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Genomic tools in Acute Myeloid Leukemia: from the bench to the bedside

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Abstract

Following its use in the initial characterization of an acute myeloid leukemia (AML) genome, next generation sequencing (NGS) has continued to molecularly refine the disease. Here we review the spectrum of NGS applications that have subsequently delineated the prognostic significance and biological consequences of these mutations. Further, we discuss the technology's role in providing a high-resolution glimpse of AML clonal heterogeneity, which may inform future choice of targeted therapy. Though obstacles remain in applying these techniques clinically, they have already impacted patient care.

Keywords

Acute Myeloid Leukemia; Genomics; High-Throughput Nucleotide Sequencing; Epigenomics; Gene Expression Profiling

Introduction

Most acute myeloid leukemia (AML) patients are cytogenetically normal (CN-AML). Their variable overall survival suggests clinical and biologic heterogeneity and a need for additional biomarkers. As many of these patients have no detectable copy number alterations, mutations in the diploid genome are likely pathogenic. Hence, lesions driving disease progression in patients without known recurrent mutations¹ may be discovered by whole-genome (WGS) and whole-exome (WES) sequencing. Indeed, the decade following the sequencing of the first cancer genome,² that of an individual presenting with CN-AML, has seen an explosion of this technology³ that has advanced our understanding of AML through the discovery of mutations in *DNMT3A*⁴ and *IDH1*⁵ and the recent finding⁶ that nearly all patients harbor at least one likely pathogenic mutation.

The prognostic significance and functional consequences of these and other recently discovered mutations are being elucidated. Translating these results to the clinic and assessing the impact of “targeted therapies” may further require determining whether mutations reside in subclones and how the latter evolve over time or following treatment.

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Just as WGS and WES have facilitated mutation discovery, these subsequent challenges are being addressed by a diverse array of next-generation sequencing technologies, including transcriptome, methylome, and targeted, custom capture sequencing.

Mutation Discovery

The capillary electrophoresis of individual fluorescently-labeled Sanger reaction products used to originally sequence the human genome over ten years at an expense of several billion dollars have since given way to “next-generation” sequencing (NGS) platforms. These can resequence the human genome at a fraction of the original cost (~\$10,000 per tumor germ line pair) in 4–6 weeks. These platforms require a library preparation phase, in which synthetic DNA (adapters) are ligated onto the ends of the fragmented DNA to be sequenced. The fragments are then subjected to a polymerase-mediated reaction, whose intermediate products can be monitored in real time by the platform’s optical instruments. For example, the Illumina platform detects the incorporation of fluorescently-labeled nucleotides during an amplification reaction, with noninterference between contiguous nucleotides ensured by a step-wise reaction in which an incorporated nucleotide’s 3’ blocking group prevents further extension until its dye has been detected and its blocking group removed by chemical cleavage. These platforms provide a nucleotide resolution view of the genome that, for example, yields precise breakpoints of chromosomal rearrangements coarsely detected by less sensitive approaches, such as spectral karyotyping. The reads have characteristic lengths [generally from 25 to 100 base pairs (bp)] and error profiles (often reflecting the error-prone polymerase) that are platform dependent. These impact a platform’s suitability for a particular application [e.g., the short reads and greater depth of the Illumina platform are suited for quantitating single nucleotide variants (SNVs), while the longer reads of the Pacific Biosciences RS platform facilitate discovery of structural variants (SVs)].

Whole-Genome Sequencing (WGS)

WGS provides comprehensive DNA sequencing of the entire genome. As such, Ley and colleagues chose⁷ it to elucidate the unknown initiating event in tumors from two CN-AML patients through a series of studies.^{2, 4, 5} These led to the discovery of mutations in *IDH1* and *DNMT3A* and have been extensively reviewed elsewhere.^{7–11} Additionally, they established several paradigms that have guided subsequent genomics studies in hematological^{12, 13} and solid tumors, including validation of sequencing results using an orthogonal platform and comparison of tumor and matched normal samples from the same patient to discover acquired, somatic variants. When matched normals are not available, putative variants may instead be filtered if they occur in a cohort of (unmatched) normal samples or are annotated in single nucleotide polymorphism (SNP) databases.

