Chapter 6
Updates in Polycythemia Vera

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Polycythemia vera (PV) is a chronic myeloproliferative neoplasm (MPN) of hematopoietic stem cells (HSC, predominantly characterized by an increase in erythrocyte mass with trilineage proliferation [1]. The blood, bone marrow, spleen, and liver are variably involved during progression through the two phases of disease progression: (1) a proliferative or polycythemic phase with increased red blood cells (RBCs); and (2) a postpolycythemic or spent phase with marked cytopenias, extramedullary hematopoiesis, hypersplenism, and myelofibrosis (MF). Hemorrhage and thrombosis are the primary clinical manifestations; the disease progresses to acute leukemia in a subgroup of patients (10–15%) who are older, with high white blood cell (WBC) count, and in those with additional somatic mutations other than Janus kinase 2 (*JAK2*) mutations [2]. The differential diagnosis includes secondary polycythemia and other chronic MPNs. Vaquez and Osler [3–6] first reported PV associated with erythremia and polycythemia, respectively. The concept of chronic myeloproliferative disorders (MPDs) emerged during the 1950s when PV was categorized with chronic myeloid leukemia, essential thrombocythemia (ET), and chronic idiopathic myelofibrosis (CIMF) based on common clinicopathological features [7]. The Polycythemia Vera Study Group (PVSG) was then formed to define the diagnostic criteria for this entity. PVSG recommendations remained the gold standard for many years [8, 9]. During the 1970s, considerable advancements in understanding PV were made due to the improvement in laboratory techniques and evolvement of scientific perspective on neoplasia. Several studies evaluating various markers such as X-chromosome inactivation, glucose-6-phosphate dehydrogenase isoenzymes, and DNA methylation demonstrated that PV arises from clonal transformation of a single hematopoietic stem cell (HSC) [10–16]. However, consensus

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with PVSG remained until 2001 when the World Health Organization (WHO) reclassified myeloproliferative disorders as neoplastic – renaming them as MPNs and modernized the diagnostic criteria using a comprehensive clinicopathological approach [17]. A point mutation in *JAK2*, resulting in substitution of valine for phenylalanine (V617F), was identified in a significant number of PV patients [18–25]. In 2008, WHO incorporated the *JAK2* mutation as one of the major diagnostic criteria [1], and in 2016, WHO lowered the cutoff values for hemoglobin and recognized the importance of bone marrow morphology (Table 6.1) [26]. Additionally, the mutually exclusive MPL proto-oncogene, thrombopoietin receptor (*MPL)* and calreticulin (*CALR)* mutations were associated with ET and primary myelofibrosis (PMF) [26, 27]. *MPL* and *CALR* mutations practically exclude PV.

Table 6.1 Classification Criteria of PV

		[a]PVSG	2001 WHO	2008 WHO	2016 WHO
Hemoglobin, g/dL	**A1**		18.5 g/dL in men 16.5 g/dL in women	>18.5 g/dL in men >16.5 g/dL in women	> 16.5 g/dL in men > 16.0 g/dL in women
Red cell mass (RCM)		36 mL/kg in men; 32 mL/kg in women	OR Increased[b]	OR Increased[b]	OR Increased[b]
Hematocrit (%)				OR Increased[c]	OR >49% in men >48% in women
Arterial oxygen saturation		**A2** ≥ 92%	–	–	–
No evident cause of secondary erythrocytosis			**A2**	–	–
Splenomegaly		**A3**	**A3**	–	–
Clonal genetic abnormality			**A4**: other than[d] Ph chromosome or BCR/ABL fusion gene in marrow cells	**A2:** presence of JAK2 V617F or functionally similar JAK2 exon 12 mutation	**A3**: JAK2 V617F or JAK2 exon12 mutation
Endogenous erythroid colony formation		–	**A5**	**A3**	–
Subnormal serum erythropoietin level		–	**B4**	**A2**	**B1**
White blood cell count >12 × 109/L		**B2**	**B2**	–	–

(continued)

Table 6.1 (continued)

	[a]PVSG	2001 WHO	2008 WHO	2016 WHO
Bone marrow (BM) histology	–	**B3:** panmyelosis with prominent erythroid and megakaryocytic proliferation	**B1:** panmyelosis with prominent trilineage proliferation	**A2**: panmyelosis with prominent trilineage proliferation with mature megakaryocytes of varying sizes
Increased leukocyte alkaline phosphatase >100 U/L	**B3**	–	–	–
Serum vitamin B12 concentration greater than 900 pg/mL or binding capacity >2200 pg/mL	**B3**	–	–	–
Requirements for diagnosis of PV	**A1 + A2 + A3** OR **A1 + A2 + any 2 from B**	**A1 + A2 and any other A** OR **A1 + A2 and any 2 from B**	**A1 + A2 and any one B** OR **A1 + any 2 from0 B**	[e]**A1 + A2 + A3** OR [e]**A1 + A2 + B**

