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MOLECULAR TESTING AND DIAGNOSTICS (J KHOURY, SECTION EDITOR)

Philadelphia-Negative Myeloproliferative Neoplasms: Laboratory Workup in the Era of Next-Generation Sequencing

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Abstract

Purpose of Review To review the impact of next-generation sequencing (NGS) on laboratory approach of myeloproliferative neoplasms (MPNs).

Recent Findings Next-generation sequencing has provided valuable information on the mutational landscape of MPNs and has been used for various applications, including diagnosis, risk stratification, monitoring of residual disease or disease progression, and target therapy. Most commonly, targeted sequencing of a panel of genes that have been shown to be recurrently mutated in myeloid neoplasms is used. Although numerous studies have shown the benefit of using NGS in the routine clinical care of MPN patients, the complexity of NGS data and how these data may contribute to the clinical outcome have limited the development of a standard clinical guideline.

Summary We review recent literature and discuss how to interpret and use NGS data in the clinical care of MPN patients.

Keywords Next-generation sequencing · Myeloproliferative neoplasms · Polycythemia vera · Essential thrombocythemia · Primary myelofibrosis · Chronic neutrophilic leukemia

Introduction

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders characterized by proliferation of at least one hematopoietic lineage, including the myeloid, erythroid and/or megakaryocytic lineages, with minimal defects in maturation. During the past decade, significant progress has been made in our understanding of the pathogenesis of Philadelphia chromosome-negative MPNs. As a result, most MPNs are now known to carry well-defined molecular abnormalities, such as *JAK2*-mutated polycythemia vera (PV), *JAK2*-, *CALR*-, or *MPL*-mutated essential thrombocythemia (ET) and primary myelofibrosis (PMF), and *CSF3R*-mutated chronic neutrophilic leukemia (CNL). The emergence of next-generation sequencing (NGS) technique has expanded the genetic landscape of MPNs. In addition to the aforementioned driver mutations which appear to be specific for MPN phenotype, somatic mutations in genes that regulate DNA methylation, histone modification, mRNA splicing, transcription, and signal transduction have been shown to play important roles in subsequent disease progression. We review recently identified, clinically important gene mutations in MPNs, their role in the diagnosis, risk stratification, monitoring of measurable/minimal residual disease (MRD) and development of target therapy, and the recommended molecular laboratory approach in the NGS era.

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Driver Mutations in Myeloproliferative Neoplasms

Constitutive activation of the JAK-STAT signaling pathway is the key to the development of MPNs. The cardinal and mutually exclusive mutations in *JAK2*, *CALR*, and *MPL* are referred to as "driver mutations" based on their role in driving the MPN phenotype (Table 1). A point mutation in exon 14 of the *JAK2* gene, *JAK2* V617F, occurs in 95–97% of PV and 50–60% each of ET and PMF cases [1–5]. The remaining 3% of PV patients carry mutations in exon 12 of the *JAK2* gene [6, 7], or rarely, mutations in *SH2B3* (previously named *LNK*) or *CBL* that also lead to JAK-STAT activation [8–12]. Mutations in *CALR* or *MPL* are detected in the majority of the *JAK2*-negative ET and PMF patients [13–16]. Approximately 10% of ET and PMF cases do not carry a known/detectable somatic driver mutation and are referred to as "triple negative."

The *JAK2* V617F mutation can drive a spectrum of MPN phenotype including PV, ET, and PMF through ligand-independent activation of receptors for erythropoietin (EPO),

