New mutations and pathogenesis of myeloproliferative neoplasms

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Myeloproliferative neoplasms (MPNs) are clonal disorders characterized by excessive production of mature blood cells. In the majority of classic MPN—polycythemia vera (PV), essential thrombocytopenia (ET), and primitive myelofibrosis—driver oncogenic mutations affecting Janus kinase 2 (JAK2) or MPL lead to constitutive activation of cytokine-regulated intracellular signaling pathways. LNK, c-CBL, or SOCSs (all negative regulators of signaling pathways), although infrequently targeted, may either drive the disease or synergize with JAK2 and MPL mutations. I2F1 deletions or TPS3 mutations are mainly found at transformation phases and are present at greater frequency than in de novo acute myeloid leukemias. Loss-of-function mutations in 3 genes involved in epigenetic regulation, TET2, ASXL1, and EZH2, may be early events preceding JAK2V617F but may also occur late during disease progression. They are more frequently observed in PMF than PV and ET and are also present in other types of malignant myeloid diseases. A likely hypothesis is that they facilitate clonal selection, allowing the dominance of the JAK2V617F subclone during the chronic phase and, together with cooperating mutations, promote blast crisis. Their precise roles in hematopoiesis and in the pathogenesis of MPN, as well as their prognostic impact and potential as a therapeutic target, are currently under investigation. (Blood. 2011;118(7):1723-1735)

Introduction

The term “myeloproliferative disorders” (MPDs) was first introduced by William Dameshek in 1951 to describe 4 different diseases with clinical and biologic similarities: polycythemia vera (PV), essential thrombocytosis (ET), and primitive myelofibrosis (PMF), and chronic myeloid leukemia (CML).1 Later on, chronic neutrophil leukemia, hypereosinophilic syndromes, systemic mastocytosis, atypical chronic myeloid leukemia, and other rare chronic hemopathies were added. Certain disorders that combine myeloproliferation and myelodysplasia features, such as chronic myelomonocytic leukemia, are closely related to MPDs. In 2008, the World Health Organization recommended changing the term myeloproliferative disorders to myeloproliferative neoplasms (MPNs).2 In this review, we will focus on the current molecular knowledge of the BCR-ABL–negative MPNs PV, ET, and PMF.

All MPNs are clonal disorders with an initial hit in the HSCs resulting in an excessive production of blood cells because of hypersensitivity or independence from normal cytokine regulation.3,4 This myeloproliferation results from the absence of feedback regulation by mature cells, decreasing cytokine levels. MPNs involve the 3 main myeloid lineages but predominate in one of them: the erythroid lineage for PV, the megakaryocyte (MK)/platelet lineage for ET, and the MK/granulocytic lineages for PMF. MPNs are associated with a normal maturation but increased numbers of mature blood cells, except in PMF, in which abnormalities in MK differentiation might be responsible for the marrow fibrosis. A continuum exists between the BCR-ABL–negative MPN and myelodysplastic syndromes (MDS), as exemplified by chronic myelomonocytic leukemia (CMLL), classified as MPN/MDS by the World Health Organization.

Several MPNs are the result of a genetic event leading to the constitutive activation of a tyrosine kinase that mimics the intracellular signaling pathways induced by hematopoietic growth factors. In CML, oligomerization of the BCR-ABL fusion protein via the BCR coiled-coil domain induces the constitutive activation of ABL that triggers many signaling pathways and drives the disease. Inhibition of the ABL kinase activity in patients results in disease regression, demonstrating that BCR-ABL is the oncogenic event responsible for the disease. Additional fusion proteins with constitutive tyrosine kinase activity involving PDGFRβ, fibroblast growth factor receptor, and PDGFRα, and more recently Janus kinase 2 (JAK2), have been described in hypereosinophilic syndromes (FIP1L1-PDGFRα) and in atypical MPNs.3 In addition, systemic mastocytosis involves gain-of-function mutations in the tyrosine kinase receptor c-KIT. Hence, it was likely that classic MPNs are also “kinase diseases.”

