Single-Cell Next-Generation Sequencing

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Contributor: Olisaemeka Ogbue , Serhan Unlu , Gogo-Ogute Ibodeng , Abhay Singh , Arda Durmaz , Valeria Visconte , John C. Molina

Single-cell DNA sequencing is a laboratory technique that analyzes the genetic content of individual cells. In the context of genetically diverse hematological cancers such as acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), the traditional approach of analyzing genetic material, which typically involves bulk samples of leukemia cells, may miss important mutations that may not be present in all cells.

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1. Introduction

Acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) are heterogeneous clinical and molecular entities that share many recurrent driver mutations [1][2][3]. The clonal architecture of these diseases creates a challenge in monitoring treatment response, particularly in the presence of measurable residual disease (MRD), which refers to the persistence of leukemic cells in the bone marrow or peripheral blood of patients in morphologic complete remission after treatment [4].

Allogeneic hematopoietic stem-cell transplant (AlloHSCT) is a potentially curative treatment for adults with high-risk/refractory AML/MDS, but disease relapse is a significant cause of transplant failure. In AML, incidence of relapse approaches 25–55% [5], with a 2 year overall survival of 14–25% in relapsed patients [6]. In MDS, a 3 year cumulative incidence of relapse (CIR) of 37% was observed in patients receiving either induction chemotherapy or demethylating agents (DMAs) before HSCT [7].

The detection of MRD in both conditions also correlates with survival and risk of relapse according to a growing body of evidence [8][9]. In the pre-transplantation timeframe, MRD status can guide therapeutic interventions, such as the intensity of pre-transplant conditioning or post-transplant chemotherapy/immunosuppression [10][11]. MRD monitoring is now recommended as routine follow-up post allogeneic HSCT to identify candidate subjects who would benefit from further treatment [12].

The prognostic significance of MRD detection is further reinforced by its inclusion in risk-stratifying patients according to European LeukemiaNet (ELN) consensus guidelines for AML diagnosis and management. In the updated 2022 ELN guidelines, persistence of MRD after complete treatment response (CRMRD+) raises the ELN risk classification [13].

Single-cell sequencing (scSeq) can reveal the clonal and subclonal architecture of diseases that are difficult to study using bulk sequencing methods. The clinical utility of scSeq in monitoring MRD has only recently been tested in AML/MDS, with promising results in contemporary studies [14][15].

2. Single-Cell Next-Generation Sequencing

Although bulk sequencing has allowed for omics mapping, resolving intratumoral heterogeneity is nontrivial, since bulk-seq is an aggregate proxy for the individual cell–omics profiles. For this purpose, single-cell sequencing methods have been developed that allow for tagging/barcoding molecular fragments specific to individual cells. However, scSeq is time-consuming, and extracted data can be noisy. Specifically, in the context of DNA sequencing, scDNASeq requires whole-genome amplification from a low DNA amount, which can result in failure to capture individual alleles (allelic dropouts) [16]. Furthermore, mutational artefacts can be introduced during amplification, which needs to be taken into account during variant calling [17]. Nevertheless, scSeq is becoming the gold standard for probing intratumoral heterogeneity in solid tumors and hematologic cancer [18][19][20].

3. Clinical Applications of scDNA-Seq in AML/MDS

Single-cell resolution of AML clonality offers several advantages over bulk NGS. Researchers discuss the mechanisms that allow for more precise characterization of clonal architecture in scDNA-seq.

