Molecular Pathogenesis and Clinical Significance of Driver Mutations in Primary Myelofibrosis: A Review

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**Key Words**
Myelofibrosis · Driver mutations · \textit{JAK2} · \textit{MPL} · \textit{CALR}

**Abstract**
Primary myelofibrosis (PMF) is a rare chronic BCR-ABL1-negative myeloproliferative neoplasm characterized by progressive bone marrow fibrosis, inefficient hematopoiesis, and shortened survival. The clinical manifestations of PMF include splenomegaly, consequent to extramedullary hematopoiesis, pancytopenias, and an array of potentially debilitating constitutional symptoms. The diagnosis is based on bone marrow morphology and clinical criteria. Mutations in the \textit{JAK2} (V617F), \textit{MPL} (W515), and \textit{CALR} (exon 9 indel) genes are found in approximately 90% of patients whereas the remaining 10% are so-called triple negatives. Activation of the JAK/STAT pathway results in overproduction of abnormal megakaryocytes leading to bone marrow fibrosis. These mutations might be accompanied by other mutations, such as \textit{ASXL1}. The commonly used prognostication scoring for PMF is based on the International Prognostic Scoring System. The subsequently developed Dynamic International Prognostic Scoring System-plus employs clinical as well as cytogenetic variables. In PMF, \textit{CALR} mutation is associated with superior survival and \textit{ASXL1} with inferior outcome. Patients with triple-negative PMF have a higher incidence of leukemic transformation and lower overall survival compared with \textit{CALR}- or \textit{JAK2}-mutant patients. The impact of genetic lesions on survival is independent of current prognostic scoring systems. These observations indicate that driver and passenger mutations define distinct disease entities within PMF. Accounting for them is not only relevant to clinical decision-making, but should also be considered in designing clinical trials.

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**Introduction**

Myeloproliferative neoplasms (MPNs) are a group of clonal hematological disorders that arise from transformation of a multipotent hematopoietic stem cell [1]. Among MPNs, chronic myeloid leukemia is characterized by the presence of Philadelphia chromosome (Ph) resulting from the translocation between chromosomes 9 and 22 \((t(9;22)(q34;q11))\) leading to the breakpoint cluster region-Abelson murine leukemia viral oncogene homologue 1 (BCR/ABL1) gene fusion associated with abnormal tyrosine kinase activation involved in the caus-
ative pathophysiology of chronic myeloid leukemia. The Ph-negative MPNs encompass 3 clinical subtypes: polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) [2]. In contrast to chronic myeloid leukemia, disease-specific genetic abnormalities have not been detected that distinguish PV, ET and PMF.

Among MPNs, PMF has the most heterogeneous clinical presentation. In the USA, the annual incidence rate of PMF ranges from 0.1 to 1 per 100,000 [3]. Therapeutic decision-making has been becoming more challenging due to the increasing use of allogeneic stem cell transplantation in the era of the availability of the approved and investigational novel agents.

Our understanding of the underlying genetic changes in PMF has significantly improved in the recent years culminating in a great impact in patient management. The current review focuses on the driver gene mutations and their clinical significance in diagnosis as well as prognosis of patients with PMF.

World Health Organization Classification of PMF

The diagnosis of PMF, as defined by the World Health Organization (WHO), is based on the combination of clinical, morphological, cytogenetic, and molecular features. Disease acceleration is recognized in the patients who show 10–19% blasts, an increased CD34+ cells with clustering, and/or endosteal location in the bone marrow (BM) histology. Patients with PMF rarely present initially in the accelerated or blastic phase [4]. Peripheral blood leukoerythroblastosis, defined as the presence of dacryocytes (teardrop cells), nucleated red cells, and immature granulocytes, is a typical but not invariable feature of PMF; prefibrotic PMF might not display overt leukoerythroblastosis [5]. In the 2016 revised diagnostic criteria, overtly fibrotic PMF is clearly distinguished from early/prefibrotic PMF, and each PMF variant includes a separate list of diagnostic criteria. The key features of prefibrotic PMF usually include increased age, matched BM cellularity, increased megakaryopoiesis of small to large megakaryocytes with atypical histotopography (endosteal translocation, dense clusters), and distinctive nuclear features (hypolobulation, clumsy-cloud-like, maturation defects) and granulocytic proliferation, reduced erythropoiesis, and presence of either normal or only minor amounts of reticulin fibers (grades 0/1) [6, 7]. The 2008/2016 WHO diagnostic criteria for PMF are summarized in table 1. The presence of all major criteria and two minor criteria are required for diagnosis of PMF [8, 9].

