







A Review on the Role of Molecular Genetics in the Diagnostic Workup of *BCR::ABL1*-Negative Myeloproliferative Neoplasms

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Abstract

The diagnostic evaluation of myeloproliferative neoplasms (MPNs) depends on the close correlation between clinical features, morphologic assessment of a trephine bone marrow biopsy, and molecular markers. Typically, MPNs have driver mutations in *JAK2*, *CALR*, or *MPL*, as well as mutations in genes related to epigenetic regulation, RNA splicing, and signaling. Mutations in these genes are a hallmark of diagnostic, prognostic, and therapeutic assessment in patients with MPNs. In line with the World Health Organization classification, all myeloproliferative disorders require molecular characterization to support diagnoses or confirm entities defined by underlying molecular abnormalities. A structured molecular analysis workflow is essential for a rapid and cost-effective diagnosis of MPN. The purpose of this review is to explore the role of molecular diagnostics in the assessment of *BCR::ABL1*-negative MPNs.

Keywords

- ▶ molecular diagnostics
- ▶ MPN
- ▶ PV
- ▶ ET
- ▶ PMF
- ▶ non-classical MPN

Introduction

The myeloproliferative neoplasms (MPNs) are a group of related clonal hematologic disorders characterized by excess accumulation of one or more myeloid cell lineages. Symptoms include fatigue, pruritus, early satiety due to splenomegaly, increased infection risk, and thrombotic events. Thromboembolic and hemorrhagic events contribute to disease morbidity. There is a greater incidence of transformation to myelofibrosis with polycythemia vera (PV) than essential thrombocythemia (ET) and secondary acute myeloid leukemia (AML) with primary myelofibrosis (PMF) than with PV or ET. These are traditionally classified into four subgroups, which include (i) chronic myeloid leukemia (CML), (ii) classical Philadelphia-negative MPNs, that is, PV, ET, PMF; (iii) nonclassical Philadelphia-negative MPNs, that is, chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL); and (iv) MPN, not otherwise specified (NOS).

Juvenile myelomonocytic leukemia (JMML) is now classified under *BCR::ABL1*-negative MPN of childhood as opposed to the earlier MDS/MPN category.^{1–3}

The initial diagnostic evaluation of MPNs depends on a close correlation between clinical features such as splenomegaly, arterial or venous thrombosis, peripheral blood indices, morphologic assessment of a bone marrow biopsy, and molecular diagnostics. Increasing knowledge of molecular alterations has influenced paradigm shift in classification, which define entities based partially or exclusively on recurrent genetic mutations.^{3,4} Hence, an integrated multimodal approach is required for precise classification.

A mutation in codon 617 of *JAK2*, resulting in the replacement of the amino acid valine with phenylalanine [*JAK2* (NM_004972.3):c.1849G > T (p.Val617Phe)], is found in the vast majority of classical MPN cases. This gain of function mutation is present in approximately 96% of PV, 55% of ET, and 65% of PMF.⁵ *JAK2* exon 12 mutations are described

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in 3-5% of cases of PV and rarely in PMF.^{5,6} Thrombopoietin receptor (*MPL*) mutations have been reported to occur in 4 to 10% of cases of *JAK2*-negative ET and PMF^{7,8} and calreticulin (*CALR*) mutations in 20 to 25% of ET and PMF cases.^{4,9}

The triple-negative MPN is a molecular entity that lacks mutations in *JAK2*, *CALR*, or *MPL* genes but still exhibits histological and phenotypic features consistent with MPNs. Triple-negative cases of ET and PMF account for approximately 20% and 5 to 10% of cases, respectively.^{10,11} Molecular investigations should be expanded in this subtype of MPN patients to identify gene mutations that are not restricted to classical MPNs and to find evidence of disease clonality.^{3,12}

Nondriver somatic mutations implicated in the disease pathogenesis of MPNs belong to several functional classes. These include mutations in the epigenetic regulator genes such as DNA methylation and chromatin modifiers, genes involved in RNA splicing, signaling pathways, transcription factors, tumor suppressors, DNA damage response pathways, and cohesin complex. Among these, the most frequent nondriver mutations are found in the epigenetic regulator genes *TET2*, *ASXL1*, *DNMT3A*, and *EZH2* and in the RNA splicing genes *SRSF2* and *U2AF1*.¹³⁻²⁰