In the latest of the trio of studies⁵, paired-end sequencing from both ends of a DNA fragment, as opposed to single reads, provided greater genomic context and facilitated alignment of reads to the reference genome. This, coupled with maturing variant calling algorithms¹⁴ that analyze mapped reads to infer SNVs in the presence of sequencing errors, (mis) alignment artifacts, and tumor/normal contamination, dramatically improved variant-calling false positive rates. These advances accentuate the innate capability of WGS to characterize the full range of mutations, including intronic and exonic SNVs, insertions and deletions (indels), copy number alterations (CNAs), and SVs (including fusions/translocations).¹⁵

Whole-Exome Sequencing (WES)

Since mutations effecting protein function are likely within coding exons, sequencing the exome (i.e., the coding exons of annotated genes) via WES is a cost-effective alternative to WGS. WES targets exons via a capture-based library preparation phase using probes whose length, number, and exonic targets vary across platforms.¹⁶ This approach captures regions flanking the probes (e.g., ~100bp, depending on fragment length), including those in introns and untranslated regions (UTRs). However, promoters, enhancers, and intronic splicing silencers or enhancers far outside these targeted regions will not be sequenced. Further, some regions, particularly those with extreme GC content.^{6, 16, 17} are difficult to capture and hence are underrepresented. Nevertheless, WES is attractive in limiting analysis to the ~1–2% of the genome most likely to be of pathogenic interest.

Several groups have applied WES to rationally-selected genotypes to minimize inter-sample heterogeneity and to enrich for subtype-specific mutations. This approach identified mutations in *DNMT3A* in acute monocytic leukemia,¹⁸ in *BCOR* in a CN-AML case molecularly screened to be free of known oncogenic mutations in *NPM1*, *CEBPA*, *FLT3*, or *MLL*,¹⁹ and in *GATA2* in CN-AML patients with biallelic *CEBPA* mutations.²⁰ WES and WGS have also discovered mutations in the splicing factors *SF3B1*, *SRSF2*, and *U2AF1* in MDS (reviewed in²¹). Recurrent mutations of the spliceosome, including in *U2AF1*, have subsequently been discovered in AML using both platforms.⁶

Transcriptome Sequencing (RNA-seq)

Unbiased sequencing of the transcriptome (RNA-seq) offers several advantages²² with respect to WGS and WES: it facilitates discovery of novel transcripts and of alternative splicing events and *trans*-splicing²³ or read-through¹⁵ fusion events that can not be detected from genomic DNA. It reduces false positives by enriching for expressed transcripts and their variants, which are more likely to be pathogenic, and quantitates this expression digitally. However, it is ineffective in discovering mutations that destabilize transcripts [e.g., by inducing nonsense mediated decay (NMD)²⁴] or are rarely expressed. Further, RNA-seq involves additional complexity during the preparation of cDNA sequencing libraries from RNA,²⁵ including the need to cope with potential degradation of unstable RNA and the sequence and structural dependence of cDNA synthesis and hybridization.²² Variations in this step accommodate different downstream analyses. For example, polyadenylation fractionation enriches for expressed mRNA relative to non-coding RNA, whereas size selection of unfractionated total RNA enriches for microRNA (miRNA).

Several groups^{26–30} have used RNA-seq to discover somatic mutations in AML at considerably reduced cost and effort relative to WGS. Greif *et al.*²⁶ found mutations in *RUNX1*, *TLE4*, and *SHKBP1*; McNerney *et al.*²⁷ discovered that *CUX1* on chromosome 7q was expressed at haploinsufficient levels in monosomy 7/del(7q) *de novo* and therapy-related AML samples; Wen *et al.*²⁸ found seven novel fusions specific to CN-AML and an additional *CIITA-DEXI* fusion that occurred in 48% (14/29) of CN-AML samples; Masetti *et al.*²⁹ discovered a recurrent *CBFA2T3-GLIS2* fusion in three of seven childhood CN-AML patients; and Walter *et al.*³⁰ identified a novel *ITGA5* splice variant as a potential relapse risk factor by RNA sequencing of relapsed patients who had been classified as low risk based on known cytogenetic and molecular markers.

