SPOTLIGHT REVIEW

Advances in next-generation sequencing and emerging technologies for hematologic malignancies

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Abstract

Innovations in molecular diagnostics have often evolved through the study of hematologic malignancies. Examples include the pioneering characterization of the Philadelphia chromosome by cytogenetics in the 1970s, the implementation of polymerase chain reaction for high-sensitivity detection and monitoring of mutations and, most recently, targeted next-generation sequencing to drive the prognostic and therapeutic assessment of leukemia. Hematologists and hematopathologists have continued to advance in the past decade with new innovations improving the type, amount, and quality of data generated for each molecule of nucleic acid. In this review article, we touch on these new developments and discuss their implications for diagnostics in hematopoietic malignancies. We review advances in sequencing platforms and library preparation chemistry that can lead to faster turnaround times, novel sequencing techniques, the development of mobile laboratories with implications for worldwide benefits, the current status of sample types, improvements to quality and reference materials, bioinformatic pipelines, and the integration of machine learning and artificial intelligence into molecular diagnostic tools for hematologic malignancies.

Introduction

Hemato-oncology has led the way for clinical implementation of molecular diagnostic testing in patients' care, starting with chronic myeloid leukemia and the pioneering work in measurable residual disease (MRD) monitoring. The detailed genomic databases that form the basis of our understanding of drivers of disease progression and treatment response are critical to how molecular pathologists and clinical variant scientists interpret variants in clinical practice today. Despite the advances of the past two decades, relapse rates remain high (~40-50%) for patients with acute myeloid leukemia (AML).[1] The current molecular standard of care for hematologic malignancies has progressed beyond diagnosis and classification to prediction of response/resistance and monitoring of MRD.[2] For example, both the International Consensus Classification and the World Health Organization emphasize the importance of molecular mutations for prognostication and guidance of therapy.[3,4]

Still more advances are coming to molecular hematopathology through: (i) improvements in the speed and footprint of next-generation sequencing (NGS) platforms; (ii) promising novel library chemistry and sequencing techniques, such as long-read and long-range sequencing; (iii) advances in bioinformatic pipelines, especially in error correction; (iv) continued efforts to bring together the numerous methods used to diagnose, manage, and monitor disease; and (v) profound advances in our conceptualization of the reference genome (Figure 1). In this review article, we touch on these new developments and discuss their implications for hematologic malignancies.

Advances in turnaround time

Rapid molecular diagnostics are particularly relevant for AML, in which the doubling time of malignant cells can be as brief as 1.5-4 days.[5] It is generally assumed that earlier initiation of induction therapy leads to better outcomes, and most patients are treated with induction chemotherapy within 4-16 days of diagnosis. However, more recent studies have demonstrated that time to treatment has less impact on long-term survival than was previously believed,[6] leaving time for comprehensive molecular genomic profiling and, therefore, a better-tailored induction regimen. However, time considerations should be balanced with the need for prognostic molecular data for informed discussions with patients.

