

Review

BCR-ABL negative myeloproliferative neoplasia: a review of involved molecular mechanisms

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Summary. The clonal bone marrow stem cell disorders essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) belong to the group of Philadelphia chromosome negative myeloproliferative neoplasia (Ph- MPN). In 2005 the *JAK2*^{V617F} mutation was discovered which has generated more insight in the pathogenetic mechanism of the MPNs. More mutations have been detected in MPN patients since. However, the underlying cause of MPN has not been discovered so far. The mechanism of increased angiogenesis in MPNs and the development of fibrosis in the bone marrow in PMF patients and in some ET and PV patients is still not known. This review will focus on the most important molecular pathogenetic mechanisms in MPN patients.

Key words: Myeloproliferative neoplasia, Essential thrombocythemia, Polycythemia vera, Primary myelofibrosis, JAK2 mutation

Introduction

Luis Henry Vaquez and William Osler wrote in 1892 the first case report of the “Osler-Vaquez disease”, nowadays known as polycythemia vera (Vaquez, 1892). In 1951 William Dameshek introduced the term “myeloproliferative disorders” (MPDs) to describe five different diseases with similarities in clinical phenotype.

He also postulated the idea of an underlying undiscovered stimulus responsible for the proliferative activity of bone marrow cells in these diseases (Dameshek, 1951). Since then, more knowledge has been gained about the MPDs; from the discovery of the Philadelphia (Ph) chromosome in chronic myeloid leukaemia (CML) via the demonstration of clonality in MPDs to the discovery of the *JAK2*^{V617F} mutation in MPDs (Nowell and Hungerford, 1960; Fialkow et al., 1967, 1981; Adamson et al., 1976; Jacobson et al., 1978; Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). Later on, the World Health Organization (WHO) recommended changing the term myeloproliferative disorders to myeloproliferative neoplasia (MPN) to underline the clonality of the diseases. According to the 2008 WHO classification the MPNs include essential thrombo-cythemia (ET), polycythemia vera (PV), primary myelofibrosis (PMF), CML, chronic neutrophilic leukaemia (CNL), chronic erythroleukemia (CEL), systemic mastocytosis (SM) and MPN unclassifiable. The classical MPNs include ET, PV, PMF and CML (Vardiman et al., 2009).

Nowadays, the classical Philadelphia chromosome negative (Ph-) MPNs include ET, PV and PMF (Murray, 2005; Campbell and Green, 2006). The MPNs are characterized by the proliferation of one or more of the erythroid, myeloid and megakaryocytic cell lineages. Proliferation of the megakaryocytic cell line is predominantly found in ET patients resulting in increased blood platelets in the peripheral blood. PV is characterized by a trilineage proliferation resulting in increased erythrocytes but often increased levels of leukocytes and blood platelets are found in the

peripheral blood. The bone marrow of PMF patients is characterized by a proliferation of the megakaryocytic and granulocytic cell line and the development of fibrosis (Murray, 2005; Michiels et al., 2007). The scope of this review is the molecular pathogenetic mechanisms involved in the MPNs. First, we will describe the JAK-STAT pathway followed by the *JAK2^{V617F}* mutation. The review will continue with a discussion of the influence of the *JAK2^{V617F}* mutation on the JAK-STAT, PI3K-Akt and Erk pathway in MPN patients. Subsequently, other recently discovered mutations in JAK2 negative MPN patients will be reviewed. Finally, the pathogenesis of angiogenesis and myelofibrosis will be discussed.

The JAK-STAT pathway

The presence of a JAK2 mutation is an important diagnostic clue to the diagnosis of Ph- MPN. The Janus Kinases (JAKs) family consists of four members: JAK1, JAK2, JAK3 and Tyk2. The binding of cytokines, growth factors or hormones on cell surface receptors of the JAK family leads to transphosphorylation between the associated JAKs. This results in tyrosine phosphorylation of the receptors and synthesis of binding sites of the downstream signalling proteins. The signal transducer and activators of transcription (STATs) in the cytoplasm of a quiescent cell are normally

unphosphorylated, but can be activated by tyrosine phosphorylation. The STATs are recruited to the previously formed binding sites, become phosphorylated, dimerize and translocate to the nucleus where they stimulate transcription (Fig. 1) (Darnell, 1997; Rawlings et al., 2004; Yu and Jove, 2004). A total of seven STATs are known: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Darnell, 1997).

The JAK/STAT pathway is inhibited by three protein families (Fig. 1):

1) Haematopoietic cells express SHP1. SHP1 belongs to the group of phosphotyrosine phosphatases (PTP); PTP dephosphorylates activated JAKs, STATs or cytokine receptors (Larsen and Ropke, 2002; Valentino and Pierre, 2006).

2) Suppressors of cytokine signalling (SOCS) interact with activated JAKs or phosphorylated receptors or they induce the JAK proteasomal degradation. CIS, SOCS1, SOCS2 and SOCS3 are members of the SOCS protein family. The synthesis of SOCS is induced by activated STATs and results in a negative feedback loop. By interacting with activated JAKs STAT recruitment to the binding sites is inhibited (Rawlings et al., 2004; Espert et al., 2005).