Ramsingh *et al.*³¹ characterized miRNA expressed in a CN-AML patient by sequencing size-selected cDNA using the SOLiD platform and discovered outlying expression of miR-233 beyond the dynamic range of miRNA microarrays and RT-PCR. Subsequent miRNA-seq studies have detected six miRNA biomarkers in circulating blood that

differentiate AML patients from controls³² and have uncovered two miRNAs whose loss leads to leukemia-related diseases in mice.³³

Frequency and Prognostic Significance of Mutations

Discovered somatic variants are frequently validated on an orthogonal platform, e.g., mutations detected on the Illumina platform may be validated using custom primer amplification followed by direct sequencing² or NGS on the Roche GS FLX system.⁵ Alternatively, deep read count scan provide validation. Deep amplicon sequencing, i.e., amplification using custom-designed PCR primers followed by deep NGS, is one such approach. Another involves liquid hybridization capture using custom sequence probes designed to cover the region of interest (e.g., spanning an SNV, an indel, or all exons within a gene) and subsequent deep NGS.^{34, 35} The scalability of custom probe approaches is attractive when validating many variants.

Both amplicon- and capture-based strategies are useful in defining mutation frequencies across a large cohort and in clinical correlation studies.¹ Ampliconpyro sequencing has been extensively used^{36–38} to determine the clinical significance of *TET2* mutations,³⁹ though the findings are inconsistent.⁴⁰ One study found little correlation between outcome and *TET2* mutations in MDS,³⁶ while another revealed their correlation with inferior event-free survival in *de novo* CN-AML cases^{37, 38} and particularly in the European Leukemia Net favorable-risk subgroup.³⁸ Further, amplicon sequencing using the Illumina⁴¹ and Roche^{42–44} platforms has associated *SF3B1* mutations with MDS characterized by ring sideroblasts and a good clinical outcome.

Many groups have demonstrated frequent recurrent mutations in multiple genes that impact prognosis. These include mutations and deletions of *TP53* in cytogenetically complex AML,⁴⁵ *MLL* fusions,⁴⁶ and *FLT3-ITD* (internal tandem duplication), *FLT3-TKD* (SNVs in tyrosine kinase domain),⁴⁷ *DNMT3A*^{48–53} and *ASXL1*^{54, 55} mutations in intermediate risk and/or CN-AML, all of which have been associated with poor outcomes in these cytogenetic subsets. However, even when large-scale patient populations are studied by different investigators, consistent correlations are not always observed. For example, not all retrospective studies have associated either the most common R882 and/or non-R882 *DNMT3A* mutations with poor outcome.⁵³

Frequently conflicting results between correlation studies may be attributed to several factors. For example, secondary mutations may modulate the effect of another mutation, as frequently seen in patients with mutations in both *SF3B1* and *DNMT3A* that have improved survival relative to patients with *DNMT3A* mutations alone.⁵⁶ Further, Damm *et al.*⁵⁷ note that their finding that *SF3B1* mutations had no effect on OS or leukemic progression, in contrast to other studies, may reflect the low-risk cohort of their study. Finally, additional heterogeneity between or within studies may be introduced by different treatment regimens.⁵⁷ Reliable clinical associations thus require multivariate analyses incorporating diverse mutations and other prognostic indicators or analyses restricted to molecularly homogeneous populations. Such studies could be conveniently accommodated by custom capture-based approaches.

Functional and Biological Consequences of Mutations

Bioinformatic analysis is useful in narrowing the sea of mutations discovered from large-scale genomic surveys to “driver” mutations likely significant in disease onset and progression. This winnowing out of irrelevant “passenger” mutations is partially achieved by the etiology of AML. Sequencing studies across cancer types have revealed that AML has a relatively low mutation burden (Fig 1), which increases likelihood of discovering

pathogenic mutations in AML. Since “cancer genes” are often mutated across multiple cancer types, their facilitated discovery has relevance beyond AML.

Genes^{58, 59} and pathways⁶⁰ mutated at a statistically significant rate above background mutation rates may be inferred from large-scale studies. Other approaches search for recurrently mutated subnetworks within protein-protein interaction networks^{61, 62} or integrate genome-wide expression and mutation data to probabilistically infer perturbations in annotated pathways.⁶³ Candidate mutations for subsequent experimental study also include those that occur at high frequency in other cancers,⁶⁴ are within annotated functional domains⁶⁵ or conserved regions,⁶⁶ or are predicted to disrupt protein function.⁶⁷ These may be further prioritized through integrated exploration of multidimensional cancer genomics data, including that describing SNVs, CNAs, DNA methylation, and mRNA, protein, and phosphoprotein expression.⁶⁸

