Molecular biology of Philadelphia-negative myeloproliferative neoplasms

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Myeloproliferative neoplasms are clonal diseases of hematopoietic stem cells characterized by myeloid hyperplasia and increased risk of developing acute myeloid leukemia. Myeloproliferative neoplasms are caused, as any other malignancy, by genetic defects that culminate in the neoplastic phenotype. In the past six years, since the identification of JAK2V617F, we have experienced a substantial increase in our knowledge about the genetic mechanisms involved in the genesis of myeloproliferative neoplasms. Mutations described in several genes have revealed a considerable degree of molecular homogeneity between different subtypes of myeloproliferative neoplasms. At the same time, the molecular differences between each subtype have become clearer. While mutations in several genes, such as JAK2, myeloproliferative leukemia (MPL) and LNK have been validated in functional assays or animal models as causative mutations, the roles of other recurring mutations in the development of disease, such as TET2 and ASXL1 remain to be elucidated. In this review we will examine the most prevalent recurring gene mutations found in myeloproliferative neoplasms and their molecular consequences.

Keywords: Polycythemia vera; Primary myelofibrosis; Thrombocytopenia, essential; Leukemia, myeloid, acute; Myeloproliferative disorders; Janus Kinase 2

Introduction

Myeloproliferative neoplasms (MNs) are clonal disorders of hematopoietic stem cells characterized by increased proliferation of myeloid cells and an increased risk of developing acute myeloid leukemia (AML). (1)

Traditionally, MNs have been diagnosed and classified according to which cell type (erythrocytes, platelets or granulocytes) predominates in the peripheral blood and to the bone marrow histology. Since the discovery that the Janus Kinase 2 (JAK2) V617F mutation is present in more than 60% of MN patients, (2-5) the identification of this and other equivalent mutations, such as JAK2 exon 12 (6) and myeloproliferative leukemia (MPL) exon 10 mutations, (7) has become an essential step in the diagnosis of these disorders. While the absence of these mutations does not exclude the diagnosis, its presence in the context of myeloid proliferation confirms the diagnosis of MNs.

Regarding the specificity of these mutations for the diagnosis of specific subtypes of MNs, polycythemia vera (PV), essential thrombocytopenia (ET) and primary myelofibrosis (PMF), the picture is very different from that of chronic myelogenous leukemia (CML), in which the presence of the hybrid gene BRC-ABL1 establishes the diagnosis. With the exception of JAK2 exon 12 mutations, which are specific for PV, JAK2V617F can occur in PV, ET and PMF, among other diseases and MPL mutations occur both in ET and PMF.

We now know that other genes are mutated in patients with MN and the physiologic consequences of each one of these mutations are starting to be unveiled. The most relevant mutations identified so far can be broadly classified into three main groups:

1 - Mutations associated with constitutive Signal Transducer and Activators of Transcription (STAT)3/5 activation.
2 - Mutations associated with transcriptional regulation.
3 - Mutations associated with progression to AML.

In this review we will examine the advances in understanding the molecular biology of the BCR-ABL1 negative MNs: PV, PMF and ET and the practical implications of these developments.

Other MNs such as mast cell disease, chronic neutrophilic leukemia and chronic eosinophilic leukemia will not be discussed here.
Mutations associated with constitutive STAT3/5 activation

The JAK-STAT signaling pathway is essential for normal hematopoiesis. This pathway is "turned on" after activation of cell receptors by its ligands, establishing the link between extracellular stimuli and the cellular effects of numerous growth factors, cytokines, interferon, etc.

Upon binding of the ligand, conformational changes in the receptor lead to JAK and STAT phosphorylation. Once phosphorylated, STATs form homodimers and translocate to the nucleus where they will promote transcription of specific genes. Constitutive STAT3 or STAT5 activation is the hallmark of most, if not all, patients with MNs. Although STAT mutations are rare in MN patients, activating mutations in STAT inhibitors [LNK and Cas-Br-M ecotropicretroviral transforming sequence (CBL)] have been documented in most patients with MN.

Janus Kinase 2 (JAK2)

JAK2 is a non-receptor tyrosine kinase downstream of a vast number of cytokine receptors, such as erythropoietin (EPO), MPL and granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR). JAK2V617F is the most prevalent mutation found in BCR-ABL negative MNs; it is present in roughly 95%, 50% and 60% of PV, ET and PMF patients, respectively. JAK2V617F affects the regulatory pseudokinase domain leading to constitutional activation of JAK2 and, as a consequence, permanently activated STAT3/5. JAK2V617F induces myeloproliferative disease in mice confirming its role as a causal mutation.

