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The Current Molecular Site of the Myeloproliferative Neoplasms - TET- à - t ê te with the JAKpot but no LNK so far to Resolve Complexity

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Abstract

The myeloproliferative neoplasms essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) are clonal haematological stem cell disorders with different clinical courses and prognosis. The predominant feature in these entities is the overproduction of at least one cellular lineage in the bone marrow. Peripheral blood cells are terminally differentiated and functional but may affect e.g. blood viscosity with a high hematocrit in PV leading to the risk for thrombotic events or bleeding. PMF, preceded by a hypercellular pre-fibrotic stage, has the highest risk to develop manifest bone marrow fibrosis with consecutive bone marrow failure. The risk for a blast crisis and transformation into secondary acute leukemia is also particularly high in PMF and PV but rather uncommon in ET.

Even though distinguishable from each other by clinical and histopathological criteria ET, PV and PMF may show overlaps, i.e. in early stages. Moreover, reactive hypercellular states in the bone marrow may mimic a myeloproliferative neoplasm (MPN) making definite molecular markers very useful.

ET, PV and PMF currently share a number of molecular defects which are detectable by accurate technologies and, in a diagnostic sense, subsequently allow a clear-cut discrimination from reactive states. Some of these molecular defects are frequent in the stage of secondary acute leukemia; others were proposed to be potential predictors for a progression of the disease course. At best, the protagonist of molecular defects in Ph- MPN, the Janus Kinase 2 with its V617F mutation, became a therapeutic target by using small molecule inhibitors. This review focuses on the hitherto discovered molecular defects in ET, PV and PMF in chronic phase and disease progression, highlights some of the current and upcoming therapies and proposes a disease model.

Keywords: Myeloproliferative neoplasms; Molecular pathobiology; Molecularly targeted therapies

Introduction

The groundbreaking discovery of the somatic point mutation in the Janus kinase 2, *JAK2* (V617F), in the so-called classical Philadelphia chromosome negative (Ph-) chronic myeloproliferative disorders (MPD) as well as a molecular defect in the thrombopoietin receptor *MPL* (W515L/K) justified a revision of the WHO classification published in 2008 [1]. The WHO classification replaced the term Ph- MPD by "myeloproliferative neoplasm" (MPN), a term that was introduced to emphasize the neoplastic nature of these disease entities. The revised classification also acknowledged the presence of molecular markers such as *JAK2* (V617F) and proposed new standards for the diagnostic algorithm, i.e. introduction of molecular diagnostics. The formerly called chronic idiopathic myelofibrosis (CIMF) was renamed to "primary myelofibrosis" to allow a better differentiation from secondary myelofibrosis demonstrable in other MPN or myeloid malignancies.

The current WHO classification gathers the four so-called classical MPN, the Philadelphia-chromosome positive (Ph+) t(9;22)(q34;q11), *BCR-ABL* positive chronic myeloid leukemia with the Ph- MPN essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) along with more rare entities such as chronic neutrophilic leukemia (CNL), systemic mastocytosis (SM), hypereosinophilic syndrome, the chronic eosinophilic leukemia not other specified as well as MPN unclassifiable [1].

Before the discovery of the gain-of-function mutation V617F in *JAK2* in a considerable number of patients with Ph- MPN was published almost 8 years ago [2-5], the knowledge on genetic defects was rather sparse over decades. Some of the cases showed demonstrable chromosomal deletions such as del(20q) or del(13q) in PMF [6] or a loss of heterozygosity (LOH) at 9p in patients with polycythemia vera (PV) [7]. The latter finding, however, was probably the most important cornerstone for the subsequent discovery of *JAK2* aberrations. Several findings like the erythroid colony (EEC) formation in PV in a cytokine-independent manner [8], the demonstration of clonality in MPN [9], cytokine hypersensitivity [10], the aberrant processing and expression of growth factor receptors such as *MPL* (Myeloproliferative leukemia virus oncogene) in PV and ET [11,12] and studies on factors involved in stroma pathology such as transforming growth factor β-1 (TGFβ-1) basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and aberrant proplatelet formation [13-17] contributed to the still leaky understanding of the nature of Ph- MPN. Moreover, some studies introduced new findings as diagnostic markers such as reduced MPL expression in certain MPD subtypes or the Polycythaemia vera rubra receptor-1 (PRV-1) mRNA expression in granulocytes from peripheral blood [18,19].

The hitherto discovered molecular defects in Ph- MPN and associated myeloid disorders can functionally be grouped in those 1)

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associated with constitutive activation of cytokine signalling driven by aberrant factors such as *JAK2, MPL, LNK, CBL, NRAS, NF1*, 2) those affecting epigenetics and regulation of transcription driven by alterations in *TET2, ASXL1, EZH2* and other *PRC2* members, *DNMTA3A* 3) those affecting the splicing machinery like *SRSF2 and SF3B1*, 4) those associated with disease progression and transformation into secondary acute leukemia like *IKZF1, RUNX1, TP53, IDH1/2, DNMTA3A*.

However, the full spectrum of intracellular alterations initiated by these molecular defects remains to be investigated. For example a protein with a new structure and folding due to a change in the amino acid sequence such as *JAK2* (V617F) may not only be constitutively activated but may provide aberrant interactions with other factors.

In the following section, the currently known molecular defects will be listed and discussed in an alphabetical order and not based on the cellular effects proposed elsewhere. Table 1 summarizes this section and allows a quick overview. At the end, the current state of therapeutic options through molecular targeting and a disease model for Ph- MPN will be introduced.

Genetic Lesions – Compilation in Table 1

Additional sex comb like 1 gene (*ASXL1*)

ASXL1 is the first of three members of the *ASXL* family (*ASXL1-3*). *ASXL1* is involved in the regulation of retinoic acid receptor mediated transcription and important for repression of *HOX* genes. Its function in mammals and especially in hematopoiesis is yet not fully understood. Besides a mild decrease in progenitor cells, mice models knocked out for *ASXL1* showed no dramatic effects on hematopoiesis [20].

Somatic mutations involving the *ASXL1* gene mainly affect exon 12 and 13 and are demonstrable in chronic MPN and at higher frequency in those showing disease progression. Up to 36% of PMF cases and post-PV/-ET showed mutations in the *ASXL1* gene whereas chronic PV and ET cases rarely showed this molecular defect (<6%) [21,22]. It was shown that *ASXL1* mutations in MPN can occur before appearance of other defects such as mutated *JAK2* and *TET2* [23]. *ASXL1* mutations are also demonstrable in subtypes of the myelodyplastic syndrome (MDS), post-MDS AML, the chronic myelomonocytic leukemia (CMML) and also *de novo* AML [24,25].

Casitas B-lineage lymphoma proto-oncogene (*CBL*)

Two studies were first in demonstration of the frequent occurrence of molecular defects in the *CBL* gene apart from already known fusion genes [26,27]. Acquired uniparental disomy (aUPD) generally leads to loss-of-heterozygosity and can induce either the loss-of-function of tumor suppressors or gain-of-function of proto-oncogenes. Interestingly, aUPD could be demonstrated in the gene of tumor suppressor *CBL* which induces a gain-of-function leading to inhibition of the wild-type *CBL* and prolonged action of tyrosine kinases.

The proto-oncogene *CBL* is a negative regulator of several receptor tyrosine kinase signaling pathways and an adaptor protein in tyrosine-phosphorylation dependent signalling [28].