Whether a mutated gene should be overexpressed or knocked down, for example, to assess its functionality is dependent on whether the lesion is likely a gain-of-function mutation in an oncogene or a loss-of-function mutation in a tumor suppressor. This distinction may be informed by the type and distribution of mutations within the gene and of the distribution of the mutation within the genome. Several types of mutations introduce premature termination codons, including nonsense point mutations and inopportune frame-shift indels, which are likely to destabilize the transcript. The scattering of these and/or consensus splice site mutations throughout a gene’s coding region, as has been detected in *BCOR*¹⁹, *TET2*,⁴⁰ and the splicing factor *ZRSR2*,^{41, 69} are a likely indicator that it acts a tumor suppressor. Homozygous deletions or the detection of the mutation within a recurrently deleted chromosomal region, as in the *CUX1* study,²⁷ provide further evidence of a tumor suppressor role. In contrast, the clustering of missense mutations within hotspots, particularly within known functional domains or conserved regions, is more suggestive of a gain-of-function mutation. These characteristics are shared by mutations in *U2AF1* (preferentially targeting residues within zinc finger domains) and *SF3B1* (preferentially targeting residues within conserved⁷⁰ HEAT domains). An oncogenic role is further supported for a gene having a missense mutation within a recurrently amplified region. For example, to enrich for potential oncogenes and tumor suppressors, Dolnik *et al.*⁷¹ designed custom DNA capture probes targeting the coding exons of 1000 genes detected within minimally deleted/gained regions via SNP analysis and used subsequent NGS to reveal mutations in *RAD21* within amplified regions.

The above approaches are particularly useful in planning experimental studies for a gene with unknown function. However, several classes of AML-relevant mutations occur in genes with ascribed function, including in epigenetic regulators and splicing factors. For these, NGS approaches can conveniently assay downstream effects of the mutations.

Effects of Mutations in Epigenetic Modifiers

The epigenome is frequently dysregulated in cancer.⁷² Indeed, epigenetic modifiers are recurrently mutated in AML and MDS,⁷³ including the DNA methyltransferase *DNMT3A*, the methylcytosine dioxygenase *TET2*, and the isocitrate dehydrogenases *IDH1* and *IDH2* discussed above, as well as the polycomb-associated *ASXL1* and the methyltransferases *EZH2* and *MLL*.⁷⁴ Gain-of-function *IDH1/2* mutations⁷⁵ antagonize *TET2* function⁷⁶ and mutations in *TET2*, *IDH1*, and *IDH2* are mutually exclusive.⁴⁰ These mutations have clinical significance⁷⁴ and the DNA methyltransferase inhibitors azacytidine and decitabine induce clinically significant complete and partial responses in patients with MDS and low-blast count AML.⁷⁷ As such, array-based strategies have been used to characterize their downstream effects genome-wide and have detected a global,

predominantly hyper-, methylation pattern induced by *IDH1/2*^{6, 76} and *TET2*⁷⁶ mutations and largely shared⁷⁶ between them.

Recent methods replace the array-based detection of earlier techniques with NGS for nucleotide resolution and reduced bias.⁷⁸ and are based on one of three approaches: methylcytosine-sensitive restriction digestion, bisulfite conversion (possibly preceded by methyl-insensitive digestion), or immunoprecipitation (IP).⁷⁸ DREAM,⁷⁹ an example of the first technique, performs serial digestion with methylation-sensitive *SmaI* and -insensitive *XmaI* restriction enzymes. Both target the same CCCGGG sequence, though the former is blocked by CpG methylation and leaves 5'-GGG blunt ends, while the latter cuts any remaining target sequences and leaves a 5'-CCGGG overhang, which acts as a unique signature for methylated sites. The second approach, sodium bisulfite treatment, converts unmethylated cytosine to uracil, but leaves methylated cytosine intact. To enrich for DNA that can be (differentially) methylated, Reduced Representation Bisulfite Sequencing (RRBS)⁸⁰ cuts bisulfite-treated DNA at CCGG sequences using the methylation-insensitive *MspI* restriction enzyme and size selects fragments to ensure the presence of at least two such sites within a defined (e.g., 300bp) sequence span. Finally, IP-based approaches are typified by MeDIP-seq,⁸¹ which uses an antibody directed against 5mC to immunoprecipitate methylated genomic regions. MethylCap-seq is a related approach that enriches for methylated DNA through capture with methyl-binding protein (MBD2).⁸² In all four techniques, the resulting DNA libraries are characterized by NGS.