The founding mutations

JAK2V617F mutation

The JAK2 locus was found affected by a 9p loss of heterozygosity in 30% of PV patients (Table 1, Figure 1).5 In 2005 a major advance in the understanding of the pathogenesis of MPN was made by the discovery of the JAK2V617F mutation.6,7 The JAK family comprises 4 kinases (JAK1, 2, and 3 and TYK2) that attach to cytokine receptors, in the cytosolic domain of cognate cytokine receptors and in atypical MPNs. The JH2 domain is a negative regulator of the JH1 kinase activity.10 At the N-terminus, the JH5-JH7 domains contain TET2, ASXL1, and EZH2, may be early events preceding JAK2V617F but may also occur late during disease progression. They are more frequently observed in PMF than PV and ET and are also present in other types of malignant myeloid diseases. A likely hypothesis is that they facilitate clonal selection, allowing the dominance of the JAK2V617F subclone during the chronic phase and, together with cooperating mutations, promote blast crisis. Their precise roles in hematopoiesis and in the pathogenesis of MPN, as well as their prognostic impact and potential as a therapeutic target, are currently under investigation. (Blood. 2011;118(7):1723-1735)
**Table 1. Genes involved in the pathogenesis of MPN**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Localization</th>
<th>Function</th>
<th>Comment</th>
<th>Disease (frequency)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK2</td>
<td>9p24</td>
<td>Tyrosine kinase, signaling</td>
<td>Gain of function</td>
<td>PV (85%-99%), ET (50%-70%), PMF (40%-50%)</td>
<td>6-9,11,12</td>
</tr>
<tr>
<td>MPL</td>
<td>1p34</td>
<td>Receptor, signaling</td>
<td>Gain of function</td>
<td>ET (4%), PMF (11%)</td>
<td>13-16</td>
</tr>
<tr>
<td>LNK</td>
<td>12q24</td>
<td>Adaptor, signal regulation</td>
<td>Loss of function</td>
<td>ET (&lt; 5%), PMF (&lt; 5%), JAK2 mutation-negative erythrocytosis (25%), post-MPN AML (13%)</td>
<td>17-19</td>
</tr>
<tr>
<td>CBL</td>
<td>11q23</td>
<td>Adaptor, E3 ubiquitin ligase, signal regulation</td>
<td>Dominant negative</td>
<td>PMF (6%), post-MPN AML</td>
<td>20,21</td>
</tr>
<tr>
<td>SOCS1</td>
<td>16p13.2</td>
<td>E3 ubiquitin ligase, signal regulation</td>
<td>Methylation</td>
<td>ET (14%-25%), PV (11%-13%), PMF (17%)</td>
<td>22,23</td>
</tr>
<tr>
<td>SOCS2</td>
<td>12q22</td>
<td>E3 ubiquitin ligase, signal regulation</td>
<td>Methylation, mutation</td>
<td>All MPN (28%)</td>
<td>24</td>
</tr>
<tr>
<td>SOCS3</td>
<td>17p25.3</td>
<td>E3 ubiquitin ligase, signal regulation</td>
<td>Methylation, mutation</td>
<td>ET (10%), PV (22%)</td>
<td>22,25-27</td>
</tr>
<tr>
<td>NAR1</td>
<td>11p33.2</td>
<td>GPase, signaling</td>
<td>Gain of function</td>
<td>post-MPN AML (7%-13%)</td>
<td>21</td>
</tr>
<tr>
<td>NF1</td>
<td>17q11.2</td>
<td>RAS signaling regulation</td>
<td>Deletion</td>
<td>PMF (0%-6%), post ET/PMF MF (14%)</td>
<td>28,29</td>
</tr>
<tr>
<td>TET2</td>
<td>4q24</td>
<td>DNA hydroxymethylation</td>
<td>Loss of function</td>
<td>PV (15%), ET (4%-11%), PMF (19%), post-MPN AML (26%)</td>
<td>30-33</td>
</tr>
<tr>
<td>ASXL1</td>
<td>20q11.21</td>
<td>Chromatin modifications</td>
<td>Loss of function</td>
<td>PV and ET (&lt; 7%) PMF (19%-40%), post-MPN AML (19%)</td>
<td>34,35</td>
</tr>
<tr>
<td>EZH2</td>
<td>7q35</td>
<td>Chromatin methylation</td>
<td>Loss of function</td>
<td>PV (3%), PMF (13%)</td>
<td>36-38</td>
</tr>
<tr>
<td>IKZF1</td>
<td>7p12</td>
<td>Transcription factor, lymphopoiesis</td>
<td>Deletion</td>
<td>post-MPN AML (21%)</td>
<td>39</td>
</tr>
<tr>
<td>RUNX1</td>
<td>21q22.2</td>
<td>Transcription factor, hematopoiesis</td>
<td>Loss of function</td>
<td>post-MPN AML (37%)</td>
<td>21,40</td>
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<tr>
<td>RB</td>
<td>13q14</td>
<td>Cell cycle, apoptosis</td>
<td>Deletion</td>
<td>PMF (19%)</td>
<td>29</td>
</tr>
<tr>
<td>TP53</td>
<td>17p13.1</td>
<td>Cell cycle, apoptosis</td>
<td>Loss of function</td>
<td>post-MPN AML (20%)</td>
<td>21,41</td>
</tr>
<tr>
<td>IDH1</td>
<td>3p33.3</td>
<td>Metabolism</td>
<td>Neomorphic enzyme</td>
<td>PMF (2%), post-MPN AML (8%)</td>
<td>42</td>
</tr>
<tr>
<td>IDH2</td>
<td>15q26.1</td>
<td>Metabolism</td>
<td>Neomorphic enzyme</td>
<td>PMF (2%), post-MPN AML (18%)</td>
<td>42</td>
</tr>
</tbody>
</table>

AML indicates acute myeloid leukemia; ET, essential thrombocythemia; JAK2, Janus kinase 2; MPN, myeloproliferative neoplasms; PMF, primary myelofibrosis; and PV, polycythemia vera.

“myeloid” receptors (erythropoietin receptor [EPO-R], myeloproliferative leukemia [MPL; TPO-R], G-CSF receptor [G-CSF-R]), to the prolactin and growth hormone receptors, to heterodimeric receptors (GM-CSF-R, IL-3-R, and IL-5-R, which share the common β chain of IL-3-R and the gp130 family of receptors), and to IFN-γ R2. JAK2 is the only JAK capable of mediating the signaling of EPO-R and MPL. JAK2 also functions as a chaperone for trafficking of these 2 receptors to the cell surface and their stability. More recently, JAK2 was also shown to promote G-CSF-R cell-surface localization. Therefore, JAK2 and the 3 “myeloid receptors” form functional units and have been shown to be required for the promotion of JAK2V617F signaling. The JAK2V617F mutation results from a guanine to thymine change at nucleotide 1849 of the cDNA, in exon 14 of the gene. This valine is located at one of the predicted interfaces between JH1 and JH2 domains, and the change to a phenylalanine appears to relieve the inhibition of the JH2 domain on the kinase domain. There is evidence that constitutive signaling by JAK2V616F requires the homodimeric receptor, explaining why the JAK2V617F-induced proliferation affects 3 myeloid lineages. Indeed, the JAK2V617F mutation has been found in the majority of BCR-ABL–negative MPNs (~ 95% of patients with PV, 50%-70% with ET, and 40%-50% with PMF), as well as in some cases of atypical MPN (30%-50% splanchnic vein thrombosis and sideroblastic anemia associated with a thrombocytosis). Beside its role in the cytokine receptor signaling cascade (Figure 1), JAK2 has been shown to influence chromatin structure (Figure 2). In hematopoietic cells, nuclear JAK2 phosphorylates histone H3Y41, thereby blocking recruitment of the repressor heterochromatin protein 1α and allowing increased expression of several genes, including the LMO2 oncogene. In the same line, JAK2V617F has been recently described to interact with and phosphorylate the protein arginine methyltransferase PRMT5 with a much greater affinity than wild-type JAK2. This property is specific (or enhanced) for the mutant protein and has been shown to disrupt the interaction between PRMT5 and its cofactor MEP50, leading to a decreased methyltransferase activity. The knockdown of PRMT5 increases colony formation and erythroid differentiation of primary cells. This emerging nuclear role of mutated JAK2 may reveal mutant–specific chromatin effects that may open a novel therapeutic window.