Table 1 Recurrent gene mutations in myeloproliferative neoplasms

Gene	Function	Frequency (%)				Prognostic impact
		PV	ET	PMF	BP	
Driver mutation						
JAK2 V617F	Signal transduction	95–97	50–60	50–60	40–50	High allele burden associated with increased risk of thrombosis and fibrosis
exon 12		2–3	NA	NA	NA	
CALR	Signal transduction	NA	20–30	25–35	25	Favorable prognosis (especially type 1)
MPL	Signal transduction	NA	3–5	5–10	5–10	Increased risk of thrombosis and fibrosis
DNA methylation						
TET2	Convert 5-mC to 5-hmC Required for myelopoiesis	10–20	10–15	10–20	20–25	Inconclusive prognostic effect
DNMT3A	DNA methyltransferase Histone methylation Transcription repression	5	1–5	5–15	10–20	Role in leukemic transformation Inferior overall survival
IDH1/2	Convert isocitrate to α-KG Regulate TET2	1–2	1–2	3–5	20–25	Inferior overall survival
Histone modification						
ASXL1	DNA methylation Transcription repression	5–10	5–10	30–40	30–50	Increased risk of fibrotic and leukemic transformation, inferior survival
EZH2	Histone methyltransferase Transcription repression	< 5	< 5	5–10	10–15	Increased risk of fibrotic and leukemic transformation, inferior survival
RNA splicing						
SRSF2	Spliceosome assembly	Rare	2	10–20	15–30	Increased risk of leukemic transformation Inferior overall survival
SF3B1	Spliceosome assembly	Rare	5	5–10	< 5	Increased risk of fibrotic transformation Associated with ring sideroblasts
U2AF1	Spliceosome assembly	Rare	Rare	5–20	5	Associated with disease progression
ZRSF2	Spliceosome assembly	Rare	3	5–10	5	Associated with fibrotic transformation
Transcription factor						
RUNX1	Regulate hematopoiesis Transcription regulation	3–5	3–5	5	15–20	Increased risk of leukemic transformation Inferior overall survival
TP53	Tumor suppressor Regulate cell cycle DNA repair, apoptosis	1	1	< 5	15–20	Very unfavorable Increased risk of leukemic transformation Inferior overall survival
Signal transduction						
SH2B3	Signal transduction	1	Rare	5	10–15	Role in leukemic transformation
CBL	E3 ubiquitin ligase Signal transduction	1	1–2	5	5–10	Role in leukemic transformation

BP, blast phase; *ET*, essential thrombocythemia; *PMF*, primary myelofibrosis; *PV*, polycythemia vera

thrombopoietin (TPO), and granulocyte-colony stimulating factor (G-CSF), respectively. *JAK2* mutation is usually associated with older age, higher hemoglobin level, leukocytosis, lower platelet count, and increased risk of thrombotic events and fibrotic transformation [17]. The variable phenotype of *JAK2*-mutated MPNs may be explained by different host characteristics (age, gender, genetic background, comorbidities), different stem cell differentiation stage at which the *JAK2* mutation arises, the presence and order of complementing genetic events (e.g., *TET2* mutation), dose effect, and bone marrow microenvironment. It has been reported that the timing of mutation acquisition may affect MPN phenotype, with "*JAK2*-first" more commonly seen in PV with an increased risk of thrombosis and "*TET2*-first" more commonly seen in ET [18]. In addition, homozygous mutation or higher mutant allele burden has been seen in most PV patients, and is associated with increased risk of thrombotic events and fibrotic transformation, whereas heterozygous mutation is usually associated with ET or PMF [4, 19]. *JAK2* exon 12 mutations, in the form of in-frame deletions and insertions, predominantly activate EPO receptor and are thus only seen in PV [7, 20]. *JAK2* V617F has also been reported in about 5% of other myeloid neoplasms, such as acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and chronic myelomonocytic leukemia (CMML), as well as in over 50% of myelodysplastic/myeloproliferative neoplasms with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) [21–23]. Moreover, *JAK2* V617F, usually at a low variant allelic frequency (VAF), can be detected in individuals without overt hematopoietic malignancy, the so-called clonal hematopoiesis of indeterminate potential (CHIP) [24].