Diagnosing nonclassical MPNs (CNL, CEL, JMML) as well as MPN, NOS requires the exclusion of *BCR::ABL1* fusion as well as molecular testing to screen for category-specific mutations such as *CSF3R*, *RAS* pathway genes, and other myeloid neoplasia-associated mutations.^{2,21}

Identifying molecular markers not only offers a simplified strategy for the diagnosis but also adds prognostic information that can guide management. Several genes, including *ASXL1*, *EZH2*, *IDH1*, *IDH2*, and *SRSF2*, are associated with shorter survival and increased risk of leukemic transformation (LT) in PMF patients.²² These mutations define a higher risk category called "high molecular risk" (HMR). Molecular analysis may, therefore, aid in treatment decisions such as allogeneic stem cell transplantation.^{22,23}

This review aims to highlight the role of molecular diagnostics in each of these entities categorized under

BCR::ABL1-negative MPN, as well as the need for a structured diagnostic testing algorithm in aiding rapid and cost-effective diagnosis.

Overview of *BCR::ABL1*-Negative Myeloproliferative Neoplasms and Screening Investigations

Polycythemia Vera

PV is a chronic MPN characterized by increased production of red blood cells independent of the erythropoiesis-regulating mechanisms. A somatic gain-of-function mutation *JAK2V617F* or other functionally similar *JAK2* mutations that result in panmyelosis is detected in most patients with polycythemia vera. A diagnosis of PV is based on composite clinical, laboratory, and bone marrow histological features and requires the exclusion of all causes of secondary erythrocytosis, heritable polycythemia, and other MPNs²¹ (► **Table 1**).

Laboratory detection of *JAK2V617F* is highly sensitive (97% sensitivity) and virtually 100% specific for identifying PV from other causes of increased hematocrit. A subnormal level of erythropoietin (EPO) is expected in 85% of PV patients. As a result of simultaneous measurement of serum EPO level, the possibility of false positive or false-negative mutation test results can be effectively avoided.²⁴

JAK2-negative cases with persisting and significant erythrocytosis are classified as "JAK2-negative PV" only after all probable causes of secondary erythrocytosis have been excluded. As the British Society of Haematology (BSH) 2019 guidelines outlined, this rare entity must meet stringent criteria to differentiate from idiopathic and secondary erythrocytosis. The World Health Organization (WHO) criteria for the diagnosis of PV are less stringent and do not define *JAK2*-negative PV separately. In contrast, the BSH criteria are more stringent regarding hematocrit requirements and other additional criteria, including splenomegaly and white blood cell counts (► **Table 2**). Although no guidelines recommend cytogenetic or molecular testing in cases with *JAK2*-negative erythrocytosis, the diagnostic workup of such cases must include the evaluation of both hereditary and acquired causes

Table 1 Diagnostic criteria for polycythemia vera (PV)³

Major criteria	
1	Elevated hemoglobin concentration (> 16.5 g/dL in men;> 16.0 g/dL in women) or Elevated hematocrit (>49% in men; >48% in women) or Increased red blood cell mass (> 25% above mean normal predicted value)
2	Bone marrow biopsy showing age-adjusted hypercellularity with trilineage growth (panmyelosis), including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size)
3	Presence of <i>JAK2</i> V617F or <i>JAK2</i> exon 12 mutation
Minor criteria	
	Subnormal serum erythropoietin level

^aMajor criterion 2 (bone marrow biopsy) may not be required in patients with sustained absolute erythrocytosis (hemoglobin concentrations of > 18.5 g/dL in men or > 16.5 g/dL in women and hematocrit values of > 55.5% in men or > 49.5% in women) if major criterion 3 and the minor criterion are present. However, initial myelofibrosis (present in as many as 20% of patients) can only be detected by bone marrow biopsy, and this finding may predict a more rapid progression to overt myelofibrosis (post-PV myelofibrosis)