Table 1. The 2008/2016 WHO criteria for diagnosis of PMF

<table>
<thead>
<tr>
<th>Major criteria</th>
<th>2008 WHO criteria for PMF</th>
<th>2016 WHO criteria for PMF</th>
<th>2016 WHO criteria for prefibrotic PMF</th>
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<td>Megakaryocyte proliferation and marked dysplasia with either reticulin and/or collagen fibrosis Not fulfilling criteria for CML, PV, MDS or other myeloid neoplasms Presence of JAK2-V617F or MPL-W515L/K or other clonal markers Absence of secondary or reactive causes of BM fibrosis</td>
<td>Megakaryocyte proliferation and marked dysplasia with either reticulin and/or collagen fibrosis ≥grade 2 Not fulfilling criteria for CML, PV, MDS or other myeloid neoplasms Presence of JAK2-V617F or MPL-W515L/K or CALR or other clonal markers Absence of secondary or reactive causes of BM fibrosis</td>
<td>Megakaryocyte proliferation and marked dysplasia with either reticulin and/or collagen fibrosis ≥grade 1 Increased marrow cellularity and granulocytic proliferation with often decreased erythropoiesis Not fulfilling criteria for CML, PV, MDS or other myeloid neoplasms Presence of JAK2-V617F or MPL-W515L/K or CALR or other clonal markers Absence of secondary or reactive causes of BM fibrosis</td>
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<td>Minor criteria</td>
<td>Leukoerythroblastosis Increased serum LDH Anemia Palpable splenomegaly</td>
<td>Leukocytosis ≥11 × 10⁹/l Leukoerythroblastosis Increased LDH Anemia Palpable splenomegaly</td>
<td>Leukocytosis ≥11 × 10⁹/l Increased LDH Anemia Palpable splenomegaly</td>
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CML = Chronic myeloid leukemia; MDS = myelodysplastic syndrome; LDH = lactate dehydrogenase.
Clinical and Laboratory Features of Primary Myelofibrosis

Signs and Symptoms
Patients usually present with splenomegaly or hepatomegaly as the main physical sign at diagnosis. Profound constitutional symptoms such as fatigue, weight loss, night sweats and fever are common. Thirty percent (30%) of the patients are asymptomatic at presentation and are detected by an incidental abnormal blood count or enlarged spleen [10]. The disease gradually evolves from the early prefibrotic to the fibrotic phase with increasing BM failure.

Laboratory Findings
A large proportion of patients present with anemia of a hemoglobin level <100 g/l require blood transfusions [11]. Other laboratory findings include leukocytosis or leukopenia, thrombocytosis or thrombocytopenia and circulating myeloblasts; increased serum lactate dehydrogenase and low cholesterol are also observed. Abnormalities on blood film examination at diagnosis may include a leukoerythroblastic feature consisting of left shifted granulocytes and red cell anisopoikilocytosis [11, 12]. Extramedullary hematopoiesis is a striking feature and refers to the presence of proliferating hematopoietic stem or progenitor blood cells outside of the BM (myeloid metaplasia). The most common sites are in the spleen or liver but may arise in the skin, lymph nodes, serosal surfaces, lungs and spine giving rise to lymphadenopathy, pleural effusion, pneumonia-like symptoms or compression of the spinal cord and nerve roots [13]. A vastly increased number of CD34+ cells are also present in the peripheral blood relative to both normal individuals and to the other MPNs. An increased number of circulating endothelial progenitors and increased vasculature, although not specific to PMF, are a notable feature [12].

