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Genetic and epigenetic alterations of myeloproliferative disorders

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Abstract The classical BCR–ABL negative myeloproliferative neoplasms (MPN) polycythemia vera, essential thrombocythemia, and primary myelofibrosis are clonal hematopoietic disorders characterized by excessive production of terminally differentiated myeloid cells. In MPN patients, the disease can progress to secondary myelofibrosis or acute myeloid leukemia. Clonal hematopoiesis, disease phenotype, and progression are caused by somatically acquired genetic lesions of genes involved in cytokine signaling, RNA splicing, as well as epigenetic regulation. This review provides an overview of point mutations and cytogenetic lesions associated with MPN and addresses the role of these somatic lesions in MPN disease progression.

Keywords Myeloproliferative disorder · Polycythemia vera · Essential thrombocythemia · Primary myelofibrosis

Introduction

Myeloproliferative neoplasms (MPN) are characterized by clonal expansion of terminally differentiated myeloid cells. According to the 2008 WHO classification, MPNs include polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic myeloid leukemia (CML), chronic neutrophilic leukemia, mastocytosis, and chronic eosinophilic leukemia not otherwise specified, as well as unclassifiable MPN [1]. The first four disease entities are also known as "classical" MPNs. Since CML is characterized by the oncogenic fusion of the *BCR* and *ABL* genes, PV, ET, and PMF are commonly known as Philadelphia-chromosome negative or *BCR–ABL* negative MPNs.

Despite their phenotypic diversity, all MPNs exhibit clonal hematopoiesis driven by acquired mutations. The most famous example of such a driver mutation is the *BCR–ABL* fusion oncogene causing CML. The discovery of the *BCR–ABL* fusion has made molecular diagnosis as well as therapeutic management of CML possible. In 2005, a somatic mutation in the *JAK2* gene was identified in the "classical" MPNs with similar significance as *BCR–ABL* in CML. The MPN-associated *JAK2* mutation (V617F) has been found in over 90 % of PV cases and in about 50–60 % of ET and PMF [2–5]. The V617F mutation is located in exon 14 of *JAK2*, but various exon 12 mutations have also been described in PV [6, 7]. Mutational analysis of *JAK2* has greatly aided the diagnosis of the "classical" MPNs, and there are several Janus kinase 2 (JAK2) kinase inhibitors at various stages of clinical development for therapeutic management of MPN.

Since the discovery of *JAK2* mutations, much effort has been made to define other genetic lesions involved in disease pathogenesis, especially in JAK2-negative ET, PMF, and PV cases. These studies have identified a striking number of other somatic lesions predominantly targeting cytokine receptor signaling and regulation of gene expression [8, 9]. As MPN has at least three recognized disease stages (chronic phase, accelerated phase, and leukemic transformation) the role of somatic mutations in disease progression has been addressed by a number of

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studies. In general, the somatic mutations found in MPN patients can be functionally classified into three groups: (1) mutations providing clonal advantage but not inducing disease phenotypes; (2) mutations capable of inducing clonal growth as well as a disease phenotype; (3) mutations causing acute disease phenotypes, such as leukemia (Fig. 1). This review will provide a comprehensive overview of MPN-associated point mutations and cytogenetic lesions. Furthermore, we will address the role of these somatic lesions in MPN disease progression and discuss the challenges arising from the genetic complexity of MPN.

Mutations of the *JAK2* gene

Janus kinase 2 is a member of a JAK family of tyrosine kinases, which additionally includes JAK1, JAK3, and TYK2. These proteins are characterized by four functional domains: FERM domain, SH2 domain, pseudokinase domain (JH2), and tyrosine kinase domain (JH1) [10]. The structure of the protein, which has two Jak homology domains JH1 and JH2, was the reason for this protein family to be named after the two-faced Roman god Janus. Although homologous to the tyrosine kinase domain, the JH2 domain lacks kinase activity and has been shown to act as a negative regulator of JAK protein kinase function [11]. The JAK proteins play important roles in the hematopoietic cell signaling induced by cytokines. Cytokine receptors, including the erythropoietin (EPOR), thrombopoietin (TPOR), and granulocyte colony-stimulating factor receptors (GCSFR), lack phosphorylation activity in their intracellular domain, and upon activation by ligand binding induce oligomerisation and phosphorylation of JAKs, which then phosphorylate further downstream targets (for example STATs) regulating transcription [12].

The *JAK2* gene encoding JAK2 tyrosine kinase is located on chromosome 9p24. Following the observation that acquired uniparental disomy (UPD) of chromosome 9p is present in 30 % of patients with PV [13], the gain-of-function somatic *JAK2*-V617F mutation was discovered as the most prominent genetic aberration in patients with MPN [2–5]. In PV, 95 % of the patients carry *JAK2*-V617F mutation, while in ET the frequency is 50–60 %, and in PMF 40–50 % (Fig. 2). This mutation can also be found in other myeloid malignancies at lower frequencies.

