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Dr. Kennedy is a Malignant Hematologist at Sunnybrook Health Sciences Centre and Assistant Professor at the University of Toronto. He completed the combined MD/PhD program in Toronto, where his graduate research focused on developing experimental models of hematologic malignancies. Building on this background, he subsequently completed residency training in Internal Medicine and Hematology, also at the University of Toronto, then enrolled in a combined clinical/research malignant hematology fellowship, splitting time between the Princess Margaret Cancer Centre and Brigham & Women's Hospital / Harvard Medical School. From a research standpoint, James is interested in understanding the genetic events that drive the leukemogenic process and how these can be targeted therapeutically. His clinical focus at Sunnybrook Health Sciences Centre encompasses acute leukemia, bone marrow failure and chronic myeloid malignancies.

NEXT GENERATION SEQUENCING FOR MYELOID MALIGNANCIES – PROGRESS AND PRACTICAL APPLICATIONS

Over the past two decades, next-generation sequencing (NGS) has revolutionized our understanding of the pathogenesis of myeloid neoplasms (MNs) and their clinical management. While traditional Sanger sequencing allows for the interrogation of single loci, NGS enables the parallel sequencing of multiple genomic locations, ranging from targeted sets of genes to the entire genome. Initially, NGS was used predominantly in research, where the ability to interrogate large regions of the genome facilitated the discovery of genes recurrently mutated in myeloid malignancies. Soon thereafter, NGS entered the clinical realm where it is now routinely utilized in diagnosis, prognostication and treatment decision-making. However, the broad availability of clinical NGS comes with a unique set of challenges. Hematologists must interpret complex molecular reports and appropriately apply the provided mutational information to their patients' care in real-time. Consequently, a systematic approach to interpreting NGS reports is crucial; the following will outline one such framework.

1) Understand the range of genetic alterations detectable by your panel

A detailed understanding of the mutational landscape of MNs has emerged over the past 20 years. Whole genome and exome sequencing of patient samples has led to the discovery of a set of ~40 genes recurrently mutated in acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and myeloproliferative neoplasms (MPN). Importantly, these genes can be organized into a limited number of biological categories, highlighting the key cellular processes whose deregulation drives pathologic myelopoiesis: RNA splicing, epigenetic regulation, the cohesin complex, transcription factors, the DNA damage response, and signal transduction (**Table 1**).[1,2]

By focusing on these recurrently mutated genes, targeted 'myeloid' NGS panels have been developed. The Association of Molecular Pathology has proposed a minimum gene list for chronic myeloid neoplasms (**Table 1, bold genes**).[3] However, the content of myeloid panels can vary with respect to the specific genes included as well as their covered regions (i.e.: hotspot vs. complete coding sequence). Early generation myeloid panels may not contain genes whose relevance to MNs has emerged more recently, such as *PPM1D* (implicated in therapy-related MNs)[4] and *DDX41* (implicated in familial MDS/AML).[5] NGS platforms can also differ from a technical standpoint, influencing their sensitivity and the types of variants that can be detected. For example, by using RNA as a starting material, some panels can detect reciprocal gene rearrangements, such as *PML-RARA, RUNX1-RUNX1T1* and *CBFB-MYH11*, which have previously required standalone RT-PCR based assays or cytogenetics/FISH for their detection.[6]

To assist clinicians, NGS reports contain a wealth of information including the genomic regions being interrogated, the assay technology, the bioinformatic pipeline, as well as the types of genetic alterations that can be detected with their associated

Molecular category	Genes
Splicing factors	***SF3B1, SRSF2, U2AF1, ZRSR2***
Epigenetic regulation	
DNA methylation	***DNMT3A, TET2, IDH1/2***
Histone methylation	***ASXL1, EZH2, BCOR, BCORL1,*** *KMT2A,* ***SETBP1***
Cohesin subunits	***STAG2, RAD21, SMC1A, SMC3***
Transcription factors	***RUNX1, ETV6, CEBPA,*** *CUX1, GATA2,* ***PHF6***
Signal transduction	
JAK-STAT	***JAK2, CALR, MPL, CSF3R***
RAS	***KRAS, NRAS, PTPN11, CBL, NF1,*** *GNAS, BRAF*
Other	***FLT3, KIT***
DNA repair	***TP53, PPM1D***
Miscellaneous	***NPM1,*** *DDX41, ETNK1*

Table 1. *Recurrently mutated genes in myeloid malignancies. Bolded genes are part of the Association of Molecular Pathology recommended minimum gene list for chronic myeloid malignancies; adapted from McClure et al, 2018*

sensitivity limits. Familiarity with these technical details is important for clinicians in order to fully appreciate the strengths and limitations of the NGS platform in use, and how this may impact the variants that are ultimately reported.