CALR encodes calreticulin, a chaperone protein in endoplasmic reticulum (ER) with a role in the regulation of protein folding and calcium homeostasis [25]. Gain-of-function mutations of *CALR* have been detected in approximately 60–80% of *JAK2*-negative ET and PMF [13, 14]. Mutated *CALR* binds to TPO, or to a lesser degree, G-CSF receptor, thereby activating JAK-STAT signaling [26, 27]. It does not bind to EPO receptor, which explains why *CALR* mutation is limited to ET and PMF, not in PV [26, 27]. *CALR* mutations have been associated with younger age, lower hemoglobin level, higher platelet count, lower risk of thrombotic events and better overall survival [28, 29]. Two common types of *CALR* mutations are reported; both are frameshift insertions or deletions in exon 9 resulting in mutant protein with the loss of ER-retention motif (KDEL) at the C-terminus: type 1 with a 52-bp deletion (c.1092_1143del p.L367fs*46) and type 2 with a 5-bp insertion (c.1154_1155insTTGTC p.K385fs*47) [13, 14]. Other mutations are classified as type 1-like or type 2-like based on their similarities to type 1 or type 2. Type 1 and type 1-like *CALR* mutations have been shown to predict favorable overall survival in PMF [30, 31]. Similar to *JAK2* mutation, *CALR* mutations have also been seen in a small subset of MDS, CMML, and MDS/MPN-RS-T [14].

Missense mutation in *MPL* represents the least common driver mutation in MPNs, with an approximate frequency of 5% [15, 16]. *MPL* mutations have been associated with higher platelet count, megakaryocyte proliferation, and increased risk of thrombotic events and fibrotic transformation [32, 33]. The two most common mutations in exon 10, *W515L/K*, constitutively activate JAK-STAT signaling through TPO receptor [16]. As a result, *MPL* mutations are also almost exclusively seen in ET and PMF, not in PV. Other *MPL* mutations have rarely been reported, such as S204P and Y591N, which have been shown to have a weak gain-of-function effect [34, 35].

Approximately 10% of MPN patients do not carry any of the above three driver mutations and are referred to as "triple negative." This is a heterogeneous group, and some of these patients may harbor non-canonical mutations [34, 35] or mutations with low VAF below the limit of assay sensitivity. Patients with "triple negative" PMF have been shown to have a higher rate of leukemic transformation and worse overall survival [36]. It has been reported that at least one non-driver mutation can be detected in nearly 90% of "triple negative" PMF patients [37]. It is recommended that a comprehensive mutation survey should be performed in patients with "triple negative" MPNs in search for a marker of clonality.

Co-operating Mutations and Clonal Evolution

Genome-wide analyses by high-throughput NGS have identified co-operating mutations with prognostic and therapeutic value (Table 1). Recurrent mutations in more than 50 genes have been reported in patients with MPNs. Among *JAK2*-mutated MPNs, approximately 80% of PMF [38] and 50% of PV/ET patients [39] carry at least one additional mutation, with increased number of co-operating mutations occurring with disease progression. The most common mutations occur in genes involved in DNA methylation (*TET2*, *DNMT3A*, *IDH1*, *IDH2*), histone modification (*ASXL1*, *EZH2*), and RNA splicing (*SRSF2*, *U2AF1*, *ZRSR2*, *SF3B1*). Other molecular abnormalities include genes involved in transcription regulation (*RUNX1*, *TP53*, *CEBPA*, *ETV6*, *SETBP1*), signal transduction (*KRAS*, *NRAS*, *KIT*, *CBL*, *SH2B3*, *NF1*), and cohesion complex factors (*RAD21*, *STAG2*). These mutations may occur in the same clones as the driver mutations or in different clones. They play a role in modifying disease phenotype and increasing stem cell self-renewal, and contribute to disease progression and clonal evolution. Unlike driver mutations, these co-operating mutations are not specific to MPNs and are also present in other hematopoietic or non-hematopoietic neoplasms [40]. Some of the co-operating mutations have also been reported in approximately 5% of healthy individuals [24].

