

# Tumor heterogeneity of acute myeloid leukemia: insights from single-cell sequencing

AiLi Chen<sup>a</sup>, ShaoYan Hu<sup>b</sup>, Qian-Fei Wang<sup>a,c,\*</sup>

<sup>a</sup>CAS Key Laboratory of Genomic and Precision Medicine, Collaborative Innovation Center of Genetics and Development, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, China; <sup>b</sup>Department of Hematology and Oncology, Children's Hospital of Soochow University, Suzhou, China; <sup>c</sup>University of Chinese Academy of Sciences, Beijing, China

## Abstract

Individual tumors comprise genetically and epigenetically heterogeneous subclones, each of which is presumably associated with a distinct function, such as self-renewal or drug sensitivity. The dissection of such intratumoral heterogeneity is crucial to understand how tumors evolve during disease progression and under the selection of therapeutic intervention. As a paradigm of cancer intratumoral heterogeneity and clonal evolution, acute myeloid leukemia (AML) has been shown to possess complex clonal architecture based on karyotype studies, as well as deep sequencing of mixed cellular populations using next-generation sequencing (NGS) technologies. The recent development of single-cell sequencing (SCS) methods provides a powerful tool to allow analysis of genomes, transcriptomes, proteomes, and epigenomes at an individual cell level. The technologies applied in AML have broadened our understanding of AML heterogeneity and provided new insights for the development of novel therapeutic strategies. In this review, we summarize the progress in the research of AML heterogeneity using SCS technology and discuss the limitations and future direction regarding how SCS can contribute to AML prognosis and treatment.

**Keywords:** Acute myeloid leukemia, Heterogeneity, Single-cell sequencing

## 1. INTRODUCTION

Peter Nowell first proposed that the formation of tumors is an evolutionary process<sup>1</sup> and that genetic mutations are traditionally recognized as molecular determinants of this process. Although unique combinations of mutations are commonly used to define different subclones, it is now widely accepted that genetic and epigenetic alterations collectively contribute to functional heterogeneity, such as self-renewal and drug resistance within individual tumors.<sup>2</sup> Intratumoral heterogeneity is a key determinant in disease progression and treatment response; therefore, a comprehensive analysis of tumor heterogeneity has important clinical implications and may provide valuable insights for the development of anticancer therapies.<sup>3</sup>

Acute myeloid leukemia (AML) is one of the best studied cancer type owing to the easy accessibility of samples and well-

defined cellular hierarchy in the normal hematopoietic system.<sup>4</sup> This unique feature with the relatively low recurrent somatic mutation rate of the disease<sup>5</sup> make AML an excellent model to explore intratumoral heterogeneity and clinical significance.<sup>6</sup> Laboratory-based technologies such as fluorescence in situ hybridization and flow cytometry as well as high-throughput next-generation sequencing (NGS) have revealed that AML comprises heterogeneous subpopulations characterized by distinct genetic and epigenetic alterations.<sup>7,8</sup> However, the multiple layers of heterogeneity have remained ambiguous until the introduction of single-cell sequencing (SCS). SCS has empowered the cancer field to achieve an unprecedented understanding of the molecular basis of intratumoral heterogeneity. In this review, we summarize how SCS technology contributes to our understanding of AML heterogeneity, with a particular focus on insights that are only revealed from single-cell analyses (Table 1).

## 2. USING SINGLE-CELL GENOME SEQUENCING TO UNAMBIGUOUSLY DEFINE THE GENETIC HETEROGENEITY OF AML

AML is thought to originate from a subset of hematopoietic stem/progenitor cells (HSCs/HPCs).<sup>9,10</sup> By gradually accumulating mutations and numerous additional genetic and epigenetic abnormalities, the found cells take a complex trajectory path to finally evolve into functionally diversified subpopulations.<sup>11-13</sup> Obtaining genomic information at the single-cell level is the ultimate solution to dissect clonal diversity. To achieve this goal, current technology requires whole-genome amplification (WGA) of DNA extracted from an individual cell to generate enough DNA for sequencing. WGA methods continue to face several technical limitations, such as a high allele dropout rate, high false-positive rate, and chimera formation, which have been well described in other reviews.<sup>14,15</sup> Currently, most single-cell

\* Address correspondence: Qian-Fei Wang, CAS Key Laboratory of Genomic and Precision Medicine, Collaborative Innovation Center of Genetics and Development, Beijing Institute of Genomics, Chinese Academy of Sciences, No. 1 Beichen West Road, Chaoyang District, Beijing, 100101, China. E-mail address: wangqf@big.ac.cn (Q. Wang).