Each year brings promises of faster turnaround times for

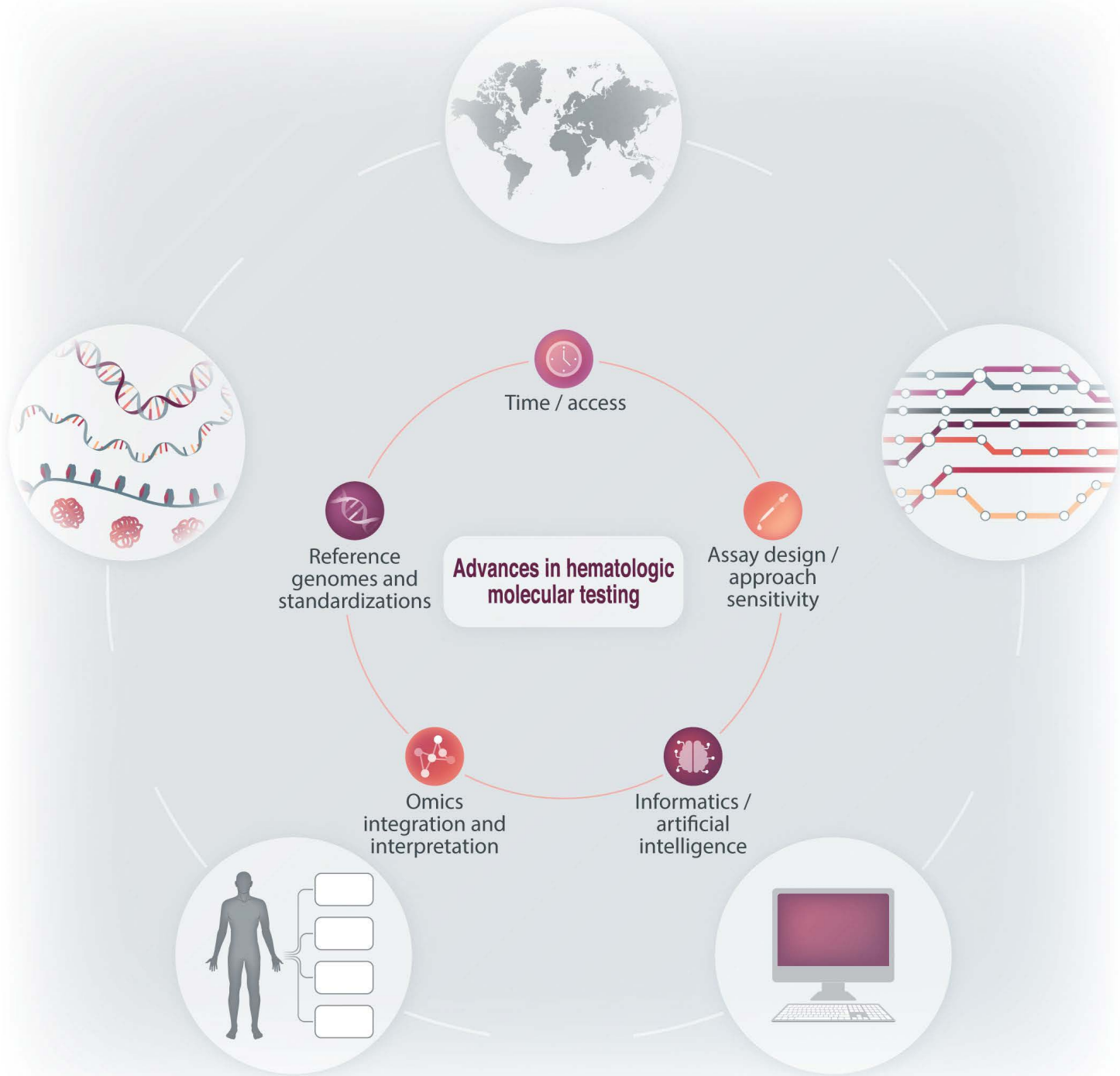

Figure 1. Advances in hematologic molecular testing. Improvements over the past decade have not only dramatically decreased the time from sample collection to reporting of results but have also made it possible for more people around the world to access this technology. Optimizations and novel chemistry have enabled better assay performance with improved sensitivity, while innovations in informatics and the addition of artificial intelligence tools have improved pipeline speed and accuracy. On the way from research to a clinical pathway is the use of "omics" data (spatial transcriptomics, proteomics, etc.), with the promise of improved diagnosis, prognosis, and theragnosis. Most importantly, with global adoption of next-generation sequencing into clinical practice, a better reference genome is needed to adequately represent diverse populations of patients.

NGS. As early as 2013, groups reported speedier NGS by replacing temperature-dependent polymerase chain reaction with isothermal amplification.[7] One example is the research by Yao *et al.*, who exploited this method to sequence the entire mitochondrial genome.[8] LAMP-sequencing, initially developed for COVID diagnostics, uses reverse transcription-based loop-mediated amplification (LAMP) primers to amplify RNA. Ion Torrent paired isothermal chemistry, with its semiconductor-based sequencing technology, is used to create an NGS system capable of faster sequencing times compared to Illumina.[9,10] An adaptation of Thermo Fisher chemistry to the Genexus instrument has yielded a myeloid mutation assay that targets both RNA fusions and common DNA hotspots in AML, providing reports within 2-3 days.[11] We cannot describe these methodologies in detail here; interested readers are referred to the relevant literature.