The discovery of JAK2V617F mutation is an important breakthrough in the understanding of BCR-ABL–negative MPN and has demonstrated the role of pathologic signaling by the JAK/STAT pathway in MPN. Two other mutations directly affecting this pathway have subsequently been described.

### JAK2 exon 12 mutations

In the rare JAK2V617F-negative PV, different somatic gain-of-function mutations in exon 12 of JAK2 have been found. These mutations span the linker region between the SH2 and JH2 domains. Although not located in the pseudokinase domain, these mutations may modify the structure of the JH2 domain in a very similar fashion as V617F. Along these lines, residue F595, located in the helix C of the pseudokinase domain, was shown to be required for both V617F and K539L mutants but not for cytokine-induced JAK2 activation. However, in contrast to JAK2V617F, exon 12 mutations are not associated with ET and PMF, although JAK2 exon 12 PV may progress to a secondary myelofibrosis.

### MPL mutations

Several gain-of-function mutations of MPL have been found in exon 10, which result in the substitution of a tryptophan 515 to a leucine, lysine, asparagine, or alanine. Amino acid 515 is located in a stretch of 5 amino acids (K/RWQFP) found inside the cytoplasm just after the transmembrane domain. These 5 amino acids play a major role in the cytosolic conformation of MPL and prevent spontaneous activation of the receptor. In addition, the MPLS505N mutation, initially described in familial ET, was also found in sporadic MPN. These MPL mutations have been found in up to 15% of JAK2 V617F-negative ET or PMF.

Subsequent modeling of the disease has shown that JAK2 and MPL mutations were able to drive the disease in mice. In mouse models of JAK2V617F, both retroviral transplantation assays and
transgenic models, including constitutive or inducible knockin approaches, demonstrated the development of a MPN, usually a PV progressing to myelofibrosis. However, in some models, an ET-like disorder, usually transient, was observed. This phenotype corresponds to a weak JAK2V617F expression, supporting the hypothesis that phenotypic heterogeneity of the JAK2V617F-induced diseases might be because of the intensity of JAK2 signaling, especially to the number of JAK2V617F copies. This assumption fits with the human situation in which most patients with ET have a single JAK2V617F mutation, whereas in most PV cases, 2 copies of JAK2V617F are present because of uniparental disomy. In addition, strain-specific variations were observed in the retroviral mouse model, suggesting that host modifier genes were involved in the phenotype of the disease. This finding also fits with recent results in humans in which JAK2V617F-heterozygous erythroid cells from PV and ET patients exhibit different gene expression profiles, with erythroid cells from ET patients displaying an IFN-γ-stimulated gene expression pattern. This is due to the fact that ET and not PV erythroid colonies are characterized by constitutive activation of STAT1, possibly via activation of IFN-γ receptor 2 or another STAT1-activating receptor. This finding plus other data suggest the existence of a modifier gene in the human disease. In MPL, BM transplantation assays with W515L or W515A mutations led to an ET-like disorder rapidly progressing to myelofibrosis. Thus, BCR-ABL-negative MPN resembles CML with some significant differences.

Genes involved in intracellular signaling

LNK

LNK, also called SH2B3, is a member of the SH2B family, which contains 2 other members (Table 1, Figure 1). This family of adaptor proteins is characterized by a conserved structure with 3 main domains (a proline-rich amino terminus, a plekstrin homology domain, an SH2 domain, and an SH3 domain) and a conserved tyrosine at the C-terminus. LNK plays an important role in hematopoiesis by negatively regulating JAK2 activation through its SH2 domain, thus inhibiting EPO-R and MPL signaling. In addition, LNK negatively regulates c-KIT and FMS signaling. LNK-deficient mice have an increased HSC pool with enhanced self-renewal properties and increased quiescence. This phenotype probably results from increased TPO/MPL signaling because TPO is
required for maintaining HSC quiescence and the HSC reservoir. In addition, Lnk−/− mice develop MPN with thrombocytosis, splenomegaly, and fibrosis and marked B-cell overproduction.