While homozygous JAK2V617F mutations are frequently found in PV and PMF patients, most TE patients are heterozygous. This finding contributes to the idea that PMF could represent an advanced stage MNs, with ET and PV representing early stages of the same disease.

Other mutations in the JAK2 gene have been described, occurring predominantly in exon 12. These mutations are specific to PV patients and are mutually exclusive with JAK2V617F.

Due to its high prevalence, the detection of JAK2 mutations has become an essential step in the diagnosis of myeloproliferative disorders. Regarding the prognostic importance of JAK2 mutations, the findings are not conclusive yet; most studies have not found differences in survival or progression to AML between patients with and without JAK2 mutations. Intriguingly, a low JAK2V617F allele burden has been associated with worse survival in PMF.

Myeloproliferative leukemia virus oncogene (MPL)

MPL is the gene encoding the thrombopoietin receptor. Exon 10 MPL mutations are found in about 6% and 3% of patients with PMF and ET, respectively, and are not mutually exclusive with JAK2V617F. No MPL mutations have been described in PV patients. Expression of MPLW515L in cell lines resulted in constitutive phosphorylation of JAK2, STAT3, STAT5, AKT and extracellular signal-regulated kinase (ERK). In a murine model assay, the presence of MPLW515L induced a PMF-like disease, confirming the role of MPL mutations as causative agents in MNs.

Cas-Br-M ecotropicretroviral transforming sequence (CBL)

The CBL protein can act either as a positive or negative regulator of tyrosine kinase signaling. It can bind to activated receptors, acting as an adaptor protein, recruiting downstream molecules, but it also has E3 ubiquitin ligase activity, marking active kinases for degradation, such as FLT3, KIT and MPL. CBL mutations are present in 6% of PMF patients and also in other myeloid malignancies. In most cases the mutation is homozygous due to uniparental disomy. To the extent of our knowledge, CBF mutations have not been described in chronic phase ET or PV.

Transfection of CBL mutants was sufficient to induce an oncogenic phenotype in fibroblast cell lines. Additionally, mice with the CBL null phenotype developed a disease characterized by splenomegaly and augmented hematopoietic progenitor pool, suggesting a role for this gene in the development of MNs. Additionally, mutated CBL inhibited E3 ubiquitin ligase activity of wild type CBL in transfected fibroblasts, revealed a dominant negative role for the mutated protein and not only a classical tumor suppressor effect.

SH2B adaptor protein 3 (SH2B3)/LNK

LNK is an adaptor protein that acts as a negative regulator of cytokine signaling. The LNK protein specifically interacts with JAK2 (wild type or mutant V617F), MPL (wild type or mutant W515L) inhibiting the downstream activation of STAT. LNK mutations are found in roughly 13% of patients with blast phase MNs but are rarely found in chronic phase ET, PMF and JAK2 wild type erythrocytosis. The few mutations described affect predominantly a hot spot at exon 2 and are heterozygous. Mutant LNK has diminished/abolished capacity to inhibit JAK2 signaling in thrombopoietin (TPO)-dependent cell lines, showing that the mutation causes a protein loss of function, making LNK a tumor suppressor gene. Primary samples from patients harboring LNK mutations presented increased STAT3/5 activation, suggesting that the loss of function caused by LNK is analogous to JAK2 or MPL gain of function mutations. Mice homozygous for LNK deficiency display a phenotype characterized by splenomegaly, extramedullary hematopoiesis and increased numbers of hematopoietic precursors. Heterozygous mice display a similar but milder phenotype consistent with a haploinsufficiency model.
Mutations associated with transcriptional regulation

Gene transcription is a complex cellular function that relies on the coordinated functioning of different classes of proteins, such as RNA polymerases, transcription factors, histone deacetylases and DNA methyltransferases.\(^{(37)}\) Recently, recurring mutations in genes involved in transcriptional regulation have been described in myeloid diseases.\(^{(38\text{-}40)}\) In MNs, the most prevalent mutations in this group of genes are found in additional sex combs-like 1 (ASXL1), enhancer of zeste homolog 2 (EZH2), TET2 and IDH1/2.