Table 2 Diagnostic criteria for *JAK2*-negative polycythemia vera according to the BSH 2019 (requires A1-A4 plus another A or two B criteria)²⁵

A1	Raised red cell mass (>25% above predicted) or Hematocrit ≥ 0.60 in men & ≥ 0.56 in women
A2	Absence of mutation in <i>JAK2</i>
A3	No cause of secondary erythrocytosis
A4	Bone marrow histology consistent with polycythemia vera
A5	Palpable splenomegaly
A6	Presence of an acquired genetic abnormality (excluding <i>BCR-ABL1</i>) in the hematopoietic cells
B1	Thrombocytosis (platelet count $> 450 \times 10^9/L$)
B2	Neutrophil leukocytosis (neutrophil count $> 10 \times 10^9/L$ in nonsmokers, $\geq 12.5 \times 10^9/L$ in smokers)
B3	Radiological evidence of splenomegaly
B4	Low serum erythropoietin

of erythrocytosis.^{25,26} Algorithm of workup of cases with polycythemia is shown in ►Fig. 1.

Essential Thrombocytosis

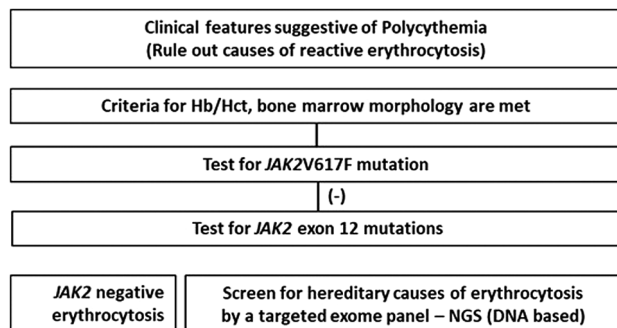
ET is a chronic MPN that primarily involves the megakaryocytes. In addition to sustained thrombocytosis (platelet count $\geq 450 \times 10^9/L$) in the peripheral blood, the disease is characterized by increased megakaryocyte counts in the bone marrow and thrombosis and bleeding.

In ET, approximately 55% have *JAK2V617F* mutation, 5 to 10% have *MPL* (exon 10) mutations, 20 to 25% have *CALR* exon 9 mutations, and approximately 20% are triple negative.^{4,5,7-9} The absence of genetic or biological markers specific to ET makes it necessary to exclude other causes of thrombocytosis, including MPNs, inflammatory and infectious disorders, hemorrhage, other hematopoietic and non-hematopoietic neoplasms (►Supplementary Table S1, available in the online version).

Studies reporting noncanonical mutations in *JAK2* and *MPL* genes in triple-negative ET warrant further investigation of these genes (not necessarily hotspot regions) and other genes associated with germline causes of thrombocytosis.^{10,11}

Primary Myelofibrosis

PMF is a clonal MPN characterized by an abnormal proliferation of megakaryocytes and granulocytes in the bone marrow, associated with fibrous connective tissue deposition and extramedullary hematopoiesis in fully developed disease.

**Fig. 1** Algorithm for molecular testing in PV.

PMF is further subclassified as prefibrotic and overtly fibrotic PMF. Furthermore, approximately 15% of patients with ET or PV might progress into a PMF-like phenotype (post-ET/PV MF) during their clinical course³ (►Supplementary Table S2, available in the online version).

Molecular investigations in a patient with PMF include screening for driver mutations in the *JAK2*, *CALR*, and *MPL* genes, followed by mutations in other myeloid genes (►Table 3). The algorithm of testing is shown in ►Fig. 2.