Ubiquitination of receptor protein-tyrosine kinases (PTKs) terminates signaling by marking active receptors for degradation. CBL is an adaptor protein for receptor PTKs. It positively regulates receptor PTK ubiquitination. Ubiquitin-protein ligases, also known as E3s, are the components of ubiquitination pathways that recognize target substrates and promote their ligation to ubiquitin. A previous study determined that the CBL protein acts as an E3 that can recognize tyrosine-phosphorylated substrates, such as the activated platelet-derived growth factor receptor [29]. It was concluded that CBL is an ubiquitin-protein ligase and thus provide a distinct mechanism for substrate targeting in the ubiquitin system.

CBL mutations were often associated with aUPD at 11q and shows involvement of *CBL* exon 8 and 9 but also of introns. PMF cases were affected by up to 6%, PV and ET were rarely affected by *CBL* mutations. Notably, an underlying molecular defect in *CBL* seems to correlate with a shorter overall survival and shorter progression-free survival in patients with (n=19) or without (n=87) *CBL* mutation [26]. However, all in all *CBL* mutations are rare genetic events in chronic MPN [22].

Cut-like 1 (*CUTL1/CUX1*)

CUX1 was initially reported to be a repressor of developmentally regulated genes because of the ability to bind and modify certain histones. In a human acute leukemia cell line (HL-60) a repressor function of the gp91-phox gene could be demonstrated *in vitro* [30]. Later on, it was proposed that *CUX1* might exhibit general tumor suppressor functions. For instance, *CUX1* showed LOH and reduced mRNA levels in tumors of the uterus [31]. Meanwhile, *CUX1* transcription factors were demonstrated to regulate a number of genes and microRNA [32]. Data on genetic aberrations in the accelerated phase or transformation to secondary acute leukemia in MPN revealed that *CUX1* on chromosome 7q was affected and involved in disease progression [33,34]. A deletion on chromosome 7q was demonstrable in 5, 17% of patients with disease progression (3/58) but only in 0,62% of patients in chronic phase (2/321) [33].

DNA Methyltransferase 3A (*DNMT3A*)

DNA methyltransferases transfer methyl groups to the cytosine at CpG islands. Whereas DNMT3 (A+B) share similar functions DNMT1 is believed to be the keeper of the methylation status [35].

DNMT3A mutations were first described in *de novo* AML in 62 out of 281 patients (~22%) [36]. Mutations comprise frameshift, nonsense, splice-site as well as deletions. *DNMT3A* mutations were demonstrable more often in the intermediate-risk cytogenetic profile but did not occur in the group of patients with a favourable cytogenetic profile. Of note, patients with *DNMT3A* mutations had a significantly shorter median overall survival [36].

In MPN, mutation rates are more frequent in PMF and cases with disease progression (~10%) than in PV (~7%) and ET (3%) [37,38]. *DNMT3A* mutations often showed co-incidence with other defects such as in *ASXL1, IDH1/2, JAK2 and TET2*. Because the definite function of mutated *DNMT3A* is yet unclear and also the mix of defects might be predictable for the molecular fate further studies will elucidate if *DNMT3A* acts as an oncogene or tumor-suppressor in MPN.

Enhancer of Zeste, homolog 2 (*EZH2*)/Polycomb repressive complex 2 (*PRC2*)

EZH2 together with EZH1 is part of the polycomb repressor complex 2 (PRC2). PRC2 itself is involved in many cellular processes such as proliferation, differentiation, senescence and cell fate [39]. PRC2 methylates certain histones thereby regulating gene expression. Apart from EZH1 and EZH2 PRC2 is composed of other factors: SUZ12, RBBP4, RBBP7, EED, AEBP2, JARID2, PHF1, PHF19, MTF2 [40].

EZH2 as part of PRC2 is a methyltransferase with high activity

Gene symbol	Gene	Cytogenetic location	Molecular defect*	Frequency in Ph- MPN (%)*
ASXL1	Additional sex comb like 1 gene	20q11.21	Truncating mutations mainly in exon 12	~36% PMF, post-PV-MF, post-ET-MF ≤ 6% chronic PV/ET
CBL	Casitas B-lineage lymphoma protooncogene	11q23.3	Missense mutations (exon 8 and 9), nonsense and splicing alterations (also intron regions)	~6% PMF Rare in PV, ET
CUX1 (CUTL1)	Cut-like 1 (CCAAT displacement protein)	7q22.1	Deletion, aUPD	<1% PMF, PV, ET ~5% in post-MPN sAML
DNMT3A	DNA Methyltransferase 3A	2p23.3	Frameshift mutations, non-sense mutations, deletions	~10% PMF ~10% post-MPN sAML ~7% PV, ~3% ET
IDH1	Isocitrate dehydrogenase 1	2q33.3	Point mutations	~4% PMF, ~2% PV, ~1% ET >20% in post-MPN sAML
IDH2	Isocitrate dehydrogenase 2	15q26.1		
IKZF1	Ikaros family zinc finger 1	7p12.2	Deletion	<1% in chronic MPN, ~20% in post-MPN sAML
JAK2	Janus kinase 2	9p24.1	aUPD, point mutation in exon 14 (V617F)	>95% PV, ~50% PMF, ~50% ET ~50% in post-MPN sAML
JAK2	Janus kinase 2	9p24.1	Mutations in exon 12 (K539L and others)	~3% PV, rare in PMF and ET
LNK (SH2B3)	Lymphocyte adaptor protein (SH2B adaptor protein 3)	12q24.12	Mutation, Loss-of-function	~5% PMF and ET ~13% in post-MPN sAML Must be considered to be detectable in *JAK2* (wild-type) erythrocytosis
MPL	Myeloproliferative leukemia virus oncogene	1p34.2	Point mutations in the juxtamembrane domain (W515K/L/A) & transmembrane domain (S505N)	~10% PMF ~3-5% ET
NF1	Neurofibromin 1	17q11.2	Deletion	~1% PMF
NRAS	Neuroblastoma RAS viral oncogene homol.	1p13.2	Gain-of-function	Up to ~13% in post-MPN sAML
PRC2	**Polycomb repressive complex 2 (without RBBP4/-7, PHF1/-19, MTF2)**			
EZH2	Enhancer of Zeste, drosophila, homolog 2	7q36.1	LOH, deletions	~13% PMF ~3% PV None in ET ?!
SUZ12	Suppressor of Zeste 12, drosophila, homolog of	17q11.2	Mutations, deletions	~1% MPN (and MDS)
EED	Embryonic ectoderm development protein, mouse, homolog of	11q14.2	Mutations, deletions	~1% MPN (and MDS)
JARID2	Jumonji, AT-rich interactive domain 2	6p22.3	Deletions	~2% MPN (and MDS)
AEBP2	Adipocyte enhancer binding protein 2	12p12.3	Deletions	Rare in chronic MPN
RUNX1	Runt-related transcription factor 1	21q22.12	Deletion, non-sense alterations	<1% in chronic MPN 27-37% in sAML
SF3B1	Splicing factor 3B, subunit 1	2q33.1	Mutations in exon 12 to 15 (K700E)	~6% PMF
SRSF2	Splicing factor, serine/arginine -rich	17q25.1	Point mutation or insertion/deletion (P95H/L/R others)	~17% PMF ~19% sAML from chronic MPN
SOCS1 *SOCS2* *SOCS3*	Suppressor of cytokine signalling	16p13.13 12q 17q25.3	Altered methylation status	SOCS1: ~15% SOCS2: ~28% SOCS3: ~28% in chronic MPN
TET2	TET oncogene family, member 2	4q24	Point/frameshift mutations (mainly exon 3 and 11), rare splicing alterations and deletions, missense mutations	~17% PMF ~16% PV ~5% ET
TP53	Tumor protein p53	17p13.1	Loss of function	Rare in chronic MPN ~27% in sAML

*=For References see Text section.

