel approach, oligonucleotide barcoded antibodies (or "hashtags") that ubiquitously recognize cell membrane proteins are used to label individual cell populations before pooling, where each sample is labeled with a different oligonucleotide sequence and the hashtag oligonucleotides are sequenced alongside the cDNA libraries to permit sample assignment of each cell.

Scale

Handling samples on a cell-by-cell basis from isolation to sequencing can be prohibitively cumbersome and labor-intensive, so a number of approaches have been developed that permit multiplexing of cells to increase the efficiency of this process. Incorporation of cell barcoding allows mRNA from many cells to be sequenced in one batch, where de-multiplexing can be performed computationally in the analysis phase; the approach has been incorporated into single-cell sequencing methods such as single-cell RNA barcoding and sequencing (SCRB-Seq),[39] massively parallel single-cell RNA sequencing (MARS-Seq),[40] and cell expression by linear amplification sequencing (Cel-Seq).[41] Furthermore, as described previously, the addition of microfluidics has increased the handling efficiency, and capacity of several techniques. Although conceptually Drop-seq relies on microfluidics to enable droplet loading, companies such as 10X Genomics (Pleasanton, CA), BioRad (Hercules, CA), and Fluidigm have engineered platforms that combine microfluidic devices and cell barcoding to enable cDNA generation from hundreds to thousands of single cells at once.

Data Handling/Analysis

As single-cell sequencing data becomes more cost-effective, the shift from molecular and biologic bottlenecks to data handling is apparent. For example, single-cell RNA sequencing typically targets 50,000 to 100,000 transcript reads per cell to enable differential expression analysis.

Transcripts are counted by the addition of UMIs and aligned against a reference for gene calling. Most commonly, data are filtered to remove background (too few UMIs), unhealthy/dead cells (as determined by the percentage of mitochondrial reads from a given cells), and doublets/multiplets. Data normalization is then performed by scaling count data to correct relative gene abundances between cells, followed by correction methods (to remove effects of technical batch or cell-cycle variation). Given that up to approximately 25,000 genes are part of the original single-cell data set, it becomes necessary to reduce the analysis to genes that are most interesting, and are most variable between cells, which is done by feature selection to reduce the dimensionality of the dataset to the most interesting approximately 500 to 5000 genes. Further dimensionality reduction is done using principal component analysis and a nonlinear reduction method, such as t-SNE,[42] or uniform manifold approximation and projection (UMAP)[43] to facilitate cluster visualization. The resulting clusters are then annotated based on genes expressed, both for identity (ie, clusters with high CD4 expression are T cells) and, to some extent, for function (ie, clusters with high granzyme B and lysozyme are likely cytotoxic). Additional downstream analyses, such as trajectory analysis, in which clusters are related to each other sequentially by differential gene expression profile, are also becoming more common.[44] Also, machine learning approaches have been leveraged to dig deeper into large (typically bulk) sequencing data sets to try to find correlations between clinical metrics and gene expression.[45,46]

There are still many challenges associated with single-cell RNA sequencing data analysis. With increasing experimental throughput, single-cell datasets have become prohibitively large for the average personal computer. Access to high-performance computing options has become a necessity in many cases. Data analysis methods have exploded in parallel with single-cell experimental approaches, and it has become important for investigators to optimize and select the most appropriate filtering, normalization and dimensionality reduction strategies for their data sets. Finally, annotation of clusters by gene expression is still largely manual in many cases, which presents challenges in both bandwidth and consistency.

EMERGING TECHNOLOGIES

Other –Omics

As single-cell sequencing technologies develop, investigators have had the opportunity to ask increasingly challenging research questions that require integration of complex cellular phenotype data with sequencing data. Approaches, such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), where markers for cellular protein expression are combined with Drop-seq–based cell isolation and processing, have begun to address these challenges, allowing sequencing information to be linked to characterized cells.[47] However, the types of phenotypic data that can be linked to sequencing data are currently limited to surface protein expression that can be labeled with an antibody. Other types of characteristic phenotypic data, such as growth kinetics, secretion, or cellular function, are not accessible by these methods, and require more sophisticated approaches.[48]