[a]*PVSG* Polycythemia Vera Study Group, *WHO* World Health Organization; **A** = major criterion; **B** = minor criterion

[b]RCM > 25% above mean normal predicted value) or

[c]Hb or Hct > 99th percentile of method-specific reference range for age, sex, altitude of residence or Hb > 17 g/dl men or 15 g/dl women if associated with a sustained increase of 2 g/dl or more from baseline that cannot be attributed to correction of iron deficiency

[d]*Ph* Philadelphia chromosome

[e]Exception: A2 is not required in 2016 criterion when there is sustained absolute erythrocytosis, Hb >18.5 g/dL in men (Hct 55.5%) or >16.5 g/dL in women (HCt 49.5%) if criterion A3 and B are present

Epidemiology

A recent meta-analysis reported an annual worldwide incidence of PV ranging from 0.01 to 2.61 per million per year in various studies, and a pooled incidence rate of 0.84 per million per year [28]. PV has been reported in patients from all ethnic backgrounds, and is more common among Europeans and North Americans. Apparent geographic variation is possibly influenced by other heterogeneous variables [28]. Although several studies suggest a slightly higher incidence in males than females, a recent meta-analysis did not support a gender difference [1]. In general, PV is a disease encountered in older individuals, occurring most frequently between 50 and 70 years of age [1]; occurrences during childhood and adolescence are exceptionally rare [28]. Familial cases have been documented, but their significance remains uncertain.

Clinical Manifestations

Patients with PV may be identified by chance when they present with nonspecific symptoms such as mild hypertension, fatigue, intense itching, and headache [29]. Symptoms of hyperviscosity associated with increased red cell mass (RCM) and thrombocytosis may cause the clinician to first suspect PV. Other presenting conditions include arterial and venous thrombosis with cardiovascular events, Budd–Chiari syndrome, or mesenteric ischemia, bleeding complications from nonspecific ecchymoses, epistaxis to major hemorrhage, hypertension, headache, dizziness, visual disturbances, vertigo, tinnitus, claudication, and erythromelalgia [30–32]. Another distinctive symptom of PV is pruritus, which occurs after exposure to warm water, resulting from increased histamine release from activated basophils and functionally different mast cells [33]; the classic symptom is intense itching following bathing. Gouty arthritis and hepatosplenomegaly may also be observed. Leukemic transformation often follows progression to end-stage disease (relative risk 1.4–6.3).

Diagnostic Criteria

Considerable advances in the diagnostic criteria for PV were reported as the scientific community made new discoveries bearing on PV biology (Table 6.1). Presently, molecular testing is standard for PV diagnosis. The PVSG was originally formed with the support of the National Cancer Institute to evaluate treatment modalities. Eligibility criteria for entering PV clinical trials were defined, and these became known as the PVSG diagnostic criteria [8, 9], which remained the gold standard for decades [34, 35]. In 2001, the WHO classification for PV was published [17]. This classification represented a modernized approach for diagnosis and took into consideration several new laboratory studies. These studies included in vitro bone marrow endogenous erythroid colony (EEC) formation, the presence of clonal genetic abnormalities other than the Philadelphia chromosome (*BCR-ABL1* fusion gene and/or protein), revised histological findings for bone marrow, and low serum erythropoietin (EPO) levels (Table 6.1). The 2008 WHO further revised the adapted classification by incorporating molecular criteria for the presence of *JAK2* V617F, additional *JAK2* exon 12 variants, or other functionally similar mutations. The revised 2016 WHO lowered the diagnostic cutoff of hemoglobin to >16.5 g/dL from >18.5 g/dL in men, and >16.0 g/dL from 16.5 g/dL in women (Table 6.1). Moreover, bone marrow morphological examination has been established as a major mandatory criterion for diagnosis of PV. The practical utility of this morphological criterion stems from its reproducibility, the diagnostic features present in both overt and latent PV, and its ability to distinguish PV from JAK2V617F-positive ET [26, 36–39].