The *JAK2*-V617F mutation affects the JH2 domain of the protein, abrogating its function and leading to constitutive activation of the kinase. In order to induce transformation and activate downstream targets, the activated kinase requires the coexpression of homodimeric cytokine receptors EPOR, TPOR, or GCSFR [14, 15]. Studies of mouse models have provided deeper insight into how one mutation can cause three different phenotypes. In mouse transplantation models the *Jak2*-V617F mutation causes erythrocytosis [3]. Further studies of *Jak2*-V617F knock-in mice showed that gene dosage has an effect on phenotype, which is closer to PV in cases of heterozygous expression of the mutant *Jak2*, while the homozygous mutation leads to a more severe phenotype and progression from PV to

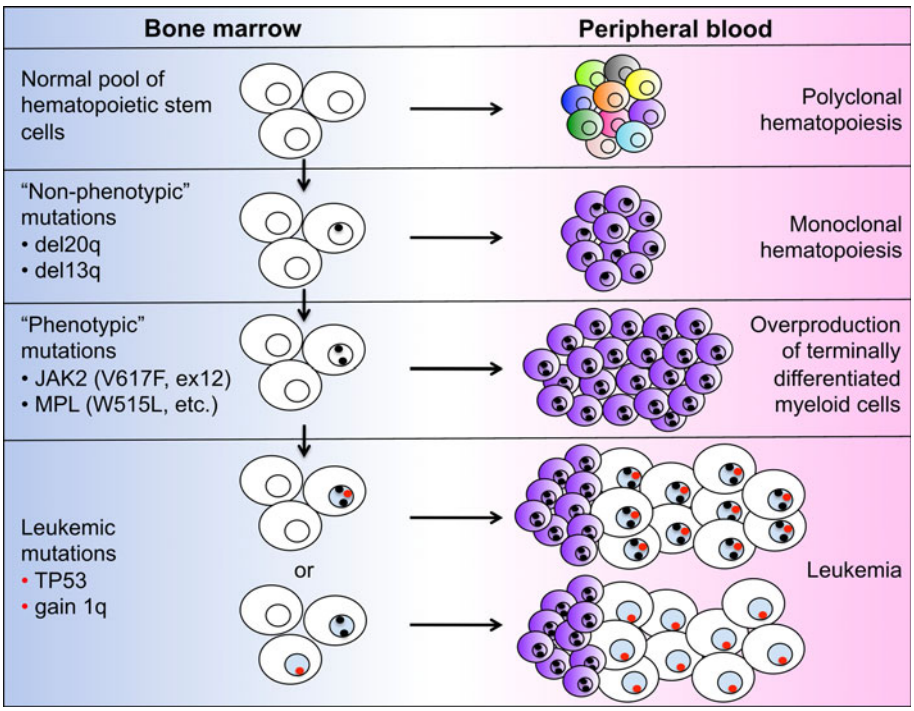

Fig. 1 Model of disease initiation and progression in myeloproliferative neoplasms. Role of genetic lesions with different phenotypic effects

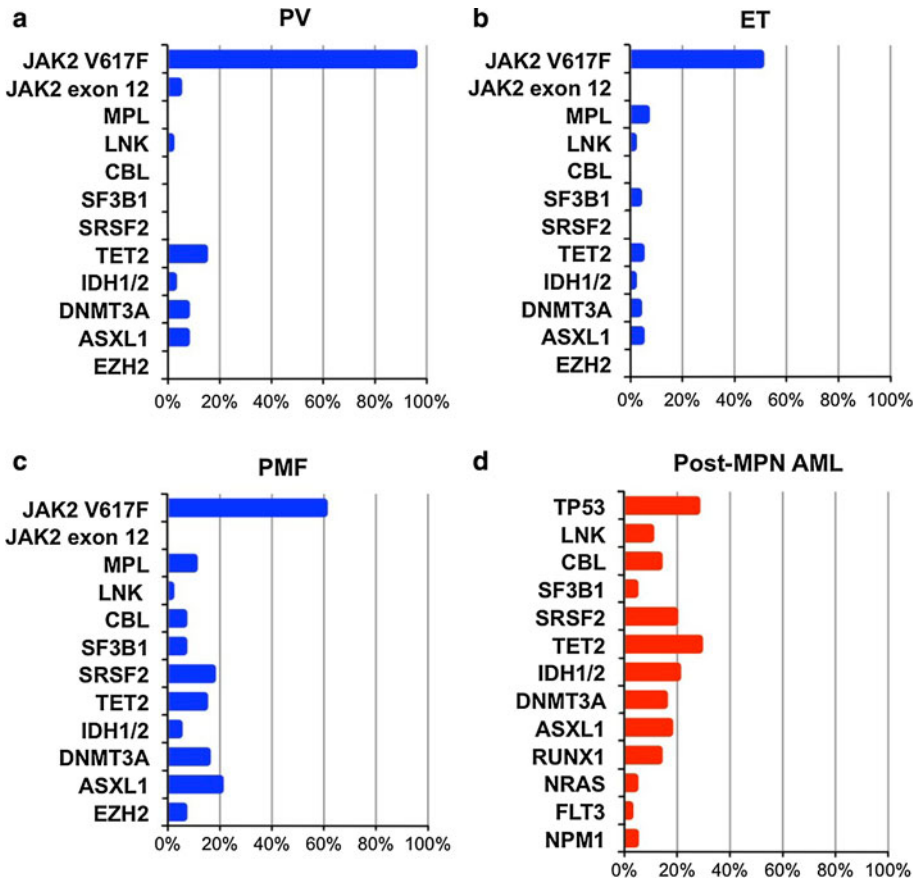

Fig. 2 Frequencies of mutations in commonly affected genes in patients during chronic myeloproliferative phase (**a**–**c**) and acute leukemic phase (**d**) of the disease. *PV* polycythemia vera, *ET* essential thrombocythemia, *PMF* primary myelofibrosis, *MPN* myeloproliferative neoplasm, *AML* acute myeloid leukemia

myelofibrosis [16]. Other mouse model studies yielded similar results, confirming that *JAK2*-V617F causes an MPN phenotype [17–21].

In PV, but not ET or PMF, a small percentage of *JAK2*-V617F-negative patients carry mutations in exon 12 of the *JAK2* gene [6, 7]. Most exon 12 mutations target the K539 residue, with a similar functional consequence on the JH2 domain, leading to a myeloproliferative phenotype in a mouse bone marrow transplant model [7].