TET2 is the most commonly mutated epigenetic regulator gene in MPNs, seen in 10–15% MPN patients [40]. The impact of loss-of-function *TET2* mutations on MPN progression is inconclusive. Some studies suggested that *TET2* mutations confer a poor prognosis in terms of leukemic transformation and overall survival [41], whereas other studies failed to show a correlation between *TET2* mutations and adverse clinical outcome or increased risk of thrombosis [42, 43]. More recently, it was reported that the timing of mutation acquisition may affect MPN phenotype, with "*JAK2*-first" being more commonly seen in PV and "*TET2*-first" being more commonly seen in ET [18]. *DNMT3A* mutations occur in 5–15% of MPN patients, with the most frequent mutation being R882H [44]. *DNMT3A* mutation has been shown to cooperate with *JAK2* mutation in inducing myelofibrosis and leukemic transformation [44, 45]. Similar to *TET2* mutation, "*JAK2*-first" is more likely to develop PV whereas "*DNMT3A*-first" is more likely to develop ET [46]. Mutations affecting the isocitrate dehydrogenase genes, *IDH1* and *IDH2*, are reported in up to 5% of chronic-phase MPNs and 20–25% of blast-phase MPNs [47, 48]. *IDH1* and *IDH2* mutations are heterozygous and occur mostly at residues R132 in *IDH1* and R140 or R172 in *IDH2* [47, 48]. Mutant IDH1 and IDH2 proteins convert alpha-ketoglutarate (α-KG) to 2-hydroxyglutarate (2-HG), leading to epigenetic dysregulation of genes involved in leukemogenesis [49]. *IDH1* and *IDH2* mutations in MPNs have been shown to predict fibrotic and leukemic transformation [50, 51].

Mutations in the two histone modification genes, *ASXL1* and *EZH2*, are more commonly reported in PMF cases. *ASXL1* mutations (mostly in exon 12) occur in 30–40% of PMF patients [52, 53] and 5–10% of PV/ET patients [39]. *EZH2* mutations are observed in 5–10% of PMF cases [54] and < 5% of PV/ET patients [39]. Both mutations tend to occur as an early event in hematopoetic stem cells and tend to co-exist in the same clone [53]. Patients with *ASXL1/EZH2* double mutations demonstrate significantly decreased hemoglobin level and increased leukocytosis [53]. Both mutations have a positive correlation with increased risk of myelofibrosis, leukemic transformation, and poor clinical outcome [52, 55–57].

Somatic mutations in genes encoding core spliceosomal proteins and accessory regulatory splicing factors have been described in 3–5% of MPN cases, with a higher frequency in PMF [38, 58]. Interestingly, nearly all mutations occur as heterozygous missense rather than nonsense or frameshift mutations, suggesting that the mutations may confer an alteration of function, such as dominant negative activity, affecting RNA splicing [59]. *SRSF2* mutations have been reported in nearly 20% of PMF and are associated with increased risk of leukemia transformation and poor overall survival [58, 60]. *SF3B1* mutations have been reported in up to 10% of MPNs and are enriched in PMF and fibrotic stage of PV and ET [61]. Unlike its prognostically favorable role in MDS, *SF3B1* mutations have been associated with disease progression to myelofibrosis in ET [61].

Mutations affecting transcription regulation, *RUNX1* and *TP53*, occur at a low frequency in chronic-phase MPNs, 2–5% and 1–2%, respectively [38, 39]. However, the frequency for both mutations significantly rises to nearly 20% in blast phase [57], and the mutations are positively correlated with adverse survival [57, 62].