2) Review the reported variants and evidence supporting their pathogenicity

Though practices vary, molecular labs follow general guidelines for the reporting of mutations.[7] Genetic variants are listed using Human Genome Variation Society (HGVS) nomenclature (**Table 2**).[8] The detected variants can range from benign germline polymorphisms, to pathogenic driver mutations, to incidental passenger mutations lacking a discernible impact on leukemogenesis. Given this complexity, evidence-based variant annotation performed by molecular diagnostics specialists is a critical upstream analytical step.

For MNs, the ideal method to distinguish between tumor-associated alterations and germline changes is to compare mutation patterns in skin fibroblasts to those present in the blood. However, such analysis is usually limited to the investigation of inherited predisposition syndromes. Instead, probable germline polymorphisms are identified using data from large databases that have pooled genetic information from healthy populations, such as the Genome Aggregation Database (gnomAD).[9] In practice, variants with greater than 1% frequency in the general population are presumed to represent germline polymorphisms and are filtered out prior to clinical reporting. Certain variant allelic frequencies (VAF) can also be suggestive of a germline alterations (i.e.: 40-60% for assumed heterozygosity); however, VAF is not a fully reliable estimate of zygosity as it can be influenced by copy number as well as the relative proportion of the mutant cell clone.[11] Myeloid NGS panels, though primarily focused on the detection of somatic variants, do include genes such as *TP53, RUNX1, GATA2, CEBPA* and *DDX41* whose alteration in the germline can predispose to the development of MNs.[5,10] Identification of such variants in the blood of patients with a suggestive family/clinical history should prompt sequence analysis of skin fibroblasts for confirmation of germline status and genetic counselling.

A second challenge centers around evaluation of the pathogenicity of detected variants. In general, this is performed by pooling evidence from sources including large scale cancer databases (i.e.: The Catalogue of Somatic Mutations in Cancer, COSMIC)[12], healthy population databases (i.e.: gnomAD), clinically annotated mutation databases (i.e.: ClinVar), in silico tools that predict the functional consequences of a given mutation (i.e.: SIFT) and primary scientific literature.[11] An evidence-based tiered system for categorizing variants is in broad use (**Table 3**), and facilitates the identification of variants of clinical significance for hematologists.[7]

3) Clinical application of the provided mutational data

Diagnosis: The 2016 World Health Organization (WHO) MN diagnostic criteria rely heavily on CBC parameters, morphologic assessment of the bone marrow (BM) and cytogenetics, with a relatively smaller role for gene mutations.[13] Though a common cadre of genes are mutated in MNs, disease-defining genetic alterations are rare, with certain notable exceptions. Activating mutations in *JAK2* are present in ~99% of polycythemia vera cases, and the majority of patients with essential thrombocythemia and