In addition to its prominent role in cytokine signaling, mutant JAK2 can act as an epigenetic regulator, mainly by phosphorylating histone H3 or an arginine methyltransferase PRMT5, in the nucleus. Phosphorylation of H3 at position Y41 inhibits the binding of heterochromatin protein 1α (HP1α), known to be involved in transcriptional repression, DNA repair and other cellular processes [22–24]. JAK2 mutants (V617F or K539L) display higher affinity for binding arginine methyltransferase PRMT5. The phosphorylation of PRMT5 by mutant JAK2 prevents its association with methylosome protein 50 (MEP50) in an arginine methylation protein complex and leads to decreased methylation of histones H2A and H4 [25]. It also seems to play an important role in erythroid differentiation and progenitor cell proliferation [25].

Mutations affecting the thrombopoietin receptor gene *MPL*

The proliferation and differentiation of megakaryocytes and development of platelets is regulated by thrombopoietin, which upon binding to its receptor encoded by the myeloproliferative leukemia virus oncogene (*MPL*) induces signaling through the JAK–STAT pathway. Not long after the discovery of *JAK2* mutations in MPN, somatic mutations affecting the exon 10 of *MPL* were found in 9 % of patients with myelofibrosis, who were *JAK2*-V617F negative [26]. The mutation described was a gain-of-function substitution of tryptophan to leucine at codon 515, which was shown to lead to cytokine-independent growth and thrombopoietin hypersensitivity in the 32D, UT7, and Ba/F3 cell lines due to constitutive phosphorylation of JAK2, STAT3, and STAT5 as well as of the ERK and AKT proteins [26]. Study of the thrombopoietin receptor protein structure revealed that the amino acid residue W515, which is affected by mutations, is located within the cytoplasmic juxtamembrane region of the protein, which prevents spontaneous activation of the receptor in the absence of ligand [27]. In mouse bone marrow reconstitution experiments, *Mpl*-W515L expression induced a PMF-like

phenotype with thrombocytosis, leukocytosis, hepatosplenomegaly, and bone marrow fibrosis, leading to a fatal outcome.

Several other somatic mutations in *MPL* were later found at lower frequency in MPN patients, affecting either the same amino acid, including W515K, W515A, W515S, W515R [28–30], or S505N, first described in familial thrombocytosis [31]. Exon 10 *MPL* mutations account for up to 15 % of *JAK2*-V617F negative cases of ET and PMF [26, 28, 29, 32]. Studies of the clonal structure in *MPL*-positive patients revealed that the *MPL* mutations arise early in the disease evolution, affecting progenitors of both myeloid and lymphoid lineages [33]. Amplification of *MPL* mutations to homozygosity through mitotic recombination leading to acquired UPD of chromosome 1p, as well as biallelic mutations has been reported in a number of patients [34–36].

Mutations affecting *LNK*

Mutations affecting *LNK* (*SH2B3*) are rare and were first described in patients with *JAK2*-V617F negative MPN [37, 38]. LNK is an adaptor protein known to be involved in the down-regulation of thrombopoietin-mediated signaling [39, 40]. It inhibits the constitutive activity of JAK2-V617F, as well as downstream MPL-W515L signaling [41, 42]. *Lnk* knockout mice develop an MPN phenotype, characterized by splenomegaly with marked fibrosis, due to accumulation of abnormal levels of erythrocytes, megakaryocytes, and B lymphocytes [43]. Mutations mainly affect exon 2, but can also be found throughout the gene. Mutations in *LNK* were also described in context of disease progression in 9.8 % of post-MPN acute myeloid leukemia (AML) cases [44]. However, two cases were also reported with *LNK* mutations only during the chronic phase, not the leukemic phase of the disease. *JAK2*-V617F positive patients with *LNK* mutations have also been reported, although the possibility of the presence of two different clones was not excluded [44]. SH2B protein family contains two members in addition to SH2B3, SH2B1, and SH2B2. A somatic mutation affecting *SH2B2* was found in one case of post-MPN AML [45].

Mutations of *CBL*

Somatic mutations of *CBL* have been described in a variety of myeloid malignancies, including AML, MPN, myelodysplastic syndromes (MDS), and others [46–51]. The *CBL* gene is located at 11q23.3 and mutations show a strong association with acquired UPDs of chromosome 11q. Mutations in this gene are found in 6 % of PMF patients, and rarely in ET and PV [50]. The CBL protein acts as a regulator of signal transduction, either as an adaptor protein or as an E3 ubiquitin ligase that can target cytokine receptors (EPOR, TPOR), tyrosine kinases (JAK2, ABL), and signaling adaptors (GRB2), leading to their proteasomal degradation [52, 53]. *Cbl* knockout mice show hyperproliferation, enhanced thrombopoietin sensitivity and STAT5 phosphorylation, and enhanced repopulating capacity of HSCs, but do not develop a malignant phenotype [54]. However, deletion of a related member of the CBL protein family, *Cbl-b*, in addition to *Cbl* in HSC leads to an aggressive myeloid phenotype in mice [55], indicating that these two proteins may have a redundant function, although *CBL-B* mutations are rarely found in patients and by themselves do not induce hematological disorders in mice. Mutations in *CBL* mainly affect exons 8 and 9, encoding the ring finger domain and the adjacent linker region to the tyrosine kinase-binding domain of the protein [49–51]. The mutated CBL does not have E3 ubiquitin ligase activity, and is thought to act as a dominant negative, impairing the function of the wild-type CBL if present, as well as CBL-B [51]. In vitro studies of CBL mutants show that they exhibit cytokine-independent growth and prolonged activation of the FLT3 signaling [46, 51, 56]. Mice transplanted with transduced mutant *c-Cbl* bone marrow cells develop myeloproliferative disease, mastocytosis, and myeloid leukemia [57]. The *CBL* mutations have been described during disease progression in 13 % of post-MPN AML patients [58, 59]. However, the prognostic value of *CBL* mutations is unclear and will need to be evaluated in prospective studies.

