Molecular Genetics in Diagnosis and Prognosis of Philadelphia Chromosome Negative Myeloproliferative Neoplasms

Molecular genetics in MPNs

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Chronic myeloproliferative neoplasms (MPNs) represent a heterogeneous group of myeloid neoplasia whose molecular asset is marked by the presence of a JAK2^{V617F} mutation. Following this seminal discovery, several other mutations have been identified highlighting an unexpected molecular complexity. Deregulation of the JAK/STAT pathway is a central feature of MPNs and, although it has raised much interest for the possibility of targeted therapy with JAK2 inhibitors, it has been realized that JAK2 mutations are secondary mutational events in most, if not all, cases. Another recurrent theme is the involvement of genes intervening in the epigenetic control of gene expression and, more recently, in RNA splicing. Most of these mutations are shared by patients with myelodysplastic syndromes as well. Studies focusing on the complex clonal hierarchy of MPNs suggest a condition of genetic instability, that could be either acquired or inherited. At this regard, the discovery of a specific germline haplotype in JAK2 provided an explanation for the phenomenon of familial clustering of MPNs, although other still unknown haplotypes are likely involved. The aim of this review is to summarize current knowledge of molecular abnormalities of MPNs and discuss their role for diagnosis and prognosis.

Key words: myeloproliferative neoplasms, mutations, JAK2, prognosis.

Molekulární genetika v diagnostice a prognóze Ph-negativních myeloproliferativních neoplázií
Molekulární genetika u MPN

Chronické myeloproliferativní neoplázie (MPN) představují různorodou skupinu myeloidních neoplázií, jejichž molekulární podstata je charakterizovaná přítomností mutace JAK2^{V617F}. Po tomto zásadním objevu bylo zjištěno několik dalších mutací, což jen zdůraznilo nečekanou molekulární složitost. Ústředním rysem MPN je deregulace dráhy JAK/STAT a i když vzbudila velký zájem vzhledem k možnosti cílené léčby inhibitory JAK2, dospělo se k závěru, že ve většině případů, ne-li ve všech, jsou mutace JAK2 sekundární mutační událostí. Další opakující se otázkou je postižení genů ovlivňujících epigenetickou kontrolu genové exprese a nově také sestřih (splicing) RNA. Většinu těchto mutací mají rovněž pacienti s myelodysplastickými syndromy. Studie zaměřené na složitou klonální hierarchii MPN svědčí o stavu genetické nestability, který by mohl být buď získaný, nebo dědičný. V tomto ohledu nám objevení specifického zárodečného haplotypu u JAK2 poskytlo vysvětlení jevu „familiárního clusteringu" MPN, přestože se na něm pravděpodobně podílejí i jiné, dosud neznámé haplotypy. Cílem tohoto přehledu je shrnout současné poznatky o molekulárních abnormalitách MPN a probrat jejich úlohu v diagnostice a prognóze.

Klíčová slova: myeloproliferativní neoplázie, mutace, JAK2, prognóza.

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Introduction

The Philadelphia-chromosome negative (Ph'-neg) classic, chronic myeloproliferative neoplasms (MPNs) include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) (1); also included are post-polycythemic or post-thrombocythemic myelofibrosis (PPV/PET-MF) that originate from usually late evolution of a previous PV or ET and are substantially indistinguishable from PMF. The molecular lesion(s) at the basis of MPNs has remained unknown until 2005, when a point mutation in exon 14 of Janus kinase 2 gene (JAK2^{V617F}) was reported by four groups almost at the same time (2–5); additional studies showed that the V617F allele is harbored by more than 95 % of patients with PV and about 60 % of ET or PMF (6–13). Other JAK2 mutations were soon described in JAK2 exon 12 in some PV patients lacking the V617F allele (14), while mutations at codon 515 of MPL (15) were discovered in 3–8 % of patients with ET and PMF (16–19). These mutant alleles all result in constitutive activation of tyrosine kinase-dependent cellular signaling pathways, particularly the JAK-STAT pathway (gain-of-function mutations) (20). However, evidence of activation of the JAK/STAT pathway is found also in patients with wild-type JAK2 or MPL, pointing to possibly other unknown