Red Blood Cell Mass, Hemoglobin, and Hematocrit

The RBC mass is determined by comparing the total blood volume to the plasma volume. Originally, it was considered a sensitive marker for PV, but subsequent studies reported a relatively low predictive value as several confirmed PV cases were demonstrated to have a lower RBC mass than the reference limit [40, 41]. An actual measurement of RBC mass is no longer performed in daily clinical laboratory practice due to its high cost, labor intensity and suboptimal diagnostic accuracy [36, 41]. The 2016 revision to the WHO added a clearly-defined gender-specific criterion for hematocrit in addition to lowering the diagnostic cutoff of hemoglobin (Table 6.1). This allows diagnosis of PV in patients with an actual increase in RCM above 25% from the mean predicted value with borderline increases in hemoglobin and hematocrit; these cases were missed with the previous WHO classification. Diagnostically, this higher sensitivity captures a group that may be in the latent or prepolycythemic phase of PV. These patients are typically identified as younger males with high platelet and low white blood cell (WBC) counts; they are more prone to arterial thrombosis with poor overall survival when compared to those with apparent PV [36, 38, 39, 42, 43]. Studies have shown the benefit of early treatment in this clinically latent group that could have latent PV or a disease with different biology and prolonged latency [43, 44]. Additionally, the new guidelines assist in distinguishing PV from *JAK2* V617F-positive ET; this distinction has therapeutic and prognostic implications because PV has lower overall survival rates and higher risk of leukemic transformation [38, 45, 46]. It is notable that maintaining a hematocrit below 45% is beneficial in preventing thrombotic complications [44].

Bone Marrow Pathology

Bone marrow morphology is a major mandatory criterion in the 2016 revision to the WHO. It has been duly acknowledged despite the fact that it was not included in the original PVSG criteria and was a minor criterion in the 2001 and 2008 WHO. In PV, the bone marrow should be hypercellular (Fig. 6.1a) with respect to patient age, with proliferation in all three lineages (panmyelosis – erythroid, myeloid, and megakaryocytic proliferation). Megakaryocytes display the most distinguishing features. In PV, megakaryocytes appear mature, but are pleomorphic with variability in size. Based on the WHO criteria, concordance rates for morphology of PV range between 71% and 82% among different pathologists. The utility of bone marrow morphology in cases of overt PV has been established [1, 47]. However, cases of "prodromal/early PV" have been recognized, and it is necessary to distinguish them from the clinical phenotype for ET. A large study showed that all the patients with *JAK2*-mutated PV, including overt and "masked PV" (mPV), met the 2008 WHO bone marrow morphology criteria [36, 37]. This supports the clinical utility of histological examination in patients with borderline or mild increases in RBC mass.

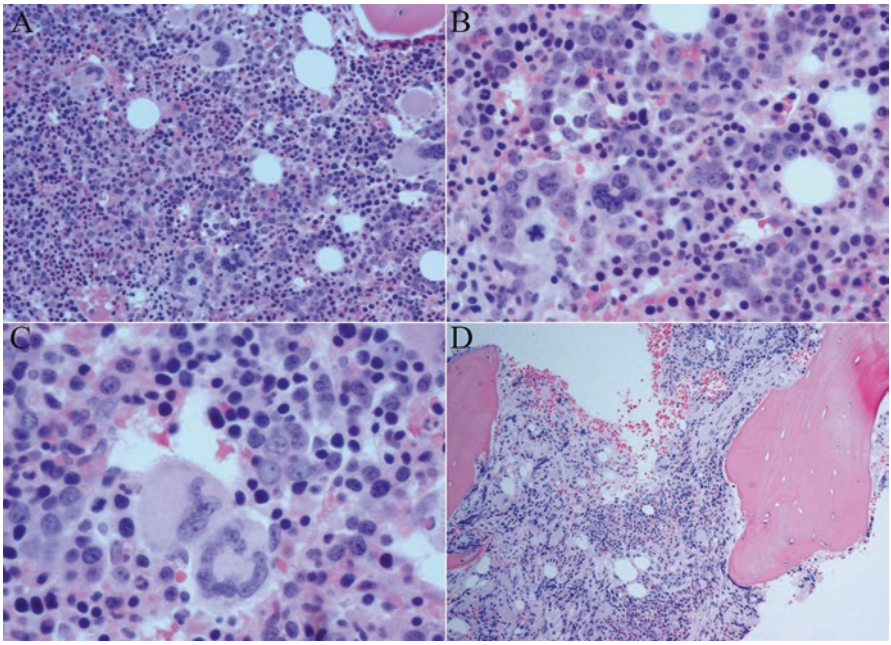

Fig. 6.1 Bone marrow biopsy of polycythemia vera. (**a**) Hypercellularity (hematoxylin–eosin, original magnification ×200). (**b**) Prominent erythroid as well as myeloid hyperplasia (hematoxylin–eosin, original magnification ×400). (**c**) Loosely clustered pleomorphic megakaryocytes with hyperlobulation (hematoxylin–eosin, original magnification ×600). (**d**) Fibrosis change in post-polycythemic stage (hematoxylin–eosin, original magnification ×100)

Several large histopathological studies demonstrated that PV could be differentiated from secondary polycythemia in approximately 96% of patients who presented with mild to significant erythrocytosis [47–49]. PV specimens typically lack a prominent inflammatory stromal reaction, including only sparse numbers of perivascular plasma cells, eosinophils, hemosiderin-laden macrophages, and cellular debris.