Single-Cell Multi-Omic Integration

Active efforts are under way to enable integration of data from multiple single-cell analytical methods, including single-cell DNA and RNA sequencing, with single-cell proteomics. Such multilayered analysis will provide the most complete characterization of the tumor and its associated microenvironment, both during the development of disease and in response to therapy. Some of the most exciting examples of these approaches include CITE-Seq (described previously, which pairs single-cell RNA sequencing and surface protein analysis),[47] Genotyping of Transcriptomes, or GoT (which pairs DNA sequencing and RNA sequencing for the same cell),[49] and combining single-cell DNA methylation analysis with RNA sequencing.[50]

Spatial Sequencing

One very significant caveat to single-cell sequencing is that it requires isolated cells that, by definition, have been removed from their anatomic context. Given the increasing appreciation for spatial and temporal heterogeneity as well as the role of dynamic cellular interactions in disease progression, it has become critical to develop methods that retain the spatial context of individual cells. To this end, several spatial sequencing approaches (such as Slide-Seq,[51] multiplexed error-robust fluorescence in situ hybridization [MERFISH],[52] spatially resolved transcript amplicon readout mapping [STARmap][53] and the Visium assay from 10X Genomics) have been developed and are now approaching single-cell resolution. The methods are similar to single-cell transcriptomic approaches, but are used on a slide-mounted intact sample (such as a tissue slice) and leverage a spatial barcode

that allows the registration of transcripts to their position on the slide.

This novel approach was used to characterize spatially resolved gene expression in 6750 tissue regions within a Gs 3 + 4 adenocarcinoma prostate tumor section. Berglund and colleagues[54] were able to resolve extensive intratumor heterogeneity and identify distinct expression profiles for tissue compartments, including normal, cancer, stroma, immune cell, and prostatic intraepithelial neoplasia glands. Moreover, they were able to resolve the high degree of inflammation and immune response in tumor and adjacent normal tissue, compared with normal tissue. In short, this approach (particularly when combined with large-scale analysis of matched single cells) will add spatial complexity and dimensionality to single-cell sequencing and even further increase the promise of this method.

SUMMARY

Cancer is a highly complex and heterogeneous disease, and immunotherapy has shown promise as a therapeutic approach. Although bulk NGS has helped to broadly inform treatment of cancer (including with immunotherapy), the increased resolution afforded by single-cell analysis offers the hope of finding and characterizing previously underappreciated populations of cells that could prove useful in understanding the progression and treatment of cancer. In evaluating immunotherapeutic approaches, single-cell analysis is uniquely suited to (1) identifying known, rare, or novel tumor targets; (2) characterizing patient-specific populations of immune cells that can be leveraged for immunotherapy; and (3) assessing the effects of immunotherapy on subpopulations of cells within a tumor to understand sensitivity or resistance. Urologic and prostate cancers are inherently heterogeneous diseases, even as far as cancer is concerned, and the potential for single-cell analysis to help understand and develop immunotherapeutic approaches to treat these diseases is very exciting. Further advances in these technologies to interweave multiple –omics methods with spatial analysis of the tumor immune microenvironment promises to bring our understanding of the evolution of cancer and effects of immunotherapy to an entirely new level, which will hopefully translate to great improvement in clinical outcomes.

CLINICAL COMMENTS

- Heterogeneity of a tumor and its microenvironment (including the immune compartment) likely contributes to differences in disease development and patient response to treatment.
- NGS of bulk tissue does not always capture heterogeneity.
- Single-cell sequencing of cells from tissue allows for better resolution of different minor cell populations, which can help identify tumor targets, identify cells that can be leveraged for immunotherapy, and to understand the effects of therapy on tumor development.

DISCLOSURE

The authors declare no commercial or financial conflicts of interest.