Table 1. A working functional classification of most common mutations occurring in MPNs

Mutations affecting the JAK/STAT signaling	Mutations affecting epigenetic gene regulation	Mutations affecting the splicing machinery	Mutations preferentially associated with leukemic transformation
JAK2^{V617F}	TET2	SF3B1	IDH1/2
JAK2 exon 12	EZH2	SRSF2	IKZF
MPL	ASXL1		TP53
LNK	IDH1/2		NF1
C-CBL	DNMT3A		RUNX1
SOCS1-3	Members of the PCR2		NRAS
	JAK2^{V617F}		KRAS
			DNMT3A

mutations insisting on the same pathway. More recently, the spectrum of MPN-associated mutations expanded to include several others genes such as genes involved in epigenetic gene regulation and RNA splicing machinery (Table 1). Many of these abnormalities are shared by other myeloid malignances, including myelodysplastic syndromes (MDS), myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and acute myeloid leukemias.

As a whole, these information have significantly advanced our understanding of the pathophysiology of MPNs, although their intrinsic complexity is still far from being entirely understood within an unitary biomolecular picture. However, mutation discoveries have been instrumental for prompting a modification of established diagnostic criteria that finally resulted in the 2008 revised WHO classification (21, 22), and stimulated intensive research to understand how they could impact on disease phenotype and prognosis. Finally, the understanding of a direct or indirect dysregulation of the JAK/STAT signaling pathway as a central theme of MPN pathogenesis and phenotype put the basis for the development and clinical exploitation of JAK2 inhibitors, resulting in one of these small molecules, Ruxolitinib, as becoming the first drug ever approved for the treatment of myelofibrosis (23, 24). In this short review we will focus on the most common genetic abnormalities reported in MPNs, their impact on diagnosis and their use as prognostic biomarkers; a brief discussion of genetic predisposition haplotype is also included.

Mutation abnormalities in MPNs

The number of mutations that are being discovered in MPNs is steadily increasing (25, 26). As a working approach, those mutations could be categorized as the most typically MPN-associated mutations involving genes of the JAK/STAT pathway, mutations in genes regulating gene expression at the epigenetic level, the last discovered mutations in the spliceosome machinery, and finally mutations that have been mostly, but not exclusively, associated with leukemic transformation (Table 1).

The JAK2^{V617F} mutation, due to a G–>T change in exon 14 is located in the JH2 pseudokinase domain; it is detected in greater than 95 % of PV and about 60 % of ET and PMF. Mutations in JAK2 exon 12, found in rare patients with a WHO-based diagnosis of PV who lack the JAK2^{V617F} mutation and in some cases of "idiopathic erythrocytosis", are heterogenous and present as complex insertion/deletions in a short region between the SH2 and JH2 domain (27). Both mutation abnormalities result in a constitutive activation of the phosphorylase activity of JAK2 due to the relief of inhibition exerted by the JH2 domain on the catalytically active JH1 domain. Other mutations that affect the JAK signaling involve LNK (28), that encodes for a member of a family of adaptor proteins involved in the negative regulation of JAK/STAT signaling. Mutations in LNK have recently been reported in patients with JAK2-negative MPN (28) including subjects with erythrocytosis (29), with apparently higher rate after blastic transformation (30). CBL is a protein that controls tyrosine kinase signaling both by serving as an adaptor recruiting other signaling components and controlling protein ubiquitinilation. Missense mutations at CBL exon 8 and 9 have been reported in approximately 10 % of PMF patients while they are very rare in PV and ET (31).