Mutations in Driver Genes in PMF
A driver mutation confers growth advantage on the cancer cell and most likely is selected in the tissue microenvironment within which the neoplastic cells arise. A driver mutation may or may not be present in the final stages of cancer but it is selected at some point along the lineage of neoplastic development.

The MPNs comprise clonal hematologic diseases that are thought to arise from a transformation of a hematopoietic stem cell. A major characteristic of Ph-negative MPNs is an increased signaling through the Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway as well as through the phosphatidylinositol 3-kinase (PI3K)-AKT (also known as protein kinase B) pathway in erythroid and myeloid cells [14–16]. The most significant evidence of molecular pathology was reported in 2005 with the identification of the somatic mutation JAK2-V617F [17–20]. This mutation in JAK2 exon 14 gene occurs in approximately 95% of patients with PV and about 60% of those with PMF and ET [17–20], and results in a valine (V) to phenylalanine (F) substitution at codon 617 [21]. This codon is located in the JH2 pseudokinase domain of JAK2, and the mutation is generally considered to negatively affect the JH2-mediated autoinhibitory functionality of the enzyme, resulting in constitutive activation of the tyrosine kinase function. This in turn results in dysregulation of JAK-dependent signal transduction and activation of multiple downstream effectors, including STAT3 and STAT5. Dysregulated JAK-STAT signaling is now recognized as the central mechanism of PMF pathobiology beyond aberrant myeloproliferation [22].

JAK2-V617F Mutation
The JAK family comprises 4 kinases (JAK1, 2, and 3 and TYK2) that attach to cytokine receptor cytosolic domains. JAK kinases possess two highly homologous domains at the carboxyl terminus: an active kinase domain (JAK homology, JH1) and a catalytically ‘inactive’ pseudo-kinase domain (JH2). The JH2 domain is a negative regulator of the JH1 kinase activity [23]. At the N terminus, the JH5-JH7 domains contain a FERM (Band-4.1, ezrin, radixin, and moesin)-like motif, which plays a role in the binding to the cytosolic domain of cognate cytokine receptors.

JAK2 plays a central role in the signaling from ‘myeloid’ cytokine receptors. It binds to the 3 homodimeric myeloid receptors including erythropoietin receptor, thrombopoietin receptor (or myeloproliferative leukemia, MPL), and granulocyte colony-stimulating factor receptor. It also binds to the prolactin and growth hormone receptors as well as to heterodimeric receptors including receptors for granulocyte-macrophage colony-stimulating factor, interleukin 3 (IL-3), IL-5, and interferon-γ. JAK2 is the only JAK capable of mediating the signaling of erythropoietin receptor and MPL. JAK2 also functions as a chaperone for trafficking of these two receptors to the cell surface and their stability [24]. More recently, JAK2 was also shown to promote granulocyte-colony-stimulating factor receptor cell surface localization [25].
In mouse models of JAK2-V617F, both retroviral transplantation assays and transgenic models, including constitutive or inducible knocking approaches, demonstrated the development of an MPN, usually a PV progressing to myelofibrosis [26–32]. However, in some models, an ET-like disorder, usually transient, was observed [18, 30–33]. The discovery of a JAK2-V617F mutation is an important breakthrough in the understanding of BCR-ABL1-negative MPNs and has demonstrated the role of pathologic signaling by the JAK/STAT pathway in MPN.

**MPL Virus Oncogene Mutations**

One year after the discovery of the JAK2-V617F mutation, somatic activating mutations in the MPL virus oncogene (MPL) were identified in patients with JAK2-non-mutated ET and PMF but not in patients with PV [34, 35]. The **MPL** gene is located on chromosome 1p34, encodes the thrombopoietin receptor and is a key factor for growth and survival of megakaryocytes. Acquired mutations at codon W515 constitutively activate the thrombopoietin receptor by cytokine-independent activation of the downstream JAK-STAT pathway. **MPL**-W515 somatic mutations are stem cell-derived events that involve both myeloid and lymphoid progenitors [35].