Table 1: Compilation of genetic lesions in Ph- MPN and secondary AML.

in contrast to EZH1 which has a rather low activity [41]. In myeloid disorders *EZH2* mutations apparently lead to inactivation or aberrant function of the protein. *EZH2* mutations can be found in ~3% of PV and ~13% of PMF but not in ET cases [42]. Mutations of *EZH2* might be associated with a poor clinical course especially in PMF where *EZH2* mutations together with co-existing mutations in *ASXL1*, *JAK2* (V617F), *CBL* or *TET2* define high-risk patients according to the International Prognostic Score System (IPSS) [43].

EZH2 together with SUZ12 and EED builds the core complex of PRC2 to enable full function. These and the other PRC2 factors *EZH1, SUZ12, EED* and *JARID2* were investigated for aberrations as well. Apart from *EZH2*, one study found that genetic aberrations in other PRC2 members are not common after screening a large number of myeloid malignancies [44]. Another study found that *SUZ12* and *EED* are affected in ~1% of cases and *JARID2* in ~2% of cases with MPN or MDS [45]. *JARID2* and *AEBP2* like the other PRC2 factors appear to

be affected later in the disease course of MPN and MDS and might be associated with transformation in secondary AML [46].

Ikaros Family Zinc Finger 1 (*IKZF1*)

This gene encodes for a transcription factor important for the sufficient development of the lymphoid lineage. IKFZ1 is involved in gene regulation through chromatin remodelling [47]. Animal models deficient in IKZF1 action showed defects of B cells, T cells and Natural killer cells or, in case of the heterozygosity for the dominant-negative allele, an acute lymphoblastic leukemia (ALL). Of note, the dominant-negative isoform Ik-6 was shown to be substantially involved in the development of B-cell malignancies [48]. Ph^+ *BCR-ABL* positive ALL frequently showed deletions of the *IKZF1* gene [49]. Secondary AML evolved from MPN showed an *IKZF1* deletion in up to 20% while chronic MPN rarely showed this defect [50]. Deletions of *IKZF1* in this study were proposed to appear later in the disease course and which is related to severe disease progression.

Isocitrate Dehydrogenase 1 and 2 (*IDH1/2*)

IDH1 and IDH2 are catalytic enzymes involved in conversion of isocitrate to alpha-ketoglutarate by concomitant production of NADPH [51].

Initially, Parsons et al. describe the occurrence of somatic point mutations in *IDH1* (R132H) and *IDH2* (R172K) as exclusive for glioblastoma [52]. Later on, by using parallel DNA sequencing a large study found these molecular defects also in *de novo* AML [53] and in chronic MPN evolving to leukemic transformation [54]. The occurrence of mutant *IDH* is highest in secondary AML developed in chronic MPN (~21%) and rare in PMF (~4%), PV (~2%) and ET (~1%). In our study from 263 MPN and 43 MDS samples none showed the expected R132H mutation in *IDH1* or the R172K in *IDH2*, respectively [55]. These negative findings were supported also by others [22]. However, in *de novo* AML we found up to 7% of cases showing the *IDH1* (R132H).

Mutant *IDH* was shown to be a gain-of-function defect which abnormally uses alpha-ketoglutarate instead of isocitrate as a substrate. As a consequence, alpha-ketoglutarate will be converted to 2-hydroxyglutarate which accumulates in the affected cell and also in the sera where it could serves as a clinical biomarker [56]. A knock-in mice model more precisely revealed that *IDH1* (R132H) increased the number of hematopoietic progenitors, provoked massive splenomegaly and, in the end, was responsible for a severe anaemia [57]. In addition, the myeloid lineage in this model showed aberrant hypermethylation of histones thereby paralleling the findings from human AML samples [53]. Interestingly, a leukemogenic consequence of the above mentioned conversion from alpha-ketoglutarate to 2-hydroxyglutarate through mutant *IDH* is a decreased *TET2* activity. *TET2* normally acts as a 2-oxoglutarate/Iron ($Fe2^+$)-dependent dioxygenase which converts 5-methylcytosine to 5-hydroxymethylcytosine. *TET2* therefore is involved in the demethylation process of DNA and its inhibition could substantially contribute to the leukemogenic potential of an affected cell [58,59].

Janus Kinase 2/*JAK2* (V617F)

Janus kinases belong to the non-receptor tyrosine kinases with JAK1, JAK2, JAK3 and TYK2 being the currently known family members. JAKs like other family of kinases play important roles in signal transduction pathways that govern cellular proliferation, differentiation, survival and apoptosis [60]. Many hematopoietic growth factors and cytokines such as erythropoietin (EPO), thrombopoietin (TPO), G-CSF, GM-CSF, interferon-γ, interleukins-2/-3/-5/-6/-11/-12/-13 but also the growth hormone and insulin recruit *JAK2* after binding to their receptors [60]. Some of these factors such as TPO recruit different non-receptor tyrosine kinases which activates *JAK2* but also TYK2.

The relatively large proteins of the JAK family (120-140 kDa) possess 7 homology domains (JH1-7). JH1 is the catalytically active domain with tyrosine kinase activity whereas the adjacent neighbour domain JH2 is a pseudokinase with auto-inhibitory function [61].

Following the intriguing finding that in patients with PV ~30% showed aUPD of chromosome 9p [7], 4 groups independently published the discovery of a gain-of-function mutation in *JAK2* [2-5]. Among MPN, PV showed the highest *JAK2* (V617F) frequency (~95%) followed by PMF (~50%) and ET (~50%) [62-67].

The molecular architecture is a hotspot in exon 14 where the wild-type G is exchanged by a mutant T with replacement of the amino acid valine by phenylalanine at position 617 (V617F) The bulky amino acid phenylalanine changes the conformation of the protein thereby no longer allowing the interaction of the autoinhibitory JH2 pseudokinase domain with the catalytic domain JH1. This disables control of its catalytic activity and leads to a constitutively activated kinase. Subsequently, downstream targets of this kinase will become activated in an affected cell [68]. The effects of mutated *JAK2* (V617F) showed a considerable diversity with respect to cellular lineages and MPN subtypes affected by this mutation. *In vitro* the *JAK2* (V617F) clone is autonomous and highly proliferative even in the absence of growth factors. This is demonstrable by higher numbers of endogenous erythroid colonies (EEC) in all subtypes harbouring the *JAK2* (V617F). Interestingly, cellular lineages are additionally hypersensitive to growth factors such as EPO, G-CSF or interleukins. In mouse models the mutation is powerful enough to mediate a PV-like phenotype with development of myelofibrosis [4,69,70]. The first mice models showed strain-specific differences. Whereas both the Balb/C and the C57Bl/6 mice showed an increase in hemoglobin and hematocrit, the number of leukocytes as well as the degree of myelofibrosis was strikingly higher in the Balb/C mice [69]. It became clear that the dosage of the mutant *JAK2* gene was responsible for the phenotypic degree of the developing MPN. A heterozygous *JAK2* (V617F) provokes a more chronic PV-like phenotype whereas homozygous expression led to a more severe course with the development of myelofibrosis [71]. This was in contrast to more previous *in vivo* findings showing no stringent correlation of the *JAK2* status with the clinical course and degree of fibrosis [72,73]. However, *JAK2* (V617F) - mutated ET patients show PV-like features, e.g. elevated hemoglobin and hematocrit levels and PV with a homozygous mutation have been suggested to have a higher risk for development of myelofibrosis [63-65,74].

An intriguing study unmasked the cell cycle regulator CDC25A as one cellular target of mutated *JAK2* (V617) with high impact on the proliferative capacity of affected cells *in vitro* and *in vivo* [75]. CDC25A was shown to be regulated by STAT5 on the translational level and its inhibition in *JAK2* (V617F) affected cells markedly reduced proliferation and extent of clonality. Therefore, CDC25A generally represents a potential target for new therapeutic strategies.