Variant Type	Example	Breakdown	Description
Substitution	JAK2 (NM_004972.3) c.1849G>T p.(Val617Phe)	*Gene:* JAK2 *Transcript ID:* NM_004972.3 *cDNA change:* c.1849G>T *Amino acid change:* p.(Val617Phe)	cDNA nucleotide 1849 (G) changed to T Amino acid 617 (Val) changed to Phe
Nonsense	TET2 (NM_001127208.2) c.5298C>G p.Tyr1766*	*Gene:* TET2 *Transcript ID:* NM_001127208.2 *cDNA change:* c.5298C>G *Amino acid change:* p.(Tyr1766*)	cDNA nucleotide 5298 (C) changed to G Amino acid 1766 (Tyr) changed to stop codon
Insertion - frameshift	CALR (NM_004343.3) c.1154_1155insTTGTC p.(Lys385Asnfs*47)	*Gene:* CALR *Transcript ID:* NM_004343.3 *cDNA change:* c.1154_1155insTTGTC *Amino acid change:* p.(Lys385Asnfs*47)	Insertion of TTGTC between cDNA positions 1154 & 1155 Lys385 changed to Asn & reading frame altered with a stop codon 47 amino acids later
Deletion - frameshift	EZH2 (NM_004456.4) c.928delA p.Thr310Leufs*11	*Gene:* EZH2 *Transcript ID:* NM_004456.4 *cDNA change:* c.928delA *Amino acid change:* p.(Thr310Leufs*11)	Deletion of A at cDNA position 928 Thr310 changed to Leu & reading frame altered with a stop codon 11 amino acids later
Duplication – in-frame	SRSF2 (NM_001195427.1) c.281_283dupGCC p.(Arg94dup)	*Gene:* SRSF2 *Transcript ID:* NM_001195427.1 *cDNA change:* c.281_283dupGCC *Amino acid change:* p.(Arg94dup)	Duplication of cDNA nucleotides 281-283 (GCC) Duplication of amino acid 94 (Arg)
Deletion – in-frame	CALR (NM_004343.3) c.1191_1199del p.(Glu398_Asp400del)	*Gene:* CALR *Transcript ID:* NM_004343.3 *cDNA change:* c.1191_1199del *Amino acid change:* p.(Glu398_Asp400del)	Deletion of 9 nucleotides between cDNA positions 1191 & 1199 Deletion of amino acids 398 to 400
Splice site	CBL NM_005188.3 c.1096-1G>C	*Gene:* CBL *Transcript ID:* NM_005188.3 *cDNA change:* c.1096-1G>C	cDNA nucleotide 1096 is the start of exon 8. In the corresponding genomic sequence, 1 nucleotide prior to nt 1096 (the -1 position) is part of the splice acceptor site (AG). Substitution of G to C disrupts the splice acceptor site, resulting in deletion of exon8.[43]

Table 2. *Variant nomenclature examples; adapted from den Dunnen et al, 2016*

Tier		Level of Evidence	Description
I	Variants of Strong Clinical Significance	A	Included in professional guidelines related to disease diagnosis, prognosis and/or therapy Targeted by an FDA-approved therapy
		B	Described in well-powered studies with consensus from experts in the field
II	Variants of Potential Clinical Significance	C	Described in multiple small, published studies with some consensus Targeted by either FDA-approved therapies in different tumor types or investigational therapies
		D	Described in preclinical trials or a few case reports without consensus
III	Variants of Unknown Clinical Significance		Not observed at significant allele frequency in the general or specific subpopulation databases, or pan-cancer or tumor-specific variant databases No convincing published evidence of cancer association
IV	Benign or Likely Benign Variants		Observed at significant allele frequency in the general or specific subpopulation databases No existing published evidence of cancer association

Table 3. *A four-tiered system to categorize somatic sequence variations – based on consensus recommendations from the Association for Molecular Pathology; adapted from Li et al, 2016*

myelofibrosis carry a driver mutation in one of *JAK2, CALR* or *MPL*.[14] In the pediatric realm, more than 90% of cases of juvenile myelomonocytic leukemia carry an activating mutation in genes involved in RAS pathway regulation (*KRAS, NRAS, PTPN11, CB*L or *NF1*), leading to their incorporation into its diagnostic criteria.[13] Lastly, mutations in the splicing factor *SF3B1* are enriched in MN patients with ringed sideroblasts (RS).[15] The specificity of this association is reflected in the WHO diagnostic criteria for MDS-RS, where, in the presence of cytopenias and dysplasia, detection of an *SF3B1* mutation can establish this diagnosis when RS comprise as few as 5% of all nucleated erythroid cells, compared to the traditional cutoff of 15%.[13]

Conversely, the majority of genes recurrently mutated in myeloid malignancies are not specific to a particular disease entity; for example, *TET2* mutations are prevalent in AML, MDS, MPNs and MPN/MDS overlap syndromes.[16] Complicating matters further, recurrent somatic mutations in MN-associated genes have been identified in the blood of individuals without hematologic disease.[17–19] These mutations are a strong independent predictor for the future development of MNs. However, the absolute risk of malignant transformation is low, approximately 0.5-1% per year, leading to this entity being termed "clonal hematopoiesis of indeterminate potential" (CHIP).[20] Thus, clonality, as defined by the presence of MN-associated somatic mutations, should not be considered as definitive evidence of a frank hematologic malignancy in the absence of supporting CBC alterations or BM pathology.