Additional sex combs-like 1 (ASXL1)

ASXL1 is a member of the polycomb group of proteins involved in transcriptional repression and activation. ASXL1 represses retinoic acid receptor-mediated transcription via its N-terminal region by the modulation of histone 3 demethylation.\(^{(41)}\) Mutations in ASXL1 are mainly heterozygous, nonsense and frameshift, occurring predominantly in exon 12 and disrupting the C-terminal region of the protein. ASXL1 mutations are found in 32%, 36%, 50% and 18% of patients with PMF, post-PV MF, post-ET MF and post-MN AML, respectively.\(^{(42,43)}\) Only rarely found in ET and PV.\(^{(44)}\) If the mutations generate loss or gain of function is still unknown. The prognostic significance of ASXL1 mutations is not established in the literature.\(^{(43)}\) In animal models, loss of function of ASXL1 expression caused only mild defects in hematopoiesis, suggesting it may have a modulating, rather than causal effect in MNs.\(^{(45)}\)

Enhancer of zeste homolog 2 (EZH2)

EZH2, like ASXL1, is a member of the polycomb group of proteins. It is part of a protein complex (called polycomb repressive complex 2) which is a histone 3 methyltransferase associated with transcriptional repression.\(^{(46)}\) EZH2 mutations in myeloid diseases can be homozygous or heterozygous, nonsense or frameshift, resulting, in most cases, in truncation of the protein.\(^{(40)}\) Additionally, it has been shown that EZH2 mutations in myeloid malignancies cause absence of histone 3 methylation at Lysine 27, revealing a loss-of-function phenotype compatible with a tumor suppressor function for EZH2.\(^{(48)}\) Mutations in EZH2 are present in 13% and 3% of patients with MF (primary or secondary) and PV, respectively.\(^{(40)}\) Moreover, the presence of EZH2 mutations has recently been associated with decreased overall survival in patients with PMF.\(^{(39)}\)

Tet oncogene family member 2 (TET2)

TET2 encodes a ketoglutarate-dependent methylcytosine dioxygenase that converts methylcytosine to 5-hydroxymethylcytosine (5-HMC).\(^{(47)}\) 5-HMC is widely distributed in all tissues, but its precise function is unknown.\(^{(48)}\) There is some evidence suggesting that 5-HMC could be either an intermediate step in the process of cytosine demethylation\(^{(49)}\) or a modification associated with gene de-repression, without going through demethylation.\(^{(50)}\) TET2 mutations occur over all exons and can be missense or nonsense substitutions, splice site, insertions or deletions resulting in frameshift or stop codons. Most mutations are expected to result in loss of protein function thus classifying TET2 as a tumor suppressor gene. Additionally, the mutations can be heterozygous, homozygous or compound heterozygous.\(^{(47,51)}\) The prevalences of TET2 mutations are 5%, 16%, 17% and 17% in ET, PV, MF and blastic phase MNs, respectively.\(^{(52)}\) TET2 knockout mice present reductions in 5-HMC levels and develop myeloid malignancies resembling MNs, myelodysplastic syndromes and AML.\(^{(53)}\) The presence of TET2 mutations has added no prognostic significance in MNs in most studies.\(^{(54)}\)

Isocitrate dehydrogenase 1 and 2 (IDH1/2)

IDH1 and 2 are enzymes that participate in the citric acid cycle catalyzing the conversion of isocitrate to a-ketoglutarate. Mutations in either IDH1 or IDH2 are present in: 0.8%, 1.9%, 4.2% and 11% to 21% of ET, PV, PMF and blast-phase MNs, respectively. IDH1 and IDH2 mutations are mutually exclusive and affect three specific arginine residues: IDH1-R132, IDH2-R140 and IDH2-R172.\(^{(55,56)}\) It has been shown that IDH1/2 mutations are associated to lower leukemia-free survival and overall survival in patients with JAK2V617F PMF.\(^{(57)}\) In addition, the documentation of chronic phase IDH mutations in two patients that progressed to AML suggests that IDH may be used as a surrogate marker for AML progression.\(^{(57)}\) Mutated IDH1/2 have a neo-enzymatic activity. Instead of converting isocitrate to a-ketoglutarate, the mutated enzyme converts a-ketoglutarate to 2-hydroxyglutarate, resulting in a reduction of a-ketoglutarate (essential for TET2 activity) and an accumulation of 2-hydroxyglutarate in the neoplastic cells.\(^{(58\text{-}60)}\) Moreover, recent evidence suggests that 2-hydroxyglutarate is an inhibitor of a-ketoglutarate.\(^{(61)}\) In conclusion, these findings suggest a mechanistic link between IDH and TET2, in which IDH mutations would cause (like TET2 mutations) decreased TET2 activity and may explain the low incidence of concurrent mutations in IDH and TET2.\(^{(55,61,62)}\)

Mutations associated with disease progression to AML

Mutations associated with disease progression are, by definition, found more frequently in post-MN AML and rarely in chronic phase disease. It has yet to be determined if these mutations happen before disease acceleration, contributing to it, or only at the time of progression to AML. If the former proves to be the case in large cohorts, these mutations could serve as surrogate markers for transformation and consequently could help in the indication of more aggressive treatment strategies such as...
hematopoietic stem cell transplantation. The genes whose mutations are associated with blastic phase MNs are RUNX1, TP53, IKZF1, LNK and IDH1/2. Since LNK and IDH1/2 have already been discussed, we will now focus on the former three.