Mutations affecting the splicing machinery

Mutations affecting various proteins involved in RNA splicing are common across all myeloid malignancies although they are predominantly found in MDS patients [60]. In MPN, they affect 9.4 % of patients [60]. Several genes encoding the components of splicing machinery were found mutated in a mutually exclusive manner, such as *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *SF3A1* and others. *SF3B1* mutations are found in 4–6.5 % of patients with PMF and 3 % of patients with ET (Fig. 2), but no association with clinical features or other mutations has been clearly established although association with thrombotic events has been proposed [61–63]. As *SF3B1* mutations show high specificity for MDS with increased ring sideroblasts ($\sim$80 % of the cases), it is not surprising that this feature has also been observed in MPN patients with this mutation [60]. Mutations affecting *SRSF2* are found in 17 % of patients with PMF and 18.9 % of post-MPN AML and are associated with *IDH1/2* mutations (28 % of *SRSF2*

mutated cases carry *IDH1/2* mutations in addition) [64, 65]. Analysis of paired samples from leukemic and chronic phases showed that *SRSF2* mutations when present at transformation were also present during the chronic MPN phase of the disease [65]. These are associated with poor overall and leukemia-free survival [64, 65]. *SRSF2* mutations target predominantly the P95 residue of the protein either by point mutations or insertion/deletions (P95H, P95L, P95R, etc.).

Mutations affecting *U2AF1* and *ZRSR2* were reported at low frequencies (~2 %) in MPN patients. The mechanism by which mutations in the splicing machinery lead to an impaired hematopoiesis is unclear. In vitro studies of U2AF1 mutants indicate that they are dominant negative, leading to aberrant splicing, inhibition of proliferation, and apoptosis induction [60].

The role of epigenetic changes in MPN

Regulation of transcription can be achieved through epigenetic mechanisms, which involve DNA and histone modifications. Methylation and hydroxymethylation of cytosines in DNA, as well as methylation, acetylation, and other modifications of histones, are the most common mechanisms of achieving transcriptional repression. These processes play an important role in cell differentiation, as they can regulate the expression of particular genes without affecting the DNA sequence. These changes are therefore reversible, which makes them potential therapeutic targets. Drugs targeting some of these changes are already in clinical use. Several genes known to be involved in DNA methylation, or histone modifications, are affected by somatic mutations in myeloid malignancies [66]. It has even been shown that patients with AML can be clustered into 16 different populations according to their aberrant DNA methylation profiles, most of which correspond to current classification subtypes of AML according to the fusion genes or mutations present [67]. Somatic mutation in epigenetic modifiers are shared among all myeloid malignancies and affect *TET2*, *IDH1/2*, *DNMT3A*, and *ASXL1* as well as members of the polycomb repressor complex 2 (PRC2). In MPN they are predominantly found in patients with PMF, or patients who show disease progression to AML; however, their specific role remains unclear, as these mutations are widely detected in different cancers. They may exert a cooperative effect with different combinations of other affected genes to cause a phenotypic readout. Recent evidence of JAK2 involvement in epigenetic regulation underlines the significance of epigenetic mechanisms in disease development [22]. In one recent study, DNA methylation profiling of 29 MPN patients was performed in an attempt to reveal differences at the epigenetic level between the three disease entities [68]. Studies of larger patient cohorts will be necessary to further delineate the methylomes of MPN patients.

Mutations affecting *TET2*

The TET proteins (TET1, TET2, and TET3) are enzymes that catalyze the conversion of 5-methylated cytosine into the hydroxymethylated cytosine and require Fe(II) and α-ketoglutarate as cofactors [69]. Deletions of chromosomal region 4q24 mapping to the *TET2* gene are observed in various myeloid malignancies [45, 70]. Sequence analysis of *TET2* revealed that this gene is also commonly affected by point or frameshift mutations, targeting mainly exons 3 (41 %) and 11 (29.1 %) [70–73], leading to the loss of its enzymatic function [74].

It appears that each of the TET protein family members has a unique function, as mutations in *TET1* or *TET3* are extremely rare. Haploinsufficiency of *TET2* seems to be enough to contribute to the disease phenotype, as in most cases only one allele is affected, although biallelic mutations have been reported. *TET2* mutations can arise in both *JAK2*-V617F-positive and -negative patients, and several studies have shown that the order of acquisition of these two genetic defects is random [70, 75]. Although association of *TET2* mutations with disease progression has been observed, no prognostic relevance of these mutations in MPN has been established.