CSF3R and Chronic Neutrophilic Leukemia

Chronic neutrophilic leukemia (CNL) is characterized by persistent absolute neutrophilia without significant left-shift or dysplasia. Approximately 80–90% of CNL cases carry mutations in *CSF3R*, a gene responsible for neutrophil proliferation and differentiation [63, 64]. *CSF3R* mutations were also initially reported in 45% of atypical chronic myeloid leukemia (aCML) [63]. However, subsequent studies showed that *CSF3R* mutations occur in virtually all cases of CNL and no or rare cases of aCML [64, 65]. The discrepant results may be largely explained by the difference in diagnostic criteria and classification. Two major types of *CSF3R* mutations have been reported: the majority of patients carry a point mutation in the membrane proximal region (most commonly T618I in exon 14) that activates JAK-STAT pathway and is sensitive to ruxolitinib; a small number of patients harbor nonsense or frameshift mutations (in exon 17) leading to truncation of the cytoplasmic tail resulting in SRC pathway activation and are sensitive to dasatinib. Of note, the latter usually occur in conjunction with the former [64]. Co-operating mutations that are commonly reported in CNL include *ASXL1* (50–60%) and *SETBP1* (30–40%); both have been associated with disease progression and poor clinical outcome [66]. *SETBP1* mutation has also been implicated in resistance to ruxolitinib [67].

The presence of *CSF3R* mutations has been included as one of the diagnostic criteria for CNL in the 2016 WHO classification. The diagnosis of CNL requires exclusion of other entities such as reactive neutrophilia and other myeloid neoplasms. The recommended clinical workup should include, in addition to review of clinical information and morphologic features, cytogenetic and molecular studies for *BCR-ABL1* fusion and mutations in *JAK2*, *CALR*, *MPL*, and *CSF3R*, as well as rearrangements of *PDGFRA*, *PDGFRB*, *FGFR1*, and *PCM1-JAK2* fusion. *CSF3R* mutation can be assessed as a part of the NGS panel or by a standalone test such as conventional Sanger sequencing or pyrosequencing (for T618I). In the absence of *CSF3R* mutation, targeted NGS for genes that are commonly mutated in myeloid neoplasms, such as *ASXL1* and *SETBP1*, or karyotypic analysis may be helpful in search for proof of clonality.

Clinical Applications

Understanding the molecular landscape of MPNs has several clinical implications. Molecular testing at the time of initial evaluation can aid in establishing a diagnosis. The presence of driver mutations has been included in the 2016 revised 4th edition of WHO diagnostic criteria. It sometimes can be difficult to differentiate MPN from reactive myelopoiesis based solely on hematologic and morphologic data. In patients with an equivocal MPN, such as persistently elevated blood cell count but not meeting the diagnostic criteria for MPN, the identification of a typical driver mutation and/or co-operating mutation, combined with relevant morphologic features, will support the diagnosis. However, due to the presence of "CHIP," gene mutation data must be interpreted with caution. The most commonly reported "CHIP" genes include *DNMT3A*, *TET2*, *ASXL1*, *JAK2*, *TP53*, *SF3B1*, *SRSF2*, and *CBL* [24]. Whereas the majority of individuals with "CHIP" never develop a hematologic neoplasm, they do have a 10–15-fold increased risk. The high frequency of "CHIP" in the elderly limits the diagnostic value of somatic mutations. Nevertheless, certain mutations, when used in an appropriate clinical setting, have been shown to be useful diagnostic adjuncts. For example, an isolated mutation in *DNMT3A*, *TET2*, or *ASXL1* in an elderly person without hematologic and morphologic features of MPN may not be diagnostically helpful. However, mutations in some of the less frequently mutated "CHIP" genes, especially when present at high VAF and/or with more than one "CHIP" gene coexisting, in younger patients with hematologic and morphologic suspicion for MPN, may raise the likelihood of MPN. On the other hand, the absence of mutation in an adequately broad gene panel in patients with mild hematologic and morphologic abnormality may render a diagnosis of MPN less likely.