REFERENCES

1. Yadav SS, Stockert JA, Hackert V, et al. Intratumor heterogeneity in prostate cancer. Urol Oncol 2018; 36(8):349–60.
2. Riaz N, Havel JJ, Makarov V, et al. Tumor and Microenvironment Evolution during Immunotherapy with Nivolumab. Cell 2017;171(4):934–49.e16.
3. da Costa JB, Gibb EA, Nykopp TK, et al. Molecular tumor heterogeneity in muscle invasive bladder cancer: Biomarkers, subtypes, and implications for therapy. Urol Oncol 2018. https://doi.org/10.1016/j.urolonc.2018.11.015.
4. Cote RJ, Esrig D, Groshen S, et al. p53 and treatment of bladder cancer. Nature 1997;385(6612): 123–5.
5. Stadler WM, Lerner SP, Groshen S, et al. Phase III study of molecularly targeted adjuvant therapy in locally advanced urothelial cancer of the bladder based on p53 status. J Clin Oncol 2011;29(25): 3443–9.
6. George B, Datar RH, Wu L, et al. p53 gene and protein status: the role of p53 alterations in predicting outcome in patients with bladder cancer. J Clin Oncol 2007;25(34):5352–8.
7. Tolkach Y, Kristiansen G. The heterogeneity of prostate cancer: a practical approach. Pathobiology 2018;85(1-2):108–16.
8. Djavan B, Susani M, Bursa B, et al. Predictability and significance of multifocal prostate cancer in the radical prostatectomy specimen. Tech Urol 1999; 5(3):139–42.
9. Trujillo KA, Jones AC, Griffith JK, et al. Markers of field cancerization: proposed clinical applications in prostate biopsies. Prostate Cancer 2012;2012: 302894.
10. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer 1953;6(5):963–8.

11. Andreoiu M, Cheng L. Multifocal prostate cancer: biologic, prognostic, and therapeutic implications. Hum Pathol 2010;41(6):781–93.
12. Boyd LK, Mao X, Xue L, et al. High-resolution genome-wide copy-number analysis suggests a monoclonal origin of multifocal prostate cancer. Genes Chromosomes Cancer 2012;51(6):579–89.
13. Kobayashi M, Ishida H, Shindo T, et al. Molecular analysis of multifocal prostate cancer by comparative genomic hybridization. Prostate 2008;68(16): 1715–24.
14. Lindberg J, Klevebring D, Liu W, et al. Exome sequencing of prostate cancer supports the hypothesis of independent tumour origins. Eur Urol 2013; 63(2):347–53.
15. Ribas A. Releasing the brakes on cancer immunotherapy. N Engl J Med 2015;373(16):1490–2.
16. Shin DS, Ribas A. The evolution of checkpoint blockade as a cancer therapy: what's here, what's next? Curr Opin Immunol 2015;33:23–35.
17. Schepisi G, Farolfi A, Conteduca V, et al. Immunotherapy for prostate cancer: where we are headed. Int J Mol Sci 2017;18(12).
18. Chevrier S, Levine JH, Zanotelli VRT, et al. An immune atlas of clear cell renal cell carcinoma. Cell 2017;169(4):736–49.e18.
19. The molecular taxonomy of primary prostate cancer. Cell 2015;163(4):1011–25.
20. Barbieri CE, Baca SC, Lawrence MS, et al. Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. Nat Genet 2012;44(6):685–9.
21. Blattner M, Lee DJ, O'Reilly C, et al. SPOP mutations in prostate cancer across demographically diverse patient cohorts. Neoplasia 2014;16(1):14–20.
22. Grisanzio C, Werner L, Takeda D, et al. Genetic and functional analyses implicate the NUDT11, HNF1B, and SLC22A3 genes in prostate cancer pathogenesis. Proc Natl Acad Sci U S A 2012;109(28): 11252–7.
23. Pflueger D, Rickman DS, Sboner A, et al. N-myc downstream regulated gene 1 (NDRG1) is fused to ERG in prostate cancer. Neoplasia 2009;11(8): 804–11.
24. Pflueger D, Terry S, Sboner A, et al. Discovery of non-ETS gene fusions in human prostate cancer using next-generation RNA sequencing. Genome Res 2011;21(1):56–67.
25. Yadav SS, Li J, Lavery HJ, et al. Next-generation sequencing technology in prostate cancer diagnosis, prognosis, and personalized treatment. Urol Oncol 2015;33(6):267.e1-13.
26. Mian OY, Tendulkar RD, Abazeed ME. The evolving role of molecular profiling in prostate cancer: basal and luminal subtyping transcends tissue of origin. Transl Cancer Res 2017;6(Suppl 9):S1441–5.
27. Rubin MA, Girelli G, Demichelis F. Genomic correlates to the newly proposed grading prognostic groups for prostate cancer. Eur Urol 2016;69(4): 557–60.
28. Zhang L, Dong X, Lee M, et al. Single-cell whole-genome sequencing reveals the functional landscape of somatic mutations in B lymphocytes across the human lifespan. Proc Natl Acad Sci U S A 2019; 116(18):9014–9.
29. Horning AM, Wang Y, Lin CK, et al. Single-cell RNA-seq reveals a subpopulation of prostate cancer cells with enhanced cell-cycle-related transcription and attenuated androgen response. Cancer Res 2018; 78(4):853–64.
30. Prideaux SM, Conway O'Brien E, Chevassut TJ. The genetic architecture of multiple myeloma. Adv Hematol 2014;2014:864058.
31. Buenrostro JD, Wu B, Chang HY, et al. ATAC-seq: a method for assaying chromatin accessibility genome-wide. Curr Protoc Mol Biol 2015;109:21. 29.1–21.29.9.
32. Buenrostro JD, Wu B, Litzenburger UM, et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 2015;523(7561):486–90.
33. Smallwood SA, Lee HJ, Angermueller C, et al. Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. Nat Methods 2014;11(8):817–20.
34. Klein AM, Mazutis L, Akartuna I, et al. Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. Cell 2015;161(5):1187–201.
35. Macosko EZ, Basu A, Satija R, et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell 2015;161(5):1202–14.
36. Gupta I, Collier PG, Haase B, et al. Single-cell isoform RNA sequencing characterizes isoforms in thousands of cerebellar cells. Nat Biotechnol 2018. https://doi.org/10.1038/nbt.4259.
37. Kolodziejczyk AA, Kim JK, Svensson V, et al. The technology and biology of single-cell RNA sequencing. Mol Cell 2015;58(4):610–20.
38. Stoeckius M, Zheng S, Houck-Loomis B, et al. Cell hashing with barcoded antibodies enables multiplexing and doublet detection for single cell genomics. Genome Biol 2018;19(1):224.
39. Soumillon M, Cacchiarelli D, Semrau S, et al. Characterization of directed differentiation by high-throughput single-cell RNA-Seq. BioRxiv 2014. https://doi.org/10.1101/003236.
40. Jaitin DA, Kenigsberg E, Keren-Shaul H, et al. Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types. Science 2014;343(6172):776–9.
41. Ziegenhain C, Vieth B, Parekh S, et al. Comparative analysis of single-cell RNA sequencing methods. Mol Cell 2017;65(4):631–43.e4.