3.1. Identification of Variants Associated with Clonal Hematopoiesis

Single-cell analysis provides for better resolution of age-related clonal hematopoiesis (ARCH) compared to bulk NGS at remission. Dillon et al. first described the application of scDNASeq with simultaneous single-cell antibody–oligonucleotide sequencing to distinguish nonmalignant ARCH from leukemia [21]. This method was applied during remission to delineate rare ARCH variants from clones associated with relapse [15]. ARCH mutations are typically mutations in epigenetic regulators that are observable at a lower VAF than by bulk NGS. These mutations can complicate molecular assessment of MRD and are collectively known as DTA mutations, including *TET2*, *DNMT3A*, *IDH1/2*, and *ASXL1*. Preleukemic ARCH clones are common in AML and increase with age [22]. Successful alloHSCT is expected to eliminate ARCH-related genetic abnormalities, and their persistence after transplantation may indicate the persistence or relapse of the leukemic clone. However, any prognostic significance of DTA mutations in AML is yet to be demonstrated by studies. Heuser et al., using NGS-based MRD monitoring, found DTA mutations that were not eliminated in 17.6% of their patient cohort with no prognostic impact on incidence of relapse or OS [23]. In contrast, MRD with non-DTA mutations was highly predictive of outcomes in a separate analysis. Therefore, ARCH mutations alone may not necessarily predict relapse risk in AML.

Further incorporation of scDNA-seq in peri-transplant MRD detection could significantly improve its predictive power due to the inherent ability to better detect and exclude these rare ARCH variants not associated with relapse.

3.2. Phylogenetics

ScDNA-seq allows for better determination of mutation rank in AML compared to bulk-seq methods, resulting in more definitive phylogenetic models. Two studies used high-throughput single-cell proteogenomics on large AML datasets to provide insights into the evolutionary trajectory of AML. Their findings suggest that epigenetic mutations precede mutations in genes related to signaling pathways such as FLT3 and the RAS family. With the notable exception of TET2 mutations, there was very little clonal trajectory when the initial mutation involved genes of the signaling pathway [24]. One probabilistic phylogenetic prediction model known as the single-cell inference of tumor evolution (SCITE) demonstrated both linear and branching models of clonal evolution in AML, with significantly higher clonal diversity occurring among samples with branching clonal evolution [25].

ScDNA-seq can also predict clonal relationships, on the basis of the individual clone composition and frequency of each clone, to deduce that clones of lower frequency evolve from clones with higher frequency [26]. This principle has been leveraged to describe the clonal trajectory of MDS driving its progression to secondary AML [27]. Recent work also correlated mutational frequency with both clinical outcomes and phenotypic differences such as white blood cell counts, LDH levels, and blast abundance in PB [28].

3.3. Deconvolution of Mutation Co-Occurrence

The clonal milieu in AML samples often comprises dominant clones that outcompete other minor clones [24], and it has been observed from prior studies using bulk NGS assays that ≥2 allelic variants are associated with reduced leukemia-free survival and OS [29]. Indeed, single-cell analysis of myeloid malignancies has shed more light on which mutational combinations promote clonal expansion. ScDNA-seq can identify the mutational co-occurrences that lead to dominant clones not previously delineated from bulk sequencing.

Miles et al. observed that specific mutational combinations involving NPM1c + FLT3ITD or DNMT3A + IDH2 resulted in clonal dominance, whereas other co-occurring alleles such as NPM1c + RAS did not promote clonal expansion [24]. The number of variants also has an impact on clonal size, as it was observed that samples with double mutant *DNMT3A*/*IDH1* or *IDH2* clones had significantly larger clonal size compared to respective single-mutant clones [24]. Ediriwickrema et al., using targeted scSeq, identified a significantly greater number of co-occurring number of variants at diagnosis in relapsed patients [15]. In addition, the driver mutations in AML were found to be mutually exclusive in occurrence at cellular level [25]. These findings may inform therapeutic strategies during the peri-transplant period to target these clones before they achieve clonal dominance.

3.4. Monitoring Clonal Evolution during Treatment

Significant alterations occur in the clonal architecture of AML when the disease becomes resistant to initial treatment. These so-called “clonal sweeps” involve the emergence of a new dominant clone that affects response to further therapy [30]. Prior studies using bulk-seq methods implicated signaling pathway mutations (particularly RAS/RTK/MAPK mutations) as potential mechanisms of resistance [31]. These clonal mechanisms of resistance have also been studied using scDNA-seq methods. In the context of FLT3-mutated AML, for instance, by

examining pre- and post-therapy treatment samples of patients with FLT3 mutations on gilteritinib (selective FLT3 kinase inhibitor), an emergent outgrowth of clones with RAS mutations and suppression of clones with FLT3-ITD mutations was observed. This finding has been supported by other studies elucidating the clonal mechanisms of resistance with other chemotherapeutic agents [32][33][34].