Some gene mutations have shown strong correlations with disease phenotype and may aid in the classification of MPNs. As mentioned above, homozygous *JAK2* V617F is usually associated with PV and heterozygosity is more commonly seen in ET and PMF [4]. Another example is that genes involved in DNA splicing are more commonly mutated in PMF [38, 58, 61]. The mutation profile may also aid in the differential diagnoses between blast-phase MPN and de novo AML. Unlike de novo AML which has *FLT3*, *NPM1*, and *DNMT3A* as the most commonly mutated genes, genes that are frequently mutated in blast-phase MPN, in addition to the driver mutations, include *ASXL1* (30–50%), *TET2* (20–25%), *IDH1/2* (20–25%), *SRSF2* (15–30%), *RUNX1* (15–20%), *TP53* (15–20%), *DNMT3A* (10–15%) and *EZH2* (10–15%) [57, 62].

More importantly, molecular aberrations have been incorporated into current prognostic models in refining risk stratification. Type 1 and type 1-like *CALR* mutations have been shown to predict favorable overall survival in PMF [30, 31]. In contrast, most of the co-operating mutations have been associated with inferior survival. Five genes, *ASXL1*, *EZH2*, *SRSF2*, *IDH1*, and *IDH2*, have been used to define a high molecular risk (HMR) group [68], and the presence of HMR mutations has been included in prognostic scoring systems, such as MIPSS70 and MIPSS70-plus for PMF patients [69•] (Table 2). Moreover, it has been shown that the presence of two or more mutations predicts shortened leukemia-free survival and overall survival [70]. Other prognostic models that have adopted mutation data include GIPSS for PMF [71] and MYSEC-PM for secondary myelofibrosis [72•] (Table 2). Using a 27-gene panel NGS, Tefferi et al. have identified *ASXL1*, *SRSF2*, and *IDH2* as "adverse variants/mutations" in PV and *SH2B3*, *SF3B1*, *U2AF1*, *TP53*, *IDH2*, and *EZH2* in ET [39]. In addition, mutations in genes such as *ASXL1*, *TET2*, *SRSF2*, *RUNX1*, *TP53*, and *EZH2* have been reported to be positively correlated with increased risk of leukemic transformation [57, 62]. Recently, it has been proposed that classification of MPNs based on underlying genomic lesions, instead of clinical and morphologic features alone, shows promise in personalized diagnosis, risk stratification and treatment [73••]. As a result, a prognostic model with eight genomic subgroups has been created and shown to predict distinct clinical phenotypes, risk of fibrotic and leukemic transformation, and survival [73••].

Periodic monitoring of mutation status of genes that have previously been shown to be mutated at the time of initial diagnosis may be helpful in the assessment of response to treatment and residual disease. MRD analysis allows for early detection of impending relapse and timely therapeutic intervention, and has significantly improved clinical outcome in patients with hematopoietic neoplasms [74]. Assessment of MRD requires molecular technologies with sufficient sensitivity to detect mutations with low VAF. Quantitative mutation-specific PCR, with a sensitivity of 0.01–0.001%, has been developed for single-nucleotide variants in genes with "hotspot" mutations such as *JAK2*, *MPL*, *DNMT3A*, *IDH1*, and *IDH2* [75]. However, such method requires the design of two primer sets for mutant and wild type, respectively, for each individual point mutation and it fails to detect clonal evolution. NGS-based assays, though with limited sensitivity (~ 1%), can detect multiple mutations simultaneously and identify emerging clonal evolution. *JAK2* V617F has been considered as the most attractive molecular marker for assessment of MRD in MPNs. However, it has been shown that the rate of reduction in mutant *JAK2* allele burden remains modest, which limits its role in MRD monitoring [76]. The role of other gene mutations in MRD monitoring awaits further evaluation.