3) Protein inhibitors of activated STATs (PIAS), they interact with activated STATs and inhibit their

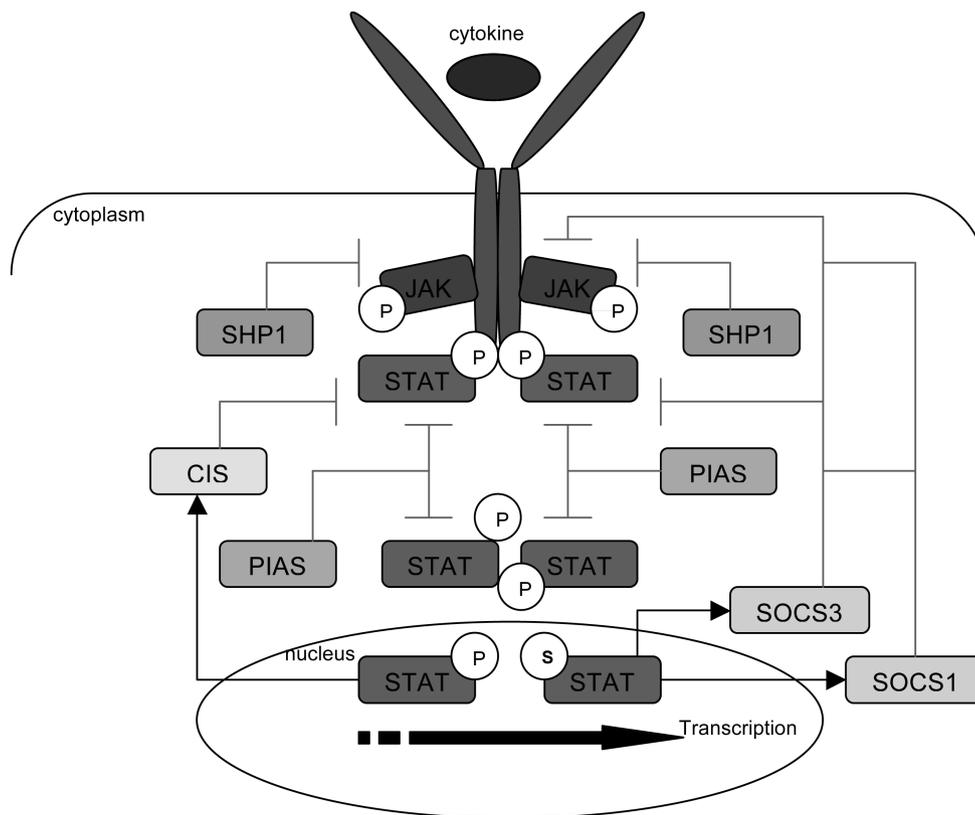


Fig. 1. Positive and negative regulation of the JAK-STAT signal transduction pathway. Binding of cytokines, growth factors or hormones on cell surface receptor transphosphorylates associated JAKs (Janus Kinase) creating binding sites for downstream signalling proteins. STATs (signal transducer and activator of transcriptions) become activated upon tyrosine phosphorylation, are recruited to the binding sites and become phosphorylated resulting in stimulation of transcription. The SOCS (suppressors of cytokine signalling), activated by STATs, provide a negative feedback loop to JAKs and STATs. PTPs (phosphotyrosine phosphatases) and PIASs (protein inhibitors of activated STATs) inhibit JAKs, STATs or cytokine receptors.

dimerisation (Larsen and Ropke, 2002).

The *JAK2*^{V617F} mutation

Until 2005 little was known about the aetiology of MPN. The discovery of the *JAK2*^{V617F} mutation in MPN patients (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005) has given a better understanding of the pathogenesis of MPNs. The *JAK2*^{V617F} mutation is present in granulocytes, erythroblasts and myeloblasts and in all erythropoietine (EPO)-independent erythroid colonies. The *JAK2*^{V617F} mutation causes an increased JAK2 kinase activity by destabilizing the inhibitory interaction (Baxter et al., 2005).

The erythroid colonies with the *JAK2*^{V617F} mutation are able to grow in the absence of EPO. Therefore, the *JAK2*^{V617F} mutation leads to factor independent growth of the erythroid cell line (Toyama et al., 2007). The receptors of bone marrow progenitor cells are hypersensitive to thrombopoietine (TPO, stimulates proliferation and differentiation of megakaryocytes), EPO (stimulates erythroblasts), stem cell factor (SCF, induces proliferation and self-renewal of multipotent haematopoietic progenitors) and granulocyte stimulating factor (GSF, stimulates proliferation and differentiation of granulocytes). The hypersensitivity of these cytokines results in monoclonal stimulation of the megakaryopoiesis, erythropoiesis and granulopoiesis. However, due to *JAK2*^{V617F} and other mutations, haematopoietic progenitor cells can proliferate without the presence or induction by cytokines (Michiels and Thiele, 2005).

