The Current Molecular Site of the Myeloproliferative Neoplasms - TET-à-tête 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 MD 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 otherwise 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 (TGF-β-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].

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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, DNMT3A 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, DNMT3A.

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 myelodysplastic syndrome (MDS), post-MDS AML, the chronic myelomonocytic leukemia (CML) 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. This indicates 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 g91-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...
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

| 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 (CCAT 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 | Deletion | <1% in chronic MPN, ~20% in post-MPN sAML |
| IKZF1       | Ikaros family zinc finger 1 7p12.12 | 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% 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, PHF11-19, 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 8p22.3 | 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 2q33.3 | 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 |

Table 1: Compilation of genetic lesions in Ph- MPN and secondary AML.
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. IKZF1 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-hydroxoglutarate 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-hydroxoglutarate through mutant IDH is a decreased TET2 activity. TET2 normally acts as a 2-oxoglutarate/Fe(2+)-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 belongs 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 a UDP 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 [4,67,70]. The first mice model 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 (V617F) 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 (XCIIP) in female ET patients harbouring the JAK2 (V617F) mutation [76-78]. As shown by XCIIP, 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 occurrence 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 - SHL3B)**

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
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 thrombopoietin (TPO) showed no molecular defect, a point mutation in the juxtamembrane domain of the thrombopoietin (TPO) and its receptor MPL 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 to 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 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 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 codelections of NF1 with SÚZII2 as a member of the polycymbo repressor complex 2 (PRC2) [46].

**Neuroblastoma RAS (NRAS)**

Mutations in NRAS 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 CBFβ 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 anemia with ring sideroblasts (RARS). In addition to SF3B1 a landmark study found other affected factors involved in RNA splicing such as SRSF2, U2AF1, ZRS2 and SF3A1 [125]. The effects of abnormal RNA splicing due to these aberrations compromised normal hematopoiesis and revealed a relevant insight into MDS pathology.

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 knockout mice 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 aspirin 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), TGI101348 (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 MP 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 (IC50) of 3.3 nM and 2.8 nM, respectively. TYK2 and also JAK3 could be inhibited by ruxolitinib as well but the IC50 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-a) 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 well tolerated. General side effects in the verum group were ecchymosis, dizziness, and headache along with haematological side effects such as thrombocytopenia and anaemia. 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 LY2788544 (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 tascotinib 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 immunomodulators 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 (K599L) 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 pathology 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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