As expected from its negative role in JAK2 signaling, LNK is also capable of attenuating the signaling induced by MPLW515L or JAK2V617F. Loss of LNK accelerates the development of MPN induced by JAK2V617F in murine models. In JAK2V617F-positive patients, LNK expression is increased and modulates the myeloproliferative process. Recently, Oh et al identified 2 mutations in LNK exon 2, one in a patient with PMF and the other with ET; both MPNs were JAK2V617F negative. The first mutation leads to a premature stop codon resulting in the absence of the PH and SH2 domains, whereas the second (E208X) leads to absence of the PH and SH2 domains. In the chronic phase of classic MPN, c-CBL mutation occurred after JAK2V617F. However, during acute myeloid leukemia, c-CBL induces a mastocytosis phenotype and myeloid leukemia, albeit with a long latency. Similarly, c-CBL knockout mice develop a mild MPN with an increase in HSC. Cbl-b−/− mice lack a hematologic phenotype. The double knockout leads to a rapidly lethal MPN with leukocytosis and excess of monocytes, a phenotype close to myelo-monocytic leukemia.

In the chronic phase of classic MPN, c-CBL mutations have been found in a low percentage of PMF patients (6%) but were not detected in a small series of PV and ET patients. In one case, the c-CBL mutation occurred after JAK2V617F. However, during the chronic phase of classic MPN, c-CBL mutations have been found in a low percentage of PMF patients (6%) but were not detected in a small series of PV and ET patients. In one case, the c-CBL mutation occurred after JAK2V617F. However, during
progression of the disease, JAK2V617F was outcompeted by the CBL mutant, suggesting that the 2 mutations had occurred in 2 different cells. 20 Similarly, a c-CBL mutation has been detected in blasts from a JAK2V617F-positive MPN, which became JAK2V617F negative during transformation. 21 Presently, c-CBL seems to be involved more in progression toward myelofibrosis or acute leukemia than in the chronic phase of the disorder, but further studies are required to establish its precise role.

**Suppressor of cytokine signaling 1, 2, and 3**

Suppressor of cytokine signaling (SOCS) proteins are also important negative regulators of JAK signaling through a classic feedback loop. 22 Loss of SOCS activity induces excess signaling by cytokines. SOCS1-inactivating mutations have been described in B-cell lymphoma. 23 Some mutations in the different SOCS have been found in MPNs, but they seem rare. 24 In contrast, hypermethylation of Cpg islands in SOCS1 and SOCS3 associated with a decrease in expression was found in JAK2V617F PV and ET as well as in JAK2V617F and MPLW515-mutation negative ET. 22,23 Treatment with 2'-deoxyazacytidine restored SOCS1 and SOCS3 expression. SOCS1 not only regulates cytokine signaling 20 but may also be implicated in direct activation of p53-dependent senescence. 25 It was suggested that the association between SOCS3 promoter methylation and diminution of transcription is found specifically in JAK2V617F-negative patients with PMF. 26 However, the role of SOCS3 in regulating JAK2V617F signaling is controversial. On the one hand, it has been reported that SOCS3 inhibits JAK2V617F signaling through proteosomal degradation. 23 On the other hand, when JAK2V617F-transformed hematopoietic cell lines were used, it was reported that JAK2V617F mutant kinase escapes SOCS3-negative regulation by inducing hyperphosphorylation of the SOCS box, thereby blocking its interaction with Elongin C and stabilizing JAK2V617F. 24 Similar results have been obtained with exon 12 JAK2 mutant kinases. 25 SOCS2 may also inhibit JAK2V617F signaling, and its promoter is hypermethylated in some MPN. 25 Presently, the role of SOCS proteins in the pathogenesis of MPN remains unclear: Is the epigenetic silencing a cooperating event with JAK2V617F 27 or is it at the origin of some MPNs such as ET?

**Genes involved in leukemic progression**

Leukemic transformation, or blast phase, occurs in approximately 15% of PMF patients and in < 10% of PV and ET patients (Table 1). 26-29 To identify the main actors of leukemic transformation in MPN, several teams have conducted comprehensive genotypic analyses of post–MPN-AML cells, with particular attention to both MPN- and AML-related genes. 26-29 To fit the definition of a blast phase-transforming event, the candidate event had to be detected in all blast cells but not in the majority of cells from the preceding MPN clone. 21

**IDH1/2 mutations**

IDH1 and IDH2 encode isocitrate dehydrogenase 1 and 2, which are NADP+ enzymes that catalyze the conversion of isocitrate to α-ketoglutarate (αKG). 26,27 Heterozygous mutations of IDH1 (R132S, R132G) and IDH2 (codon R140 and R172) have been identified in AML. 28-30 Mutated IDH1/2 are neomorphic enzymes that catalyze the reduction of αKG to (R)-2-hydroxyglutarate. A subsequent overproduction of (R)-2-hydroxyglutarate has been proposed to affect the function of αKG-dependent enzymes such as TET2, resulting in a decrease of 5hmC. 26 Although the effects of IDH1/2 mutations on HSCs and progenitors remain unknown, these mutations may belong to a set of initiating events of the same nature as the TET2 mutations. The analysis of 1473 patients with MPN revealed a low incidence of IDH1/2 mutations in chronic phase ET, PV, and MF (0.8%, 1.9%, and 4.2%, respectively) contrasting with a 21.6% frequency in blast phase. 22 Thus IDH1/ IDH2 mutations are associated with MPN transformation, but clonal hierarchy and follow-up studies are needed to better define whether they promote the initiation or the progression of MPN to AML.

**IKZF deletion**

The IKZF1 gene encodes for the Ikaros transcription factor, which regulates the development of B and T cells. 23 IKZF1 deletions are frequent in acute lymphoblastic leukemia, especially in BCR-ABL-positive cases. 24 Hemizygous deletions of the IKZF1 gene region on chromosome 7p were observed in 1 of 437 patients with chronic-phase MPN and 6 of 29 patients with post–MPN-AML. 39 Clonal hierarchy analysis showed that in some cases IKZF1 deletion was a late event in the progression of the disease and was frequently related to a complex karyotype. Thus, like in post–CML acute lymphoblastic leukemia, IKZF1 deletions in post–MPN-AML are late events in the progression of MPN to AML.