Recent studies have found evidence that the TET2 protein can catalyze stepwise oxidation of 5-methyl-cytosine into not only 5-hydroxymethyl-cytosine (5hmC), but also 5-formylcytosine and 5-carboxylcytosine (5caC) and that the presence of these nucleotides in mouse embryonic stem cells DNA is dependent on the level of TET protein expression [76]. However, 5hmC can also be deaminated by AID/APOBEC deaminases into 5-hydroxymethyluracil (5hmU). The importance of these findings lies in the fact that 5caC and 5hmU can be recognized, excised, and replaced with an unmethylated cytosine through the base-excision repair mechanism, in which thymine DNA glycosylase (TDG) plays an important role [77–79]. Therefore, TET2 is a key player in active demethylation of DNA, which has many implications in the role of *TET2* haploinsufficiency in development of myeloid malignancies. Studies of TET2 in mouse models provided evidence that TET2 deficiency reduces the levels of 5hmC in genomic DNA, impairs differentiation of hematopoietic stem cells (HSC), and increases their repopulation capacity in competitive transplant experiments [73, 80–82]. Deletion of *Tet2* causes a CMML-like phenotype in mice after ~4–5 months, with evidence of extramedullary hematopoiesis, splenomegaly, and increases in HSC and

multipotent progenitor populations [73, 80–82]. This latency in disease development suggests that *TET2* mutations require additional genetic events to lead to a malignant phenotype.

TET2 mutations are found in ~4 % of ET, 9.8–16 % of PV, and 8–14 % of PMF [71, 72]. A recent study showed, however, that somatic *TET2* mutations are present in 5.6 % of elderly individuals with clonal hematopoiesis, who do not have a hematological malignancy [83]. In a 5-year follow-up of seven such patients, one patient acquired a *JAK2*-V617F mutation and developed ET. Although mutations in several other genes were found to be overlapping with *TET2* mutations in patients, further studies will be needed to explain the exact involvement of TET2 in the disease initiation process.

Mutations of *IDH1/2*

Isocitrate dehydrogenase (IDH) 1 and 2 are $NADP^+$ dependent enzymes, which catalyze the conversion of isocitrate to α-ketoglutarate, in cytoplasm and mitochondria, respectively. Somatic mutations affecting *IDH1* and *IDH2* genes were first described in gliomas and AML [84–86]. Their incidence in MPN is estimated 0.8 % in ET, 1.9 % in PV, ~4.2 % in PMF, and ~21 % of post-MPN AML cases [87, 88] (Fig. 2). It has also been shown that in addition to association with leukemic transformation, *IDH* mutations predict poor survival in MPN patients [89]. Mutations in *IDH1/2* can occur on both *JAK2*-V617F positive and negative background, suggesting that leukemia can arise from the MPN clone, or as a separate clonal disorder [90]. Co-occurrence with other MPN-specific mutations has been observed.

Mutations in *IDH1/2* are associated with cytogenetically normal de novo AML, and much effort has been made to better understand their contribution to leukemia initiation [91]. The mutations in *IDH1* affect the arginine residue on position 132 in exon 4, and its analogue in *IDH2*-R172, indicating a gain of function. Mutations in *IDH2* at position R140 have also been reported. *IDH2* mutations are more common than *IDH1*, and the two are always mutually exclusive [59, 91]. IDH1-R132H has recently been characterized in a knock-in mouse model, in which mice expressing mutant IDH1 show an increase in the early hematopoietic inhibitors, while developing a progressive splenomegaly, and ultimately anemia [92]. Several research groups have demonstrated that mutant IDH converts α-ketoglutarate into 2-hydroxyglutarate (2HG) in a NADPH-dependent manner, while losing the ability to use isocitrate as a substrate [86, 91, 93]. Accordingly, all AML patients carrying *IDH1/2* mutations show elevated 2HG levels [91]. As TET2 requires α-ketoglutarate as a cofactor, mutations in IDH1/2 inhibit TET2 activity, leading to a decrease in hydroxylation of 5-methyl-cytosines and demethylation [94, 95]. Evidence that mutations in *IDH1/2* and *TET2* rarely co-occur and that patients with these mutations show overlapping hypermethylation signatures [94] indicate that these lesions act through the same mechanism in causing leukemia. Increased 2HG levels can also lead to inhibition of histone demethylases [96], which may be an additional mechanism for leukemogenesis.

Mutations of the *DNMT3A* gene

DNMT3A is a DNA methyltransferase responsible for de novo addition of a methyl group to the cytosine within CpG dinucleotides. It belongs to a methyltransferase family of proteins, together with DNMT3B, which has a similar function, and DNMT1, which is thought to play a role in maintenance of the methylated state. Although mutations in *DNMT3A* are frequently found across myeloid malignancies, mutations in *DNMT3B* or *DNMT1* have not been reported. *DNMT3A* mutations were first described in 22 % of AML cases [97]. Subsequently, it was shown that these mutations are an independent prognostic factor of poor survival.

It is unclear if DNMT3A functions as an oncogene or a tumor suppressor. Most of the mutations described affect one amino acid residue (R882) suggesting a gain-of-function mechanism, while the rest of the mutations are mainly frameshifts or premature stop codons scattered throughout the gene, suggesting a loss of function [97–99]. However, in all mutated cases the mutations are heterozygous, implying that the presence of the wild-type allele is necessary and that the mutated protein might act as a dominant negative. This notion is supported by in vitro studies showing decreased catalytic activity of the mutated protein [100].

DNMT3A mutations were reported in ~10 % of MPN patients having higher prevalence in PMF and patients progressing to AML (~15 %) than ET and PV (3 and 7 %, respectively) [98, 99] (Fig. 2). Patients harboring *DNMT3A* mutations often carry mutations in *IDH1/2*, *TET2*, *ASXL1* or *JAK2*. It is unclear how mutations in this gene contribute to disease pathogenesis. A conditional *Dnmt3a* deletion in the mouse hematopoietic system affects the self-renewing capacity of long-term HSC, causing their expansion while impairing their differentiation without development of a malignant phenotype, as demonstrated by a serial transplantation model [101]. Hypomethylation and up-regulation of genes involved in the pluripotency of HSC in this model suggests that DNMT3A plays an important role in their silencing, thus permitting differentiation [101]. However, effects on gene expression in patients carrying *DNMT3A* mutations have not been studied.