The natural progression of PV involves a latent/prodromal phase, followed by the overt polycythemic phase and the "spent" or postpolycythemic myelofibrosis phase (post-PV MF) [1]. In the latent or prepolycythemic phase, bone marrow findings are similar to those in the polycythemic phase [36, 37]. Findings are specific and do not overlap with secondary erythrocytosis [1, 47]. In the polycythemic phase, the bone marrow is hypercellular with trilineage proliferation (panmyelosis). Hyperplastic normoblastic erythropoiesis predominates, but maturing granulopoiesis and megakaryopoiesis without dysplasia remain intact (Fig. 6.1a–c). In comparison, in the postpolycythemic or spent phase, erythropoiesis is markedly decreased, granulocyte maturation is delayed, and pleomorphic megakaryocytes without atypia are frequently observed. Overlapping features are typically observed with advancement of the disease, and MF progresses from a dense meshwork of reticulin with thickened sinuses (sinus wall sclerosis) to deposition of coarse collagen bundles to end-

stage myeloid scarring (Fig. 6.1d). More than 10% blasts in the peripheral blood or bone marrow herald disease acceleration, whereas 20% or higher is regarded as acute leukemia [1].

Cytogenetic Abnormalities

Absence of the Philadelphia chromosome or the *BCR-ABL1* fusion product is essential for exclusion of chronic myeloid leukemia, *BCR-ABL1* positive. Various mutations in the hematopoietic progenitor cells of PV patients have been detected by conventional cytogenetic and fluorescence in situ hybridization methods, and these abnormalities have been shown to accumulate over time [50–53]. Fewer than 20% of cases have an identifiable cytogenetic clone at diagnosis, whereas more than 80–90% have one at 10 years [54–57]. The most frequent genetic aberrations include deletion or translocation of chromosome 20, trisomy 8, and trisomy 9. Abnormalities in 13q, 5q, 7q, 1q, 5, and 7 are less common. Because similar karyotypes are also observed in patients with other MPNs, they do not convey specific data. Although complex karyotypes are more common in PV with transformation, a significant number of patients with fibrotic transformation have a normal diploid karyotype [54, 57–59]. Hence, cytogenetic abnormalities are not necessarily a good predictor for post-PV MF transformation. In several studies, the transformation was not found to be associated with mutant *JAK2* V617F burden [57, 60]. However, a recent study identified significant impact of *JAK2* V617F mutant allele burden on fibrotic transformation [61]. The group demonstrated that when *JAK2* V617F is regarded as a continuous variable, a difference of 10% in mutant allele burden between two subjects increases the risk of post-PV MF by 40% in the subject with a higher mutant allele burden [61]. Currently, the use of the mutant allele burden to guide treatment strategies is under investigation.

Serum EPO Levels

The vast majority of PV patients express very low levels of serum EPO (Table 6.1). This is a key diagnostic feature that differentiates PV from other polycythemic conditions [62, 63]. The molecular mechanism causing low EPO levels in PV is likely due to normal compensatory feedback loops in the setting of elevated erythropoiesis.

Molecular Pathogenesis and JAK2 V617F Mutation

The most significant discovery in *BCR-ABL1* negative MPNs has been the presence of an acquired *JAK2* V617F mutation in the *JAK2* gene on chromosome 9p24 (Table 6.2). It has been established as a driver mutation in MPN mouse models

Table 6.2 Mutations in PV

Gene	Location	Function	Type of mutation	Frequency (%)	Prognostic significance
JAK2	9p24 exon 14	Tyrosine kinase, protein coding	Point mutation at codon 617, Gain of function	96–99	Allele burden single most risk factor for thrombosis; higher allele burdens are more responsive to ruxolitinib therapy
	9p24 exon 12		Across multiple codons from 533 to 547 as substitutions, deletions, duplications or insertions, gain of function	3	Prognosis is similar to PV due to JAK2 V617F mutation
MPL	1p34 exon 10	Thrombopoietin receptor, signaling JAK-STAT pathway	Single amino acid substitution at codon 515, loss of function	Rare (0)	–
CALR	19p13 exon 9	Calcium binding protein associated with endoplasmic reticulum, transcriptional regulator	Insertions, deletions, indels with frameshift, resulting in truncated protein	Rare (0)	–
TET2	4q24 all codons	Epigenetic transcriptional regulator, hydroxymethylation	Insertions, deletions, nonsense; loss of function	10–20	Lower[a] OS; often acquired at the time of[b] LT
ASXL1	20q11 exon 13	Encodes chromatin binding protein	Frameshift and stop, loss of function	2–10	May indicate fibrotic transformation
DNMT3A	2p23 exons 7–23	Epigenetic modification, methylation of histones	Inhibits differentiation	5–10	Higher risk of[b] LT
EZH2	7q 35 all codons	Epigenetic modifier, transcriptional repressor via histone methylations	Loss of function	≤2	Infrequent[b] LT