Prognosis: Given the central pathogenic role of gene mutations in MNs, it follows that they have the potential to provide insight into disease risk. The European Leukemia Net (ELN) has proposed a risk stratification schema for AML based on cytogenetic and NGS findings (**Table 4A**).[21] Biallelic mutations in *CEBPA* or *NPM1* alterations confer a favorable prognosis, whereas mutations in *ASXL1, RUNX1, TP53* and *FLT3-ITD* (particularly at a high allelic ratio), confer an adverse risk, prompting clinicians to consider consolidative allogeneic stem cell transplant (allo-SCT) in eligible patients.

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3-ITD* or with *FLT3-ITD*^{low*} Biallelic mutated *CEBPA*
Intermediate	Wildtype *NPM1* without *FLT3-ITD* or with *FLT3-ITD*^{low**} Mutated *NPM1* with *FLT3-ITD*^{high} t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(q23;q34.1); *DEK-NUP214* t(v;11q23.3); *KMT2A* rearranged t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM (EVI1)* -5 or del(5q); -7; -17/abn(17p) Complex or monosomal karyotype§ Wildtype *NPM1* with *FLT3-ITD*^{high} Mutated *RUNX1*¶ Mutated *ASXL1*¶ Mutated *TP53*

Table 4A. *ELN AML risk stratification; adapted from Döhner et al, 2016.*
** Allelic ratio, calculated as FLT3-ITD/FLT3-wildtype; low < 0.5; high ≥ 0.5*
*** without adverse-risk genetic lesions*
§ Complex cytogenetics: 3 or more unrelated chromosomal abnormalities; Monosomal karyotype: 1 single monosomy in association with at least 1 additional monosomy or chromosomal abnormality
¶ Unless occur with favorable-risk AML subtypes

NGS-based risk stratification is also emerging for chronic myeloid malignancies. Mutations in *ASXL1, EZH2, IDH1/2, SRSF2* and *U2AF1* (at Q157) define a high molecular risk group in myelofibrosis,[22,23] and are integrated alongside traditional risk factors in prognostic scoring systems such as the MIPSS70[24] and MIPSS70 plus version 2.0[25] (**Table 4B/C**) which strive to identify patients where allogeneic SCT should be considered. Similarly, for MDS, several groups have developed scoring systems that improve upon the traditional International Prognosis Scoring System (IPSS) and IPSS-R by integrating mutational data.[26–28] While the exact molecular features of these scoring systems vary, common themes have emerged. For example, a higher absolute number of mutated genes as well as TP53 alterations (particularly bi-allelic) confer negative prognostic impact,[29] while SF3B1 alterations are generally associated with lower risk disease, though this can be modulated by co-mutation.[28] A notable strength of these novel scoring systems is that instead of simply classifying patients into broad categories, personalized outcome predictions are generated for each patient, enabling a more refined estimate of disease risk.

***Therapy*:** The genetic profile of a MN can also provide key information regarding responsiveness to therapy. For example, IDH1/2 mutant AML blasts have an intrinsically lower apoptotic threshold, rendering them particularly sensitive to depletion of the anti-apoptotic protein, BCL2.[30] Consequently, IDH mutant AMLs are highly responsive to therapeutic regimens containing the BCL2 inhibitor venetoclax.[31,32] In MDS, there has been much interest in using molecular data to predict responsiveness to hypomethylating agents (HMA). In some studies, TET2 mutations have predicted a favorable treatment response, particularly among individuals where it is an early, clonal mutation.[33–35] However, in a recent study using a machine-learning approach, no single or combination of gene mutations predicted HMA responsiveness; instead, eight genomic combinations predicting HMA resistance were identified. While further validation is required, such analyses