Up to now, it is unclear how one mutation can be responsible for three phenotypically different diseases. Besides that, not every MPN patient carries the *JAK2*^{V617F} mutation; the *JAK2*^{V617F} mutation is found in >95% of the PV patients and in about 50% of the ET and PMF patients. This generated three hypotheses. The first hypothesis is the 'gene-dosage' hypothesis, which postulates a correlation between phenotype and allele burden, which is the ratio between mutant and wild type JAK2 in haematopoietic cells; between MPN patients there is a variability in the number of cells carrying the mutation and there is a variability in the alleles that carry the *JAK2*^{V617F} mutation. Most PV patients are homozygous for the *JAK2*^{V617F} mutation, while ET patients are heterozygous. Some ET patients with increasing allele burden transform over time to PV or PMF. More important, ET patients with the *JAK2*^{V617F} mutation have a "PV-like" phenotype compared to ET patients without the mutation. MPN patients carrying the *JAK2*^{V617F} mutation do not have a higher risk of developing post-PV and post-ET myelofibrosis compared to patients without this mutation (Tiedt et al., 2008; Vannucchi et al., 2007, 2008; Passamonti and Rumi, 2009). Controversy exists about the allele burden and the risk of developing acute myeloid leukaemia (AML) (Barosi et al., 2007; Tefferi et al., 2008). It was

shown that some patients with a *JAK2*^{V617F} positive MPN can lose this JAK2 mutation in the progression to AML. A possible explanation for this phenomenon is the concept of a "pre-JAK2" phase in which other mutations are responsible for the progression to AML. This 'pre-JAK2 phase' is a second hypothesis which postulates additional somatic mutations or inherited predisposing alleles present before the *JAK2*^{V617F} mutation, which are responsible for the clonal haematopoiesis and determine the phenotype. This pre-JAK2 phase might even be responsible for generating the *JAK2*^{V617F} mutation or they might act synergistically (Darnell, 1997; Larsen and Ropke, 2002). The 'gene-dosage' and 'pre-JAK2 phase' hypotheses do not entirely explain the phenotypic heterogeneity of the MPNs. Differences in mice with different genetic backgrounds were observed leading to a third hypothesis of 'host genetic factors'. This hypothesis postulates that host genetic characteristics might act as modifiers of the phenotype in combination with the *JAK2*^{V617F} mutation. For instance, single nucleotide polymorphisms (SNPs) and even gender is an independent modifier with women having a lower *JAK2*^{V617F} allele burden than men (Pardanani et al., 2008; Delhommeau et al., 2009; Stein et al., 2010).

The coexistence of independent JAK2-mutant and JAK2 wild-type clonal expansions in the same patient can be an explanation. It is observed that bone marrow of JAK2 positive AML patients are preceded by transformation to myelofibrosis during their disease course, in contrast to bone marrow of JAK2 wild-type AML which is preceded by chronic-phase ET and PV patients. The mechanism of this disease evolution remains to be elucidated (Beer et al., 2010).

Recent studies with JAK(1/2) inhibitors have shown that the symptoms in MPN patients carrying the *JAK2*^{V617F} mutation dramatically improve. At the same time a similar improvement is seen in patients with the wild type JAK2 gene. Ruxolitinib (INCB018424), SAR302503 (TG101348) and CYT387 are promising JAK(1/2) inhibitors judging by the improvement of symptoms and the moderate side effects. These side effects include thrombocytopenia, anaemia and neutropenia and gastro-intestinal symptoms like diarrhoea, nausea and vomiting. A withdrawal syndrome has been reported after discontinuation of Ruxolitinib intake characterized by acute relapse of disease symptoms, accelerated splenomegaly and worsening of cytopenias (Ostojic et al., 2011; Pardanani and Tefferi, 2011; Pardanani et al., 2011; Tefferi and Pardanani, 2011; Verstovsek et al., 2012; Harrison et al., 2012). Although the *JAK2*^{V617F} mutation is an important underlying mechanism, it is not the cause of the MPN. Therefore, when using JAK inhibitors a balance should be found between the improvement of symptoms and the occurrence of side effects for each individual patient. The fact that patients with wild type JAK2 also benefit from JAK(1/2) inhibitors indicates that other, still unknown, underlying mechanism(s) are responsible for

the increased JAK/STAT pathway activity in MPN patients.

The JAK-STAT, PI3K-Akt and Erk pathway and MPN

Sustained activation of JAK2 increases STAT3 and STAT5 phosphorylation. Activated STAT3 regulates cell growth through regulation of cyclin D1 and induces Bcl-2 resulting in an anti-apoptotic signal. STAT3 is also known to play an important role in megakaryopoiesis, mainly through the expansion of megakaryocytic progenitor cells (Fukada et al., 1996; Bromberg et al., 1999; Kirito et al., 2002). Activated STAT5 up-regulates Bcl-xL, inhibiting apoptosis of megakaryocytes (Fig. 2), and mediates cell growth through induction of cyclin D1 (Matsumura et al., 1999; Socolovsky et al., 1999). The net result of STAT3 and STAT5 activation is apoptosis inhibition and a proliferative activity.

Due to *JAK2^{V617F}* mutation the sustained JAK2 activation suggests an up-regulation in phosphorylated STAT3 (pSTAT3) and STAT5 (pSTAT5) in MPN

patients. This is probably more explicit in JAK2 positive patients, while STAT3 and STAT5 are activated by JAK2. Indeed, pSTAT5 had been shown to be up-regulated in *JAK2^{V617F}* positive MPN patients (Grimwade et al., 2009).