Though the *JAK2* (V617F) was a breakthrough in the field, it became clear that this molecular defect is not the definite initiator in MPN pathogenesis. This was demonstrated by X-chromosome inactivation patterns (XCIP) in female ET patients harbouring the *JAK2* (V617F) mutation [76-78]. As shown by XCIP, the proportion

of cells with *JAK2* (V617F) was smaller than the total number of clonal cells in a given patient [76]. In addition to classical XCIP approaches, one study elegantly investigated the portion of cells affected by the *JAK2* (V617F) mutation in comparison to the total number of clonal cells in a given patient by using the deletion 20q (del20q) as an autosomal, XCIP-independent clonality marker [77]. A clear discrepancy between a higher proportion of cells carrying the del20q and a lower percentage of cells carrying the *JAK2* (V617F) could be demonstrated. These data indicate that the hematopoietic stem cell (HSC) must have acquired at least one molecular defect which precedes the occurence of *JAK2* (V617F). Subsequent discoveries of newer molecular defects indeed showed that ASXL1 belong to the events which can occur before *JAK2* (V617F) was acquired [23].

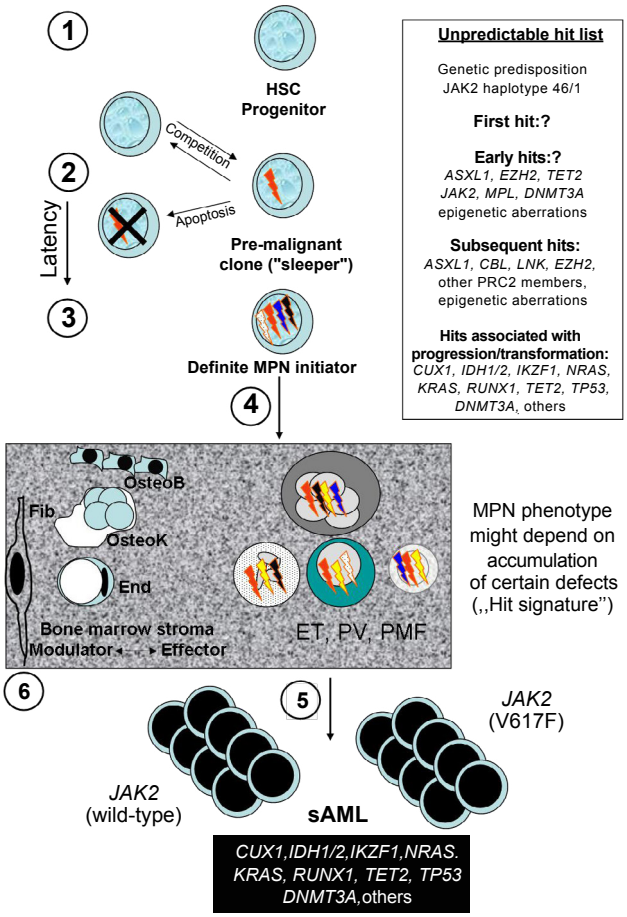
A very important prerequisite for the acquisition of the *JAK2* (V617F) mutation seems to be a certain combination of single-nucleotide polymorphisms (SNPs) on one allele of the *JAK2* gene which confers susceptibility for the V617F mutation [79,80]. Patients with PV, ET and myelofibrosis (n=311) all had the so-called *JAK2* haplotype 46/1 and in most of the cases the V617F mutation arose on this allele [79]. This genetic constitution as shown by a specific SNP profile probably justifies the term "predisposition" for development of a Ph- MPN (Figure 1) [81-83].

Janus Kinase 2/ *JAK2* (K539L and other Aberrations in Exon 12)

In the diagnostic setting patients may show high haemoglobin levels, high hematocrit and also low serum EPO levels but do not harbour the *JAK2* (V617F) mutation. These patients must be considered to be affected by a rare but important defect in exon 12 of *JAK2*. These molecular aberrations are diverse and may be point mutations leading to a K539L substitution or comprise insertions/deletions leading to various amino acid substitutions [84]. The bone marrow in patients affected by exon 12 aberrations shows a prominent erythropoiesis, but otherwise no true peculiarities, because also the megakaryocyte population appear normal in size, number and distribution. However, another study found the bone marrow with *JAK2* exon 12 mutations more peculiar with also the megakaryocyte population being affected [85]. Interestingly, with regard to *JAK2* exon 12 aberrations a large retrospective study found two features which were noteworthy to stress: 1. Gender did matter because more women than men were affected when compared to idiopathic erythrocytosis showing the *JAK2* wild-type status, 2. Patients presented at younger age when compared to *JAK2* (V617F), [86]. However, compared to *JAK2* (V617F) the frequency of *JAK2* exon 12 aberrations is low, molecular diagnostics is laborious, and molecular testing should be restricted to individuals which are *JAK2* (V617F) negative and whose clinical parameters strongly suggest a clonal erythropoiesis.

LNK (SH2B adaptor protein 3 - SHLB3)

LNK has a central role in the control of *JAK2* activity and involves receptors which recruit *JAK2* like EPO-R and MPL [87]. Mice deficient in LNK showed an affected pool of hematopoietic stem cells (HSC), i.e. an increase of cells in numbers, but also extended self-renew capacity and quiescence [88]. LNK deficient cells showed an increased sensitivity to several cytokines and activated downstream pathways involving *JAK2* [89]. LNK knock-out mice developed a disease with MPN-like features such as thrombocytosis, bone marrow fibrosis and extramedullary hematopoiesis. *JAK2* (V617F) mutated animal models with a concomitant *LNK* deficiency showed a faster progress to a full-blown MPN-like disease [90]. As shown for both, *JAK2* (V617F) and *MPL* (W515L), LNK is capable of hindering their aberrant constitutive signalling. Interestingly, in *JAK2* (V617F) mutated MPN patients LNK expression was increased and correlated with a higher burden of the



Abbreviations: Fib=Fibroblast, End=Endothelial cell, OsteoK=Osteoklast, OsteoB=Osteoblast

Figure 1: Proposed steps in the development of Ph- MPN: 1. A genetically normal hematopoietic stem cell (HSC) and/or early progenitor cells might be predisposed for a genetic lesion which is currently unknown. Predisposition as shown for the *JAK2* haplotype 46/1 may exist for other genes but are yet undiscovered 2. Cells affected by a genetic defect may undergo apoptosis. An affected cell that survives is in continuous competition with the larger pool of normal HSC. A small pre-malignant clone is clinically unapparent. 3. The latency until clonal hematopoiesis in Ph- MPN become clinically apparent is unknown and may span decades. During this time single cells may acquire additional molecular hits such as ASXL1, *TET2*, *JAK2* and/or epigenetic aberrations which lastly drive the clone to clinical appearence. 4. The composition of molecular defects ("hit signature") and the occurrence in progenitors of particular cellular lineages definitely initiate the disease and form the type of Ph- MPN. 5. Occurrence of additional genetic lesions associated with sAML is rarely demonstrable before disease progression takes place. Most of the genes are not specific for sAML evolved from Ph-MPN. Secondary AML may or may not harbour the *JAK2* (V617F) mutation; up to 50% of sAML evolved in a chronic Ph- MPN with a pre-existent *JAK2* (V617F) show the *JAK2* (wild-type) in the leukemic cells [81]. 6. The bone marrow stroma including cell populations forming the connective tissue (e.g. fibroblasts, adipocytes, endothelial cells, osteoblasts, osteoklasts) and the so-called vascular niche and the endosteal niche apparently play a relevant role in the process not only of physiological stem cell homeostasis but also in clonal evolution of malignant stem cells and progression of disease, e.g. myelofibrosis, neoangiogenesis, and aberrant stem cell trafficking in Ph- MPN. Endothelial cells in *JAK2* (V617F) Ph- MPN may belong to the malignant clone [82,83].

mutant *JAK2* (V617F) allele [91]. This work also showed that LNK is an important negative controller of signals mediated through the TPO/*MPL* axis. Up-regulation of LNK in *JAK2* (V617F) patients can be interpreted as a counteracting mechanism.