Mutations in **MPL** cluster around amino acid 515 which is located in a stretch of 5 amino acids (K/RWQFP) found in the cytoplasmic section of the transmembrane domain. These 5 amino acids play a major role in the cytosolic conformation of MPL and prevent spontaneous activation of the receptor [36, 37]. Recurrent pathogenic mutations include the common W515L and W515K and the rare W515A, W515R and W515S mutations [38, 39]. The S505N mutation was first described in familial thrombocythemia but has subsequently been identified as a somatic mutation in ET and PMF [38, 39]. The 2 most recurrent mutations W515L and W515K are found in approximately 15% of JAK2-V617F-nonmutated MPN that is 5% of ET and up to 10% of PMF [35]. The 2 mutations were independently assessed because the 2008 WHO diagnostic criteria for MPNs highlighted the function of the **MPL**-W515L/K mutations in the diagnosis of ET and PMF. Alternative mutations have also been reported in rare cases including V501A, S505C, A506T, V507I, G509C, L510P, R514K and R519T, although the pathogenic significance of some of these mutations is not clear [40–42]. The median overall survival of patients was approximately 9 years in both **MPL**-mutated and JAK2-mutated PMF [43].

**Calreticulin Gene Mutations**

Calreticulin (CALR) was originally identified as a Ca$^{2+}$-binding protein in the endoplasmic reticulum lumen of most cells of human origin. Its main function is to play a critical role in quality control processes during protein synthesis and folding, through binding to misfolded proteins. The CALR is found at multiple subcellular localizations outside of the endoplasmic reticulum, where it mediates a variety of cellular processes, including apoptotic cell clearance, cell adhesion, and cell migration [44–46]. Moreover, CALR is implicated in a variety of cellular roles, including modulation of activation of the unfolded protein response and Ca$^{2+}$ signaling and storage, regulation of steroid-sensitive gene expression, chaperoning in protein folding, autoimmune response, and neuromodulations [47, 48].

In total, 50 different types of mutation in **CALR** are reported which results in a frame shift to the same alternative reading frame, generating a novel C terminus of the mutated protein. More than 80% of **CALR** mutations constitute 1 of 2 variants: type 1, a 52-bp deletion (L367fs*46), or type 2, a 5-bp TTGTG insertion (K385fs*47) [49, 50]. Emerging data suggest functionally relevant structural differences between type 1 and type 2 **CALR** variants, including a higher α-helix content of the mutant C terminus in type 2 compared with type 1 [51]. Overexpression of the most common **CALR** mutation (52 bp deletion) in IL-3-dependent Ba/F3 cells led to IL-3-independent growth and hypersensitivity to IL-3. Cells overexpressing the mutant were sensitive to the JAK family kinase inhibitor SAR302503 and showed elevated STAT5 phosphorylation in the absence of IL-3. This indicates that JAK/STAT signaling is involved in the observed cytokine-independent growth of mutant CALR-expressing Ba/F3 cells [49]. Cell line models demonstrated that CALR mutants activate the JAK2 downstream pathway via its association with MPL. The mutant-specific carboxyl terminus portion of CALR interferes with the P domain of CALR to allow the N domain to interact with MPL, leading to the phosphorylation of JAK2 and constitutive activation of JAK2/STAT/PI3K and mitogen-activated protein kinase pathways [52–54].

Patients with CALR-mutated PMF are generally younger than their JAK2-mutated counterparts, and they display a higher platelet count, lower leukocyte count, higher hemoglobin level, lower incidence of spliceosome mutations, and longer survival [55]. The prognostic benefit of **CALR** mutations may be limited to type 1 (52-bp) or type 1-like **CALR** variants; patients with type 2 (5-bp insertion) or type 2-like **CALR** variants exhibit compara-
ably worse survival, which is comparable with that of PMF patients with JAK2-V617F.