The *JAK2^{V617F}* mutation also activates the MAPK and PI3K-Akt pathway (Fig. 3) (James et al., 2005; Levine et al., 2005). The *JAK2^{V617F}* mutation and pSTAT5 can both activate PI3K by interacting with p85, a regulatory subunit of PI3K. Activated PI3K activates Akt, which in turn activates mTor on Ser2448 which directly phosphorylates ribosomal p70S6Kinase (p70S6k) (Bjornsti and Houghton, 2004; Huang and Manning, 2009). The phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTor) pathway is known to be commonly activated in leukaemia and lymphoma and is also known to play a role in inhibiting apoptosis in normal human erythroblasts (Skorski et al., 1997; Kashii et al., 2000). The PI3K/Akt pathway induces the phosphorylation of BAD, a pro-apoptotic member of the Bcl-2 family, via

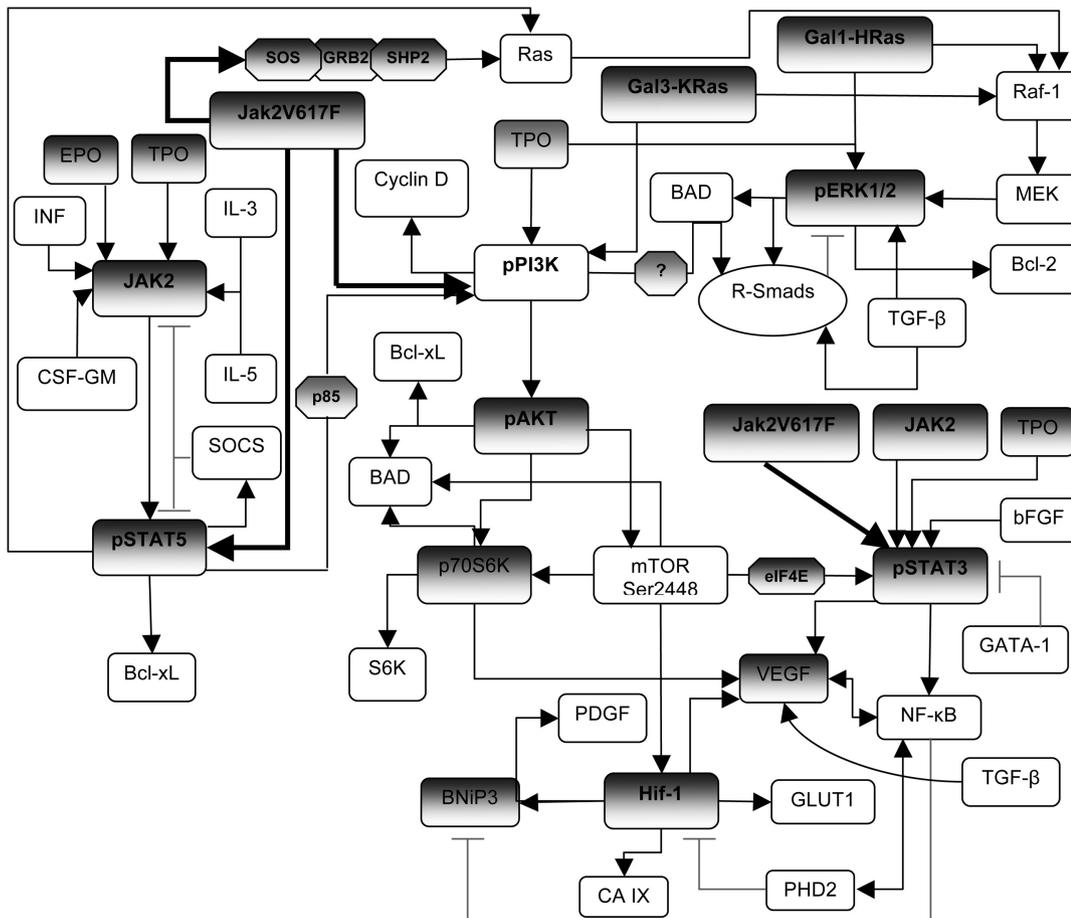


Fig. 2. Signalling pathway of JAK-STAT, Erk and PI3K/Akt. JAK2 (Janus Kinase) is activated by different cytokines, growth factors and hormones (erythropoietine (EPO), thrombopoietine (TPO), interleukin (IL)-3 and -5, stem colony stimulating factor-granulocyte-macrophage (CSF-GM) and interferon (INF)). JAK2 phosphorylates STAT5 (signal transducer and activator of transcription) and STAT3, however the *JAK2^{V617F}* mutation induces a sustained activation of STAT5, STAT3, PI3K (phosphatidylinositol-3 kinase) and indirect of extracellular signal-regulated kinase (Erk) resulting in increased activation of their downstream proteins. Phosphorylated STAT5 (pSTAT5) and pSTAT3 induces Bcl-xL resulting in megakaryocyte apoptosis. STAT3 is not only activated by JAK2 and the *JAK2^{V617F}* mutation but also by TPO and fibroblast growth factor (FGF). Phosphorylated PI3K (pPI3K) phosphorylates AKT (pAKT) activating Bcl-xL and inhibits BAD resulting in inhibition of apoptosis. pAKT also activates the mammalian

target of rapamycin complex 1 (mTORC1) which can phosphorylate STAT3 as well and activates ribosomal p70S6Kinase (p70S6K) and hypoxia-inducible-factor (HIF-1α). HIF-1α, p70S6K and mTORC1 are involved in angiogenesis by activation of vascular endothelial growth factor (VEGF).