Mutations affecting *ASXL1*

Additional sex comb like 1 gene (*ASXL1*) encodes a nuclear polycomb protein that can act both as activator and repressor of transcription through various mechanisms, mainly involving histone modifications [102, 103]. Absence of the homologue of this protein, Asx, in *Drosophila* prevents deubiquitination of histone H2A and repression of HOX genes [104]. Although mutations in *ASXL1* have been reported in various myeloid malignancies, *Asxl1* knock-out mice present only mild defects in myelopoiesis, without exhibiting a phenotype related to myeloid malignancies other than splenomegaly [105]. A recent study showed evidence that loss of *Asxl1* enhances the effects of oncogenic *NRas*-G12D expression in vivo in causing a myeloproliferative disorder phenotype [106]. However, we cannot exclude the possibility that *ASXL1* mutations described in myeloid malignancies that lead to truncation of the plant homo domain (PHD) of the ASXL1 protein act as gain-of-function or dominant negative mutations.

Mutations affecting exon 12 of *ASXL1* have been described in 7.8 % of 64 MPN cases in one study [107]. Further studies on larger patient cohorts revealed that *ASXL1* mutations are more frequent (up to 36 %) in patients with PMF and patients showing disease progression to secondary myelofibrosis, than in PV or ET [108, 109].

Aberrations of the polycomb repressive complex 2

Polycomb repressive complex 2 (PRC2) is a protein complex that plays an important role in the regulation of development, cell proliferation, and differentiation through histone modifications that lead to transcriptional repression [110]. The enzymatic core of the PRC2 complex consists of EZH2 (alternatively EZH1), SUZ12, RBAP46/48, and EED. The other members of this complex are AEBP2, JARID2, PHF1, PHF19, and MTF2. EZH1, and EZH2 are methyltransferases that can di- or trimethylate lysine 27 of histone 3 (H2K27me2/3) [111, 112]. Although inactivation of PRC2 complex members leads to reduced levels of H3K27me2/3, this is not the case for EZH1, which displays lower methyltransferase activity [113]. This may be the reason why *EZH2*, but not *EZH1*, is commonly affected by somatic mutations in cancer. In myeloid malignancies, it is mainly a target of deletions or truncating loss-of-function mutations [114, 115], although a gain-of-function mutation has been described in B cell-lymphomas [116] and overexpression has been linked to various cancers [117]. In vitro studies have shown that reduced levels of H2K27me3 were also observed after ASXL1 knock-down, and that ASXL1 physically associates with EZH2, indicating that loss of ASXL1 function inhibits recruitment of PRC2 complex to target loci [106].

Somatic mutations in *EZH2* are found in 12 % of patients with MPN/MDS [114] and 6–13 % of patients with myelofibrosis, in whom they predict poor survival [118]. Mutations affecting *SUZ12* are found in 1.4 %, *EED* in 1 % and *JARID2* in 2 % of MDS/MPN cases [119]. Focal homozygous deletions affecting single genes *JARID2* and *AEBP2* have been described in patients with PV and post-MDS AML, respectively [120]. Inactivation of members of PRC2 complex is often achieved through homo- or hemizygous deletions, as well as acquired losses of heterozygosity. Overall, they seem to be more frequent in patients that show disease progression towards leukemia. However, further functional studies will be necessary to delineate the mechanism by which inactivation of PRC2 complex leads to myeloid malignancies, and their relation to other gene mutations.

The role of cytogenetic aberrations in MPN

Diagnostic work-up of patients with myeloid malignancies often includes classical cytogenetic karyotype analysis, which enables detection of translocations and large-scale chromosomal aberrations. Many of the aberrations detected are used for characterization and classification of hematological malignancies, such as the most famous case of the *BCR–ABL* fusion ("Philadelphia chromosome") in CML. Development of microarray technology enabled analysis of copy number changes, as well as the detection of acquired losses of heterozygosity across the entire genome, at resolutions unreached by classical cytogenetic analysis. The importance of uniparental disomies in cancer development is that these aberrations lead to homozygosity through mitotic recombination of non-sister chromatids, whereby mutated tumor suppressors and oncogenes can be amplified, conferring an additional clonal advantage to affected cells [121]. Microarray technology was used to detect recurrent cytogenetic aberrations, which may point to novel genes involved in disease pathogenesis [45, 122, 123]. The largest cohort study of 408 MPN patients, using SNP arrays that provide 1.8 million data points per genome, revealed that 37.5 % of MPN patients show no detectable cytogenetic aberration, while in 62.5 % at least one aberration was present [45]. Karyotype complexity did not differ between patients with ET, PV, or PMF. However, higher cytogenetic complexity was observed in patients showing disease progression to secondary myelofibrosis or AML. Several cytogenetic aberrations were recurrent in the MPN patient cohort, such as 9p UPD, 1p UPD, del20q, del13q, 14q UPD, 1q gain, trisomy 8, del4q, and 7q UPD [45]