IDH1/2	2q33/15q26	Cell cycle, metabolism	Gain of function	≤2	Decreased leukemia free survival
TP53	17p13.1	Cell cycle, apoptotic tumor suppressor	Loss of function	≤2	Frequent[b] LT
SOCS1	16p13.2	E3 ubiquitin ligase, negative regulation	Methylation, loss of function	≤2	Unclear
LNK (SH2B3)	12q24 exon 2	Encodes adaptor protein directly binding to phosphorylated JAK2	Loss of negative regulation function	≤2	Unclear
CBL	11q23 exons 8–9	E3 ubiquitin ligase, negative regulation	Loss of negative regulation function	Rare	Infrequent[b] LT
SF3B1	2q23 exons 12–16	Splicing factor 3b subunit 1	Loss of function	Rare	Infrequent[b] LT
IKZF	7p12	Encodes transcription factor, lymphopoiesis	Deletion	Not reported	Unclear
NRAS	1p13.2	GTPase	Gain of function	≤2	Possibly associated with LT
SRSF2	17q21 exon 1	Splicing factor	Unclear	Rare	Decreased OS

[a]*OS* Overall survival
[b]*LT* Leukemic transformation

[64, 65]. *JAK2* V617F is the most frequent molecular abnormality found in almost all patients (>95%) with PV, and was independently identified by four different groups [18, 20, 21, 66]. A guanine to cytosine mutation occurs in codon 617, which resides in the JH2 pseudokinase domain of exon 14, resulting in a valine to phenylalanine substitution (V617F) [20, 21, 66]. The *JAK2* V617F is a somatic Class I mutation, which modifies growth factor signaling [67]. Damage in the JAK2 JH2 domain results in loss of autoregulatory inhibition of JH1 kinase exerted via JH2 pseudokinase, leading to phosphorylation of the JAK2 tyrosine kinase and its constitutive activation, with downstream activation of JAK-signal transducer and activator of transcription (STAT)/RAS/MAPK/PI3K/AKT pathways (Fig. 6.2). This results in myeloid proliferation, which occurs at decreased levels or in the absence of cytokines [69].

It is known that cytoplasmic domains of cytokine receptors are docked to JAK2 dimers, one of the Janus kinases, a nonreceptor tyrosine kinase. At the NH_2 terminus, protein 4.1, ezrin, radixin, and moesin (FERM) and SH2 domains secure JAK2 to the cytokine receptors. The JH1 kinase domain is located at the COOH terminal, adjacent to the JH2 pseudokinase domain (Fig. 6.3) [69]. The classical view of the JAK-STAT (signal transducer and activator of transcription proteins) pathway activation is the following: binding of cytokine to the receptor brings the JAK2 molecules into proximity, cross-activation leading to phosphorylation of their tyrosine kinase residues; subsequently, this causes phosphorylation and dimerization of STATs, which translocate to the nucleus for further transcriptional downstream activity. However, recent X-ray crystallographic studies, conducted by molecular dynamic simulations, support a contrary currently prevalent view [64]. Studies on the growth hormone (GH) receptor-JAK2 activation have showed that the cytoplasmic regions of the receptor are in proximity in the basal state. Binding of GH causes activation of the JAK2 receptor; the cytoplasmic regions of the receptor crossover resulting in separation of JH2 pseudokinase domains. This change eliminates the autoinhibitory effect of the JH2 pseudokinase on the JH1 kinase of the other JAK2 in the dimer, thus bringing the JH1 kinase into close proximity with JH2, which results in JAK2 phosphorylation at multiple amino acid residues through unknown mechanisms [70].

Crystallographic modeling shows that the V617F mutation causes destabilization of the JH2-SH2 linker, which then destabilizes the JH2-JH1 interaction (Fig. 6.4) [64, 71]. It is also postulated that the V617F mutation may overstabilize the positive regulation mediated by JH2 by an unknown molecular mechanism [64]. The V617F mutation straightens and rigidifies the αC helix within the JH2 domain [64, 72, 73], whereas the wild type (WT) allele has a kink without considerable structural difference [64, 73]. It has been shown that JAK2 JH2 binds adenosine triphosphate (ATP) and phosphorylates negative regulatory sites involved in JAK2 activity [64, 74]. Understanding these mechanisms may provide potential therapeutic targets such as αC, ATP binding pockets; for example novel small molecules can inhibit JAK2 V617F activity without affecting the activity of the WT allele [64]. Currently, the approved JAK2 inhibitors, such as ruxolitinib are type I; these bind to the ATP binding pocket in the kinase domain in its active configuration, thus also