Advances in sequencing platforms and library preparation

Long-read sequencing, which offers average read lengths of 10,000 to 100,000 base pairs or longer, was first realized in 1980 with the passage of an unbroken fragment of nucleic acid through a nanopore enzyme, creating an energy

differential recorded on a semiconductor membrane.[12] Although long-read sequencing by nanopore technology has been commercially available since 2015, early iterations had low accuracy (~68%).[13] Steady improvements, such as the addition of a helicase enzyme to control sequencing speed, flow cell alterations, and improved informatics, have enhanced the accuracy to 99% or better, albeit in limited studies.[14]

Long-read data shed light on the implications of phasing; that is, whether mutations are *in cis* (on the same chromosome) or *in trans* (on homologous chromosomes). Such differences may result in either reduced or enhanced protein activity, depending on whether the gene is a tumor suppressor or an oncogene.[15] Studies have shown the significance of phasing information in hematologic malignancies. For example, Intlekofer *et al.* noted that acquired *IDH2* mutations lying *in trans* are an important mechanism of acquired mutations in AML.[16]

Optical genome mapping (OGM) platforms use probes or tags to "barcode" a genome so that the high molecular weight DNA can be rapidly scanned and then aligned to a reference. OGM can potentially improve upon current clinical cytogenetics tests in three ways: first, by decreasing turnaround times; second, by using molecular techniques, thereby eliminating the need for highly skilled cytogenetic technologists; and third, by offering higher resolution than high-resolution karyotyping or single nucleotide polymorphism arrays.

Significant advancements in library preparation and analysis have made OGM platforms clinically ready - or nearly so. The BioNano OGM platform uses a short probe to label a sequence that is repeated throughout the genome. Once tagged, long DNA molecules are linearized and imaged, allowing for rapid genome scanning and alignment of chromosomal regions.[17] Hi-C sequencing exploits the cross-linking action of formaldehyde on DNA segments that are in close proximity to each other *in vivo*, and standard NGS is then used for data generation.[18] Multiple layers of whole genome and epigenetic data are produced by Hi-C, and analysis requires advanced software and bioinformatic pipelines and novel artificial intelligence algorithms, which could prove challenging for some laboratories to implement. Duncavage *et al.* describe refinements to whole-genome sequencing that could position this sequencing technique as an alternative to conventional cytogenetics in myeloid diseases. Their method uses a highly efficient 8-hour library preparation and automated data analysis pipeline to generate 50x mean genome coverage.[19] Although only a few studies have explored the potential of low-pass, cell-free, DNA-based whole genome sequencing, Shao *et al.* demonstrated in 103 AML patients that aberrant changes in 5-hydroxymethylcytosine sre a unique, sensitive, and specific informative marker in AML for diagnosis and adverse prognosis.[20]

Although most research efforts over the past two decades have focused on genetic mutations, it has been made clear that the epigenome represents an important level of biological and regulatory function. Methylation-based biomarker assays have demonstrated the clinical significance of epigenetic variation, but implementation has been impeded by the lack of a gold-standard comparator and standardized control materials. The SEQC2 epigenomics quality-control study addresses these problems by providing three different whole-genome bisulfite sequencing protocols in addition to a comparison of the methylation signatures of several cell lines.[21] Methylation sequencing holds the potential for exciting advances in hematologic malignancies, such as the ability to be used in combination with long-read sequencing to identify "dead zone" structural variants and methylation defects.[22] A summary depicting new sequencing platforms is shown in Figure 2, and the current status of these technologies is detailed in Table 1.