In chronic MPN, *LNK* mutations affect only ~5% of ET and PMF patients, but in post-MPN AML *LNK* mutations were detectable in up to 13% of cases [92]. Accordingly, *LNK* aberrations appear to be late genetic events in the disease course of MPN. Interestingly, one study found *LNK* mutations in two patients with isolated erythrocytosis and wild-type *JAK2* status and expanded the hitherto sparse knowledge on so-called idiopathic erythrocytosis [93].

Myeloproliferative Leukemia Virus Oncogene (*MPL*)

Thrombopoietin (TPO) and its receptor MPL is indispensable for hematopoietic stem cell survival, differentiation of hematopoietic progenitor cells, and is the key regulatory element of megakaryopoiesis and platelet formation [94]. The MPL receptor is expressed by hematopoietic tissues, hematopoietic stem cells, erythroid progenitors, megakaryocytes, and platelets [95].

Early studies in MPN showed that reduced megakaryocyte MPL expression is due to impaired post-translational modifications [11,96,97]. Demonstration of reduced platelet MPL expression and weakly labelled megakaryocytes by immunohistochemistry were found to be of diagnostic value in MPN [12,18,96,98,99]. Several studies demonstrated that megakaryocytes and the erythroid lineage co-express MPL and the erythropoietin receptor (EPO-R) as well as transcription factors like NF-E1 and -E2 [100-102]. Because of diverse structural homologies between MPL, EPO-R and their ligands, it became evident that the megakaryocytic and the erythroid lineage arise from a common progenitor cell [102]. Therefore, abnormal expression and function of MPL or EPO-R could induce proliferation of both the megakaryocytic and erythroid lineage [103,104].

In 2006, the discovery of another gain-of-function mutation in MPN was published [105]. Whereas mutation screening of the granulocyte-colony stimulating factor receptor (G-CSF-R) and the EPO-R in this cited work showed no molecular defect, a point mutation in the juxtamembrane domain of the thrombopoietin receptor *MPL* (exon 10) was discovered [105]. In *MPL* (W515L), like with the *JAK2* (V617F) mutation, a single nucleotide is changed (G to T), leading to a substitution of the amino acid tryptophane by leucin. Both substitutions apparently change the folding of the protein or drive new interactions of the mutant protein with others. In the end, the function is substantially perturbed. Cell lines transfected with *MPL* (W515L) showed cytokine-independent growth and hypersensitivity for TPO. The *MPL* (W515L) mice model showed exaggerated number of platelets, a massive hepatosplenomegaly and splenic infarctions. This study and subsequently performed analysis in a large cohort of 1182 patients showed a *MPL* (W515L) frequency of ~5% in patients with PMF and ET [106]. It is of note that the latter study revealed another mutation *MPL* (W515K) as well as the occurrence of *MPL* mutations together with *JAK2* (V617F) in individual patients. Correlation of the molecular status with clinical presentation has revealed no specific phenotype and analysis of larger trials was anticipated in order to find peculiarities of patients carrying the *MPL* (W515L/K) mutation. Beer et al. [107] investigated the occurrence of *MPL* mutations in 776 samples from ET patients [107]. The overall frequency of *MPL* exon 10 mutations was 8.5% in patients with unmutated *JAK2*. One patient with concomitant *JAK2* (V617F) and *MPL* mutation could be identified. Patients with the W515K mutation had have a higher mutant allele burden than those with W515L. Compared with *JAK2* (V617F) *MPL* mutated ET patients showed lower haemoglobin levels, higher EPO levels, higher platelets, an endogenous megakaryocyte colony growth but no EEC and an overall less bone marrow cellularity. However, *MPL* mutations lacked prognostic significance with regard to haemorrhage, thrombosis, development of myelofibrosis and survival.

The *MPL* (W515L/K) mutation was detectable in CD34-positive cells from patients with PMF which *in vitro* gave rise to multiple hematopoietic lineages carrying the mutation [108]. *MPL* (W515L/K) is like *JAK2* (V617F) an event demonstrable in HSC and early progenitors.

Other *MPL* mutations such as the *MPL* (S505N) were found initially in familial MPN and later on also in sporadic MPN [109,110]. In a study of 221 patients with *JAK2* (wild-type) ET and PMF, 33 (15%) carried a *MPL* mutation [110]. Only 1 ET patient carried both the *MPL* mutation and the *JAK2* (V617F) mutation. Interestingly, 3 of the patients showed *MPL* double mutations on the same chromosome.

Neurofibromin (*NF1*)

The *NF1* gene encodes for neurofibromin, a cytoplasmic protein that is predominantly expressed in neurons, Schwann cells, oligodendrocytes, and leukocytes. *NF1* has the capacity to regulate several intracellular processes, e.g. the RAS-cyclic AMP pathway, the ERK/MAP serine/threonine kinase cascade, adenylyl cyclase, and cytoskeletal assembly [111]. Apparently 5 neurofibromin isoforms are expressed in different tissues of which isoform 10a possess a transmembrane domain. This isoform is widely expressed among human tissues.

NF1 has been suggested earlier to be a tumor suppressor because of its role in negative regulation of the Ras pathway and in the development of certain types of neurofibromatosis [112,113]. Later on, aberrations of *NF1* in other cancers such as glioblastoma [114] and certain neurotropic melanoma types [115] were described.

Deletions of *NF1* in chronic MPN are rare events with only 2 affected MPN patients in a study group of 151 patients [116]. Interestingly, a recent study found frequent codeletions of *NF1* with *SUZ12* as a member of the polycomb repressor complex 2 (PRC2) [46].

Neuroblastoma RAS (*NRAS*)

Mutations in RAS proteins (NRAS, KRAS, HRAS) were widely studied in subtypes of cancer such as colorectal cancer or lung cancer and mainly affect codon 61 in *NRAS* and codons 12/13 in *KRAS*, respectively. Mutations in *RAS* genes in the end disrupt GTPase activity with subsequent constitutive activity of pathways such as MAPK. Meanwhile *RAS* mutations predict the response of targeted therapies against growth factor receptors such as EGFR [117]. Among other genes such as *CBL, RUNX1, TET2, NRAS* was shown to be present in post-MPN AML and therefore is a genetic defect associated with disease progression [118].

Runt-related transcription factor 1 (*RUNX1*)

Alpha- and beta subunits of Runx transcription factors are encoded by *RUNX1-3* genes and the *CBFB* gene, respectively. The alpha subunit binds directly to DNA whereas the beta subunit increases the binding affinity of the alpha-subunit without direct binding to DNA.

RUNX1 (alternatively named *AML1*) is critically involved in the proper development of all hematopoietic lineages but also is involved

in development of certain neural compartments and bone formation [119]. Moreover, *RUNX1/AML1* was shown to be involved in the t(8;21) translocation of the M2 subtype of AML according to the French- American-British-Classification (FAB) [120].

RUNX1 was suggested to be one of the genes associated with disease progression because mutations are frequently detectable in secondary AML (post-MPN) in 27%-37% but rarely in chronic MPN [118,121-123]. In a more descriptive study, we previously discussed that aberrant microRNA expression might be involved in dysregulated expression of *RUNX1* in chronic MPN [124].