Detection of molecular markers is necessary to diagnose PMF and helps in these patients' risk stratification. The two new prognostic systems incorporating molecular markers in the risk stratification of PMF are GIPSS (genetically inspired prognostic scoring system) and MIPSS70+ version 2.0 (MIPSSv2; mutation- and karyotype-enhanced international prognostic scoring system).^{27,28}

The WHO 2016 recommends further screening of mutations in *ASXL1*, *EZH2*, *TET2*, *IDH1*, *IDH2*, *SRSF2*, and *SF3B1* in cases of triple-negative PMF to establish clonality.³ This heterogeneous group includes various somatic and germline mutations in driver genes and other myeloid neoplasia-associated genes.^{10,29} In a study where a larger panel of genes (where all coding regions of clinically relevant genes) was screened in a suspected case of pediatric myelofibrosis, a mutation in the *MPIG6B* gene (Megakaryocyte and platelet inhibitory receptor G6B- associated with thrombocytopenia, anemia, and myelofibrosis) was detected.³⁰ Screening for mutations in genes associated with germline causes of myelofibrosis is suggested in rare cases where the etiology of myelofibrosis remains elusive.

Chronic Neutrophilic Leukemia

CNL is a rare MPN. It is characterized by sustained peripheral blood neutrophilia, hypercellular bone marrow due to neutrophilic granulocyte proliferation, and hepatosplenomegaly. The diagnosis requires the absence of *BCR::ABL1* fusion, excluding reactive neutrophilia and other myeloproliferative and myelodysplastic/MPNs (►Supplementary Table S3, available in the online version). *CSF3R* mutations are strongly, but not exclusively, associated with CNL and are a central diagnostic feature of this disorder.³ Molecular investigations include

Table 3 Frequently mutated genes in myeloproliferative neoplasms (content adapted from ³⁴)

Gene	Mutation location	Mutation effect
Phenotype driver mutations		
JAK2	Exon 14	JAK/STAT signaling
JAK2	Exon 12	JAK/STAT signaling
MPL	Exon 10	JAK/STAT signaling
CALR	Exon 9	JAK/STAT signaling
Initiation mutations		
TET2	All coding regions	Epigenetic regulation
DNMT3A	Exons 7–23	Epigenetic regulation
Disease-modifying mutations		
IDH1/2	Exon 4/ Exon 4	Epigenetic regulation
EZH2	All coding regions	Epigenetic regulation
ASXL1	Exon 13	Epigenetic regulation
SF3B1	Exon 12–16	mRNA splicing
SRSF2	Exon 1	mRNA splicing
U2AF1	Exons 2–7	mRNA splicing
ZRSR2	All coding regions	mRNA splicing
RUNX1	All coding regions	Transcription
IKZF1	All coding regions	Transcription
TP53	All coding regions	DNA repair
CUX1	All coding regions	Cell cycle
CBL	Exons 8–9	JAK/STAT signaling
SH2B3	Exon 2	JAK/STAT signaling

screening by a targeted myeloid gene panel that helps establish a diagnosis (presence of *CSF3R* mutation) and identify adverse risk markers such as *ASXL1* and *SETBP1*. Sequential FISH analysis or a targeted fusion gene panel screening by next-generation sequencing (NGS) (RNA-based), rearrangements in *PDGFRA*, *PDGFRB*, *FGFR1*, *PCM1::JAK2*, or other tyrosine kinase fusions must be excluded.

Chronic Eosinophilic Leukemia

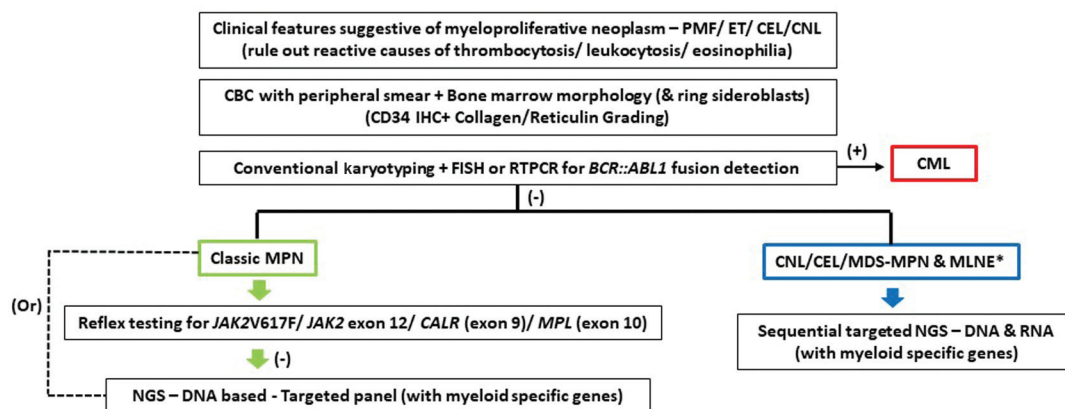
CEL is a multisystem disease characterized by persistent hypereosinophilia and clonal proliferation of eosinophil precursors. It is clinically essential to distinguish between CEL and idiopathic hypereosinophilic syndrome (HES) clearly. The WHO defines idiopathic HES as eosinophilia (eosinophil count $\geq 1.5 \times 10^9/L$) persisting for more than or equal to 6 months (updated to 4 weeks in the WHO 2022) for which no underlying cause can be found, associated with signs of organ involvement and dysfunction and there is no evidence of eosinophil clonality.^{2,3}