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**Table 1**  
**Applications of Single-cell Sequencing technologies in AML studies.**

Technology	Applications in AML	Comparison with bulk sequencing		Reference
		Advantage	Disadvantage	
Single-cell genome sequencing	Identifies residual pre-leukemic HSCs	–Higher accuracy (in defining clonal structure complexity) –Suitable for rare sample study	–High technical errors	16
	Unambiguous resolve the complex subclonal structure		–Biased genome coverage	17
	Recognize heterozygous and homozygous FLT3-ITD and NPM1 mutations within the same patient		–Mainly used for genotyping limited target region	20
	Reveal the different differentiation ability of genetic heterogeneity AML subclones		–High cost	21
Single-cell transcriptome sequencing	Resolve transcriptional heterogeneity of AML blast with hierarchical organization	–Capable for identifying heterogenous populations	–Limited expressed-gene number detected per cell	28
	Correlate AML transcriptional signature with genetic alterations	–Rare cell population discovery	–Require high quality of input cells	28
	Uncover the heterogeneity of the conventional marker defined LSC population (in mouse model)		–High cost	29
	Trace the drug response of LSC populations			30
	Identify functional AML subpopulations			28

AML = acute myeloid leukaemia, LSC = leukemic stem cell.

genomic studies of AML have relied on genotyping of somatic mutations pre-detected by genome sequencing of a mixed cell population, with the genotyping libraries constructed from single-cell WGA or colony-forming units derived from a single cell.<sup>16–19</sup>

Single-cell genotyping of limited somatic mutations (usually <10) in a small number of cells (several to several hundreds) allows the determination of a relatively clearer clonal structure of de novo AML compared with NGS of bulk samples. AML was originally viewed as evolving from a linear accumulation of multiple driver mutations. However, the estimates of subclone size in deep bulk sequencing indicate that AML is likely evolved with a complex branching architecture.<sup>6</sup> Despite this improved understanding, it remains difficult to determine the exact evolutionary trajectory and clonal structure based on the variant allele frequency (VAF), particularly when the VAFs of two subclonal mutations are similar. Single-cell DNA sequencing can resolve the combined pattern of multiple mutations in an individual cell and subsequently provide evidence for either linear or branched structures in individual AMLs. Remarkably, single-cell genotyping of pre-detected mutations identifies residual preleukemic HSCs that exist in bulk AML cells and constitute a cellular reservoir with linearly accumulated founder mutations. These pre-leukemic cells are predisposed to acquire additional mutations and evolve into a distinct clonal hierarchy observed in the full-blown disease.<sup>16</sup> Single-cell gene capture of 1953 somatic single nucleotide variants (SNVs) from patients with AML unambiguously demonstrates the origin of two separate subclones by detecting mutually exclusive SNVs, which represent an evolutionary branch point from the founding clone.<sup>17</sup> Interestingly, most VAF in bulk sequencing is calculated under the assumption that all mutations occur heterozygously in single cells, and a study using single-cell genome analysis has revealed the existence of both heterozygous and homozygous FLT3-ITD and NPM1 mutations in an individual patient.<sup>20</sup> These data suggest that bulk sequencing overestimates the number of leukemic cells but underestimates the clonal diversity of the blast. In an intriguing study, single-cell genotyping was performed on flow-sorted AML blasts and mature myelomonocytes.