Splicing Factor 3B, Subunit 1 (*SF3B1*)

Splicing factor, serine/arginine-rich, 2 (*SRSF2*)

Genetic aberrations of factors involved in RNA splicing were previously reported for *SF3B1* mainly in the MDS subtype refractory anaemia with ring sideroblasts (RARS). In addition to *SF3B1* a landmark study found other affected factors involved in RNA splicing such as *SRSF2, U2AF1, ZRSR2* and *SF3A1* [125]. The effects of abnormal RNA splicing due to these aberrations compromised normal hematopoiesis and revealed a relevant insight into MDS pathobiology.

SF3B1 and *SRSF2* mutations were detected in ~6% and ~17% of patients with PMF, respectively [126,127]. *SRSF2* mutations in PMF patients were independently associated with poor survival, the concomitant affection by *IDH1*/2 mutations, and leukemia-free survival [127]. In another study post-MPN AML were investigated for *SRSF2* mutations and showed a mutation rate of ~19% compared to secondary AML evolved from MDS (~5%) and *de novo* AML (~6%) [128]. *SRSF2* mutations were associated with poor overall survival in univariate and multivariate analyses. It was concluded that *SRSF2* is a relevant contributor to leukemic transformation in MPN.

Suppressor of Cytokine Signalling (*SOCS1,-2,-3*)

The suppressors of cytokine signalling (SOCS) are critically involved in the regulation of cellular proliferation, survival, and apoptosis via cytokine-induced JAK/STAT signalling. The suppressor of cytokine signalling -1 (SOCS-1) interacts with various components of cytokine and hematopoietic growth factor signalling including those activated by interleukin-6 or steel factor (c-kit) [129]. It has been demonstrated that SOCS-1 is a specific inhibitor of Janus kinase 2 (JAK2) signalling through binding of a conserved regulatory tyrosine in the activation loop of the catalytic domain JH1 of *JAK2* [130]. Furthermore, at least 2 domains, the pre-SH2 domain or kinase inhibitory region (KIR), and the SH2 box, are responsible for effective binding and inhibition of signals transmitted by *JAK2* [131].

In the accelerated phase of Ph^+ *BCR-ABL* positive CML an overexpression of SOCS-2 was shown and it was suggested that the normally intact negative feedback loop should be overcome by SOCS-2 up-regulation [132]. We previously found an overexpression of *SOCS1* mRNA in bone marrow cells of Ph- MPN [133]. This study involved an overall of 89 MPN cases and except for PMF showed an overexpression independent of the *JAK2* status (V617F vs. wild-type). Another study showed hypermethylation of the *SOCS1* promoter region in 6/39 MPN patients (3 with and 3 without *JAK2*-V617F) [134] which suggested decreased mRNA expression in these 15% of cases. Another work performed studies on the methylation status of CpG islands of *SOCS1- SOCS3* in MPN and correlated successfully hypermethylation of *SOCS1* or *SOCS3* in 23/81 MPN cases (28%) with decreased mRNA expression in a given case [135]. Hypermethylation of *SOCS2* was then demonstrated by another group in cytokine-independent cell lines and 2 out of 7 MPN patients [136].

Tet Oncogene Family Member 2 (*TET2*)

The first larger study on *TET2* defects showed considerable variations including frameshifts, nonsense or missense mutations in exon 4 and 12 of patients with MPN [137]. The overall mutation frequency in MPN was ~13% with ~5% in ET and up to 16 % and ~17% in PV and PMF, respectively. *TET2* mutations occur in both *JAK2* (V617F) and *JAK2* (wild-type) cases with frequencies of ~17% and ~7%, respectively. Interestingly, occurrence of *TET2* aberrations significantly increase with age because ~23% of affected patients were above 60 years of age compared to only 4% of *TET2* positive cases in younger individuals. The presence of *TET2* mutations did not correlate with prognostic factors such as overall survival, rate of leukemic transformation or risk of thrombosis in PV and PMF. However, in patients with PV another study showed that *TET2* mutations may occur in *JAK2* (V617F) and *JAK2* (wild-type) clones in an individual patient [138]. Interestingly, different *TET2* alterations appear to occur in the same individual suggesting molecular dynamics probably due to genetic instability. Of note, this study did not observe a correlation of *TET2* mutations and older age.

Following their first discovery in MPN *TET2* mutations were also found in other myeloid malignancies with considerable high frequencies in *de novo* AML (42%) [139], in systemic mastocytosis (29%) [140], in MDS with rearranged 4q24 (19%), in CMML, in secondary AML (up to 32%) and in familial MPN [139-141].

Alterations in the *TET2* gene therefore appear to be a common event in myeloid malignancies including those showing dysplastic features, chronic myeloproliferation and leukemic transformation. Haploinsufficiency of *TET2* is thereby sufficient enough to contribute to the phenotype of disease because in the majority of cases only one allele is affected.

Before the discovery of *TET2* alterations the function of this gene was almost unclear. However, TET1 was shown to catalyze the reaction from 5-methylcytosine in the DNA to 5-hydroxymethylcytosine [142] suggesting a role for TET proteins in epigenetic regulation. Recent studies in mice revealed that *TET2* is a major player in the demethylation process of DNA. Animals deficient in *TET2* showed decreased levels of 5-hydroxymethylcytosine along with high levels of 5-methylcytosine in the DNA, they showed less differentiation capacity of the hematopoietic stem cell compartment but increased potential of its self-renewal capacity [143, 144]. Interestingly, in the *TET2* knock out models the remaining differentiation capacity mainly affected the monocytic and granulocytic lineage. Following a latency of several months, the animals died from disease phenotypes very similar to CMML, secondary AML or even MDS [144]. These data underline the function of *TET2* as a tumor suppressor which normally maintain normal hematopoiesis.

Tumor Protein p53 (*TP53*)

Among the genes affected by molecular defects in solid cancer and other malignancies *TP53* is one of the best studied gene so far [145,146]. Normal function comprises involvement in DNA repair, control of cell cycle and cell fate, i.e. apoptosis and senescence. Accordingly, a loss-of-function defect in *TP53* substantially affects homeostasis of a given cell.

TP53 mutations are frequently detectable in post-MPN AML but are occasionally detectable also in chronic MPN earlier before

transformation becomes clinically overt [118]. Mutation of *TP53* in post-AML was shown in ~27% of cases under investigation and affected mainly both alleles [34]. In one study *TP53* aberrations were associated with gains of chromosome 1q, a locus harbouring the TP53 inhibitor MDM4 [147]. Post-MPN AML patients in this study were affected by *TP53* or 1q gains in ~45% of cases.

Taken together, *TP53* mutations are uncommon in chronic MPN but are very frequent in post-MPN AML suggesting this molecular defect as a potential predictor of disease progression and transformation. In one study *TP53* mutations were the only independent prognostic factor of poor survival [34].

Therapeutic Targets

The following section does not claim to give a complete overview of currently established and up-coming therapies in MPN. However, it aims to summarize briefly the standard therapies in MPN followed by introduction of some studies which investigated promising agents for molecularly targeted therapies.