Advances in single-cell genotyping: studying clonal dynamics and clonal evolution

On average, the AML cancer genome has fewer mutations than any other cancer genome. The AML testing protocol is also perhaps the most integrated of any malignancy, utilizing a broad range of tests for diagnosis, prognosis, monitoring, treatment guidance, and response prediction. However, we continue to see relapse rates of approximately 40-50%, even after transplantation.[23]

In contrast to bulk tumor sequencing, single-cell analysis offers the ability to track clonal diversity and observe clonal dynamics and trends. Different groups have pursued single-cell genotyping as a means of understanding the evolutionary events that allow specific leukemic clones to evade treatment. Although still in its infancy, the technique shows promise. Early work on *FLT3* ITD and *NPM1* mutations by Pagurian *et al.* showed tumor heterogeneity at the subclonal level, which could contribute to the high rates of relapse and resistance in AML.[24] These single-cell studies have shed light on the complex interplay between the immune microenvironment and the heterogeneous mutant clones that drive disease progression in AML.[25]

The extensive clonal diversity of the AML genome was further substantiated in a study by Morita *et al.*, who performed single-cell DNA sequencing on 123 AML patients and were able to show the evolutionary dynamics underlying therapeutic resistance in some of their patients.[26] Yeaton *et al.* used single-cell transcriptomic profiling to demonstrate that aberrant monocytic cell inflammatory signals are associated with poor prognosis and leukemic progression.[27]

Despite these insights, the barriers to clinical implementation are not insubstantial. Early iterations of single-cell