phosphorylated Akt (pAkt) and p70S6k. The BAD function is inhibited upon BAD phosphorylation resulting in inhibition of apoptosis. Activation of the PI3K/Akt pathway also induces the activation of Bcl-xL, leading to inhibition of megakaryocyte apoptosis (Fig. 2) (Bakin et al., 2000; Schwaller et al., 2000; Nyga et al., 2005). Due to the inactivation of the pro-apoptotic factor BAD and activation of Bcl-xL, Akt suppresses apoptosis and promotes cell survival (Datta et al., 1997, 1999; Del Peso et al., 1997; Cardone et al. 1998; Vivanco and Sawyers, 2002). Since the bone marrow of MPN patients is characterized by a pathological increase in megakaryocytes, Akt might play an important role in the up-regulation of megakaryocytes, possibly secondary to the *JAK2^{V617F}* mutation. It has already been demonstrated in previous studies that an increased phosphorylation of Akt is present in *JAK2^{V617F}* positive patients. It is also associated with increased erythropoiesis in PV patients (Dai et al., 2005; Grimwade et al., 2009; Laubach et al., 2009).

An increased activation of Ras-Erk signalling pathway has been demonstrated in PV patients (Laubach et al., 2009). Extracellular signal-regulated kinase (Erk) belongs to the mammalian MAPK families which are serine-threonine kinases (Boulton et al., 1990). The receptor tyrosine kinase (RTK)-Grb2-SOS signalling axis activates GTPase Ras which in turn activates Raf-1. Raf-1 mediates the activation of MEK which in turn activates Erk (see Fig. 2) (Matsuda et al., 1994). Erk phosphorylates BAD, resulting in inactivation of BAD with inhibition of apoptosis and activation of Bcl-2. This forms homodimers which generates anti-apoptotic responses (Zha et al., 1996). The net result of Erk phosphorylation is the inhibition of apoptosis, by blocking the function of BAD and activation of Bcl-2. Therefore, the activation of the Erk pathway is suggested to be one of the mechanisms responsible for the hypercellularity seen in the bone marrow of MPN patients, also possibly secondary to the *JAK2^{V617F}*

mutation.

Signalling in *JAK2^{V617F}* negative MPN

Before the discovery of *JAK2^{V617F}* mutation, the molecular pathogenesis of MPN was unknown. It was thought that ET, PV and PMF were clonal disorders arising from a multipotent progenitor cell characterized by independency or hypersensitivity of haematopoietic progenitors to numerous cytokines. The PV progenitors are hypersensitive to EPO, leading to the forming of erythroid progenitor cells in the absence of EPO. In PMF there is an enhanced sensitivity to TPO, with a defective TPO-receptor in which the negative regulation is disturbed.

Recently, another mutation in the *JAK2* gene was found, the *JAK2 exon 12 mutation*. It is only found in *JAK2^{V617F}* negative PV patients and contributes primarily to erythroid myeloproliferation. This mutation leads to increased levels of phosphorylated *JAK2*, *STAT5* and *Erk* compared to patients with wild type *JAK2*. This mutation has shown even higher phosphorylated *JAK2* and *Erk* levels compared to patients with the *JAK2^{V617F}* mutation. PV patients with the *JAK2* exon 12 mutation are younger and have normal leukocyte and platelet counts compared to PV patients with the *JAK2^{V617F}* mutation (Lundberg et al., 2000; Medinger et al., 2009).

An additional mutation is present in the myeloproliferative leukaemia oncogene (*MPL*), which encodes the TPO-receptor, present in 5% of *JAK2^{V617F}* negative ET patients and in 10% of *JAK2^{V617F}* negative PMF patients. This gain of function mutation also results in activation of *JAK2*, *STAT3*, *STAT5*, *Erk* and *Akt* (Boveri et al., 2008; Boiocchi et al., 2011). ET patients with a *MPL* mutation are older, have lower haemoglobin levels and higher platelet counts. *MPL*-mutated PMF patients are also of older age with lower haemoglobin levels (Medinger et al., 2010; Boiocchi et al., 2011).