Among epigenetic genes, abnormalities of TET2, EZH2 and ASXL1 are the best characterized in MPNs. TET2 (Ten-Eleven-Translocation-2) is located on 4q24 and contains 11 exons; other members of the family are TET1 and TET3. Known function of TET proteins is to accomplish 5-methylcytosine hydroxylation resulting in the generation of 5-hydroxymethylcytosine (hmC); hmC has been found enriched in actively transcribed genes and in the promoters of polycomb-repressed elements that are normally activated during development of embryonic stem cells (32). TET2 mutations are found in myeloid malignancies (33) including classic MPNs (approximately 14 %), mastocytosis, MDS, chronic myelomonocytic leukemia (50 %) and in post-MPN or de-novo AML. TET2 mutations may precede or follow JAK2^{V617F} mutation (33, 34) or occur at the time of disease transformation to AML (35). Mutations are scattered over the gene and consist of small insertions, deletions and nonsense mutations, all resulting in a loss-of-function of the protein, and missense mutations affecting conserved amino acids in catalytically active regions. TET2 alterations are most commonly heterozygous, suggesting that TET2 haploinsufficiency may be a mechanism sufficient for transformation. EZH2, located on 7q36.1, encodes for the PcG Enhancer of Zeste Homolog 2, the catalytic component of the polycomb repressive complex 2 (PRC2) that methylates histone H3 at lysine 27 (H3K27me3), a marker of inactive chromatin. Macro- and micro-deletions of the genomic region containing EZH2 have been found in about 10 % of MDS, with a few subjects presenting loss-of-heterozygosity due to acquired uniparental disomy (36, 37). Mutations of EZH2 have been reported in patients with PMF, MDS, MDS/MPN (36–38); they are scattered throughout the gene and include missense, nonsense and premature stop codons resulting in loss of function. Both monoallelic and biallelic mutations were described. ASXL1 at chr 20q11.21 encodes the Additional SeX combs–Like protein-1, which is one of the 3 mammalian homologs of Drosophila Additional Sex Comb (Asx) gene. ASXL1 consists of 12 exons; frameshift mutations, nonsense mutations, and large 20q11 deletions of ASXL1 have been described in 10–15 % of MPNs and MDSs, 40 % of CMML (particularly in the myeloproliferative subset, 60 %), in refractory anemia with ring sideroblasts and thrombocytosis, a few patients with chronic myelogenous leukemia and 15–20 % of acute leukemias (39). Among MPNs they are found mainly in patients with myelofibrosis, with frequency ranging from 5 % to 20 % depending on the series (40–42). Most ASXL1 mutations are found in exon 12, spanning the region from Tyr591 to Cys1519, and disrupt the protein downstream of the ASXH domain with loss of the PHD domain.

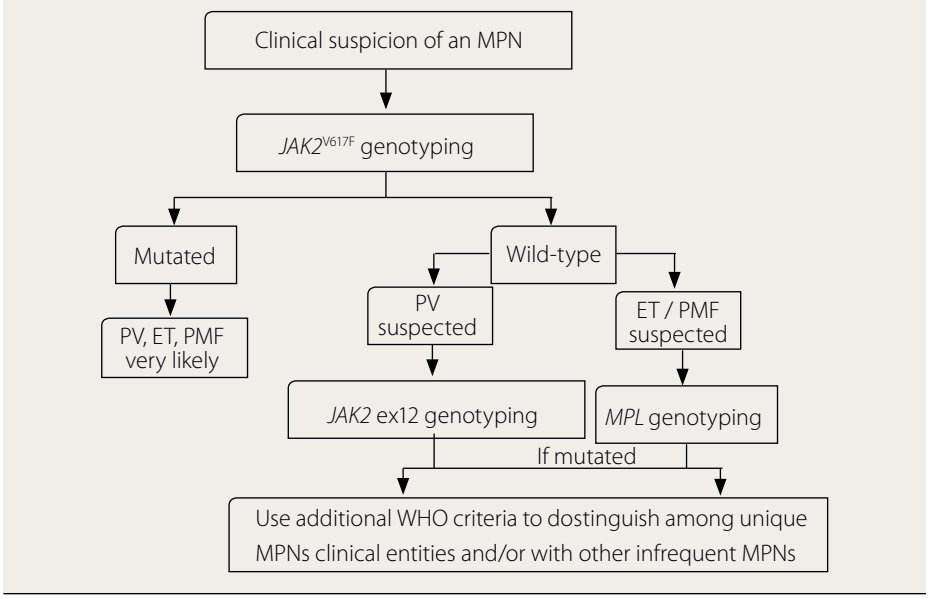
Recently, mutations in genes of the spliceosome machinery, including SF3B1, SRSF2, U2AF1 have been reported in MDS patients as well as in MPNs (43–45), particularly in myelofibrosis (46). Patients with acute leukemia developed from a previous MPN have been reported to present a high rate of mutations in SRSF2 (approximately 19 %) (47).