Approximately 10% of patients with PMF are triple-negative (nonmutated JAK2, CALR, and MPL) and have a poor prognosis and demonstrate a high rate of leukemic transformation [56–58]. Whole exome sequencing of patients with triple-negative ET or PMF identified activating mutations outside exon 10 of MPL [59].

Other Mutations
In PMF, the frequencies of nonspecific mutations are higher compared with PV and ET. Mutations with frequencies of 10% or more in PMF include ASXL1 (additional sex comb like 1), TET2 (ten eleven translocation oncogene family member 2), SRSF2 (serine/arginine rich splicing factor 2), and U2AF1 (U2 small nuclear RNA auxiliary factor 1) [60, 61]. It had been reported that PMF patients harboring a CALR+/ASXL1− mutation profile have a more prolonged survival, approximately 18 years, compared with those with CALR−/ASXL1−. While CALR+/ASXL1+ and CALR−/ASXL1+ patients were in a similar intermediate risk category [43, 62]. Other mutations that are less frequent in chronic phase PMF but with a significantly higher frequency in the blast phase include IDH1 and IDH2 (isocitrate dehydrogenase 1 and 2) [63, 64], TP53 (tumor protein P53), DNMT3A (DNA cytosine methyltransferase 3A) [65], IKZF1 (IKAROS family zinc finger) and LNK mutation (table 2) [66].

ASXL1, EZH2, SRSF2, and IDH1/2 mutations provide added value in the combined molecular and clinical prognostication of PMF. The presence of two or more of these mutations could predict the lower overall survival rate in patients with PMF: median 2.6 years versus 7.0 years for no mutations [67]. Compared to the best available therapy, a JAK inhibitor, ruxolitinib, reduced the risk of death (hazard ratio 0.57, 95% confidence interval 0.30–1.08) in patients with PMF independent of the mutation profile [68].

The Prognostic Impact of Cytogenetics in PMF
Approximately one third of patients with PMF present with cytogenetic abnormalities including del(20q), del(13q), trisomy 8 and 9, and abnormalities of chromosome 1 including duplication 1q. Other less frequent lesions include −7/del(7q), del(5q), del(12p), +21 and der(6)t(1;6)(q21;p21.3). The types of cytogenetic abnormalities are generally similar among patients with PMF, post-ET myelofibrosis and post-PV myelofibrosis. Based on the effect on prognosis, cytogenetic findings in PMF are classified as either favorable or unfavorable. The former include normal karyotype or isolated del(20q) or del(13q) and the latter includes all other cytogenetic abnormalities. An unfavorable cytogenetic profile in both PMF and post-PV/ET myelofibrosis is associated with higher JAK2-V617F mutational frequency. Patients whose PMF transforms to acute leukemia usually show complex karyotypes at transformation and a significantly decreased median survival [69, 70].

Genetic Predisposition in Primary Myelofibrosis
Ph-negative MPNs are characterized by multilineage clonal hematopoiesis with the identical somatic activating mutation in the JAK2 tyrosine kinase gene (JAK2-V617F) found in most individuals, and that is believed to be a critical driver of excess proliferation. Family members of individuals with MPN are at higher risk for the development of MPN, consistent with the existence of MPN predisposition loci, where germline variation contributes to predisposition and phenotypic pleiotropy [71, 72].

Genome-wide analysis identified the 46/1 (also called GGCC) JAK2 haplotype that predisposes individuals to V617F+ MPN. This germline haplotype is tagged by the C allele of the single-nucleotide polymorphism rs12343867 (C/T). The JAK2 haplotype 46/1 confers susceptibility to PMF, regardless of JAK2-V617F mutational status. Patients with homozygous JAK2 46/1 showed significantly higher hemoglobin and leukocyte counts, higher JAK2-V617F allelic burden but no association with other clinical or laboratory features was observed [73–75].