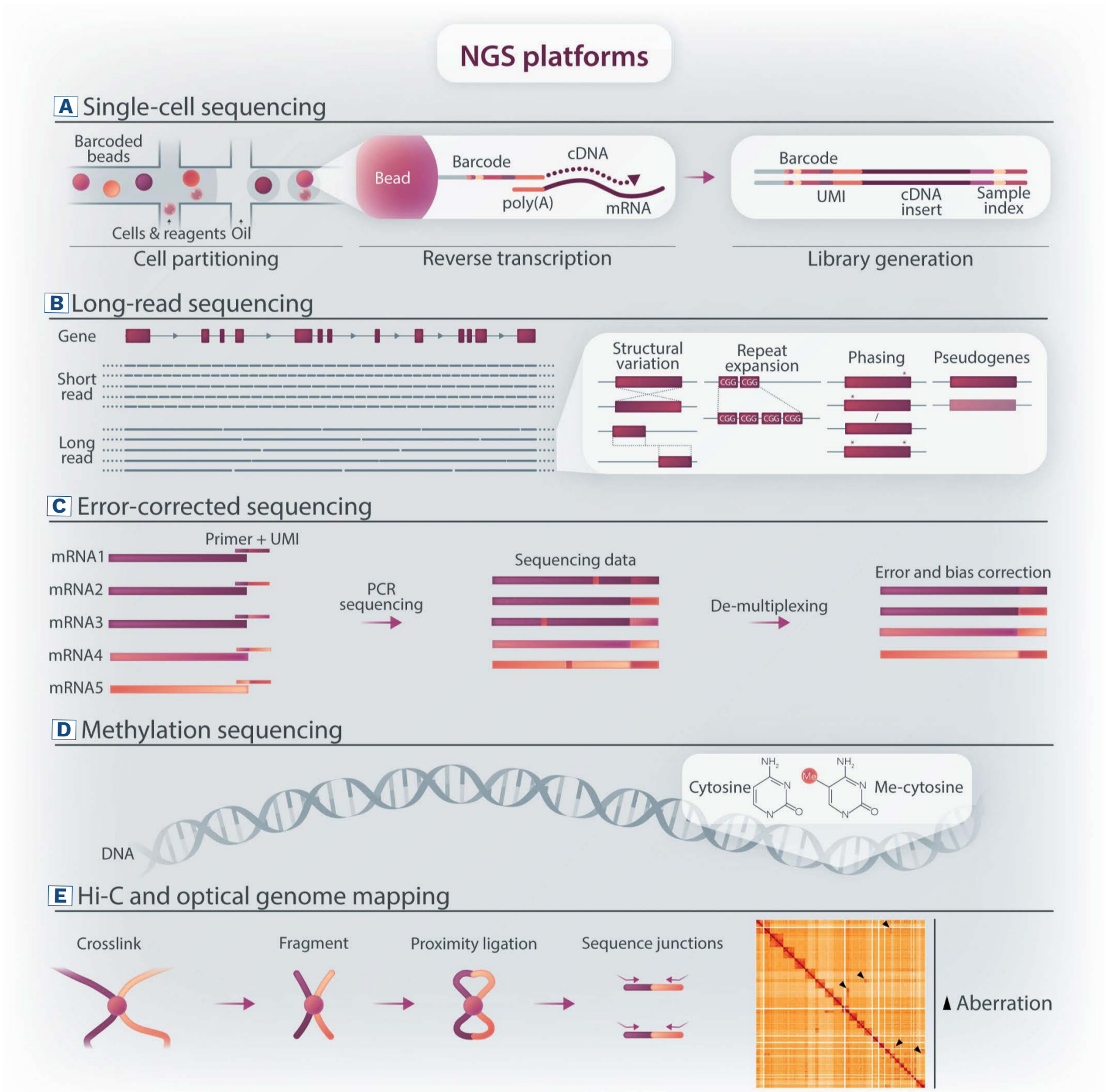

Figure 2. Advances in next-generation sequencing platforms. (A) Single-cell sequencing methods vary. Depicted here is a single-cell preparation using microfluidics, in which the cells and reagents are encapsulated with barcoded beads that tag each mRNA molecule of a single cell. (B) Long-read sequencing capitalizes on the ability to read a long string of DNA or RNA without interruptions or the need to stitch together the smaller 150-250 base pair reads typical of standard next-generation sequencing. This allows for identification of structural variations, long repeat expansions, phasing information, and pseudogenes. (C) In error-corrected sequencing, each nucleic acid molecule is tagged with a unique barcode prior to polymerase chain reaction amplification, allowing for downstream de-multiplexing and error correction as well as identification of bias errors. (D) Methylation sequencing typically relies on a bisulfite reaction, which allows the differentiation of cytosine from methylated cytosine. The purpose is to identify hyper- or hypo-methylated gene promoter regions that may contribute to tumorigenesis. (E) The illustration shows the steps of Hi-C sequencing, in which DNA that has undergone formalin crosslinking is fragmented and the fragmented ends are ligated. Sequencing of the junctions allows for interpretation of the whole genome at a resolution that can identify small copy number alterations, indels, inversions, and translations. Optical genome mapping (OGM; not illustrated) uses light microscopy-based techniques to create a map of the genome. OGM does not sequence DNA at the resolution of next-generation sequencing (i.e., at the base level) but offers higher resolution than DNA microarray. NGS: next-generation sequencing; UMI: unique molecular identifier; PCR: polymerase chain reaction.

Table 1. New and emerging sequencing technologies. A summary of new sequencing platforms, their applicability to hematologic malignancies, and their clinical availability.

Technology	Use in blood cancers	Current status	Availability of commercial kits and products	References
Error-corrected sequencing	Improved detection of fusions More sensitive variant detection for MRD in HM (as low as ~0.001% for duplex sequencing and ~0.3% for anchored multiplex PCR sequencing)	In broad clinical use	Widely available commercial and laboratory-developed tests (e.g., Invitae, TwinStrand Biosciences)	doi:10.1016/j.cancergen.2020.02.007 doi:10.1186/s12920-020-0671-8
Long-read sequencing	Detection of fusions Detection of copy number variation, structural variants Detection of nuclei acid base modifications (e.g., methylation) Reduced turnaround time (in some cases)	Not in broad clinical use for HM; mainly research applications	RUO kits from limited companies (Oxford Nanopore, PacBio) Limited laboratories offer services for LRS Some platforms require investment in new instruments	doi:10.1038/s41587-021-01108-x doi:10.3389/fgene.2019.00426
Methylation sequencing	Detection of epigenetic signatures	Small number of targeted NGS methylation tests in clinical use for solid tumors	DNA methylation sequencing kits widely available Applicable to routine sequencers in most molecular laboratories LDT have limited availability in specialized molecular laboratories for specific markers (e.g., MGMT and MLH1 promoter methylation)	doi:10.1016/j.jmoldx.2013.05.011 doi:10.1016/j.yexmp.2023.104855
Optical genome mapping	Rapid molecular-based karyotype Higher resolution than current karyotype testing	Used in clinical trials, not yet in routine clinical practice	RUO instruments and research kits	doi:10.1016/j.ajhg.2021.06.001 doi:10.1016/j.jmoldx.2022.12.005
Single-cell sequencing	RNA-based single-cell gene expression DNA single-cell sequencing Spatial transcriptomics	Research applications in HM	Require investment in expensive components per assay and new instruments RUO hematologic-specific (Mission Bio); non-hematologic-specific kits from limited companies (NanoString, 10x Genomics)	doi:10.3390/genes12030398 doi:10.1038/s41587-020-0472-9