These two cell populations contain the same founding clone but distinct subclones, indicating that genetic heterogeneity impacts the differentiation ability of AML subclones.<sup>21</sup> A recent study adopted droplet microfluidics for single-cell DNA genotyping, which enabled a comprehensive analysis of thousands of AML cells in parallel.<sup>22</sup> This single-cell DNA sequencing has potential in the development of high-throughput genome-wide detection of genetic alterations in the future.

These single-cell genomic studies<sup>16–20</sup> strongly support the clonal architecture inferred from the analysis of bulk samples. Importantly, analyses of AML at single-cell resolution unambiguously resolve the subclonal structure that cannot be appropriately assigned through mutation frequencies or unknown mutation status (heterozygous or homozygous). Thus, these studies reveal the previously unappreciated complexity of the clonal structure of AML.

### 3. USING SINGLE-CELL TRANSCRIPTOME SEQUENCING TO REVEAL THE FUNCTIONAL HETEROGENEITY OF AML

The transcriptome is the dynamic output of all genetic information, which closely reflects the functional status of cells. Compared with genomic analyses, single-cell transcriptome sequencing (scRNA-seq) is a more developed technology that allows for a high-throughput (several hundreds to ten thousands cells) detection of tens of thousands of transcripts. Considerable technical progress has been made over the past years, including the introduction of the Moloney murine leukemia virus, which has template-switching activity to ensure amplification of full-length mRNA transcripts,<sup>23</sup> and the development of unique molecules index for reduced amplification bias.<sup>24</sup> A commercial provider (10× Genomics®, 7068 Koll Center Parkway, Suite 401, Pleasanton, California 94566) ensures the widespread use of high-throughput SCS technology. The scRNA-seq of a normal hematopoietic cell population confirms the structured hierarchy of a normal blood development of stem cell derived-myeloid and -lymphoid lineages,<sup>25,26</sup> and more importantly, leads to the discovery of a novel dendritic cell (DC) subset.<sup>27</sup> Transcriptomic

profiling of AML has significantly improved our understanding of tumor hierarchical structure and led to the identification of novel cell types, leukemic stem cells (LSCs), and drug-resistant subpopulations.<sup>28–30</sup>

Unique leukemia-enriched plasma membrane proteins (e.g., CD25 and IL1RAP) enabled the prospective isolation of two AML blast populations from individual patients, and the separate subclones were shown to be genetically and epigenetically distinct and to have different functions in their drug sensitivity, growth, and engraftment behavior.<sup>31</sup> These data demonstrate the existence of functional diverse subpopulations within individual AMLs. scRNA-seq data were used to profile 16 patients with AML and 5 healthy donors and to classify AML cells into 6 subpopulations along the HSC to myeloid axis of the normal cell hierarchy. Each population was characterized by a unique expression signature, including HSCs, progenitors, granulocyte-macrophage progenitors (GMPs), promonocytes, monocytes, and conventional DCs. Such signatures exhibit a close correspondence to the genetics of the AMLs. For example, TCGA tumors with high GMP-like and promonocyte-like scores derived from scRNA-seq perfectly overlap with PML-RARA fusions. Different AML cell signatures are further correlated with clinical outcomes such the outcomes are better in patients with a higher GMP-like score than in those with a higher HSC/Progenitor-like score.<sup>28</sup> The molecular classification of AMLs has also been explored by mass cytometry and chromatin accessibility at the single-cell level. By comparing surface and intracellular signaling proteins simultaneously in healthy and leukemic cells by single-cell mass cytometry, Levine et al<sup>32</sup> have reported that the tightly coupled surface and signaling phenotypes in healthy cells were perturbed in AML cells. Compared with cell surface markers, the signatures of intracellular signaling proteins are more reliable to predict clinical outcomes in patients.<sup>32</sup> The analysis of single-cell chromatin accessibility also reveals the regulatory heterogeneity of AML cells resembling a normal blood cell hierarchy, with a similar pattern distributed along the HSC to myeloid axis.<sup>33</sup> These studies indicate an intrinsic relationship among the transcriptomes, proteomes, and epigenomes in AML cells.