The variant rs2736100_C in the telomerase reverse transcriptase (TERT) gene at the TERT-CLPTM1L locus is one of the 8 variants that have been reported to associate with long telomeres in white blood cells, suggesting that this common variant acts on the TERT gene encoding the reverse transcriptase of the telomerase complex essential for maintaining the telomere length. The TERT rs2736100_C variant is associated with increased counts of myeloid white blood cells, red blood cells and platelets but not lymphoid cells. Hence, this variant exerts its effect on hematopoiesis by increasing proliferation of cells derived from a common myeloid progenitor. A recent genome-wide association study of more than 3,000 MPN cases and more than 10,000 controls identified 2 single-nucleotide polymorphisms with genome-wide significance in JAK2-V617F-negative MPN, rs12339666 (JAK2) and rs2201862 (MECOM). rs2736100 (TERT) and rs9376092 (HBS1L/MYB) single-nucleotide polymorphisms were reported to have genome-wide significance when JAK2-V617F-positive cases were included. The variant rs9376092 had a stronger effect in JAK2-V617F-
negative cases with CALR and/or MPL mutations, whereas in JAK2-V617F-positive cases rs9376092 was associated with ET rather than PV [76, 77].

Prognostic Scoring in Primary Myelofibrosis

Robust prognostic modeling in PMF started with the development of the International Prognostic Scoring System (IPSS) in 2009. The IPSS for PMF identifies 5 independent predictors of inferior survival at the time of initial evaluation: age >65 years, presence of constitutional symptoms, hemoglobin <100 g/l, leukocyte count >25,000/μl, and circulating blasts ≥1%. The presence of 0, 1, 2, and ≥3 adverse factors defines low, intermediate-1, intermediate-2, and high-risk disease, respectively. The corresponding median survivals are 11.3, 7.9, 4, and 2.3 years, respectively [78].

The International Working Group-Myeloproliferative Neoplasms Research and Treatment subsequently developed a dynamic prognostic model (Dynamic IPSS; DIPSS) that uses the same prognostic variables used in IPSS but can be applied at any time during the disease course. DIPSS assigns 1 point to each of the adverse features of
IPSS except for hemoglobin <100 g/l, which receives 2 points. The risk categorization is modified to low (zero adverse points), intermediate-1 (1 or 2 points), intermediate-2 (3 or 4 points), and high (5 or 6 points). The corresponding median survival includes not reached, 14.2, 4, and 1.5 years, respectively [79]. IPSS- and DIPSS-independent risk factors for survival in PMF were subsequently identified and included unfavorable karyotype, (i.e. complex karyotype or 2 abnormalities that include +8, i(17q), −7/7q−, −5/5q−, inv(3), 12p−, or 11q23 rearrangement [80, 81], red cell transfusion requirement [82, 83], and platelet count <100,000/μl [84]. These have resulted in the DIPSS-plus prognostic scoring system, which employs clinical as well as cytogenetic variables. The 4 DIPSS-plus risk categories based on the aforementioned 8 risk factors are low (no risk factors), intermediate-1 (1 risk factor), intermediate-2 (2 or 3 risk factors), and high (4 or more risk factors) with respective median survivals of 15.4, 6.5, 2.9, and 1.3 years [85].

Conclusion

Prognostic modeling of PMF began with the IPSS system, followed by the development of DIPSS and DIPSS-plus. Genetic mutations have an independent role in DIPSS-plus system. CALR mutations are inclined to lower the score and are less likely to cause anemia or to require blood transfusions. With regard to survival, the presence of CALR mutations is more effective on the overall survival independent from the DIPSS-plus risk stratification, compared with the JAK2 mutation or the JAK2/MPL/CALR triple-negative profile, but they show a similar effect on survival as compared to the MPL mutation. Patients with triple-negative PMF also display an inferior leukemia-free survival among all the genetic variants. In summary, the knowledge of driver and subclonal mutations can provide valuable information for diagnosis and prognosis, which can ultimately be highly useful for clinical decision-making for the management of patients with PMF.

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