MRD: measurable residual disease; HM: hematologic malignancies; PCR: polymerase chain reaction; RUO: research use only; LRS: long-read sequencing; NGS: next-generation sequencing; LDT: laboratory-developed test.

Table 2. Comparison of reference genomes. The progression of the human reference genome and the advantages and disadvantages of each iteration.

Genome	Released	Organization	Adoption	Advantages	Disadvantages
GRCh37/hg19	2009	Genome Reference Consortium	Widespread clinical adoption	Well-annotated	Missing sequence
				Robust community for bioinformatics tools	No heterochromatin sequence
				-	Limited input diversity
GRCh38/hg38	2013	Genome Reference Consortium	Limited clinical adoption	> 1,000 issues resolved from GRCh37	Coordinate changes
				Increased alternative loci	Not all contigs can be mapped to GRCh37
				-	Increased complexity of pipeline to manage alternative loci Missing sequence Laboratories slow to validate
T2T-CHM13	2022	T2T Consortium	Research use	Up to 99% of genome sequenced with confidence	Source material is European
				Acrocentric chromosomes mapped	Community of bioinformatics tools lags GRC assemblies
				Improved accuracy of structural variation	Lacks seasoned annotation repositories
				-	Limited reports of mapping from GRCh37/hg19
Pangenomes, various	Ongoing	Human Pangenome Reference Consortium	Research use	High-quality assemblies from diverse populations	Requires new bioinformatic methods
				Partnership with T2T Consortium	Lacks seasoned annotation repositories

References: Schneider *et al.* Evaluation of GRCh38 and *de novo* haploid genome assemblies demonstrates the enduring quality of the reference assembly. Genome Res. 2017;27(5):849-864. Nurk *et al.* The complete sequence of a human genome. Science. 2022;376(6588):44-53. GRC: Genome Reference Consortium; T2T: telomere to telomere.

RNA sequencing showed high levels of cell loss during library preparation, often with only 100 to 200 cells captured per sample processed, although more recent studies have been able to capture more than 6,000 cells in a single marrow aspirate sample.[25] In addition, as yet, there are no standardized methods or robust and verified control material, the cost of sequencing is high, and the bioinformatics complex to implement.[28]

Advances in data analysis and bioinformatic tools

With each advance in technique or change in purpose comes a corresponding need to adapt the error prevention and correction in the bioinformatics pipeline. For example, the addition of neural networks for analysis of earlier generations of more error-prone nanopore data have shown the ability to improve accuracy from 68% to 99.47%.[29]

The potential use of NGS in MRD monitoring is a prime example of advances through error-correction. Pipelines for somatic variant identification typically have error rates of about 1-2%, leading to an inability to distinguish persistent disease from sequencing artifacts. 'Error-corrected sequencing,' which modifies library preparation as well as data processing, has been successfully adapted from ultra-low variant discovery to MRD evaluation in AML with reported limits of detection (LOD95) of 0.017% variant allele fraction.[30]

The European LeukemiaNet has updated its guidelines for AML MRD detection to include the use of NGS for this purpose.[2] However, the European LeukemiaNet does not support NGS-MRD as the sole method of MRD monitoring due to higher limits of detection than those for flow cytometry and possible confounding by clonal hematopoiesis and germline mutations.[1,2] One area for further research is RNA sequencing which, although not more sensitive for variant allele frequencies, does give better results with structural variants.