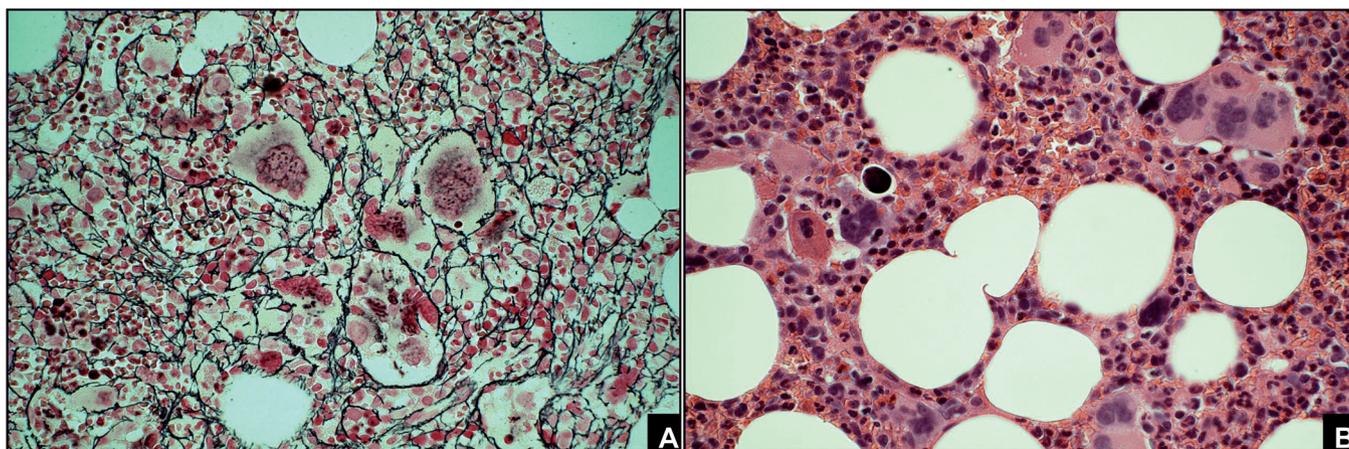


Fig. 3. Overview of bone marrow with myelofibrosis. A. Reticuline fibrosis in bone marrow. B. Primary myelofibrosis. HE, x 1,000

Therefore, these mutations seem to have different phenotypes compared to MPN *JAK2*^{V617F} positive MPN patients.

Other mutations are found in MPN patients (Table 1), involving *LNK* mutations. Wild-type *LNK* inhibits the constitutive activity of *JAK2*^{V617F} and *MPL*. Consequently, mutations in *LNK* result in neutralising this inhibition. However these mutations are also found in other myeloid malignancies and thus are not specific for MPN. *LNK* mutations include *Ten-Eleven-Translocation2 (TET2)* mutations important in DNA demethylation, *Additional Sex Comb-Like1 (ASXL1)* mutations, *Isocitrate Dehydrogenase 1/2 (IDH1/IDH2)* mutations and *Enhancer of Zeste Homolog 2 (EZH2)* mutations. *LNK* negatively regulates the *JAK2* activation via the TPO-receptor and is shown to be mutated in all MPN at a low frequency in patients with the *JAK2*^{V617F}, *TET2*, *IDH* and/or *MPL* mutations, but was found more often in MPN patients who had transformed to AML (13%) (Folkman, 1995; Niu et al., 2002; Wrobel et al., 2003; Gianelli et al., 2007; Nakayama, 2009; Milosevic and Kralovics, 2013).

Epigenetic mechanisms can be modulated via regulation of transcription involving DNA and histone modifications. The *TET2*, *ASXL1*, *IDH1/2* and *DNA cytosine methyltransferase 3a (DNMT3A)* belong to these epigenetic modifiers. Mutations in these epigenetic modifiers are predominantly found in PMF patients and blast phase MPN patients. *ASXL1*, *IDH1/2*, *DNMT3A* and *EZH2* mutations are associated with poor survival in MPN patients.

The discovery of so many mutations in MPN patients suggests that they are primarily background mutations affecting the same pathways. Some mutations

are involved in early phase MPN (*ASXL1*), some in chronic phase MPN (*JAK2*^{V617F}, *MPL*) and on others in disease progression (*LNK*, *TET2*, *IDH1/2* and *DNMT3A*). The higher frequency of some mutations in myelofibrosis patients (PMF, post-ET/PV myelofibrosis) could predict disease progression to myelofibrosis in PV and ET patients (*ASXL1*). Other mutations are found in higher frequency in AML patients preceded by a MPN (*LNK*, *DNMT3A*) (Brecqueville et al., 2012; Milosevic and Kralovics, 2013).

Except for these mutations, bone marrow cells, like megakaryocytes, stem cells, progenitor cells and myeloid cells, can also produce aberrant cytokines, activating a signalling in MPNs. These cytokines are produced in mutant and non-mutant MPN cells. In other diseases the *STAT3* and *NF- κ B* signalling have been found to regulate cytokine transcription (Porcu et al., 2012). Treatment with *JAK* inhibitors as well as *STAT3* deletion can both reduce cytokine expression in MPN cells. This explains why *JAK* negative MPN patients can still respond clinically on *JAK* inhibitory therapy (Trelinski and Robak, 2013).

The complex of the different molecular aberrations together with the production of aberrant cytokine signalling is nowadays believed to be the pathogenic mechanism initiating MPN disease.

Angiogenesis, myelofibrosis and MPN

Angiogenesis is the formation of new blood vessels and capillaries from existing blood vessels and play an important role in the pathogenesis of many human malignancies and non-malignant diseases. It was shown by several studies that the micro vessel density (MVD)

Table 1. Somatic mutations in ET, PV, PMF and blast phase MPN patients.