3.5. Mapping of Genetic–Phenotypic Evolution in AML

In AML, LAIPs on leukemic blasts may serve as therapeutic targets [35]. The prognostic significance of these immunotypes, especially when combined with genotypic analysis, is yet to be explored.

The simultaneous use of scDNA-seq and immunophenotyping (obtained from cell-surface protein expression analysis in mature hematopoietic lineages) can allow for genotype–phenotype correlation among sequenced cells to better dissect intratumor heterogeneity in AML, providing a linkage between phenotypes and the genetic mutations that drive them. Prior studies using single-cell analysis provided significant insight into the impact of genotype on cell-surface expression in leukemia samples. Morita et al. observed a significant correlation between CD34 expression and the mutations present in samples. They found that higher CD34 expression was seen in samples with TP53 mutations, while the opposite was true in samples with NPM1 and IDH1/2 mutations [25].

Demaree et al. applied DAb-seq [36], a tool for joint profiling of DNA and surface proteins in single cells at high throughput. This method yields genotypic information from DNA samples and is distinct from other tools that rely on transcriptomics and antibody sequencing methods, which only capture phenotypic features. When applied in three patients across multiple treatment timepoints, a strong correlation between the AML genotype and corresponding malignant phenotype was observed in one patient.

The potential applications include the capability to be used to identify and track specific immunophenotypic populations before or after HSCT that may be amenable to cell-surface-targeted therapies.

4. Shortcomings of Current Techniques of scDNAseq

The major shortcomings of scDNAseq techniques include false variance call rates, allelic drop out (ADO), limited throughput, lack of standardization, and cost.

Both qPCR and MFC-based MRD analyses in AML aim to detect aberrant cell populations at a one in 10,000 frequency [13]. This is particularly important in the post-transplant period, as MRD presence is associated with relapse [37][38]. However, while single-cell-based genomic sequencing techniques can detect cells with genomic aberrations in this frequency range, there are concerns about false variance call rates that may be much higher, which could significantly reduce the utility of this technology for detecting MRD. In particular, Pellegrino et al. showed that the frequency of detected variant alleles that are potential experimental artefacts can be as high as 2.5%, which is considerably higher than the MRD detection threshold [39]. Currently, spiked-in cell lines and multiple control loci with known frequency are used to detect error rates in each experimental design, and the difference in

rate between ADO in control lines and samples must be explored further to validate the use of these control methodologies in future experiments [39].

Moreover, the problems created by ADO (referring to an apparent loss in heterozygosity when an allele at a particular genetic locus fails to amplify or sequence) and false variance calls in general are exacerbated by complex karyotypes and unbalanced chromosomal abnormalities observed in AML genomic landscape, which may cause false variance call rates to be significantly different than the rates observed in commonly used control cell lines and between patient samples [40][41]. Therefore, the optimization of control methods and the demonstration of whether they can be carried over to the complex landscape of genomics in AML clinics must be shown and approved by a consensus of physicians to harmonize use of this technology as a diagnostic tool in MRD.

Inference of clonal architecture through scDNAseq produces better results compared to bulk NGS. However, false-negative variant calls can be significantly reduced by comparing the frequency of cells with variant alleles to VAF of the same mutations in bulk sequencing in the same population [39]. This approach is more reliable in detecting low-frequency MRD, with general error rates for conventional NGS around 0.1% [42][43]. Although this rate causes NGS to fall behind other PCR techniques in the sensitivity of detection of low VAF mutations, it is comparable with MCF [13]. Therefore, bulk DNA sequencing with NGS makes an ideal companion to scDNA-seq for MRD studies. The efficacy of this approach was shown with a novel in silico method called B-SCITE [44], which combined genomic data from the whole cell population using bulk sequencing and single-cell resolution data generated through single-cell genomic sequencing. The authors demonstrated that their model outcompetes multiple scDNA-seq analysis approaches in inferring clonal architecture [44]. Despite the aforementioned shortcomings of each approach, this study showed that there is a measurable benefit to combining these methods, which cannot be achieved through the application of only one.