The diagnosis of CEL is based on the exclusion of *BCR::ABL1* fusion; rearrangement of *PDGFRA*, *PDGFRB*, or *FGFR1*; or *PCM1::JAK2*, *ETV6::JAK2*, or *BCR::JAK2* fusions, (► **Supplementary Table S4**, available in the online version) which can be screened by either a sequential FISH analysis or a targeted fusion gene panel (RNA based). Extensive screening for mutations in genes associated with eosinophilia is suggested after all causes of reactive/ secondary eosinophilia are excluded.

Juvenile Myelomonocytic Leukemia

JMML is a hematopoietic stem cell-derived MPN of early childhood. Mechanisms involved in the pathogenesis in at least 90% of cases include unchecked activation of the RAS pathway. JMML diagnoses are made by clinical, laboratory, and molecular criteria (► **Supplementary Table S5**, available in the online version). There are three major changes to the diagnostic criteria in WHO 2022: (1) exclusion of *KMT2a* rearrangements; (2) removing monosomy 7 as a cytogenetic criterion; and (3) emphasizing the importance of diagnostic molecular studies, especially those that demonstrate the activation of the RAS pathway.^{2,3}

It is crucial to consider genetic factors in determining risk stratification, and treatment approaches for JMML, with cases originating from somatic mutations in *PTPN11* and germline variants linked to neurofibromatosis type 1 (*NF1*) being the most aggressive. In contrast, spontaneous remission is seen in some cases with pathogenic germline *CBL* variants. Molecular investigations include screening for gene mutations associated with the RAS pathway by a targeted



*MLNE - Myeloid, lymphoid neoplasms associated with eosinophilia

Fig. 2 Algorithm for molecular testing in ET/PMF/CEL/CNL/MPN, NOS.

myeloid gene panel. When a mutation is identified in *PTPN11*, *NF1*, or *CBL* genes, the extended investigation must include establishing a germline/somatic etiology by testing DNA from the patient's skin fibroblasts for the same mutation. Once a germline etiology is established, family screening is suggested to identify the relatives at risk/related stem cell donors.

Myeloproliferative Neoplasm, Not Otherwise Specified

MPN (NOS) exhibits definite clinical, laboratory, molecular, and morphological characteristics of a MPN. However, it does not meet the diagnostic criteria for a specific MPN entity or overlaps the characteristics of two or more MPN entities (–Supplementary Table S6).

Most cases of MPN, NOS fall into one of three groups:

- A subset of cases with a masked prepolycythemic presentation of polycythemia vera, prefibrotic/early PMF, or early-phase essential thrombocythemia in which the characteristic features are not yet fully developed and a proportion of cases presenting with a portal or splanchnic vein thrombosis that fail to meet the diagnostic criteria for any of the specific MPN entities may also be considered to belong in this group.
- MPN at an advanced stage, in which the underlying disorder is obscured by myelofibrosis, osteosclerosis, or a transformation to a more aggressive stage with increased blast counts and myelodysplastic changes.
- Cases with convincing evidence of MPN but masked clinically and morphologically by a coexisting disorder.