**NRAS/KRAS mutations and NF1 deletion**

RAS proteins are membrane-associated GTPases that in their active form trigger a variety of effector signaling pathways, such as the MAPK cascade of serine/threonine kinases. The most frequent mutations in Kras and NRAS occur at codons 12, 13, and 61, leading to an inhibition of the GTP-ase activity, thus allowing constitutive activation of effector pathways such as MAPK signaling and downstream transcriptional control. It is still unknown whether RAS mutations promote the initiation or the progression of myeloid malignancies to acute leukemia. In MPN, this question remains unresolved because NRAS mutations were found in post–MPN-AML blasts and in cells from the preceding chronic phase, but also, in some patients, exclusively in AML blasts at transformation. 21 NF1 deletions were mainly found in myelofibrosis and rarely in PV or ET. 22-29 Their frequency in leukemic transformation is unknown.

**TP53 mutations**

TP53 gene encodes p53, a major tumor suppressor protein involved in various biologic activities, including the control of cell-cycle checkpoints and apoptosis. Germline loss-of-function mutations in TP53 predispose patients to a multiplicity of cancers, and acquired mutations in p53 occur in approximately 10% of AML samples. 107 TP53 mutations are not associated with the chronic phase of MPNs. However, mutated TP53 have been found with a 20% frequency in post–MPN-AML patients. 21-41,108 In one particular case with both MPL and TET2 mutations during chronic-phase ET, multiple TP53 mutations were identified at the time of post–ET-AML that resulted in an oligoclonal pattern in progenitors, with one selected subclone giving rise to leukemic blasts. 109 Thus, the picture of TP53 mutations in post–MPN-AMLs suggests that they play a prominent role in the transformation process.

**RUNX1 mutations**

The RUNX1/AML1 gene encodes a transcription factor with a major role in hematopoiesis. It was found mutated in MDS and
AML. In a series of 34 post–MPN-AML patients, RUNX1 mutations were found in leukemic blasts of 11, most of which were localized in the RUNT domain (residues 50-177). This observation suggests that RUNX1 mutation may represent one of the most frequent genetic events implicated in MPN transformation to AML.

Mutations in “epigenetic regulator genes”: a pre-JAK2 event?

An emerging hypothesis posits that mutations in signaling molecules are not sufficient for disease development in humans and that several cooperating genetic hits might be required to induce disease and allow progression (Table 1, Figure 2). This hypothesis was determined by 4 lines of evidence:

1. Familial MPN has been described with a dominant-autosomal transmission. The phenotype of these familial forms correspond either to a classic MPNs (PV, ET) in the absence of germinal transmission of JAK2V617F or MPLW515L, or to a larger spectrum of diseases (CML, systemic mastocytosis and also acute leukemia).

2. Certain ET and rare PV were clonal as judged by X inactivation assays, whereas JAK2V617F was present only in a minority of cells, suggesting the existence of a pre-JAK2 clone, in which the JAK2V617F mutation has occurred. In some JAK2V617F MPNs associated with the 20q deletion, this deletion was present in the majority of cells, whereas the JAK2V617F burden was low; furthermore, JAK2V617F was not found in some erythropoietin-independent colonies.

3. More surprisingly, it was initially reported that approximately 50% of acute leukemia developing on a JAK2V617F MPNs was JAK2 wild type, even in the absence of previous chemotherapy. This finding suggests the existence of a pre-JAK2 clone, on the background of which 2 independent genetic events have occurred, one being the JAK2V617F mutation leading to a MPN and another one being a genetic alteration leading to leukemia.

4. Hematopoiesis of JAK2V617F MPN is quite surprising for a clonal disorder involving a HSC. In most PV and ET at diagnosis, the allele burden is extremely low in HSC, and the clonal dominance only occurs during late stages of hematopoiesis, corresponding to the maximum activity of hematopoietic cytokines. In contrast, JAK2V617F HSCs predominate in advanced MPNs such as PMF or secondary myelofibrosis. This finding suggests that JAK2V617F confers only a weak advantage to HSC.

As a consequence of the development of whole genome assays (first comparative genomic hybridization and single nucleotide polymorphism arrays and more recently whole genome sequencing), an increasing number of mutations have been observed in BCR-ABL–negative compared with BCR-ABL–positive MPNs, and these
mutations involve genes modifying epigenetic regulation. Mutations in these genes are also found in a great variety of myeloid malignancies, including MDS and AML, suggesting a common pathogenesis in the 3 disorders but implying that these mutated genes are not directly involved in the phenotype of the myeloid malignancies.

Genes that might be mutated before JAK2 in MPNs normally participate in the epigenetic control of transcription. It is not known whether all pre-JAK2 mutations yet to be identified will fall into this category.

**EZH2**

EZH1 and EZH2 proteins (enhancer of zeste homolog) belong to the polycomb repressive complex 2 (PRC2).\(^{121}\) PRC2 is involved in various cellular processes, including proliferation, differentiation, cell-identity maintenance, aging, and plasticity.\(^{121,122}\) In addition to EZH1 or EZH2, PRC2 includes EED, RbAp46/48, SUZ12, AEBP2, JARID2, and PCL. It is thought that PRC2 contributes to chromatin structure regulation. PRC2 methylates histone H3 at lysine 27, a mark of inactive chromatin, through the SET domain of EZH1 or EZH2.\(^{123}\) Its interaction with DNA in mammals is still elusive but may involve YY1 and long intergenic noncoding (ie, Linc) RNAs. It also associates to DNA-methyltransferase (DNMT) proteins to direct DNA methylation.