Long-read sequencing technology represents an enormous advancement for hematologic malignancies in particular, in which structural variants contribute to a significant proportion of disease.[3,4] Although long-read sequencing has not yet been implemented for clinical use, greater accuracy and improved computational tools will be instrumental in the development of clinical-grade assays. Third-generation techniques have also benefited, by necessity, from bioinformatic changes. For example, a comparison of 11 variant callers found that tools popular in clinical laboratories, such as GATK, Senteion, and Mutect2, generally do not perform as well on third-generation platforms,[31] and adaptations are necessary for acceptable performance. Improved computational methods at the stages of library preparation and sequencing have also helped third-generation techniques produce longer reads with fewer errors, and some investigators have found accuracy levels comparable to those of current clinical sequencing platforms.[31] Ultimately, third-generation platforms have yet to accrue the community support enjoyed by traditional sequencing methods.

A key opportunity for improvement continues to be the primary analysis of sequencing data, which comprises quality control, alignment, variant calling, and annotation. Thus far, no scientific society or consortium has released a comprehensive recommendation for primary analysis. Rather, there are dozens of tools and programs that could be chosen for each step in primary analysis; for example, one author estimated more than 60 read mappers in 2019.[32] Some of these steps are covered by the GATK Best Practices from the Broad Institute. Even so, most clinical pipelines are custom-made, proprietary platforms built out of a few of the many open-source and commercial packages available.[33]

Even with a call for guidelines, it is not clear that clinical laboratories have the resources or motivation to pursue changes that may profoundly affect their workflow. In 2017, the Association for Molecular Pathology, the American Society of Clinical Oncology, and the College of American Pathologists jointly released guidelines on the interpretation and reporting of somatic variant classifications. The Association for Molecular Pathology subsequently formed the Variant Interpretation Testing Across Laboratories (VITAL) Somatic Working Group to monitor the utilization and performance of these guidelines through a series of volunteer proficiency tests, or 'Somatic Challenges.' A follow-up published in 2023 found that only 59% of responses thus far have agreed with the guidelines' intended classifications.[34]

The lack of a standard is a natural by-product of a vigorous open-source bioinformatics community. It also speaks to the fragmentation of the field and represents a challenge for large-scale oncology studies. A change in approach may be found in nf-core, an open-source initiative that has released a number of modular pipelines for various analyses, such as single-cell RNA sequencing, gene fusions, and the B- and T-cell adaptive immune receptor repertoire.[33]

Advances in integration and interpretation

With significant advances in nearly all modalities of patient testing and interpretation over the past decade, a new grand prize has emerged in genetic testing for hematologic malignancies: the integration of data from multiple modalities. For clarity, we separate 'integration' into two concerns: first, the desire to combine disparate workflows and interfaces; and second, the desire to analyze data from multiple sources.

Hematologic malignancies are already routinely interrogated using multiple methods: fluorescence *in situ* hybridization, flow cytometry, blood counts, histomorphology, polymerase chain reaction, Sanger sequencing, and chromosomal array, not to mention clinical information contained in unstructured chart notes and documents. These assays are typically performed in separate laboratories, often with separate information systems and reporting mechanisms. The hematopathologist may be asked to organize and integrate these results into a clinically useful report. However, due to different turnaround times and the separation of laboratories, such reporting is often piecemeal, and the burden is transferred to the oncologist to place data into an interpretable timeline. Currently, no automated solution exists to integrate the results of multiple workflows into a single comprehensive genetic report.