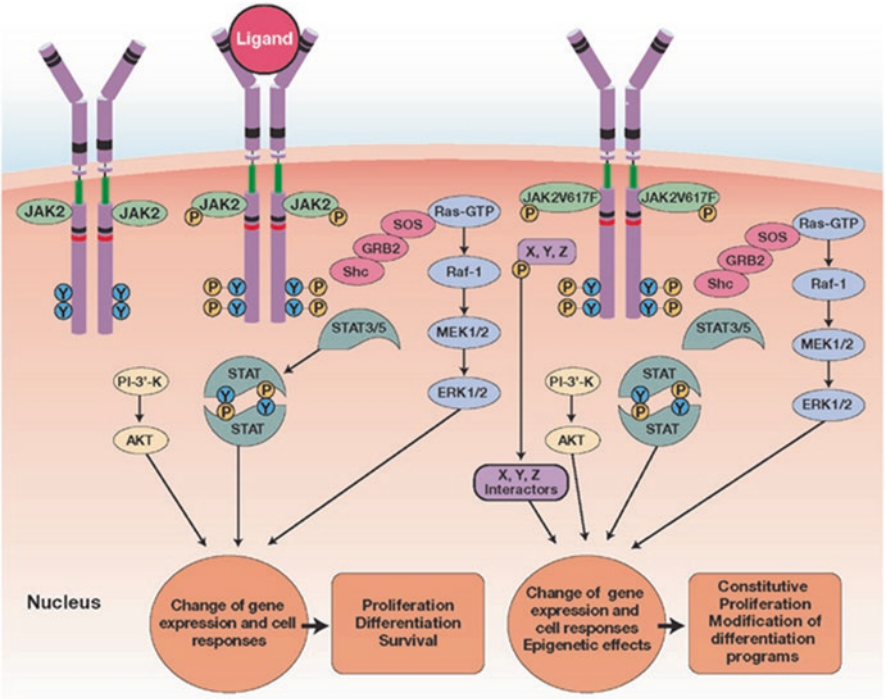

Fig. 6.2 Cytokine receptors exist on the cell surface in an inactive state bound to JAKs via their cytosolic domains. The binding of a specific ligand induces a conformational change in the preformed dimer, leading to tyrosine phosphorylation and cross-activation of JAKs, which phosphorylate intracellular receptor tyrosine residues (Y-P). In turn, the phosphorylated residues attract signaling adaptor proteins that recognize specific tyrosine phosphorylated sequences. Various adaptor proteins become substrates of JAKs, triggering signaling cascades. Cytokine receptors are linked to the STAT, Ras–MAPK, and phosphatidylinositol-3′-kinase (PI3K)–AKT pathways, which converge at the nucleus and regulate gene expression. *Left*, unliganded inactive cytokine receptor; *middle*, ligand-activated receptor, which is transient and induces anti-apoptotic, proliferative, and differentiation signals. *Right*, unliganded receptor that is constitutively active because of the attachment of JAK2 V617F, a constitutively active JAK, and is therefore recapitulating the cytokine-induced pathway, although in a persistent manner. Novel signaling molecules (X, Y, and Z) become substrates of activated JAK, initiating novel interactions (X, Y, and Z interactors) that change gene expression and/or induce novel epigenetic events (With permission from Vainchenker et al. [68])

affecting WT JAK2. Novel more effective JAK2 type II inhibitors, causing actual decrease in the mutant allele load, are currently developed; these inhibitors bind in a similar fashion to the ATP binding pocket in the kinase domain, but in its inactive configuration [64].

Among the various downstream activators, *STAT5* is also a crucial actor in disease pathogenesis. Hence, there is an opportunity to therapeutically abrogate dysregulation of its transcriptional targets [73]. These targets are PIM kinases, c-MYC and JUNB, CYCLIN D2, P27KIP, CDC25A, PU.1, ID1, BCL-XL, MCL-1, and RAD51 [73]. However, Kouzarides et al. showed that JAK2 alone can be localized

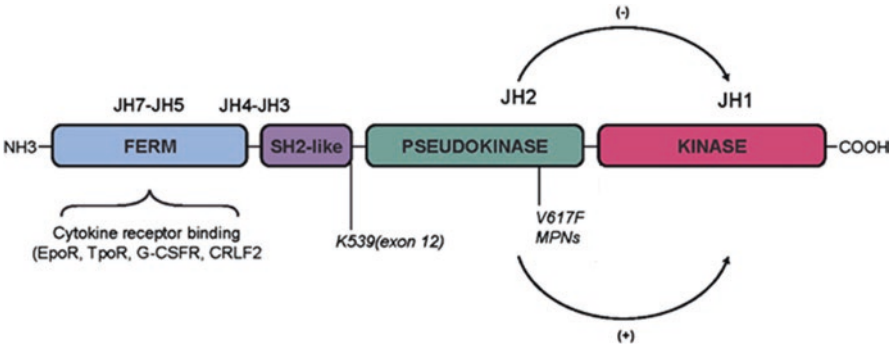

Fig. 6.3 Domain structure of JAK2. JAK2 contains a tyrosine kinase domain (JAK homology 1 (JH1)), a pseudokinase domain (JH2), SH2-like domain, and a domain that resembles protein 4.1, ezrin, radixin and moesin (FERM). The latter domain is responsible for attachment to the cytosolic domains of cytokine receptors. The pseudokinase domain, JH2, functionally prevents the activation of the kinase domain, JH1 (*curved arrow on the top*). In contrast, mutations in the pseudokinase domain (*red*) lead to activation (*arrow on the bottom*) of JH1. The V617F mutation has been identified primarily in MPNs