**EZH2** codes for 1 of the 2 possible catalytic subunits of PRC2. EZH2 proteins are methyltransferases involved in the di- and trimethylation of K27.\(^{123-125}\) EZH2 overexpression is observed in numerous solid tumors such as breast and prostate cancers and may induce dedifferentiation.\(^{126}\) EZH2 is mutated in B-cell lymphomas, but the recurrent mutation targeting Y641 results in a gain of function: the mutated enzyme preferentially generates trimethylated K27.\(^{127}\) In contrast, in myeloid disorders EZH2 mutations may be associated with deletion of the other copy or preferentially with loss of heterozygosity.\(^{36-38}\) EZH2 mutations are predicted to be inactivating through either the truncation of the protein or modification of essential amino acids. They are not associated with a specific MPN or MDS subtype but might be more frequent in MPD/MPNs and are associated with poor prognosis. EZH2 mutations are not observed in ET, but are seen in 3% of PV and 13% of MF.\(^{36}\)

**ASXL1**

ASXL1, together with ASXL2 and ASXL3, are related to the *Drosophila melanogaster* additional sex combs (Asx), a polycomb gene required for long-term repression of the HOX genes.\(^{128}\) Asx has recently been identified in a new polycomb complex (PR-DUB) able to deubiquitinate histone H2A through the activity of the BAP1 protein.\(^{129}\) This activity is the converse of the monoubiquitination mediated by the PRC1 complex, and the balance between the 2 activities is important in the regulation of target genes, such as the HOX genes.\(^{125}\) It is not known whether ASXL1 has a similar function in mammals.

Mutations in ASXL1 are frameshifts and stop mutations located within the 12th exon of the gene; they usually affect only one copy of the gene and result in the loss of the carboxyterminal PHD domain.\(^{34,130}\) The frequency is low in low-grade disorders but greater in late MDS, AML, and in proliferative CMML. However, the veracity of the most frequently reported ASXL1 mutation has been recently questioned.\(^{132}\) In MPNs, ASXL1 mutations are rare in ET and PV (< 7%) but frequent in PMF (from 19%-40%).\(^{34,35}\)

The function of ASXL1 in hematopoiesis is still poorly understood. *Asxl1* knockout mice have a mild defect in hematopoiesis, predominantly in lymphopoiesis.\(^{132}\) A marked decrease in myeloid-erythroid progenitors was observed but without detectable effect on HSC. Its role in human hematopoiesis is unknown.

**TET2**

The Ten-Eleven-Translocation2 (*TET2*) gene codes for a 2-oxoglutarate and Fe(II)-dependent hydroxylase that is able to hydroxylate methylated cytosine (methylcytosine; mC).\(^{133-135}\) This function is shared with the 2 other TET proteins known in mammals, TET1 and TET3.\(^{133,135}\) The function of the resulting modified nucleotide, hydroxymethylcytosine (5mC), is not clear yet, but it appears to be present in all cell types.\(^{136-138}\) 5mC may have a function by itself and/or represent a step toward cytosine demethylation.\(^{138}\) There is preliminary evidence that the function of *TET1* and *TET2* is mainly related to the generation of 5mC.\(^{139}\)

The founding member of the TET family is *TET1*. It has been isolated as a fusion partner of MLL in acute leukemia with the t(10;11)(q21;q32) chromosomal translocation.\(^{140,141}\) *TET2* has 9 coding exons. Exon 2 is included in half of the transcripts starting from exon 1 and contains an ATG; a second ATG is located at the beginning of exon 3. The main TET2 protein is 2002 amino acids long (starting from the ATG in exon 3), but a second product is predicted to be translated from a RNA species that is not spliced at the 3′ side of exon 3 and runs on until a polyA site downstream of exon 4.\(^{30}\)

*TET2* is mutated in a wide range of myeloid malignancies.\(^{30,33}\) Observed mutations are mainly small insertions and deletions and nonsense mutations that are expected to result in the loss of function of the protein. Missense mutations affecting conserved amino acids have been shown also to impact the catalytic activity of the protein. Accordingly, patients with *TET2* mutations have lower global 5mC content than wild type.\(^{142}\) Of note, the global level of 5mC may not directly impact the mC level as evaluated on a gene-specific base because *TET2*-mutated patients have lower mC in MDS samples and greater mC in AML samples.\(^{30,142}\)

*TET2* mutations are found in approximately 14% of MPNs ranging from ET (11%) to PMF (19%).\(^{32,143}\) In roughly 20% of the patients, 2 mutations are observed, suggesting that the inactivation of a single copy of *TET2* is sufficient for the transformation process. *TET2* mutations are not responsible for the familial MPNs identified to date, although a germline mutation has been described.\(^{144}\) In vitro studies have first shown that *TET2* mutations occur before JAK2 mutations during the natural history of sporadic MPNs.\(^{32}\) However, subsequent studies suggest that the converse may happen and also that *TET2* mutations may occur when MPNs transform to AML.\(^{144-147}\)

One caveat: every mutational event that inactivates *TET2* may not have been identified. For example, small deletions outside of the coding sequence or deletion of a single exon would have been missed by the current analyses.