Risk factor	Score
Hemoglobin < 100 g/L	1
Leukocytes > 25 x 10^9/L	2
Platelets < 100 x 10^9/L	2
Circulating blasts ≥ 2%	1
Constitutional symptoms*	1
MF fibrosis grade ≥2	1
HMR category§	1
Absence of CALR type 1 mutation	1
2 or more HMR mutations	2

Risk group	Overall score	Median OS
Low	0-1	27.7 years
Intermediate	2-4	7.1 years
High	≥ 5	2.3 years

Table 4B. *Myelofibrosis - MIPSS70; adapted from Guglielmelli et al, 2018*
** Weight loss >10% of baseline in the year before diagnosis, unexplained fever or excessive sweats persisting for more than 1 month.*
§ HMR category: mutation in any one of ASXL1, EZH2, SRSF2 or IDH1/2

Risk factor	Score
Anemia	
80-99 g/L (women); 90-109 g/L (men)	1
< 80 g/L (women); < 90 g/L (men)	2
Circulating blasts ≥ 2%	1
Constitutional symptoms*	2
HMR category§	2
Absence of CALR type 1 mutation	2
2 or more HMR mutations	3
Cytogenetics¶	
Unfavorable	3
Very high risk	4

Risk group	Overall score	Median OS
Very low	0	Not reached
Low	1-2	16.4 years
Intermediate	3-4	7.7 years
High	5-8	4.1 years
Very high	≥ 9	1.8 years

Table 4C. *Myelofibrosis - MIPSS70 Plus version 2.0; adapted from Tefferi et al, 2018*
** Weight loss >10% of baseline in the year before diagnosis, unexplained fever or excessive sweats persisting for more than 1 month*
§ HMR category: mutation in any one of ASXL1, EZH2, SRSF2, IDH1/2 or U2AF1 at amino acid Q157
¶ Cytogenetic classification as per reference 44
- Favorable: Normal karyotype; Sole 20q−; Sole 13q−; Sole +9; Sole sex chromosome abnormality; Sole chromosome 1 translocation/ duplication
- Unfavorable: Sole +8; Sole 7q−; Sole translocations not involving chromosome 1; Two abnormalities not including a VHR abnormality; Single/multiple 5q− abnormalities; Complex karyotype without a VHR abnormality; Monosomal karyotype without a VHR abnormality; Sole abnormalities not otherwise classified
- Very high risk: Single/multiple monosomy 7; Single/multiple inv(3)/3q21 abnormalities; Single/multiple i(17q) abnormalities; Single/ multiple 12p−/12p11.2 abnormalities; Single/multiple 11q−/11q23 abnormalities; Single/multiple autosomal trisomies other than +8 or +9 (e.g., +21, +19)

highlight the power of evaluating gene mutations, not in isolation, but in networks to discern their clinical relevance.

Uncovering the mutational landscape of MNs has also fueled the development of novel therapies that target specific gene mutations. The FLT3 inhibitors midostaurin and gilteritinib have emerged as efficacious therapies for newly diagnosed and relapse/ refractory (R/R) FLT3-mutant AML, respectively.[36,37] Similarly, ivosidenib and enasidenib have shown promising results for IDH1 and IDH2 mutant R/R AML.[38,39] Additional targeted therapies are currently at early stages of development. For example, eprenetapopt, a small molecule that restores wildtype p53 function to cells bearing TP53 mutations, is currently under study in TP53 mutant MNs.[40] The spliceosome inhibitor H3B-8800 is being evaluated in early phase clinical trials, hoping to exploit the inherent vulnerability of cells with heterozygous splicing factor mutations to further inhibition of the splicing machinery.[41,42] Together, these therapies portend an exciting future where NGS will inform personalized therapeutic approaches in patients with MNs.

Conclusion

The advent of NGS technology has revolutionized our understanding of MN pathogenesis while offering significant potential for clinical application. As evidence continues to accumulate highlighting its utility, physicians must learn to integrate this information into routine practice. In addition to keeping abreast of the ever-expanding literature in this field, a working understanding of the technical and bioinformatic details pertaining to the sequencing platform in use is also required. As clinicians continue to gain familiarity with NGS, the future is extremely bright, as molecular profiling will be central to ongoing efforts to provide personalized care to patients through individualized predictions of disease risk and tailored therapeutic regimens.

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