in the nucleus and phosphorylate histone Y41, and increase expression of oncogenes such as *MYC* and *LMO2* [75]. One of the mechanisms of cytokine hypersensitivity of JAK2 V617F mutant cells and disease progression is attributed to upregulation of La auto-antigen with resultant p53 inactivation [73, 76]. The *JAK2* V617F mutation simulates fibroblastic growth and proliferation by increasing oncostatin M expression (OSM), a profibrogenic cytokine stimulator of bone marrow fibroblasts. Pathogenic hypercytokinemia can be controlled by potentially targeting OSM [73, 77]. Mutant JAK2 binds more strongly, phosphorylates and downregulates activity of PRMT5, an arginine methyltransferase JAK binding protein. Abnormal histone methylation affects chromatid remodeling, increases hematopoietic stem cell progenitor population, myeloproliferation, and erythroid differentiation [73, 78]. These insights in the pathogenesis provide possible therapeutic strategies.

The *JAK2* V617F mutation is found in MPNs associated with PV (>95%), essential thrombocythemia (50%), PMF (50%) or various other disorders. It is also seen in refractory anemia with ringed sideroblasts associated with marked thromobocytosis (~50%), and rarely in other myelodysplastic syndromes (MDS), MDS/MPNs such as chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia, hypereosinophilic syndrome, and systemic mastocytosis. The *JAK2* V617F mutation is consistently absent in all secondary bone marrow conditions, unrelated leukemias, and healthy controls [79]. However, screening for the *JAK2* V617F mutation is the first diagnostic test for PV in the appropriate clinicopathological context.

The *JAK2* V617F mutation is the major diagnostic criterion for *BCR-ABL1* negative MPNs (PV, ET, and MF). The three MPNs have different phenotypes, thus this single mutation associated with different phenotypes is of great interest in

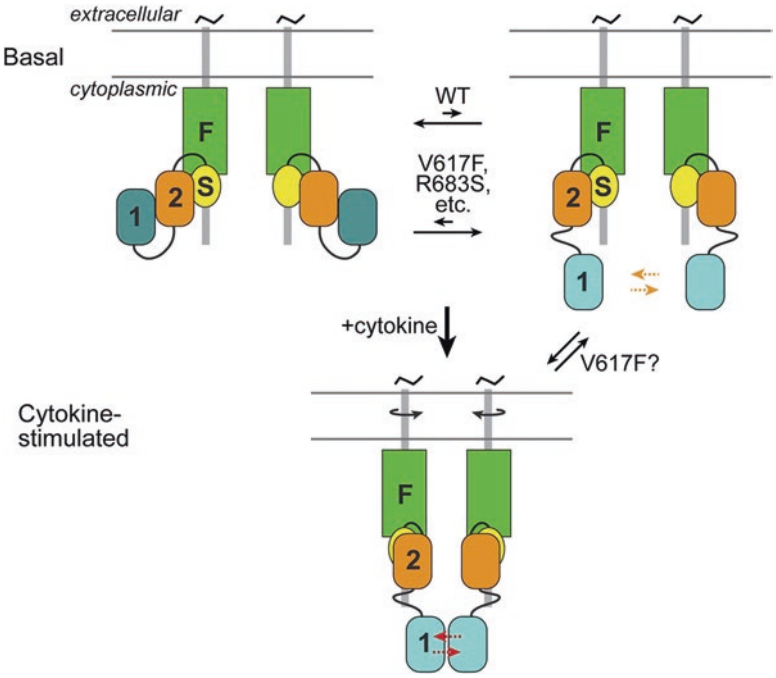

Fig. 6.4 Schematic of JAK2 activation. Top: In the basal state, two JAK2 molecules associate via their N-terminal band 4.1, ezrin, radixin, moesin (FERM or F) and SH2 (S) domains with predimerized type I cytokine receptors. The autoinhibitory interaction between JH2 [2] and JH1 [1] sequesters JH1 from each other and stabilizes an inactive state of JH1 (*dark cyan*) (*left*). This is in equilibrium with a state (*right*) in which JH1 is disengaged from JH2 (JH1 *cyan*, higher activity), which increases the probability of trans-phosphorylation (*orange arrows*) on the activation loop of JH1. By destabilizing the autoinhibitory interaction, pathogenic mutations, such as V617F, shift the equilibrium to the partially active state. *Bottom*: Cytokine binding to the extracellular region of the cytokine receptors induces a structural rearrangement in the cytoplasmic region (possibly through rotation of the transmembrane helices), which greatly facilitates (*red arrows*) JH1 trans-phosphorylation. JH2 is also necessary for cytokine-induced JAK2 activation, but the molecular interaction(s) responsible for this is not known (and not shown). As indicated, in addition to destabilizing the autoinhibitory interaction, V617F might promote the JH2-mediated positive interaction normally induced by cytokine (or possibly promote an interaction distinct from the cytokine-mediated one)