Another shortcoming of sc-DNAseq for the detection of low-frequency MRD is the necessity for ultrahigh-throughput sequencing. For a one in 10^4 detection rate, upward of 10,000 cells must be sequenced, ideally many times more to make up for losses throughout the experimental workflow. sc-DNAseq experiments of myeloid malignancies have not incorporated as many cells in experiments [15][24][25][39]. However, the robustness of MRD detection requires high-throughput sequencing, and the establishment of clonal hierarchy and phylogeny in tumor subclones does not. The frequency of studied mutations is significantly higher than the lower limit of detection of MRD. Nonetheless, established workflows can reach necessary cell read counts [45], but the benefits of using such ultrahigh-throughput sequencing in the setting of hematological malignancies must be established before the high cost of usage can be justified. The primary utility of scDNAseq in MRD detection will not be detection of ultralow-frequency residual disease. As a result, a combination of bulk and scDNA sequencing can mitigate the problem of detection of low-frequency variants through carefully prepared high-depth NGS panels [46].

The need for a good cellularity sample may pose a challenge due to hypocellularity occurring during the conditioning/recovery phase following transplantation. This is a peculiar shortcoming of single-cell studies in MDS research in cases of hypocellular MDS. This leads to a paucity of cells that will create a major challenge for all

single-cell platforms, along with the heterogeneity of disease, with multiple clones carrying different sets of somatic mutations in each patient.

Another hurdle to overcome would be to reduce the heterogeneity of sizes and ranges of the currently available panels, which range between 19 and 685 genes being used for variant allele detection in scDNAseq studies in AML so far [24][25][33][39]. If scDNAseq is to be exported to the clinical realm of MRD detection in AML, commercially available panels should include at least the most up-to-date somatic mutations that are defined by ELN, at a sensitivity that is comparable with established methods for MRD detection [13]. If clonal hierarchy can be established with the help of single-cell genomics while keeping the MRD detection rate stable through integrative genomics with bulk NGS, further research will need to establish how the information gathered through deciphering clonal hierarchy in each patient can help best utilize precision treatment options.

Lastly, researchers acknowledge the cost of scDNAseq as a barrier to the expansion of AML MRD detection for clinical use. scDNAseq is expensive, including procedural costs such as sample collection and library preparation, as well as the analysis and interpretation of the output requiring skilled personnel [47]. A lack of standardization in mutation panels adds an additional level of complication. Low insurance coverage and reimbursement rates will further hinder scDNAseq utilization, as insurance companies will still consider the application of scDNAseq MRD testing experimental. Targeted sequencing of frequent AML mutations by encapsulation and barcoding of single cells is expected to decrease the cost threshold of scDNAseq in the near future [47].

5. Single-Cell RNA Sequencing (ScRNAseq)

Researchers tried to uncover the potential of scDNA-seq in materializing the clonal architecture and hypothesize the benefits that can be observed in the clinical setting. However, scRNA-seq is an additional single-cell based analysis method that can shed even more light on the disease state beyond genomic alterations. Such transcriptomic analyses have been utilized in solid tumors [48] and are being explored in clinical settings for hematologic malignancies [49][50][51]. So far, studies in AML have successfully identified similar cell-types in different patients showing that, even with significant intratumoral heterogeneity [52], there are co-upregulated pathways in different cell subtypes in different patients that behave similarly, and the transcriptomic patterns of these different cell types correlate with underlying genetic alterations. The effect of genomic aberrations over time, especially crucial ones such as *FLT3*, *IDH1*, *IDH2*, and *NPM1*, on the transcriptomic profile of different cell subtypes can be analyzed using a combination of transcriptomic and genomic analysis, which can improve the understanding of pathways involved in relapse, development of resistance, and response to therapy. Targetable transcriptomic changes in crucial genes that regulate proliferation and survival in AML/MDS can help generate more personalized therapy and monitor response to treatment in both clonal cells and healthy immune cells prior to relapse at a much higher resolution that is obtainable by bulk sequencing methods [53][54] (**Figure 1**).