Mutations	Mutational frequency	Pathogenetic relevance
<i>JAK2</i> ^{V617F} exon 14 (janus kinase 2)	PV>95%; ET and PMF 50-60% BP-MPN±50%	abnormal myeloproliferation and progenitor cell growth factor hypersensitivity
<i>JAK2</i> exon 12	PV±3%; ET ± 3-5%	mainly erythroid myeloproliferation cytokine-independent growth
<i>MPL</i> (myeloproliferative leukemia virus oncogene)	PMF±10% BP-MPN±5%	and thrombopoietin hypersensitivity
<i>LNK</i>	ET, PV and PMF rare BP-MPN±10%	wild-type <i>LNK</i> inhibits activity of <i>JAK2</i> ^{V617F} and <i>MPL</i>
<i>TET2</i> (TET oncogene family member 2)	ET 4-5%; PV 10-16% PMF 8-14%; BP-MPN±17%	catalyzes conversion of 5-methylated cytosine into hydroxy-methylated cytosine and is therefore a key player in active DNA demethylation
<i>ASXL1</i> (additional sex combs-like 1)	ET and PV rare PMF±13%; BP-MPN±18%	wild-type <i>ASXL1</i> is involved in normal haematopoiesis and can act as activator and repressor of transcription
<i>IDH1/IDH2</i> (isocitrate dehydrogenase); <i>EZH2</i> (enhancer of zeste homolog 2)	ET, PV and PMF rare BP-MPN±20% PV±3%; MF±6-13%	converts α -ketoglutarate into 2-hydroxyglutarate (2HG) and inhibits <i>TET2</i> activity leading to decrease in demethylation wild-type <i>EZH2</i> is part of polycomb repressive complex 2 (PRC2)
<i>DNMT3A</i> (DNA cytosine methyltransferase 3a)	ET±3%; PV±7% PMF±10%; BP-MPN±15%	DNA methyltransferase responsible for the maintenance of DNA in the methylated state

ET: essential thrombocythemia, PV: polycythemia vera, PMF: primary myelofibrosis, BP-MPN: blast phase myeloproliferative, MF: myelofibrosis and includes PMF, post-ET/PV myelofibrosis.

is increased in the bone marrow of MPN patients, with the highest MVD in PMF patients. The MVD was also found to be higher in MPN patients with the JAK2 mutation compared to MPN patients who do not carry the JAK2 mutation (Lundberg et al., 2000; Medinger et al., 2009, 2010; Boiocchi et al., 2011; Boveri et al., 2008).

Angiogenesis is tightly regulated by pro- and anti-angiogenic factors. Hypoxia-inducible-factor-1 (Hif-1) is a major regulator of angiogenesis. It is activated under hypoxic conditions. Hif-1 consists of Hif-1 α , expressed in normoxic conditions, and Hif-1 β , expressed in hypoxic conditions. The Hif-1 protein becomes active only when the α - and β -subunit interact, resulting in Hif-1 heterodimer, which is an active transcription factor (Nakayama, 2009).

One of the target genes of Hif-1 is vascular endothelial growth factor (VEGF) (Fig. 2). VEGF is increased in bone marrow of MPN patients, and particular in PV and PMF patients (Lundberg et al., 2000; Wrobel et al., 2003; Medinger et al., 2009; Gianelli et al., 2007). STAT3 activation induces up-regulation of VEGF (Fig. 2). Besides that, when STAT3 is blocked, VEGF expression is inhibited (Niu et al., 2002). An increased phosphorylation of STAT3 might therefore be one of the mechanisms of the increased angiogenesis seen in the bone marrow of MPN patients. At the same time, megakaryocytes and thrombocytes are important sources of VEGF as well. Because megakaryocytes and thrombocytes are increased in ET, PV and PMF patients, the precise mechanism of increased angiogenesis in MPN patients is not fully understood (Wrobel et al., 2003).

Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (Bnip3), another target protein of Hif-1 (Fig. 2), is a pro-apoptotic protein belonging to the BH3-only subfamily. It is activated in hypoxic conditions in normal and cancer tissue by Hif-1 activation (Boyd et al., 1994; Chen et al., 1997; Giatromanolaki et al., 2004). In an immunohistochemistry study a trend of lower Bnip3 expression in PMF patients was shown, with a further decrease of the expression in PMF follow-up biopsies (Theophile et al., 2008). Down-regulation of Bnip3 due to DNA methylation and histone deacetylation was shown as well in acute lymphatic leukaemia (ALL), acute myeloid leukaemia (AML) and multiple myeloma (MM) (Murai et al., 2005). These studies indicate disturbed apoptosis mechanisms and not just an increased proliferative activity in MPN patients. Further, these studies might also indicate an angiogenesis pathway independent of hypoxia and thus independent of Hif-1 activation, while Bnip3 is expressed in hypoxic conditions.

The pathogenesis of fibrosis

The amount of vessels is correlated to the grading of fibrosis; the higher the MVD, the more severe the grading of fibrosis. This indicates a correlation between

angiogenesis and fibrosis (Lundberg et al., 2000; Medinger et al., 2009; Boveri et al., 2008). Myelofibrosis is the abnormal increase in bone marrow fibre content. There is a difference between reticulin fibrosis and collagen fibrosis with the latter showing a stronger correlation with abnormal blood counts and no response to treatment (Thiele and Kvanicka, 2006; Kuter et al., 2007). Fibrosis is subdivided into:

- MF-0; single scattered reticulin fibers with no intersections (cross-overs) with appearance of normal bone marrow.