Testing Methods for Driver Mutations and “Other/Passenger” Mutations in MPN

The molecular testing algorithm in MPN begins with the exclusion of *BCR::ABL1* fusion. However, this is not recommended in cases with PV. Detection of *BCR::ABL1* can be done either by conventional karyotyping/ FISH or reverse transcriptase polymerase chain reaction (PCR). Molecular screening investigations for driver and other mutations are usually performed on peripheral blood or bone marrow DNA.

Driver Mutations

JAK2

To detect *JAK2V617F*, there is a wide range of techniques available. Allele-specific PCR (AS PCR) is both sensitive and a specific test with a detection limit of 0.1%.⁵ Other techniques include sanger or pyro sequencing, real-time/ quantitative allele-specific PCR, and NGS. Technique selection is based on several attributes, including a low threshold of detection (at least 1% for diagnosis and 0.1% for residual disease monitoring), a high level of specificity (no false positives at minimal detection levels), and a high degree of reproducibility. In cases of *JAK2*-negative polycythemia, mutations in *JAK2* exon 12 mutations can be detected by sanger sequencing (sensitivity of 15–20%) or by NGS.⁶

CALR

There are three types of *CALR* mutations in MPN: insertions, deletions, or insertions and deletions (indels) in exon 9, which cause a +1 base pair frameshift of the coding sequence. This results in the generation of a translated protein lacking a retrieval sequence typical of normal *CALR* and endoplasmic reticulum resident proteins. *CALR* exon 9 mutations are categorized predominantly into a 52-bp deletion: (*CALRdel52*/type I; c.1092_1143del; L367fs*46) or a 5bp insertion *CALRins5*/type II; c.1154_1155insTTGTC; K385fs*47). Other types include more than 50 different variations of *CALR*.³¹ Because of the diversity and complexity of mutations that affect *CALR* exon 9, methodologies that can accommodate these mutations are most useful in the diagnostic setting. *CALR* mutation status is determined by PCR and capillary electrophoresis (fragment analysis and Sanger sequencing) of exon 9.³²

MPL

Mutations in the *MPL* gene cluster around amino acid 515, which is in a stretch of five amino acids, found in the cytoplasmic section of the trans-membrane domain. W515L and W515K are the most common pathogenic mutations, while W515A, W515R, and W515S are rare mutations.⁷ There are various detection methods available, but a multiplexed AS-PCR test that detects the four most common *MPL* exon 10 mutations (W515L, W515K, W515A, and S505N) with 100% specificity and a 1% sensitivity is the most cost-effective and currently being followed.³³

While a multiplex AS PCR and fragment analysis are sensitive and specific, only the target mutations can be screened. However, a more cost-effective method is to screen a targeted panel of genes by NGS that includes all the coding regions of the driver genes along with other myeloid-associated genes, as it can detect both canonical and non-canonical mutations.^{10,11} Conventional methods of mutation detection are represented in –Fig. 3.

Other (Passenger/Nondriver) MPN-Associated Mutations

Patients with MPNs have a highly complex genomic landscape with mutations in driver and nondriver genes conferring increased risk of disease progression and transformation to AML. As described earlier, somatic mutations in nondriver genes are frequently mutated in all MPN categories. Among these, mutations in the epigenetic regulator genes *TET2*, *ASXL1*, *DNMT3A*, and *EZH2* and in the RNA splicing genes *SRSF2* and *U2AF1* are the commonest. Identification of mutations in these genes helps in the risk stratification in MPN patients. In cases where mutations in all the three driver genes are absent, further screening for mutations in *ASXL1*, *EZH2*, *IDH1/2*, *SRSF2*, *TET2*, and *SF3B1* for evidence of clonality of hematopoiesis (triple-negative MPN) helps in differentiating clonal from reactive etiology. In nonclassical MPN, the exclusion of rearrangements in *PDGFRA*, *PDGFRB*, *FGFR1*, *JAK2*, or other TKI fusions is essential to make a diagnosis. These must be screened either by sequential FISH analysis or a targeted fusion