The rate of leukemic transformation in MPN patients is highest among those with myelofibrosis. Mutations in FLT3, NRAS (48), NPM1 (49), RUNX1 (50), DNMT3A (51), IDH1, IDH2 (52), TET2 (33, 53) and TP53 (54) have been all implicated in leukemic transformation, as well as several chromosomal aberrations, such as deletions of IKZF1 (55), JARID2, AEBP2 (56) and amplifications of MDM4 (54). However, a definite picture of the leukemogenic process is still far from being delineated.

Germline predisposition haplotypes

The well-known phenomenon of familial clustering of MPNs supported the hypothesis of a genetic predisposition (57). In 2009, it was discovered that the JAK2^{V617F} mutation is acquired preferentially on a specific constitutional JAK2 haplotype, named 46/1 or GGCC; this common, very low penetrance predisposition allele is esti-

Figure 1. Molecular-based approach to the diagnosis of MPNs



mated to account for 50 % of the population risk of developing a MPN and has a 3 to 4 odds ratio of association with JAK2^{V617F}-mutated MPN (58–60). The haplotype is associated also with JAK2 exon 12-mutated PV (61), JAK2-wild type ET (62) and possibly MPLW515-mutated PMF or ET as well (63–65); an increased frequency of 46/1 haplotype may also be detected in patients with MPN/associated splanchnic vein thrombosis (66–68). However, it is predicted that additional rare, highly penetrant predisposing polymorphisms are likely to exist. The mechanisms of the association of the 46/1 haplotype with MPNs are debated: the "hypermutability hypothesis" suggests that the 46/1 haplotype is genetically more unstable that other haplotypes and more prone to acquire V617F mutation while according to the "fertile ground" hypothesis the 46/1 haplotype would be in linkage disequilibrium with other unknown genetic variables contributing to the expansion of a JAK2^{V617F} mutated clone. This haplotype associates with Ph'-negative MPNs only (69). Recently, another population-based haplotype, the C allele of ***JAK2*** rs4495487, has been shown to contribute significantly to the occurrence of ***JAK2***^{V617F}-positive and -negative MPNs in the Japanese population, in addition to the 46/1 haplotype (70). Of particular interest, the 751 Gln/Gln genotype in XPD, a gene involved in DNA repair, was found to have a strong association with both leukemic transformation (Overall Risk, OR: 4.9) as well as the development of non-myeloid malignancies (OR: 4.2) in a cohort of MPN patients (71).