Leukemic subtypes segregate according to differentially methylated regions, detected by MeDIP-seq, not only in promoters, as might be anticipated, but also in gene bodies, CpG islands (CGIs; inside and outside promoters) and CGI shores.⁸³ These subtypes also clustered according to differential methylation in satellites, long terminal repeats, and in short (SINEs) and long (LINEs) interspersed nuclear elements. A toggling of methylation status in these repetitive regions between normal and leukemic blood cells was observed using DREAM analysis: sites with significant hypermethylation in normal cells tended to show significant hypomethylation in leukemia samples and vice versa.⁷⁹ Mutation- and drug-specific effects have also been described. Loss of *DNTM3A* in hematopoietic stem cells (HSCs) induced hyper- and hypo-methylation in CpG dinucleotides, as detected by RRBS.⁸⁴ This loss instead leads to predominant hypomethylation following differentiation to B cells, as assayed by DREAM.⁸² *IDH1/2* mutations lead to a marked increase in hyper- relative to hypo-methylated sites in mutants relative to controls, with an enrichment in promoter regions and CpG islands near transcription start sites (TSSs) detected by an enhanced RRBS.⁸⁵ Finally, decitabine treatment was found using MethylCap-seq to significantly reduce global methylation, particularly in chromosome subtelomeric regions, possibly suggesting a region-specific mechanism of drug action.⁸²

Mutations to epigenetic modifiers are anticipated to affect transcription and chromatin state, consequences which have been investigated using chromatin IP followed by NGS (ChIP-seq). ChIP-seq using antibodies targeting H3K27me3 revealed a significant reduction in genome-wide H3K27me3 TSS occupancy following *ASXL1* knockdown.⁸⁶ Specific loss of H3K27me3 at the posterior *HOXA* cluster, which is known to contribute to myeloid transformation, suggest that *ASXL1* mutations promote transformation by relieving gene repression. Saeed et al.,⁸⁷ also using ChIP-seq, identified accessible genome regions to which the oncofusion proteins *AML1-ETO* and *PML-RARA* bind.

Effects of Mutations in Splicing Factors

Minigene reporter assays of splicing mutations⁸⁸ have been extended genome-wide using gene expression arrays in expectation that the mutations' impaired ability to recognize the 3' splice site will perturb gene expression. For example, splicing-sensitive arrays indicated that

exons were significantly downregulated and introns were significantly upregulated (i.e., unspliced) following *U2AF1* mutant expression.⁴¹ RNA-seq provides a more comprehensive and quantitative alternative to gene and exon arrays. Yoshida *et al.*⁴¹ validated their above finding by observing increased read counts in likely intronic regions within *U2AF1* mutant samples, while Makishima *et al.*⁸⁹ found these mutations perturbed *TET2* splicing. To determine the role of modulated splicing in AML, Przychodzen *et al.*⁹⁰ analyzed publically available RNA-seq data from The Cancer Genome Atlas (TCGA; <https://tcga-data.nci.nih.gov/tcga>) in their comparison of six samples with *U2AF1* mutations in tAML or sAML and 14 WT samples. 35 exons found to have significantly altered expression were predominantly skipped, including genes involved in mitosis and RNA processing. In contrast, *SF3B1* mutations assayed by RNA-seq induced a high percentage of exon retention in RARS patients.⁹¹