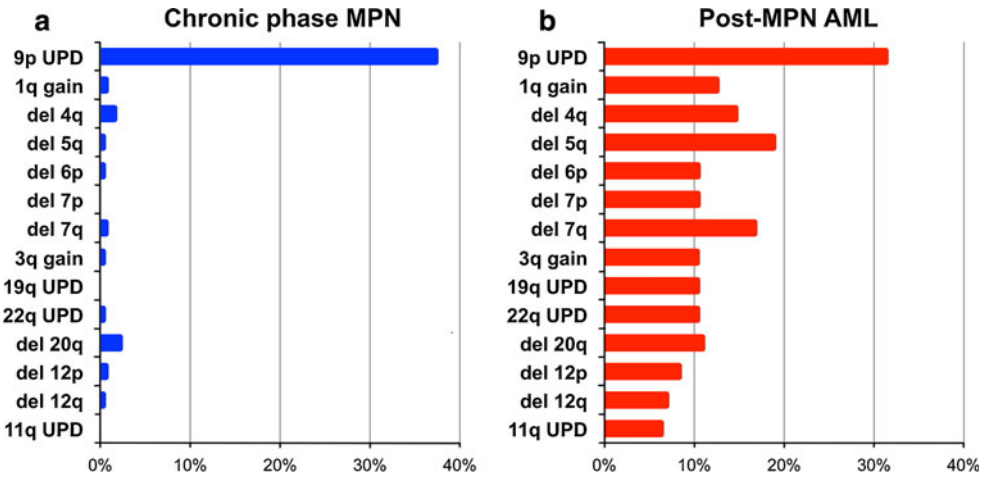

Fig. 3 Frequencies of recurrent cytogenetic aberrations in patients with myeloproliferative neoplasms during chronic phase (**a**) and acute leukemic phase (**b**) of the disease. *MPN* myeloproliferative neoplasm, *AML* acute myeloid leukemia

(Fig. 3). The most frequent cytogenetic aberration in MPN patients was UPD of chromosome 9p. All samples with 9p UPD and most samples with 1p UPD carried mutations of *JAK2* and *MPL*, respectively [45]. In PV 68 % of *JAK2*-V617F positive patients also had a UPD of chromosome 9p [45], while patients with ET exhibited low frequency of this aberration [124]. Excluding the 9pUPD, MPN patients had the same frequency of cytogenetic aberrations regardless of their *JAK2* mutational status. The high resolution of the copy number analysis allowed the mapping of common affected regions to single genes in this study, including *IKZF1*, *CUX1*, *TET2*, *FOXP1*, *ETV6*, and *RUNX1* [45]. Deletions targeting *TET2*, *FOXP1*, *ETV6*, as well as del13q and del20q, were found in similar frequencies in both the chronic and leukemic phases of the disease, suggesting a more universal role in its pathogenesis. *ETV6* has been previously implicated in different hematological cancers as a fusion partner of *ABL1*, *RUNX1* and other genes, due to translocations involving chromosome 12 [125, 126]. Deletions of *FOXP1* have previously been reported in cancer, and it is considered to be a putative tumor suppressor [127]. Since these proteins are transcription factors, it is uncertain which property of HSC is affected by their decreased dosage. Although del20q and del13q are frequent chromosomal aberrations found in various myeloid malignancies, the common deleted region contains numerous genes and the targets of these aberrations remain unknown [128]. Deletions of 7p targeting *IKZF1*, and 7q targeting *CUX1* were found to be associated with disease progression and are discussed in the following section.

Genetic aberrations associated with disease progression

Myeloproliferative neoplasms can progress to secondary myelofibrosis (also called accelerated phase in ET and PV) or AML. Many MPN patients develop anemia and progressive accumulation of blasts in peripheral blood or bone marrow, which are also characteristics of accelerated phase of the disease in all three disease entities. The current criteria for diagnosis of post-MPN AML is the presence of 20 % or more blasts in the peripheral blood and/or bone marrow [1], and the rate of leukemic transformation in MPN is 7 % [129]. Patients with secondary AML have poor prognosis, with an adverse outcome within a few months after leukemic transformation [59]. The genetic basis of transformation to leukemia remains poorly understood. Recent studies on limited patient cohorts have shown that different somatic cytogenetic lesions and mutations may lead to leukemia development.

Mutations affecting tumor suppressor p53 (*TP53*) were found to be associated with post-MPN AML comparing both with the chronic phase and de novo AML. They are detected in 27.3 % of post-MPN AML, 1.6 % of chronic phase MPN (Fig. 2), and 4.27 % of de novo AML patients [59, 130]. While mutations affecting chronic phase patients were monoallelic, they were usually biallelic in secondary AML and associated with 17p UPD or deletions. Interestingly, the 1q locus containing the gene encoding MDM4, a known inhibitor of p53 was repeatedly found to be amplified in post-MPN AML [45, 59]. As previously reported in solid tumors [131], gains of 1q were mutually exclusive with p53 mutations, indicating that these two genetic lesions affect the same pathway, which then seems to contribute to 45.5 % of the post-MPN AML cases [130]. The significance of this pathway in leukemic transformation is further supported by the finding that *TP53* mutations are the only independent prognostic factor of poor survival in secondary AML [59]. Considering the low frequency of mutations affecting *TP53* in chronic phase MPN patients, this mutation can be used as a predictor of leukemic transformation in MPN patients. The extent to which therapy administered during the chronic phase of the

disease influences inactivation of p53 pathway leading to transformation is unclear [132].