Role of mutation abnormalities in the diagnosis of MPNs

The JAK2 and MPL mutations are integral to the 2008 WHO classification (21, 22). Mutation analysis for JAK2^{V617F} mutation is currently at the first level of investigation in patients with a suspicion of MPN (Figure 1). Thus, a positivity establishes with high probability a MPN, but the individual subtype should be defined by using a combination of other major (PV) and/or minor (ET and PMF) criteria; a negative test for JAK2^{V617F} does not exclude a MPN, including PV. In particular, bone marrow biopsy is essential to differentiate ET from PMF and the prefibrotic myelofibrosis (72) as well as other unusual forms of MPN/MDS (73). A cooperative effort within the European Leukemia Net has focused on the best-standardized conditions for JAK2^{V617F} assay; results are expected soon. Assays that have at least a 1 % sensitivity are optimal for diagnosis while a greater sensitivity (down to 0.01 %) might be necessary for assessing residual JAK2^{V617F} positivity after a stem cell transplant (74). False-positive are very rare, and in this light one should probably interpret the findings of low-levels of JAK2^{V617F} in healthy subjects (75); however, this remains an open question. Whether a quantitative assay should be routinely used is a matter of discussion, but just for diagnostic purposes it should probably not. There is a trend towards higher V617F allele burden in MF and PV versus ET, with median burden levels greater than 50 % in the former versus 20 % in ET (76, 77); however, there is a wide distribution of levels, thus they are not diagnostic of MPN subtype at all. Homozygosity for the JAK2^{V617F} mutation, that originates from mitotic recombination of the short arm of chromosome 9 (78), is displayed by approximately 30 % of PV or PMF patients as opposite to 2–4 % of ET. However, in the clinical practice, the term "homozygosity" is used, somewhat incorrectly, to mean an allelic burden greater than 50 % when measured in a patient's blood cell (granulocyte, whole blood) samples by quantitative molecular assay; as a matter of fact, the MPN clone is variably comprised of JAK2^{V617F} heterozygous, homozygous as well as JAK2 wild-type hematopoietic progenitors, as demonstrated in single colony genotyping experiments (79). Homozygous progenitors are most prevalent in PV and PMF and outmost rare in the majority of ET patients (80).

In JAK2V617F negative patients with thrombocytosis and/or the suspicion of ET a search for mutations of MPL exon 10 (mainly, at codon 515, much rare at 505) should be considered; these involve about 5 % of ET and 10 % of PMF patients (16, 18). Finally, in the unusual patients with a suspicion of PV who is JAK2^{V617F} negative, a search for mutations at exon 12 might be considered (14), provided the diagnostic suspicion is high enough due to low erythropoietin levels, confirmed evidence of raised red cell mass and eventually findings of erythropoietin-independent colonies, after having carefully excluded reactive and familial forms. These considerations derive from the fact that mutation survey for JAK2 exon 12 is complex and expensive due to the several abnormalities that have been reported to occur (81); furthermore, since the clone size may be small in many patients, the sensitivity of current assays might not be adequate enough to avoid false negative results (82).

A *JAK2*^{V617F} allele burden exceeding 50 % has been reported to occur in about one quarter of patients with prefibrotic PMF as compared to none of a corresponding series of 90 ET (83). Therefore, according to this paper, a *JAK2*^{V617F} allele burden greater than 50 % would favor a diagnosis of prefibrotic PMF rather than ET (83), but in the absence of controlled studies these data should be managed with caution.

Genotypig for the 46/1 haplotype has no role in diagnosis of MPNs; in particular, it should not be used, notwithstanding it could be solicited by some patient's, to predict the risk of developing an MPN in the relatives.

Prognostic significance of mutation abnormalities in MPNs

The possibility that a JAK2^{V617F} mutated allele and/or the burden of the mutation influences disease manifestation and survival has been the objective of several studies which, in most cases, have shown that the V617F allele burden correlates with hematologic characteristics and some clinical end-points, although it is clear that the burden of mutated allele is not the only mechanism at the basis of MPN phenotypic variability.

Among 323 patients with PV (67.8% heterozygous and 32.2% homozygous) and 639 patients with ET (40.2% wild-type, 57.6% heterozygous, and 2.2% homozygous) collected in a multicentre Italian study (6), homozygosity was associated with evidence of a stimulated erythropoiesis and myelopoiesis, lower platelet count, a higher incidence of splenomegaly, larger spleen size, and a greater proportion of patients requiring cytoreductive therapy. Homozygous PV patients had also a higher incidence of pruritus. The rate of major thrombosis was not increased in homozygous patients with PV compared to heterozygous, similar to previous findings in smaller series (13). On the contrary, thrombotic events were definitively more frequent among homozygous ET patients, with an hazard ratio 3.97-fold higher than in JAK2wild-type, that remained significant after multivariate analysis including other established risk factors and leukocytosis as covariates (84, 85). Meta-analyses including more than 2,000 patients, have confirmed that the mere presence of a $JAK2^{V617F}$ mutated status is associated with a predicted overall risk of about 2 when compared to wild-type subjects (86, 87). A mutated allele burden greater than 75% was associated with a 3.56-fold higher relative risk of total thrombosis in a prospective study in 173 patients with PV (88); thus, patients with PV included within the highest V617F allele burden quartile may represent a subgroup at particularly higher risk of thrombosis. Furthermore, there is evidence that the proportion of PV patients with progression to post-PV myelofibrosis resulted significantly higher among homozygous than heterozygous (6, 13, 89). The risk of having a fibrotic transformation was significantly higher also among homozygous ET patients (14.3% of homozygous vs. 4.7% of heterozygous vs. 1.6% of wild-type patients $P < 0.001$) in the Italian study (6). As a whole, these data indicate that the burden of $JAK2^{V617F}$ allele is associated with the magnitude of myeloproliferation, clinical manifestations, the risk of thrombosis in ET and possibly PV, and with the probability to evolve to myelofibrosis, therefore representing a negative prognostic factor for disease severity.