**Run-related transcription factor 1 (RUNX1)**

The protein encoded by this gene is the alpha subunit of the transcription factor called core binding factor (CBF). CBF is essential for hematopoiesis and its disruption by either translocations or point mutations is a frequent event in AML and MDS.\(^{(63)}\) It has been shown that RUNX1 C-terminal mutations impair the DNA-damage repair response.\(^{(64)}\) RUNX1 mutations in chronic phase MNs are rare\(^{(63,65)}\) but its acquisition in blastic phase of patients with wild type RUNX1 has been documented in 27% (5 out of 18) of patients.\(^{(65)}\) In another study, 37.5% (6 out of 16) of post-MN AML patients were positive for RUNX1 mutations.\(^{(66)}\) The mutations are mostly located in the RUNT domain, are heterozygous and are probably associated with loss of function.\(^{(66)}\)

**Tumor protein p53 (TP53)**

TP53 encodes a tumor suppressor gene that has a central role in the cellular response to stress. It can activate DNA repair, induce cell cycle arrest, apoptosis and senescence after genomic damage. TP53 is one of the most frequently mutated genes in a range of cancers. TP53 mutations are rarely found in chronic phase MNs, but they have been found in 20% (4 out of 16) of post-MN AML patients.\(^{(66)}\) The mutations cause loss of function of the protein and in all cases both alleles were affected. In another study, TP53 was found mutated in 27.3% and 3.1% of post-MN AML and chronic phase (heterozygous mutations) patients, respectively.\(^{(67)}\) In both studies, TP53 mutations were documented in the chronic phase before progression to AML, suggesting that it may be useful as a predictor of progression to AML. Additionally, amplifications of chromosome 1q have been documented in 0.32% and 18.18% of patients with chronic phase and post-MN AML.\(^{(67)}\) The amplified region harbored MDM4, an inhibitor of p53, amplified in other malignancies.\(^{(68,69)}\) In this study, TP53 mutations and 1q amplifications were mutually exclusive, suggesting a similar role for both genetic changes. In total, 45.5% of the patients with post-MN AML had an abnormality in either TP53 or chromosome 1q.\(^{(67)}\)

**IKAROS family zinc finger 1 (IKZF1)**

IKZF1 encodes a transcription factor associated with chromatin remodeling, essential for normal lymphopoiesis. Mutations in IKZF1 have been described in lymphoid malignancies, with deletions being particularly frequent in Ph\(^{+}\) acute lymphoblastic leukemia (ALL).\(^{(70)}\) IKZF1 deletions are present in less than 1% and 20% of chronic phase and post-MN AML, respectively.\(^{(71)}\) The absence of IKZF1 deletions in the chronic phase of three patients with AML suggests that IKZF1 mutations may be a late event in the progression to AML and therefore not useful as a surrogate marker.\(^{(71)}\)

**Discussion**

Medicine is evolving towards the complete molecular characterization of diseases. In few years, all the DNA-associated changes, both genetic or epigenetic, causing diseases will be characterized and consequently used for diagnostic, prognostic stratification or therapeutic purposes. With the advent of the new generation of DNA sequencers, the rate of discovery of new mutations has been greatly accelerated, and our understanding regarding the molecular events associated with distinct phenotypes has followed the same trend. It is reasonable to believe that in the near future, with the identification of other mutations, we will be able to detect the multiple molecular events associated with each disease in the majority of patients. The challenge now is to define disease entities that are better associated with prognosis and response to treatment than the classic histopathological classification could predict. Regarding MNs, the use of RUNX1, TP53 and IDH mutations will probably be included soon in the clinical evaluation, since they may predict transformation to AML and therefore the need for more aggressive treatment.\(^{(57,65-67,72)}\) Additionally, mutated IDH1 and 2 are good candidates for targeted therapy, since both are neo-enzymes with definite functions.

In summary, the molecular events causing MNs and the progression to AML are just starting to be unveiled. The complete characterization of all steps involved in this process will substantially change disease classification and certainly lead to better treatment strategies.

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