- MF-1; a loose network of thin reticulin fibers with many intersections, especially in perivascular areas.

- MF-2; a diffuse and dense increase in reticulin forming with extensive intersections, occasionally with only focal bundles of thick collagen fibers and/or focal osteosclerosis.

- MF-3, a diffuse and dense increase in reticulin with extensive intersections with coarse bundles of collagen and often associated with significant osteosclerosis (Thiele et al., 2005).

This classification is a subjective scoring system with limitations due to interobserver variability and inconsistency of use. Further, in daily practice it remains difficult whether a case is MF-0 or MF-1 and MF-2 or MF-3 and how much collagen is tolerable to diagnose a case as MF-2. The same is true for ET and the prefibrotic form of PMF which shows phenotypic similarities (Thiele et al., 1996, 1999a,b). However, the presence and grading of fibrosis is important to diagnose MPN and is used as a guide for treatment decisions.

Bone marrow fibrosis is a major feature of PMF patients, but can also develop in ET and PV patients, known as post-essential thrombocythemia myelofibrosis (post-ET MF) and post-polycythemia vera myelofibrosis (post-PV MF), respectively (Mesa et al., 2007; Vardiman et al., 2009). Myelofibrosis is characterized in the peripheral blood by tear-drop erythrocytes and the presence of myeloid precursor cells. The symptoms these patients display consist of splenomegaly, progressive anaemia and constitutional symptoms like fatigue, weight loss, night sweats, bone pain and shortness of breath. Survival in myelofibrosis varies from 2 to 11 years due to an increased risk of leukemic transformation. Treatment of myelofibrosis is mainly palliative; blood transfusion, radiotherapy, chemotherapy, splenectomy or stem cell transplantation. The latter is the only curative option (Tefferi, 2000).

The development of fibrosis has been studied widely, although a clear underlying mechanism has not been identified yet. Platelet-derived growth factor (PDGF) is a potent stimulator of fibroblast growth present in megakaryocytes and platelets (Groopman, 1980), resulting in abnormal numbers of megakaryocytes and platelets in disorders with increased bone marrow fibrosis. Although PDGF stimulates fibroblast growth, studies from Kimura et al. and Terui et al., showed little effect of PDGF on reticulin or collagen production (Kimura et al., 1989; Terui et al.,

1990).

Transforming growth factor β (TGF- β) is another potent stimulator of fibroblast collagen synthesis found in megakaryocytes and platelets. There are 3 isoforms of TGF- β ; TGF- β 1, - β 2 and - β 3. Megakaryocytes in humans produce and are negatively regulated by, TGF- β 1. It was shown that megakaryocyte colony-forming units (CFU-MK) in ET patients are less sensitive for the negative regulation of TGF- β 1 due to a reduced expression of Smad4 (an intracellular effector of TGF- β) (Dong and Blove, 2006; Kuroda et al., 2004). In PV and PMF patients it was shown that one of the TGF- β high-affinity cell surface receptors, T β RII, has a reduced expression. This results in loss of sensitivity to TGF- β 1 with resistance to its growth-inhibitor and apoptotic effect, resulting in clonal expansion of megakaryocytes and fibroblasts (Le Bousse-Kerdiles et al., 1996; Rooke et al., 1999; Chou et al., 2003; Wang et al., 2006).

In mice models it was shown that megakaryocytes and leucocytes are both responsible in the development of fibrosis. A pathological emperipolesis of polymorphonuclear (PMN) leucocytes within the megakaryocytes results in the destruction of megakaryocyte organelles and leakage of PDGF and TGF- β into the bone marrow microenvironment. This indicates that there is an inappropriate release of stored substances in megakaryocytes. The net result is activation and proliferation of fibroblasts leading to fibrosis. This phenomenon is supported by the correlation between the amount of reticulin deposition and the rate of emperipolesis (Schmitt et al., 2000, 2002). However, it remains unknown what drives the emperipolesis. Other cytokines and haematopoietic growth factors and the interplay between them might be involved in the development of fibrosis in the bone marrow of MPN patients.

Concluding remarks

Although important progress has been made in the understanding of the MPN pathogenesis with the discovery of the *JAK2*^{V617F} mutation and other mutations, the underlying mechanism of MPN is still not understood. The development of JAK inhibitors has improved the quality of life of MPN patients significantly, although a survival advantage has not been proven yet due to too short follow up time. Besides that, the current data set is from a small population.

Although progress in the understanding of the pathophysiological mechanism of fibrosis has been made in the last couple of years, discussion remains whether bone marrow fibrosis in MPN patients is of clonal MPN origin, or reactive and polyclonal. But the most important question remains: what is the main underlying mechanism driving the development of fibrosis? When we have the question to this answer life of MPN patients can be prolonged. Therefore, more research is needed to shed light on the underlying mechanism of myelofibrosis in order to generate better therapeutic options.

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