In the case of primary myelofibrosis, a statistically significant association of the $JAK2^{V617F}$ mutated status with a more pronounced myeloproliferative phenotype was found in some studies (8, 9, 90), at variance with others (10, 11). A study conducted in England (90) reported a hazard ratio of shortened survival of 3.3 (95% confidence interval, 1.26–8.68) in patients harboring the V617F allele; these results were not confirmed in other series. However, Tefferi et al. (10) first described a poorer survival in patients harboring a low (ie, within the first quartile) mutated allele burden, and those findings were confirmed by Guglielmelli et al. (9). In the latter study patients included in the lower quartile had significantly shorter progression time to anemia, leukopenia, and a longer time to large splenomegaly compared to patients in the upper quartiles; furthermore, patients in the lower quartile had a significantly reduced overall survival compared both to upper quartiles and JAK2 wild-type patients. On the other hand, neither a mutated $JAK2^{V617F}$ status nor the mutated allele burden appeared to have prognostic relevance in a study of 65 patients with post-PV or PET-MF (91). In summary, a low $JAK2^{V617F}$ allele burden at diagnosis seems to be a strong surrogate marker associated with shortened survival in PMF, possibly because it points to the prevalence of another clone harbouring more detrimental mutations.

It is debated whether a nullizygous status (i. e., the absence of) for the 46/1 haplotype has a negative prognostic role in PMF (92, 93).

Other mutated genotypes, including MPLW515L/K (19, 94), CBL (31), TET2 (33), ASXL1 (40), LNK (28, 30), IDH1/IDH2 (95) have not been shown to be prognostically informative, although most patient series analyzed to date were too small to allow reliable statistical analyses (53). On the other hand, we found that EZH2 mutational status had a significant negative impact on disease outcome among PMF patients. EZH2 mutated subjects preferentially clustered in the IPSS high-risk category and presented shortened overall survival and leukemia-free survival compared to their wild-type counterparts; of importance, EZH2 mutated status maintained a negative prognostic significance in a multivariate analysis together with the IPPS score and a low $JAK2^{V617F}$ allele burden.

Conclusions

Due to their strong, though not exclusive, association with MPNs and their frequency, finding a mutation in JAK2, both the V617F and exon 12 mutations, or in MPL represents a very useful criterion for supporting the diagnosis of MPNs; these molecular abnormalities have been incorporated as major criterion in the revised 2008 WHO classification, and they are currently assayed in most reference hematology laboratories. Other criteria are necessary, however, to distinguish among the different clinical entities, although JAK2 exon 12 mutations are associated typically with PV and MPL mutations with ET and PMF. Support to the prognostic role of JAK2 mutation and its allelic burden for thrombosis in PV and ET and progression to PPV/PET MF has been substantiated by several studies, while its role in disease progression and survival in PMF is still debated. On the other hand, other mutations have little diagnostic impact, owing to both their low specificity and frequency, but they might deserve a stronger prognostic role, as shown for EZH2 in PMF and p53 alterations at the time of leukemic transformation. On these premise, we can expect a progressively greater and meaningful impact of molecular diagnostics in MPNs over the next years.

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