Clonal Evolution

NGS has revealed that most AML tumors are oligoclonal. Variant discovery by WES and their subsequent capture-based targeted deep sequencing in hematopoietic stem/progenitor cells (HSPCs) from healthy donors indicate that the cells accumulate random mutations during aging.³⁵ If a HSPC transforms to a leukemic blast, these passenger mutations are “captured” in its progeny as it clonally expands and serve as a genetic signature identifying the clone. In particular, the variant allele frequency (VAF, or ratio of reads supporting the variant to total reads at the locus) acts as a molecular clock indicating when in the clonal hierarchy the mutation was acquired: heterozygous, clonal mutations within a pure sample have a VAF of ~50%, whereas subclonal mutations, acquired later, are present in fewer cells and have lower VAFs. Aggregations of VAFs thus reflect clones (Fig. 2). Since, both passenger and driver mutations are valuable clonal markers, the most comprehensive perspective on clonal architecture is provided by first discovering variants using WGS and then quantifying VAFs using deep, targeted sequencing.^{34, 92, 93} Discovering clonal markers with WES^{35, 94, 95} or even through limited candidate gene resequencing⁹⁶ have also revealed subclonal architecture, though their more limited number relative to WGS almost certainly (further) underestimates clonal heterogeneity. Hence, WGS-based discovery is particularly important for diseases with few protein coding mutations, such as AML, whereas WES may suffice to assess clones in diseases with high mutation burden, such as melanoma and chronic lymphocytic leukemia (CLL).⁹⁵ Additionally, sensitivity in detecting low-frequency clones is improved with increased sequencing depth. For example, a sequencing depth of 100× is likely to detect VAFs as low as ~4% (95% binomial confidence interval).

A study of tumor/relapse pairs has shown two patterns of clonal evolution during AML relapse: (1) the founding clone in the primary tumor gained additional mutations and evolved into the relapse clone or (2) a subclone escaped therapy, gained additional mutations, and expanded into the relapse clone (Fig 2).³⁴ A likely model describing this subclonal evolution, in which AML develops through serial acquisition of mutations in HSPCs, has been supported by isolating and sequencing preleukemic HSPCs, found to have a subset of mutations of their leukemic progeny.⁹⁴ A similar persistence of mutations from an antecedent disease accompanied by acquisition of additional mutations during progression to AML has been observed in serial studies tracking evolution from severe congenital neutropenia⁹⁷ or MDS⁹² to AML. However, recurrently mutated genes display a wide range of VAFs across sAML samples, indicating that none are consistently associated with the founding clone and that the disease progresses through a variety of acquired mutations across patients.⁹³

Clonal heterogeneity is frequently discernible from a single tumor sample. However, multiple samples derived from a single patient are often required to disambiguate subclones

overlapping in the original sample and to appreciate the tumor's complexity (Fig 2). These may be relapse samples, physically-isolated biopsies taken at the same time point, or tumor cells exposed to some manipulation (e.g., passage in culture or through immunodeficient mice) that could induce a distinct (fitness) phenotype in a subpopulation of cells. Characterizing⁹⁸ this intra-tumor heterogeneity and clonal architecture is important as they may have clinical implications⁹⁹ and contribute to therapy resistance.¹⁰⁰ For example, subclonal mutations may be correlated with poor clinical outcome in CLL.⁹⁵

Clinical Application of NGS

Current genetic testing in AML is inadequate to detect the clinically-relevant mutations of this heterogeneous disease.¹⁰¹ Metaphase cytogenetics and FISH lack resolution, while Sanger-based sequencing is cost and time prohibitive. Mass spectrometry genotyping identifies mutations at specific residues (e.g., in *N/KRAS* and *IDH1/2*), but is unable to detect mutations scattered throughout the gene body (e.g., in tumor suppressors *TP53*, *TET2*, and *ASXL1*). Further, genes such as *N/KRAS*¹⁰² and *DNMT3A*,⁴ which are expected to be targeted at well-characterized residues, have exhibited noncanonical, but oncogenic, mutations that would have been missed by SNP-directed approaches.¹⁰² In the latter case, up to 40% of patients with *DNMT3A* mutations harbor non-R882 mutations, which are also associated with poor outcome.⁴ Comprehensive WGS is an attractive diagnostic platform, but suffers from high cost and analysis time and moderate- to low-coverage insensitive to low-frequency, subclonal mutations. Deep-coverage, targeted NGS of panels of candidate genes ameliorates these concerns and leverages the community's investment in large-scale sequencing efforts, particularly through TCGA, in cataloging somatic cancer mutations. For example, a "pan-cancer" panel assays the entire coding sequence and selected introns of 236 cancer-related genes (www.foundationone.com). 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 active in AML, including translocations, SNVs, and indels.¹⁰³ Further, the deep read counts of targeted NGS are well suited to minimal residual disease monitoring.¹⁰⁴