Finally, insight into the molecular pathogenesis of MPNs has led to the development of novel therapeutic agents. Ruxolitinib, a tyrosine kinase inhibitor that inhibits JAK1 and JAK2, has been shown to improve overall survival in PMF patients carrying HMR mutations [76, 77]. *TET2*

Table 2 Prognostic scoring systems in myelofibrosis which has incorporated molecular data

Genetic variables	Non-genetic variables	Risk group (scores)	Median survival
MIPSS70 (Guglielmelli et al. 2018)			
≥2 HMR mutations (2 points)	Leukocytes > 25×10^9/L (2 points)	Low (0–1)	27.7 years
1 HMR mutation (*ASXL1*, *EZH2*, *SRSF2*, *IDH1/2*) (1 point)	Platelets < 100×10^9/L (2 points)	Int (2–4)	7.1 years
Absence of *CALR* type 1-like mutation (1 point)	Circulating blasts ≥ 2% (1 point)	High (≥ 5)	2.3 years
	Hemoglobin < 100 g/dL (1 point)		
	Constitutional symptoms (1 point)		
	Bone marrow fibrosis grade ≥ 2 (1 point)		
MIPSS70-plus (Guglielmelli et al. 2018)			
Unfavorable karyotype (3 points)	Hemoglobin < 100 g/dL (1 point)	Low (0–2)	20.0 years
≥ 2 HMR mutations (2 points)	Circulating blasts ≥ 2% (1 point)	Int (3)	6.3 years
Absence of *CALR* type 1-like mutation (2 points)	Constitutional symptoms (1 point)	High (4–6)	3.9 years
1 HMR mutation (*ASXL1*, *EZH2*, *SRSF2*, *IDH1/2*) (1point)		Very high (≥ 7)	1.7 years
GIPSS (Tefferi et al. 2018)			
Very high risk karyotype (2 points)	None	Low (0)	26.4 years
Unfavorable karyotype (1 point)		Int-1 (1)	8.0 years
Absence of *CALR* type 1-like mutation (1 point)		Int-2 (2)	4.2 years
ASXL1 mutation (1 point)		High (≥ 3)	2 years
SRSF2 mutation (1 point)			
U2AF1 Q157 mutation (1 point)			
MYSEC-PM (Passamonti et al. 2017)			
CALR-unmutated genotype (2 points)	Hemoglobin < 110 g/dL (2 points)	Low (< 11)	Not reached
	Circulating blasts ≥ 3% (2 points)	Int-1 (11–< 14)	9.3 years
	Platelets < 150×10^9/L (1 point)	Int-2 (14–< 16)	4.4 years
	Constitutional symptoms (1 point)	High (≥ 16)	2.0 years
	Age at secondary MF (0.15 point/year)		

MIPSS70, Mutation-enhanced International Prognostic Scoring System for patients ≤ 70 years; *MIPSS70-plus*, MIPSS70 includes cytogenetic data; *GIPSS*, genetically inspired prognostic scoring system; *MYSEC-PM*, myelofibrosis secondary to polycythemia vera and essential thrombocythemia prognostic model; *HMR*, high molecular risk

mutations have been shown to increase the response rate of patients with MPN-associated myelofibrosis to pomalidomide [78]. Other examples include IDH1/IDH2 inhibitors in *IDH1/IDH2*-mutated cases. A recent study has demonstrated therapeutic advantage of combined JAK2 and IDH2 inhibition in patients with *JAK2* and *IDH2* double mutations [79]. With the rapid advances in molecular biology and drug development, it is hoped that more and more of these mutations may soon become targetable.

Algorithms of Molecular Laboratory Workup

Screening for the presence of *BCR-ABL1* fusion transcript and mutation in one of the three drivers (*JAK2* V617F and exon 12, *CALR* and *MPL* W515L/K) is recommended for all new presentation of possible MPNs. For the diagnosis of PV, ET, and PMF, depending on the clinical settings, sequential testing with initial assessment for *JAK2* V617F followed by detection of either *JAK2* exon 12 mutation for patients with suspected PV, or *CALR* and *MPL* mutations for patients with suspected ET or PMF is a reasonable approach. NGS allows for high-throughput, simultaneous detection of multiple mutations in a short time and at an affordable cost. In the absence of the driver mutations, a comprehensive search for other co-operating mutations may provide proof for clonality.