Standard therapies for PV patients focus on reduction of the high hematocrit by phlebotomy to avoid or minimize risks for thrombotic events or haemorrhage. Patients with high-risk PV may receive aspirine to improve microvascular events as well as hydroxyurea or pegylated interferon to control disease-related symptoms and improve quality of life [148]. Low-risk ET usually undergoes a "watch and wait" therapy, intermediate-risk ET may treated with aspirin, and patients with a prominent thrombocytosis and a high risk for thrombotic events must be treated with platelet-lowering agents such as anagrelide or hydroxyurea with or without aspirin [149,150]. In PMF, therapy can be adjusted to risk scores like the dynamic international prognostic scoring system (DPSS) [151], the IPSS or the International Working Group for Myelofibrosis Research and Treatment consensus criteria. It aims to improve constitutional symptoms which arise from organomegaly of spleen and liver but also from insufficient hematopoiesis leading to cytopenia. Drugs used in PMF treatment comprise erythropoiesis-stimulating agents, androgens, danazol, corticosteroids, thalidomide, lenalidomide, hydroxyurea, and cladribine [152]. In combination these drugs may be induce longer lasting improvement of the disease course, e.g. when lenalidomide and corticosteroids were combined. Patients in one study showed a reduction of splenomegaly (42%), improvement of anemia (30%), a decline of myelofibrosis as evidenced by bone marrow histopathology in 10 out of 11 patients, and a lower *JAK2* (V617F) mutant allele burden in 8 patients (decline of up to 50% in 4 patients, no longer detectable mutant allele burden in 1 patient) [153]. However, none of the aforementioned therapies was powerful enough to change the overall median survival in PMF which ranges from 3 – 5 years [152]. The only curative therapeutic option in PMF is the allogeneic stem cell transplantation which by implementation of intensity reduced chemotherapy regimen showed an event-free overall survival of 67% after 5 years [154].

Soon after discovery of the predominant molecular defects of *JAK2* and *MPL* in MPN the first molecularly targeted therapies were initiated by using low molecular mass ATP-competitive inhibitors of either *JAK2* or against JAK1 and *JAK2* [155]. A selection of currently studied JAK kinase inhibitors comprise ruxolitinib (Incyte in the U.S., Novartis elsewhere), Lestaurtinib (Cephalon), Pacritinib (S*Bio), TG101348 (Sanofi), CYT387 (YM BioSciences), AZD1480 (AstraZeneca) and LY2784544 (Lilly) [156]. Some of these drugs are now in clinical phase I-III studies but the only drug approved for therapy in patients with intermediate to high risk MF by the Food and Drug Administration (FDA) in the U.S. so far is ruxolitinib. Ruxolitinib is an orally administered potent inhibitor of JAK1 and *JAK2* with half maximal concentrations (IC_{50}) of 3.3 nM and 2.8 nM, respectively. TYK2 and also JAK3 could be inhibited by ruxolitinib as well but the IC_{50} must then 6-fold and 130-fold higher, respectively [157]. Ruxolitinib inhibited downstream signalling by *JAK2* (V617F) and wild-type *JAK2* with reduced phosphorylation of *JAK2* effectors STAT5 and ERK1/2 *in vitro*. Cell lines transfected with the *JAK2* (V617F) showed reduced cellular proliferation and induction of apoptosis when treated with ruxolitinib [157]. Primary cells from PV patients with a high mutant *JAK2* (V617F) allele burden showed decreased formation of endogenous erythroid colonies and a decline of erythroid/myeloid progenitor cells. In a mice model with a *JAK2* (V617F) induced MPN-like disease, ruxolitinib (formerly called INCB018424) reduced splenomegaly, decreased peripheral cytokine levels (IL-6, TNF-α) to baseline, and increased survival of the animals [157].

Following promising phase I/II studies which showed a notable improvement of clinical symptoms without severe toxicity [158], the double-blinded placebo-controlled (phase III) COMFORT-I study included a total of 309 patients with PMF, post-PV-MF and post-ET-MF having an IPSS score of intermediate to high risk. In the verum group (initial n=155) receiving 15 or 20 mg ruxolitinib (BID=twice daily) ~42% of patients showed a reduction of ≥ 35% spleen volume after 24 weeks when compared to 0.7% in the placebo group (initial n=154) [159]. While the placebo group showed a modest increase in the *JAK2* (V617F) allele burden at 24 and 48 weeks, the ruxolitinib treated patients showed a decline of the mutant allele burden by ~10.9% and 21.5%, respectively. Notable benefits from clinical discomfort and relief of symptoms such as abdominal pain, itching, night sweats, bone/muscle pain, inactivity/fatigue could be demonstrated in many patients treated with ruxolitinib in contrast to the placebo group. Ruxolitinib was all in all well tolerated. General side effects in the verum group were ecchymosis, dizziness, and headache along with haematological side effects such as thrombocytopenia and anemia. Discontinuation from the study was a rare event. After a median follow-up of 52 and 51 weeks, respectively, 13 patients in the verum group and 24 patients in the placebo group were dead [159].

In a randomized phase III study the COMFORT-II investigators compared ruxolitinib with the best available therapy (comprises hydroxyurea, interferon, corticosteroids, EPO, androgens and others; combination allowed) in patients with PMF, post-PV-MF and post-ET-MF having an IPSS score of intermediate to high risk and a defined size of palpable splenomegaly [160]. Patients were randomized 2:1 to ruxolitinib (n=146) and the best available therapy group (n=73). Primary and secondary efficacy outcome measures were a reduction of ≥ 35% spleen volume at week 48 and 24, respectively. In the ruxolitinib group 28% (week 48) and 32% (week 24) of patients showed a ≥ 35% spleen reduction compared to 0% (week 48) and 0% (week 24) receiving the best available therapy. 80% of the ruxolitinib treated patients still showed a therapy response after 12 months. As also shown in the COMFORT-I study the ruxolitinib group had a significantly better relief from clinical symptoms compared to the best available therapy regimen. After a median time of 61.1 weeks, 8% and 4% deaths were reported in the ruxolitinib and the best available therapy group, respectively [160]. Notable cross-over to the ruxolitinib group and incomplete survival follow-up was suggested to hinder reasonable interpretation at this time point. Main adverse effects on hematopoiesis were thrombocytopenia and anaemia with more severe cases in the ruxolitinib group compared to the best available therapy group.

Ruxolitinib was also investigated in PV and ET patients refractory for hydroxyurea treatment and in PV patients to test for superiority over the best available therapy [155].

Apart from ruxolitinib other molecularly targeted therapy studies were conducted or are under way by using lestaurtinib (inhibitor of FLT3, JAK2, RET = rearranged during transfection) in patients with PMF, post-PV-MF and post-ET-MF [161], pacritinib (SB1518, a *JAK2* and FLT3 inhibitor) in patients with myelofibrosis [162], TG101348 (a JAK2-, FLT3-, RET- inhibitor) in patients with PMF, post-PV-MF and post-ET-MF [163], CYT387 (an inhibitor of JAK1, JAK2, TYK2) [164], AZD1480 (a JAK1 and *JAK2* inhibitor) [165,166] and LY2784544 (*JAK2* inhibitor) [167].

Especially FLT3 inhibitors were also widely investigated for their potency in *de novo* AML whereas some others like the newer JAK3 inhibitor tasocitinib seems to be also potent in diseases with a strong inflammatory background like rheumatoid arthritis [156]. Moreover, these small molecule inhibitors seem to act as immunmodulators and may help to prevent organ rejection after transplantation [168].

Other drugs investigated for targeting aberrant molecular pathways in MPN and other myeloid malignancies are inhibitors of the mTOR pathway such as everolimus [169], the EGFR inhibitor erlotinib [170] and the proteasome inhibitor bortezomib affecting the NFκB pathway [171]. Because a breakthrough by using a single agent is pending, investigators proposed combination therapies such as the mTOR pathway inhibitors RAD001 and PP242 with AZD1480 and ruxolitinib [166].

Compounds developed for targeting especially epigenetic changes in MPN were givinostat (ITF2357), panobinostat (LBH589), vorinostat and decitabine (5-aza-2'-deoxycytidine) and might reverse changes such as histone H3 phosphorylation or aberrant methylation [156].