Technical challenges remain, however, including the inefficient capture of GC-imbalanced targets such as *CEBPA*^{103, 105} and potentially limited genomic DNA, though whole-genome amplification may address the latter without introducing appreciable bias.¹⁰⁶ Bioinformatic analysis¹⁴ is a bottleneck in the clinical sequencing pipeline and software tools need to be validated.¹⁰⁷ For example, alignment is computationally demanding, potentially sensitive to noisy reads, and problematic in repetitive regions.¹⁰⁸ Fully leveraging NGS clinically will require carefully considered informed consent. For example, WGS of a patient with multiple primary tumors revealed the presence of a cancer susceptibility, germline mutation in *TP53* with clinical implications for the patient's children.¹⁰⁹ Because the informed-consent document included a provision to communicate clinically relevant information to family members, the treating physician contacted the next of kin to inform them of the mutation and to encourage genetic counseling. In a second case, WGS detected a cryptic fusion within a clinically relevant timeframe, which impacted the patient's treatment plan.¹¹⁰ Diagnosis was facilitated by a "movable firewall" within the IRB-approved protocol that maintained the patient's anonymity, yet allowed the research team to communicate relevant findings to the treating physician.

Despite the remaining obstacles, the two above cases demonstrate the clinical impact of NGS. These successes and earlier studies have significantly advanced the state of the art in clinical diagnosis, with at least ten open clinical trials utilizing NGS (clinicaltrials.gov; keywords: "next generation sequencing" and "cancer"). Further, large, clinically-annotated data sets have already been accumulated by prior studies and offer a rich opportunity for

retrospective analysis. As technological trends continue to reduce sequencing cost, integrated⁶ clinical analysis and sequencing of the genome, transcriptome, and methylome, coupled with queries to drug-gene interaction databases (<http://dgidb.genome.wustl.edu>), will become a practical approach to comprehensively interrogating and treating leukemia.

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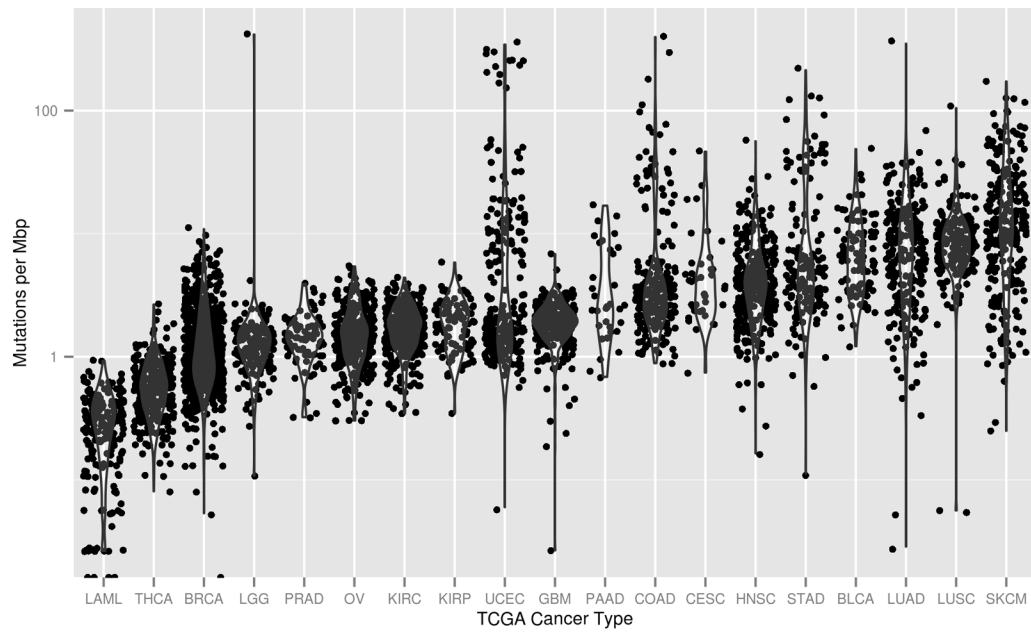


Fig 1. AML has a reduced mutation burden relative to other cancer types. Labels indicate TCGA cancer codes (<https://tcga-data.nci.nih.gov/datareports/codeTablesReport.htm>). Mbp: million base pairs.