AML LSCs have been recognized as the apex of the AML cellular hierarchy within the heterogeneous population, with properties of self-renewal, cell cycle quiescence, and chemoresistance.<sup>34</sup> It is believed that a cure for AML depends on eradicating the LSCs. However, no definitive phenotypic markers are known to purify LSCs. Intriguingly, the LSC-enriched population can be recognized by characteristic transcriptional signatures, such as the “LSC score.”<sup>35</sup> The use of the LSC score in a single-cell classification can help identify and trace the LSC population during treatment and provide opportunities to improve identification of the LSC population. Indeed, the LSC expression signature has been applied in single-cell analysis of the MLL-AF9 mouse model and has uncovered the heterogeneity of the conventional marker-defined LSC population (Lin-Il7r-Kit+Sca1-CD34+CD16/CD32+) that contains two independent self-renewing lineages with different clonal activities.<sup>29</sup> In contrast, LSCs have metabolic features that require active oxidative phosphorylation status.<sup>36</sup> scRNA-seq has been used to trace the response of the LSC population during venetoclax with azacitidine treatment, which can disrupt the energy metabolism in AML patients.<sup>30</sup> Interestingly, SCTS also leads to the identification of a differentiated monocyte-like AML cell population, which suppresses T-cell activation in vitro and might have immunomodulatory functions that contribute to the pathogenesis of the disease.<sup>28</sup>

#### 4. CONCLUSION: LIMITATIONS AND PROSPECTIVE

Multiple SCS technologies have been applied in cancer research and have achieved remarkable progress. However, significant technical improvements are needed to achieve a comprehensive view of tumor heterogeneity. First, SCS technology is still limited by amplification bias and extensive technical errors, particularly in single-cell genome sequencing.<sup>37</sup> Second, the need for simultaneous acquisition of multiomics data of a single cell has not been fulfilled. For example, the co-existence of leukemic, preleukemic, and normal blood cells in AML samples requires the integration of mutational spectrum and transcriptional states to optimally classify the functional heterogeneous subpopulations. To date, most attempts at systematically detecting both genetic and RNA expression information from the same single cell are either expensive or have low sensitivity,<sup>38–40</sup> thus restricting the widespread use of integrative analysis. However, some limited success has been achieved with a relatively low throughput in the studies of hematopoietic malignancies. These include single-cell transcriptome analyses in combination with successful detection of the BCR-ABL breakpoint in chronic myeloid leukemia<sup>41</sup> and a novel method that allows mutational analysis (up to 12 different mutations) and parallel RNA sequencing from the same single cell in myeloproliferative neoplasm.<sup>42</sup> Furthermore, efforts have been made to combine transcriptome detection with surface-marker protein analysis<sup>43</sup> or assay for transposase-accessible chromatin using sequencing<sup>44</sup> within a single cell. These studies hold promise for the future development of multiomics integrated at the single-cell level.

Dissecting AML heterogeneity using SCS has provided novel insights into the molecular mechanisms underlying drug resistance and disease progression. Single-cell targeted mutation analysis in AML treated with the FLT3 inhibitor quizartinib has revealed highly complex drug-resistance clones at relapse, demonstrating marked clonal complexity associated with therapeutic resistance.<sup>45</sup> By comparing clonal structures identified by single-cell DNA sequencing at diagnosis and relapse, multiple bypass pathways have been identified in patients with AML having IDH2 mutations that can potentially be targeted for treatment.<sup>46</sup>

While these studies serve as excellent examples of how new knowledge from SCS can aid in the development of new drugs and treatment options, dissecting intratumoral heterogeneity holds a relatively greater potential for clinical application in the era of precision medicine. For example, new biomarkers identified from previous unknown drug-resistant subpopulations could be used to better stratify patients with AML. Moreover, SCS in combination with a more sensitive targeted mutation detection method can be used to monitor functionally diverse subclones with different drug-sensitivities and stem cell potential during treatment and disease progression. This information will be invaluable in improving prognostic prediction and detecting minimal residual disease. With rapid development of these advanced technologies, it is conceivable that SCS will have a tremendous impact on the clinical practice of AML.

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