Mutations have been shown to be present in the HSC/progenitor populations.\(^{32}\) In vivo analyses demonstrated that the *TET2*-mutated cells from MPN samples are able to engraft into immunodeficient mice.\(^{32}\) Clone-amplification studies have shown that *TET2* mutations and JAK2V617F act synergistically to induce a clonal dominance at the level of hematopoietic progenitors in PV patients (Figure 3).\(^{32,145}\) TET1 and 2 proteins appear to play a crucial role in stem cell biology as shown in the control of pluripotency of murine embryonic stem cells. Initially, it was suggested that TET1 was involved in the self-renewal of ES cells.
by controlling Nanog, but recent evidence suggests that both TET1 and TET2 are necessary for pluripotency by controlling cell fate through the regulation of distinct sets of genes. Thus, TET2 may have a similar role on HSC. Preliminary results show that in the mouse, in vitro knockdown of TET2 by shRNAs increases monocytic differentiation in the presence of GM-CSF, causing an excess of immature forms. In human hematopoiesis, knocking down TET2 transiently favors monocytic differentiation at the expense of the granulocytic differentiation and decreases the proliferation of erythroid progenitors (F.D., unpublished data, June 2011).

Mutations in EZH2 and in ASXL1 point at deregulation of polycomb activity in MPN. In addition, LMBTL1, a polycomb gene, may be one of the important lost genes in chromosome 20q deletion frequently seen in MPN. It is known that DNMT3A interacts with EZH2, allowing the coupling between PRC2 and DNA methylation. Because DNMT3A interacts with EZH2 and is mutated in AML and MDS, mutational analyses of this gene in MPN may identify a new “epigenetic player” in MPN pathogenesis. Mutations in TET2, ASXL1, and EZH2 genes are observed in a wide range of myeloid malignancies. Furthermore, virtually all the other genes mutated in MPNs are in fact not specific to these disorders. Mutations in JAK2 and MPL have also been described in other diseases, such as acute leukemia, but they affect different amino acids and their frequency is lower than in MPNs.

**Discussion**

Initially, MPNs were considered as simple, single-hit diseases that lead to an increase in mature blood cells. ET and PV, disorders with a pure excess of mature blood cells, fit with this hypothesis, whereas PMF, which also has abnormal MK differentiation, is likely more complex. Thus, as with BCR-ABL in CML, a single mutation in a signaling protein would be at the origin of classic MPNs. The discovery of JAK2 and MPL gain-of-function mutations in classic MPN and the recapitulation of the human disorder in mouse models support this hypothesis. However, other lines of evidence indicate that JAK2V617F mutation may not be the initial event in certain MPNs and that other genetic events may be required for disease development. These data led to the concept of a pre-JAK2 event. At least 3 different genes involved in epigenetic regulation may precede JAK2 mutation in MPNs. It is likely that mutations in other genes have yet to be discovered. Thus, instead of being a single-hit disorder, MPN would result from the combination of several genetic events as exemplified by the finding of EZH2, TET2, and JAK2 mutations in the same MPN patient. Thus, the pathogenesis of the MPN has evolved from a simple to a complex model. At a first glance, this appears to sharply contrast with CML, where BCR-ABL is thought to fully explain the disease, although this is not the case for all CML patients.

**Why would several genetic events be required for the development of MPN?**

The hallmark of these disorders is an increased production of mature blood cells. In some instances, a single germline mutation can induce thrombocytosis, erythrocytosis, or neutrophilia. Inherited diseases, all HSC carry the mutation, whereas in sporadic MPNs the signaling mutation (JAK2V617F, MPLW515L, or BCR-ABL) is acquired by a HSC that must compete with wild-type HSCs.

Mathematical models predict that at least 1%-2% of the HSC need to carry the JAK2V617F mutation to produce the clinical phenotype. Given an estimate of 1 × 10⁶ HSCs in humans the initial JAK2V617F-positive HSC must expand 10⁴ fold more than wild-type HSC before inducing a clinical phenotype. Evaluation of the JAK2V617F burden at different hematopoietic differentiation steps in humans has shown that the clonal expansion occurs mainly in late differentiation steps in PV and ET. In addition, CD34⁺ cells from PV or ET patients at diagnosis generate essentially JAK2 wild-type hematopoiesis in xenograft experiments, showing that JAK2V617F HSC are still in minority in early disease. Furthermore, JAK2V617F HSC did not outcompete JAK2 wild-type HSC in one case of allogeneic human BM transplantation. This mild effect of JAK2V617F on HSC biology is also observed in mouse models. Transgenic JAK2V617F HSCs displayed only a slight advantage on JAK2 wild-type HSC in transplantation assays. Furthermore, in one knockin model, JAK2V617F HSCs were at a disadvantage. These results are unexpected because the TPO/MPL axis plays an important role in HSC self-renewal and quiescence in the BM niche. Thus, it is possible that MPL signaling differs between HSC and MKs and/or that JAK2V617F only partially activates normal MPL functions in HSC.

Therefore, a crucial (and presently unknown) factor in MPN pathogenesis is the latency between the mutation of JAK2 and the appearance of the disease. On the basis of the log-log incidence of MPN with age (that increases after 60 years), mathematical models suggested that a single mutation, such as JAK2V617F, although providing a minor advantage to HSC, may cause a MPN with a very long latency (>45 years). Another prediction of the model is that >95% of the JAK2V617F clones die by exhaustion and disappear. Therefore, cooperation of JAK2V617F with other genetic events modifying HSC biology will greatly facilitate the development of a clinical phenotype. It cannot be excluded, however, that JAK2V617F may give a significant advantage to HSC in specific conditions such as inflammation, as suggested by preliminary studies. It is thus conceivable that JAK2V617F HSC remain harmless during a long period, until genetic or environmental changes such as hematopoietic stress or aging allow clonal dominance and MPN emergence. Of note, clonal dominance occurs at the HSC level in PMF that frequently carry several mutations.