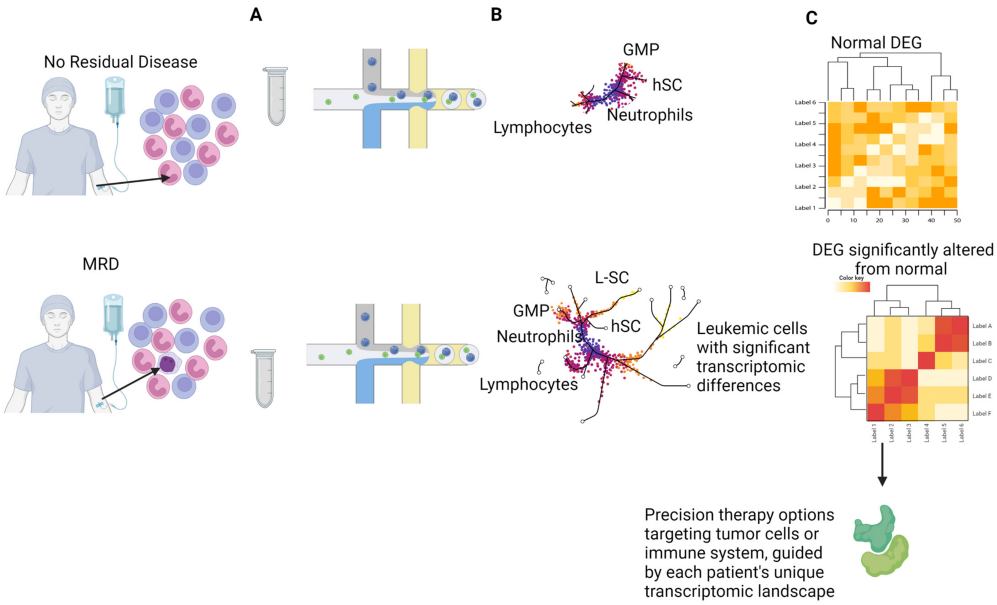


Figure 1. MRD scRNA diagnostic advantage. DEG: differentially expressed genes, GMP: granulocyte monocyte progenitor, HSC: hematopoietic stem cell, L-SC: leukemia stem cell, MRD: minimal residual disease. (**A**) Microfluidics system barcoding cells. (**B**) Upper panel, patient without MRD with transcriptome of regular peripheral blood; no clonal lines detected. Bottom panel, clustering of cell lines from peripheral blood of patient with MRD through dimensional reduction of high-dimensional transcriptional data; clusters include pathological clonal lines including L-SC. (**C**) Panel colors represent gene expression. Upper panel, no differential expression of genes. Bottom panel, differentially expressed genes in MRD. Significantly elevated expression of targetable genes might increase the benefit derived from precision medications.

scRNAseq can observe the effect of mutations, such as *RUNX1*, *NPM1*, *CBFB*, and other genetic abnormalities, on therapy response. Differences in transcriptomic profiles of cellular subgroups with distinct expression profiles can be analyzed to observe the effect of targeting these mutations on the transcriptomic landscape. During the post-transplant period, activation of proliferative transcriptomic signatures in healthy immune cells may indicate a favorable prognosis; however, in cell populations carrying the aforementioned mutations, it could be an early sign of relapse.

In MDS, studies utilizing scRNAseq have generated transcriptomic profiles of hematopoietic stem cells and multipotent progenitor cells from MDS patients and identified differentially expressed genes regulating myeloid differentiation patterns, RNA metabolism, and ribosome biogenesis compared to healthy donors [55]. Understanding the transcriptomic landscape can provide insights into the progression patterns of cells with somatic mutations in MDS.

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