42. van der Maaten L, Hinton G. Visualizing data using t-SNE. Journal of Machine Learning Research 2008;9:2579–605.
43. Becht E, McInnes L, Healy J, et al. Dimensionality reduction for visualizing single-cell data using UMAP. Nat Biotechnol 2018. https://doi.org/10.1038/nbt.4314.
44. Qiu X, Mao Q, Tang Y, et al. Reversed graph embedding resolves complex single-cell trajectories. Nat Methods 2017;14(10):979–82.
45. Glaab E, Bacardit J, Garibaldi JM, et al. Using rule-based machine learning for candidate disease gene prioritization and sample classification of cancer gene expression data. PLoS One 2012;7(7):e39932.
46. Singh D, Febbo PG, Ross K, et al. Gene expression correlates of clinical prostate cancer behavior. Cancer Cell 2002;1(2):203–9.
47. Stoeckius M, Hafemeister C, Stephenson W, et al. Simultaneous epitope and transcriptome measurement in single cells. Nat Methods 2017;14(9):865–8.
48. Beaumont KG, Hamou W, Bozinovic N, et al. Multiparameter cell characterization using nanofluidic technology facilitates real-time phenotypic and genotypic elucidation of intratumor heterogeneity. bioRxiv 2018. https://doi.org/10.1101/457010.
49. Nam AS, Kim KT, Chaligne R, et al. Somatic mutations and cell identity linked by genotyping of transcriptomes. Nature 2019;571(7765):355–60.
50. Zhou F, Wang R, Yuan P, et al. Reconstituting the transcriptome and DNA methylome landscapes of human implantation. Nature 2019;572(7771):660–4.
51. Rodriques SG, Stickels RR, Goeva A, et al. Slide-seq: a scalable technology for measuring genome-wide expression at high spatial resolution. Science 2019;363(6434):1463–7.
52. Xia C, Babcock HP, Moffitt JR, et al. Multiplexed detection of RNA using MERFISH and branched DNA amplification. Sci Rep 2019;9(1):7721.
53. Wang X, Allen WE, Wright MA, et al. Three-dimensional intact-tissue sequencing of single-cell transcriptional states. Science 2018;361(6400).
54. Berglund E, Maaskola J, Schultz N, et al. Spatial maps of prostate cancer transcriptomes reveal an unexplored landscape of heterogeneity. Nat Commun 2018;9(1):2419.