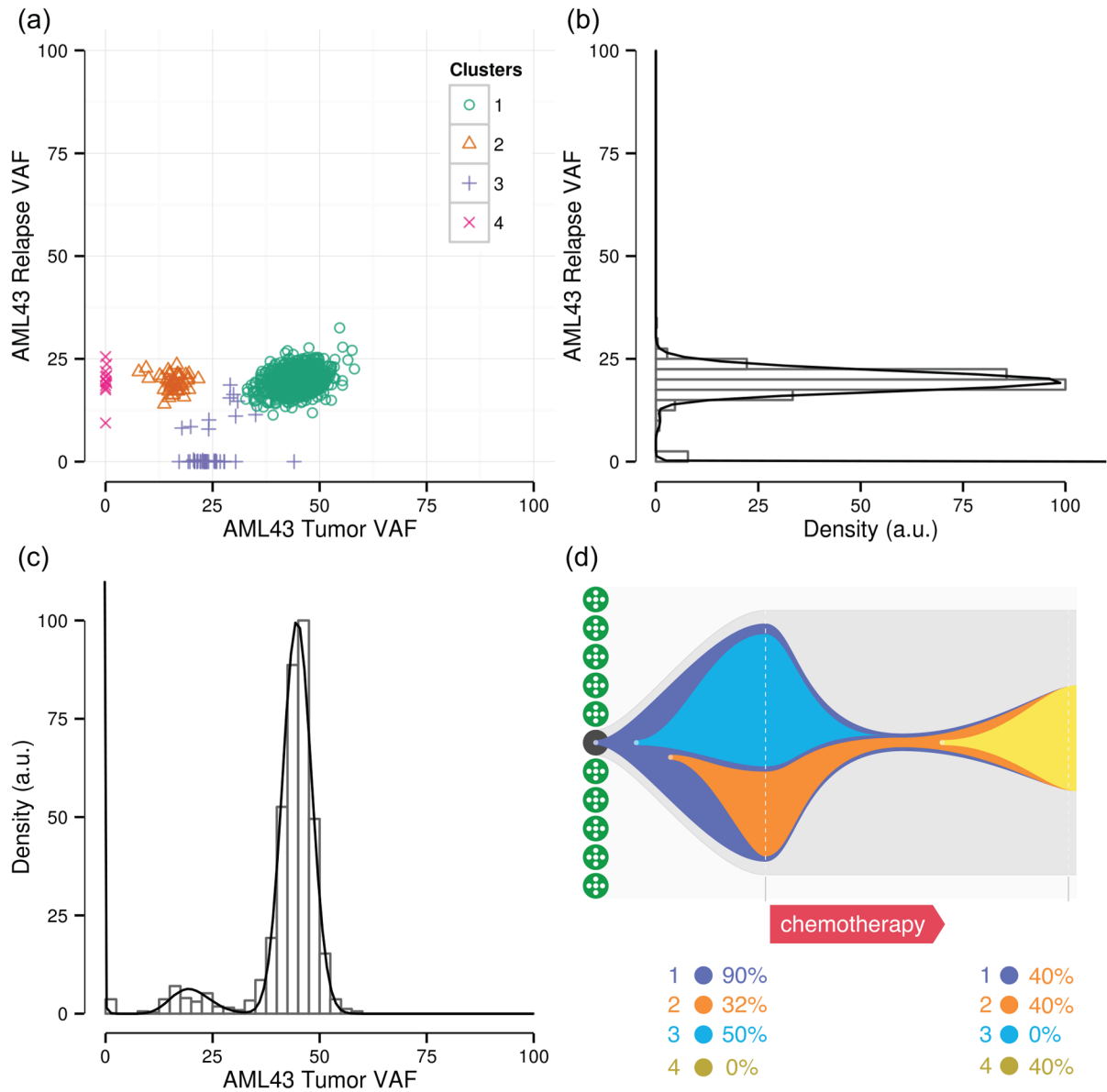


Fig 2. Subclone escapes therapy to expand into relapse clone. (a) Simultaneous (SciClone clustering⁹³) analysis of tumor and post-treatment relapse samples³⁴ reveals a subclone that was eradicated by therapy (cluster 4) and another that evaded therapy and became dominant during relapse. (b) Analysis of relapse only VAFs can not disambiguate subclones corresponding to clusters 3 and 5 from founding clone (cluster 2). (c) Analysis of tumor only VAFs. (d) Clonal evolution inferred from (a).