It is noteworthy to underline that above mentioned targeted therapies in MPN so far do not directly hit any of the molecular defects discovered up to date. The number of *JAK2* inhibitors all target both mutant and wild-type *JAK2* and based on the fact that *JAK2* (V617F) is not the founder of the malignant clone *JAK2* inhibitors will not cure the MPN. On the other hand *JAK2* inhibitors decrease cytokine signalling and the pro-inflammatory response which is apparently responsible for a number of clinical complaints in MPN patients. The above reviewed effects of *JAK2* inhibitors such as ruxolitinib are indeed a notable relief from clinical symptoms which cannot be underestimated. The rationale for a molecularly targeted therapy with one of the established inhibitors is therefore apparent.

Cytokine signalling and mainly secondary changes in the bone marrow environment due to inflammatory processes, hypoxia, pro-angiogenic signalling by VEGF with increased micro vessel density as demonstrable in PMF induce some of the clinical symptoms in MPN. Therefore, the inhibition of cytokine signalling by JAK inhibitors affect cellular homeostasis and reduce MPN-typical features. On the other side the relevant adverse effects of JAK inhibition affect hematopoiesis leading mainly to anaemia and low platelet counts. Accordingly, a continuous therapy will compromise the clinical course in other ways.

It is likely that upcoming therapies will be composed of different compounds directed against the variety of molecular defects demonstrable in a given patient. Future targeted therapy in Ph- MPN therefore could be a personalized therapy based on the individual set of affected genes.

Summary

First of all, the discoveries of molecular defects in the Ph- MPN over the recent few years were awesome. Research groups and networks composed of experts in haematology, pathology and molecular biology in the U.S., Europe and all over the world cooperated to pave the way for one of the most impressive progress in myeloid malignancies. Only 7 years after the somatic mutation in *JAK2* (V617F) was detected this breakthrough not only proved to be indispensable for a more accurate diagnosis. *JAK2* in Ph- MPN is meanwhile target for small molecule inhibitors and stands for a new era of molecular therapeutics.

After discovery of *JAK2* (V617F) and *MPL* (W515L/K) in 2005 and 2006, respectively, many other molecular aberrations were found in Ph- MPN and associated myeloid malignancies to better discriminate from reactive lesions and, at least in part, allow a more distinct differential diagnosis. For example the *JAK2* (K539L) mutation is probably another prototype for an entity-specific molecular defect in patients with PV-like clinical presentation but lack of *JAK2* (V617F) and bone marrow features uncommon for PV. Moreover, these findings expand the knowledge on the fundamental pathobiology in chronic diseases which for many decades were more on the sidelines. Accordingly, the publication record for PMF, PV and ET climbed up enormously. However, there is probably no direct correlation between the number of molecular defects discovered in Ph- MPN so far and the extent of true understanding of the nature of the three entities in question.

We have to realize that 3 distinct diseases in terms of histopathological and clinical presentation exist even though overlaps are not uncommon.

We realize that many molecular defects except for those associated with leukemic transformation are demonstrable in all 3 diseases at the chronic stage.

It is widely agreed that *JAK2* (V617F) is an important event which substantially contributes to the phenotype of a Ph- MPN. However, it is well accepted that other somatic molecular defects precede *JAK2* (V617F) in a HSC to found clonal hematopoiesis. Accordingly, Ph- MPN phenotypes are the result of different genotypes, i.e. accumulating somatic molecular hits form a signature responsible for what clinically become apparent. Aberrations in *ASXL1, TET2*, *EZH2* and probably in yet unknown genes are important examples for early hits in Ph- MPN before occurrence of *JAK2* (V617F). On the other hand, a molecular hit through aberrant *TET2* function can also occur later in the disease course when *JAK2* (V617F) is already a driver over a long time.

Even though Ph- MPN pathobiology starts on the level of a HSC it is likely that progenitor cells of different lineages (all affected by the "master defect") later on acquire a different signature of additional molecular hits. This also could explain the different phenotype with more demonstrable hits underlying PMF pathobiology compared to the more mild disease course in ET showing less genetic lesions. Moreover, the hit signature probably explains the different risks for transformation into secondary AML.

Accordingly, a comprehensive molecular profiling for a much better discrimination of subtypes in Ph- MPN would be ideal. An elegant study recently performed a so-called genome-wide methylome/epigenome profiling which showed aberrant promoter hypermethylation in PV and ET whereas PMF was epigenetically a distinct subgroup with aberrant hyper- and hypomethylation [172]. Methylated genes in PV and ET affected signalling pathways enriched for binding sites for transcription factors such as GATA-1. By contrast, aberrantly methylated genes in

PMF were more involved in inflammatory pathways and were enriched for transcription factors other than PV and ET. Interestingly, within PMF cases those with *ASXL1* mutations formed a distinct subgroup showing all in all increased methylation [172]. As expected *TET2* mutated MPN cases showed decreased levels of hydroxymethylation and, much more intriguing, another distinct set of hypermethylated genes.

The latter study is another example for the importance and impact of molecular techniques which should be applied to gain a better insight into the pathobiology of the Ph- MPN. Once discovered, molecular marker or sets of marker not only allow a better understanding in an intellectual sense but preferably generate markers for a more accurate diagnosis.

We have robust and evidenced criteria for the diagnosis of a given Ph- MPN. Fortunately, this still includes histopathological evaluation of a bone marrow biopsy which in my opinion is a very important instrument for diagnosis of MPN and most other myeloid malignancies. Accordingly, the doctor should convince the patient not only about the necessity of a core biopsy for initial diagnosis but also for the agreement to take sequential biopsies during the time course. This, because many important features especially of Ph- MPN like the first slight increase of blasts or any changes in stroma pathology, e.g. a regression of myelofibrosis during therapy, is detectable only through this invasive procedure. Because of the progress in laboratory technologies over the last 10 -15 years the bone marrow biopsy, when adequately preserved and gently decalcified, is almost unrestricted stable and useful for molecular analysis of DNA, mRNA and microRNA [173-175]. Moreover, the correlation of histopathology and molecular data in one biopsy sample optimize diagnosis finding.

However, the demonstration of some of the hitherto discovered molecular markers in Ph- MPN is somewhat challenging with regard to techniques which are required for optimal sample analyses. While a *JAK2* (V617F) assay meanwhile is part of the standard molecular diagnostics in Ph- MPN, a comprehensive analysis for *TET2* aberrations is more elaborated and time-consuming. The molecular nature of *TET2* aberrations and other genes such as *CBL* can be more complex (Table 1) and will probably not be unmasked by a single laboratory assay. Accordingly, the introduction of appropriate assays which are still robust even when performed in different laboratories is of utmost necessity. For example, the limited accuracy of standard SANGER sequencing for detection of some of the currently used molecular markers in Ph- MPN and other myeloid malignancies is an important issue which has to be considered [176]. For both the diagnostic setting and basic clinical research the most sensitive and the best workable technique must be established.

Where do we currently stand with the Ph- MPN?

The number of molecular defects detectable in Ph- MPN continuously increases. All the markers lack specificity because they are demonstrable also in a variety of other myeloid malignancies. Yet no marker stands definitely for a beginning transformation into secondary leukemia, yet no definite marker may indicate a beginning myelofibrosis and last not least, standardized laboratory assays for many markers are pending. In other words, the complexity of Ph- MPN became more complex and not easier to look through.

Nevertheless, we now have a variety of markers which can be studied in more detail not only to describe their occurrence in a given entity but to reveal more relevant insights into pathobiology. Technical progress should allow a more comprehensive determination of molecular defects present in a given patient and in the end might lead to an individualized molecularly targeted therapy.

Notice

The term “chronic MPN” as used throughout the manuscript comprises disease stages without hints of disease progression and leukemic transformation.

If not otherwise cited this review was inspired by a previously published book chapter contributed by this author and colleagues [177].

The author apologize that many excellent experimental data and clinical research as well as comprehensive reviews were not cited due to the large number of publications in the field and due to space restriction.

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