**Does a pre-JAK2 event really exist?**

There is clear evidence that TET2 mutations, and also ASXL1 and EZH2 mutations, may precede JAK2V617F but that the converse may also occur. This finding suggests that the important issue is not the order of appearance of the mutations but the fact that they all occur in a HSC with the MPN phenotype being mainly driven by the JAK2V617F mutation. There is also evidence that secondary acquisition of TET2 mutations may be associated with disease progression and transformation. In support of this notion, it has been shown that TET2, EZH2, or ASXL1 mutations are far less frequent in PV and ET than in PMF, which combine at least 2 mutations.

Two classes of MPNs may thus exist; a simple one, in which a single JAK2 or MPL mutation induces PV or ET, and a complex class, with 2 or more mutations producing PMF. For this reason, as previously suggested, PMF might be the accelerated phase of classic MPN. Next-generation sequencing may help establish whether a large fraction of ET and PV at diagnosis is the consequence of a single genetic event.
In several MPN patients, the coexistence of 2 oncogenic events, such as JAK2V617F, MPLW515L, JAK2 exon 12, BCR-ABL, and c-CBL mutants, has been demonstrated.\(^{18,20,174-178}\) In nearly all these cases, the 2 events have occurred in 2 different cells; moreover, independent acquisitions of JAK2V617F in a single patient have been reported.\(^{108,179}\) In one case it has been demonstrated that the MPLW515L and JAK2V617F subclones were derived from the same ancestral TET2-mutated clone.\(^{21}\) Thus, a reasonable hypothesis is that TET2, ASXL1, and EZH2 mutations favor the occurrence of secondary genetic events. From the original clone, a complex combination of mutations that does not follow a linear dynamics may give rise to different subclones. Depending on the type of dominant clone, different hematopoieses evolving in time and appearing in different sequences will be induced. Such a model will explain the occurrence of JAK2 wild-type leukemia in JAK2V617F-positive MPNs, whereas, in contrast, the linear accumulation of mutations in the JAK2V617F-dominant clone will favor the progression of MPN to myelofibrosis and leukemia. This hypothesis has been validated only in one case of wild-type JAK2 AML, which had emerged from an original TET2 mutated clone found in the initial MPN.\(^{180}\)

In other examples, TET2 mutations were only found in the leukemic cells.\(^{21,147}\) There are 2 possible scenarios for these patients:

1. The ancestral clone exhibits a mutation different from a coding sequence mutation on TET2 as exemplified by the EZH2 mutation preceding TET2 mutation.\(^{56}\)
2. AML and MPN are independent disorders. This would mean that a “mutator” phenotype exists in some MPN patients. This phenotype may be acquired, for example, related to changes in the hematopoietic environment\(^{181,182}\) or in the germline. This last possibility is supported by a marked increased risk (\(~5\) to \(7\)-fold) among first-degree relatives of MPN patients.\(^{183}\) Such a risk has been at least partly related to 3 SNPs in the JAK2 gene, including one in the exon 14 defining the haplotype 46/1. The haplotype 46/1 is present with an allelic prevalence of approximately \(10\%\) in the normal populations and confers a \(3-\) and a \(1.4\)-fold increase in the risk of developing a JAK2V617F or exon 12 or a MPLWS15 MPN, respectively.\(^{184-187}\) The mechanism of this low penetrance susceptibility is unknown. This predisposition is different from the familial forms of MPN with a Mendelian transmission.\(^{112}\) However, none of the genetic events at the origin of this familial form is presently known, and this could correspond either to a “mutator” phenotype related to an alteration in gene repair or to a haploinsufficiency in a tumor suppressor gene. The precise understanding of the molecular mechanism of these familial forms will be certainly be critical for understanding the pathogenesis of MPN.

What are the consequences for therapy?

New targeted therapies against JAK2 have been developed.\(^{188-191}\) In contrast to the initial results, which showed a targeted effect of JAK2 inhibitors on JAK2V617F cells, including HSC,\(^{192,193}\) there is increasing evidence that they also affect JAK2 wild-type and JAK2V617F cells although having less effect on JAK2V617F HSC.\(^{59}\) This finding supports the clinical results that have shown good clinical responses in constitutional symptoms like asthenia and splenomegaly in myelofibrosis but a weak effect on the JAK2V617F clone.\(^{180}\) However, in cases in which JAK2V617F is not the initiating event, this approach may revert the MPN hematopoiesis to a pre-JAK2 clonal hematopoiesis (pre-JAK2 disorder) with the risk of developing other malignant blood disorders. The proof of this potential was demonstrated in one PV patient treated by pegylated IFN-\(\alpha\), where, after treatment, the JAK2V617F burden was at the threshold of detection, but the hematopoiesis was clonal because of a preexisting TET2 mutation.\(^{194}\) More recently, studies of “epigenetic” therapies in which the authors use either HDAC inhibitors or “demethylating” agents have been initiated.\(^{195-198}\) It is unknown whether these therapies will be able to target the alterations induced by ASXL1, EZH2, or TET2 mutations.

Future studies aimed at identifying mutations present in MPN in which the authors use new-generation sequencing will be crucial not only to characterize the many cases of MPNs with no known molecular cause but also to better understand the evolution of the clone and the synergistic effects of the different mutations. This will be very important for understanding MPN pathogenesis and develop new therapies, and also, more generally, to understand the stepwise mechanisms leading to cancer development in humans.

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References


111. Molteno AR, Williams DM, Rogers Q, Leacons MA, Spivak JL. Phenotypic variability within the JAK2 V617F-positive MPD: roles of progenitor cell and...


140. Lonsdorf RB, Moore J, Mathew S, Raimondi SC, Mukhtar ST, Downing JR, TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia. 2003;17(3):637-641.


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