The second type of integration is the ability to analyze data from multiple sources and return an interpretation. Additional data sources for hematologic malignancies include transcriptomics, proteomics, epigenomics, chromatin mapping, single-cell sequencing, and long-read sequencing. Many research studies have examined the yield of combining two or more modalities, e.g., combining multiparameter flow cytometry with NGS to reconstruct clonal evolution in patients with AML or using spatial data analysis to enable the correlation of cell subsets on glass slides with genetic and/or gene expression data. Of particular interest are efforts to associate transcriptomes with cell identity through the use of single-cell sequencing and antibody-epitope labeling. This has led to a number of promising techniques in both chemistry and computational analysis, such as genotyping of transcriptomes, CITE sequencing, and TARGET sequencing.[35]

Numerous computational and statistical challenges to integrated data analysis exist, including multiple-hypothesis errors and the alignment of mixed data types. It is unsurprising that advances in machine learning are central to multi-omic analysis. A group recently demonstrated the implementation of a deep learning model to derive 12

unique molecular subgroups of multiple myeloma using data from NGS, chromosomal array, and RNA-sequencing assays.[36] Phenotype acquisition through natural-language processing provides another layer of data for integration. Researchers used this method in conjunction with multiparameter flow cytometry, cytogenetics, and NGS data to identify a new subgroup of AML. Finally, several new commercial ventures are focused on the use of multiplex results for clinical trial eligibility, including Strata Oncology and OM1.

Advances in the reference genome

The reimagining of the reference genome may be the most revolutionary bioinformatic advancement in the past 5 years. Table 2 shows the evolution of the reference genome. The *de facto* standard in use today, GRCh37/hg19, is in fact no standard when one considers the many releases, patches, and alternate allele files that are cobbled together to create the true "normal" to which we compare all patients' sequences. The Genome Reference Consortium (GRC) consolidated these amendments and addressed many gaps and errors in its latest version, GRCh38/hg38. Despite being released nearly a decade ago, however, GRCh38 has had little uptake in the clinical realm, with a 2020 survey finding that only 7% of the 28 academic and commercial laboratories surveyed had migrated to the newer reference genome.[37] The most cited reasons for not having migrated were insufficient staff and a perceived lack of benefit for the effort required.

A range of strategies for improving or replacing the reference genome have been suggested, including: (i) a consensus genome comprising the population-wide majority allele at each position; (ii) population-specific consensus genomes; and (iii) the pangenome, in which the reference would no longer be a linear structure but a complex many-to-many representation of all known variants at each locus.[38] The pangenome, commonly conceived as a De Bruijn graph, has been pursued vigorously from a bioinformatics perspective, requiring as it does new methods of representing, traversing, and interpreting sequences.

Finally, the critical achievement of 2022 was the publication of the results of the Telomere to Telomere (T2T) Consortium's genome, which provided clarity on the additional 8% of the human genome not previously characterized.[39] The T2T project characterized regions of the genome not included in the GRC assemblies, including the short arms of chromosomes 13, 14, 15, 21, and 22.[39] The sequence as well as the technologies developed for the project will be used to realize the goal of a human pangenome, increasing the accuracy of molecular diagnostics for diverse populations.

Conclusion

New innovations in the past decade have greatly advanced care in patients with hematologic malignancies. Cloud-based systems assisted by artificial intelligence have improved data analysis, and the development of mobile laboratories has expanded these benefits worldwide, bringing the possibility of precision-driven therapies to all patients. Advances in molecular testing have swept across developed countries and established targeted NGS as a standard of care in most hematologic malignancies. However, despite the vast amounts of new knowledge, leukemia relapse and mortality rates remain high overall. The scientific community continues to innovate to push the field of molecular diagnostics forward, seeking a deeper understanding of how patients develop resistance and what factors contribute to relapse, with the hope that these discoveries may lead to better therapies. The future will bring improved monitoring strategies with highly accurate molecular-based MRD testing, single-cell sequencing technologies to assist with clone-tracking of a patient's disease, as well as methylation-based sequencing to help predict better and more tailored treatment regimens as a part of the routine clinical care of leukemia patients.

Disclosures

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Contributions

CCSY and RK contributed equally to the research for and writing, editing, and revision of the review.

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