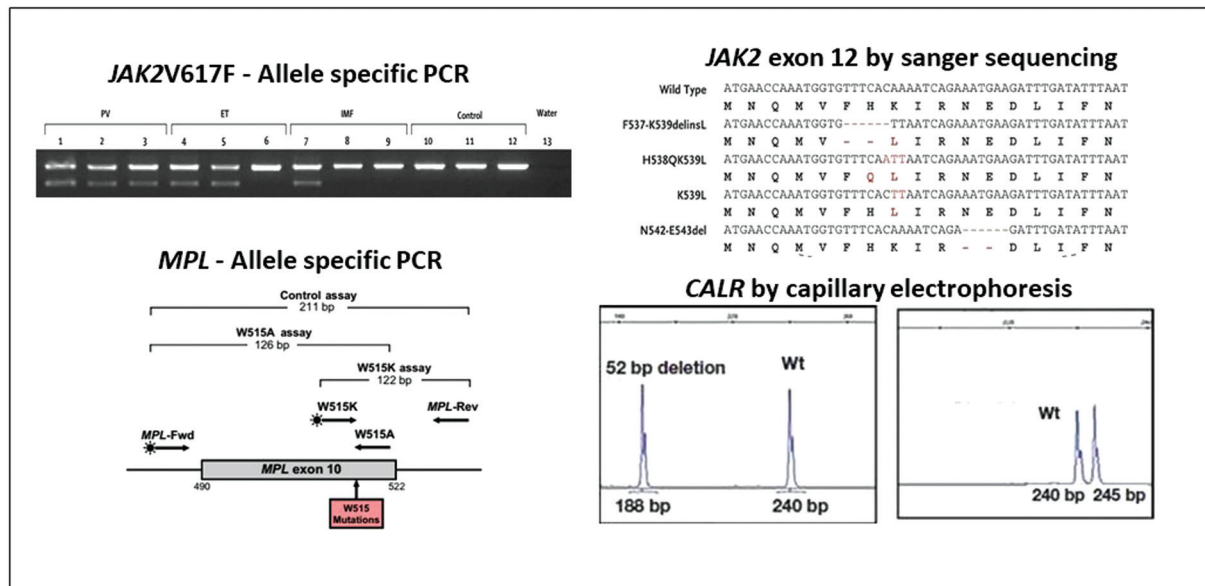


Fig. 3 Conventional methods of detecting *JAK2V617F*, *MPL* exon 10, and *CALR* exon 9 mutations.

gene panel. Myeloid-associated genes included in most targeted panels are represented in (► **Table 3**).³⁴

Discussion and Conclusion

With the ability to sequence multiple genes simultaneously and identify mutations within these genes with high specificity and sensitivity, NGS is ideal for the molecular diagnosis of MPNs.³² Single-target assays may be employed sequentially, but multiplex assays, typically using NGS, sequence several targets in parallel and are more cost-effective. Either approach is acceptable if laboratory turnaround times and assay sensitivity are satisfactory [detection of 1–3% variant allele frequency (VAF) or lower for *JAK2* c.1849G > T (p.Val617Phe), and 5% VAF for *JAK2* exon 12, *CALR* exon nine or *MPL* exon ten variants]. The British Society for Hematology recommends that the use of broad myeloid NGS panels to screen cases with suspected MPN is unlikely to be cost-effective.³⁵ Still, if larger panels are used, the initial analysis and report should be limited to common MPN driver mutations.

The discovery of mutations in the three driver genes (*JAK2*, *CALR*, and *MPL*) has significantly contributed to understanding the classical *BCR::ABL1*-negative MPN. The introduction of mutations in one of the driver genes as a major WHO criterion highlights the importance of testing for these markers in the diagnosis, classification, and prognosis of various MPN subtypes. While the diagnosis of MPN remains multidisciplinary, requiring consideration of clinical features, histomorphological appearances, and other laboratory parameters, molecular diagnostics has become, and will continue to be, an increasingly integral part of this process.

Authors' Contributions

All the authors approve the submitted version of the manuscript. M.M. and A.K.A. wrote the paper. A.B.K.,

U.P.K., and B.P. contributed to the critical revisions of the manuscript.

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Ethical Disclosure

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Conflict of Interest

None declared.

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