IDH1/2 mutations are present in ~12 % of patients with leukemic transformation, and their role has been described in a previous section of this review, as well as mutations affecting *TET2* which are found in 28.3 % of post-MPN AML patients [65]. The frequency of *IDH1/2* mutations does not differ between de novo and secondary AML patients [59]. Large-scale changes in the methylation patterns that enable classification of AML indicate the significance of epigenetic regulators in disease pathogenesis [67]. Mutations in *RUNX1*, which encodes a transcription factor involved in hematopoiesis, have been reported in AML, post-MDS AML, and post-MPN AML [59, 133–136]. Regarding the cytogenetic aberrations linked to the disease progression, the first observation was that patients with ET and PV who carry a 9p UPD, and consequently a higher *JAK2*-V617F mutational burden, show higher incidence of progression to secondary myelofibrosis. In general, patients with post-MPN AML have more complex karyotypes compared with chronic phase, with complex karyotype being present in ~45 % of patients with post-MPN and post-MDS AML [59]. Other cytogenetic lesions associated with post-MPN AML include del5q, del6p, del7p, del7q, gain3q, 19q UPD, and 22q UPD (Fig. 3). Monosomy of chromosome 7 is one of the most common cytogenetic aberrations in myeloid malignancies, often linked to poor prognosis. Deletions of 7p were found in six of 23 post-MPN AML patients and target transcription factor IKAROS encoded by *IKZF1* gene [137]. This genetic aberration was rare in the chronic phase (<1 % of cases) and was described as a late genetic event in the evolution of the leukemic clone. In vitro studies showed that knock-down of Ikaros causes cytokine hypersensitivity in hematopoietic progenitors [137]. Overexpression of a dominant negative isoform of this protein was linked to activation of JAK–STAT signaling pathway [138]. This transcription factor is known to be involved in regulation of differentiation of lymphoid cells, and is frequently affected in ALL, indicating its general role in leukemogenesis. Deletions of 7q target the *CUX1* gene. CUX1 is a transcription factor involved in regulation of cell cycle and hematopoiesis [139, 140]. Although the exact function of CUX1 in leukemic transformation remains to be described, a recent study reported that CUX1 deficiency negatively regulates the expression of members of the ATM/p53 pathway [141]. Deletions of chromosome 17q11.2 targeting *NF1* are also found in patients showing disease progression. *NF1* encodes neurofibromin 1, which negatively regulates RAS and is involved in granulocyte–macrophage colony-stimulating factor signaling [142]. Biallelic mutations of *NF1* in MPN including frameshift mutations and deletions of the second allele have been reported, and there is evidence that loss of *NF1* leads to development of a progressive myeloproliferative disorder [143]. Mutations in *NRAS* affecting the same pathway have also been described in post-MPN AML [58].

There are two hypotheses on the emergence of the leukemic clone in MPN patients (Fig. 1). The first argues that the MPN clone acquires additional genetic lesions that lead to impairment of differentiation and accumulation of blasts, while the second postulates that AML can arise as a separate disease in the same patient from an independent clone. The second hypothesis is supported by recent evidence that the leukemic blasts in MPN patients are often lacking the mutations found in the MPN clone [58, 144]. In line with this finding, it would be expected that secondary AML share the genetic features with AML arising de novo; however, recent finding suggest otherwise. Mutations commonly detected in de novo AML such as *FLT3* and *NPM1*, occur at much lower frequencies in secondary AML. Several gene mutations such as *TET2*, *RUNX1*, *IDH1/2*, and *NRAS*, and deletions of *IKZF1* and *NF1* were found in similar frequencies between post-MPN and de novo AML [59, 108], indicating that these genes are involved in the general leukemogenesis pathway, which is not dependent on the disease etiology. In contrast, mutations in *TP53*, gains of 1q and deletions of 7q targeting *CUX1* seem to be specific for secondary AML. It is important to stress that no somatic genetic lesions are detectable in approximately one-third of post-MPN AML patients. Application of next generation sequencing technologies in the analysis of their genomes will help elucidate the underlying cause of leukemic transformation in these patients.

Familial myeloproliferative neoplasms

Despite many somatic mutations and chromosomal aberrations described in MPN, there are several lines of evidence that germline genetic factors also contribute to MPN pathogenesis and disease progression. Familial clustering of MPN has been reported in various studies [145–150]. In general, 5–10 % of MPNs account for familial cases [150] and it has been shown that relatives of MPN patients are at higher risk of developing the disease [151]. The causative germline mutations in many of the familial cases are unknown [152]. Germline mutations in *MPL*, and recently in *JAK2*, have been reported in several familial cases of thrombocytosis [31, 153–155]. Another interesting finding was that the GGCC (also known as 46/1) haplotype in the *JAK2* locus predisposes to *JAK2*-V617F positive MPN [156]. Further studies demonstrated that the same haplotype represents a susceptibility factor for ET and PMF regardless of the *JAK2* status [157, 158]. However, the

frequency of the GGCC haplotype was found to be similar for the familial and sporadic MPN cases, and thus, it cannot explain familial clustering of MPN [159]. With respect to disease progression, a coding polymorphism in the codon 751 of *XPD* gene was shown to be associated with leukemic transformation as well as development of non-myeloid malignancies in patients with PV and ET [160]. Furthermore, germline mutations in regions of somatic loss of heterozygosity may exert a phenotypic effect in blood cells and modulate the MPN phenotype [161].

Conclusion

The diversity of somatic lesions in patients with the diagnosis of MPN indicates that the clonal evolution of MPN stem cells involves a number of cellular pathways. In general the mutations identified in MPN either target cytokine receptor signaling or involve aberrant gene expression via epigenetic regulation, reduced function of transcription factors or via aberrant splicing. In addition, the leukemic transformation process of MPN targets the DNA damage response pathway. Patients often carry a number of somatic lesions that are acquired in sequential order within a single clone. However, patients with biclonal hematopoiesis have been identified with two clones evolving side by side, each having a different potential to influence the hematological phenotype. The genetic complexity of MPN will further increase with new knowledge uncovered by high-throughput sequencing. It is clear by now that each MPN patient will represent a unique entity based on the somatic genetic profile of the MPN clone. Highly individualized diagnostic and therapeutic approaches will be required for successful clinical management of this phenotypically and genetically diverse disease group.

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Conflict of interest None.

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