In our experience, triaging of cases for molecular testing by molecular hematopathologists has been proved to be cost-effective in avoiding unnecessary testing meanwhile suggesting appropriate tests that have not been ordered by the clinicians. Both bone marrow and peripheral blood are comparable samples. Sorting of granulocytes to improve detection rate is usually unnecessary. In contrast, the choice of a detection method can greatly influence the detection rate. A targeted NGS with a comprehensive panel of genes that are commonly mutated in myeloid neoplasms is a reasonable initial approach at the time of diagnosis. The panel needs to be reviewed periodically and updated with the discovery of new genes. An ideal panel should include assessment of the entire coding sequence instead of hotspot mutations, since more and more

non-canonical mutations in driver and non-driver mutations have been reported with pathogenic potential. Assays that are designed to assess only hotspot mutations may miss relevant variants/mutations, resulting in false negative results. For example, *JAK2* K539I has been shown to bind to EPO receptor with a higher affinity than TPO or G-CSF receptors [20], and *JAK2* F556V and V625F have also been shown as gain-of-function mutations [35]. *MPL* P70L and M602T mutations have been shown to be pathogenic [80], and *MPL* T119I, S204F, E230G, and Y591D are all gain-of-function mutations [35]. Quantitation of *JAK2* V617F or *MPL* W515L/K mutation levels using mutation-specific quantitative PCR (qPCR) or digital droplet PCR (ddPCR), both with high analytic sensitivity, is recommended for follow-up of *JAK2*- or *MPL*-mutated cases. Cases with only one mutation detected by initial NGS may be followed by a standalone test, whereas for cases with two or more mutations, it may be cost-effective to follow with the NGS assay. We recently reported a custom platform designated as Ultra-rapid Reporting of GENomic Targets (URGENTseq) which allows us for providing NGS results for selected genes useful for immediate diagnosis and treatment decisions in myeloid neoplasms within 48 h of sample collection [81]. The technical and bioinformatic details are beyond the scope of discussion of this review.

Data interpretation must take into consideration clinical information, hematologic data, morphologic features, cytogenetic data and other molecular data. NGS reports should include standardized HGVS nomenclature, exon location, DNA change (SNV, insertion/deletion), protein change (missense, nonsense, frameshift), dbSNP and/or COSMIC ID, VAF and interpretation of somatic versus putative germline origin, and pathogenic versus variant of uncertain clinical significance. Methodology should also be included such as test platform, software tools for variant calling and annotation, list of exons/codons covered for each gene, depth of coverage, and analytic sensitivity. It is recommended that, at least for genes that have been implicated to be useful in the monitoring of response to therapy and MRD, VAF should be provided in the molecular report. *JAK2* V617F with higher VAF has been associated with increased risk of thrombotic events and fibrotic transformation [17]. Therefore, monitoring *JAK2* allele burden may aid in early identification of patients with such risks. *TP53* is another example of a gene for which VAF should be provided. It has been shown that most *TP53* mutations are present with low VAF at initial diagnosis of MPNs; the increase in VAF or loss of the wild-type *TP53* allele heralds or accompanies leukemic transformation [41, 82].

Conclusion

The introduction of NGS into clinical laboratory has significantly changed our approach to the diagnosis, risk prediction, monitoring, and treatment of MPNs. While sequential testing of individual driver mutations is still an acceptable practice under certain clinical settings, simultaneous assessment of multiple mutations using a comprehensive NGS panel is most practical and cost-effective. NGS has allowed several major advances in the field of MPNs, such as identification of novel gene mutations, establishment of prognostic scoring systems for PMF and secondary post-PV/ET MF, and development of targeted therapy. However, many questions remain unanswered, including, but not limited to, standardization of consensus gene panels and data interpretation, efforts to reduce assay cost and improve reimbursement policies, optimization of mutation data integration into existing prognostic schemes, and discovery of more actionable therapeutic targets. Hopefully the next few years will witness translation of more research discoveries into routine clinical care and precision personalized medicine.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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