pathogenesis. Mutations primarily occur at the multipotent HSC level, and are found in granulocytic, erythroid, megakaryocytic, as well as lymphoid lineages [80]. Molecular analysis showed that the *JAK2* V617F mutation enables and perpetuates clonal proliferation distorted towards phenotypic erythrocytosis in PV [81], whereas myeloid differentiation is seen in MF [82, 83]. The same *JAK2* V617F mutation may affect subsets of HSCs with distinct anomalous differentiation to a specific MPN [80]. Preference to develop into PV may be modulated by quantitative differences in the JAK2 type. In murine engraftment experiments, James et al. found that engrafted PV primarily presents with *JAK2* WT with a small proportion that is

heterozygous for the *JAK2* V617F mutation. The authors also found an overwhelming number of *JAK2* V617F mutations that are homozygous are seen in post-PV MF or PMF. Apart from the *JAK2* V617F mutation, additional genetic or other changes and increased responsiveness towards cytokine stimulation of *JAK2* V617F-committed progenitors and HSCs (especially in PMF) may play a role in disease pathogenesis [83, 84]. Another study suggested that PV phenotype is a function of its allelic profile and mutation burden. This study suggested that clone sizes for the *JAK2* V617F mutation are small and heterozygous in PV and ET, whereas early progenitors in heterozygous *JAK2* V617F PV are more erythropoietin-sensitive, dedicated and committed as compared to those in ET. Homozygous PV was found to be a minor subset, whereas progenitors were found to be erythropoietin-independent with preferential proliferation occurring at the terminal stage of differentiation. No homozygous clones were found in ET [85]. In a different study, microsatellite mapping and analysis of clonality in the earliest erythroid progenitors of PV and ET demonstrated that both PV and ET acquire homozygous clones, but PV has a dominant single homozygous mutant subclone 8–85 times higher than the minor mutant subclones. These findings show that the homozygous subclone is a driver for erythrocytosis and hence PV phenotype. Additional genetic or epigenetic changes may also play a role in progression of the disease [86]. Overall, the prevailing view is that *JAK2* V617F alone is not a strong driver mutation with small clone sizes and can remain clinically stable disease over years [73, 85, 87].

Homozygous *JAK2* V617F has been detected in approximately 30% of patients with PV [18, 19, 21, 88]. Homozygosity in PV is associated with older age groups, higher hemoglobin and leukocyte counts, splenomegaly, thrombosis and cardiovascular events, and symptomatic disease with consequent therapy [73, 89]. Chromosome 9p with the mutation *JAK2* V617F is frequently involved in loss of heterozygosity (LOH at 9p) in PV [20, 90]. Thus, the lost allele 9p with the *JAK2* V617F mutation is duplicated by mitotic recombination which itself likely causes LOH at 9p and its duplication by uniparental disomy (UPD), resulting in two copies of *JAK2* V617F allele in the affected cell [20, 73, 90]. This change confers variability, heterogeneity, variable levels of mutant allele with dosage effect, with a progression from heterozygosity to homozygosity in clinical presentation of PV [89]. As discussed earlier, homozygosity appears to be a driver of erythrocytosis (PV phenotype) and adverse prognostic variables. The *JAK2* V617F mutant allelic burden, quantified by PCR, is routinely measured in granulocytes. Generally, mutant allele burdens lower than 50% and higher than 50% are reported as heterozygous and homozygous, respectively. This routinely used terminology can cause confusion and deception regarding mutant *JAK2* V617F genotype because the mutant can comprise either sole or combinations of heterozygous, hetero-homozygous or homozygous cell populations [91]. Nevertheless, it has been demonstrated that a higher allelic load in granulocytes (>50%) corresponds to the adverse effects seen in homozygous population, helps risk stratification by molecular quantification, and is a threshold for initiation of therapy with JAK2 inhibitors [73, 89, 92]. Low allelic burdens can be managed with phlebotomy and aspirin to reduce risk of thrombosis [92].

The mutation *JAK2* V617F appears to confer a slower rate of disease progression, a higher risk for thrombotic or hemorrhagic complications, and an increased degree of MF. In addition, homozygous versus heterozygous clones for *JAK2* V617F may confer a poorer overall prognosis [89]. Several studies showed the absence of correlation between *JAK2* V617F allelic burden and fibrotic risk of transformation [57, 60].

The mutant allelic burden is the single most important factor for risk of thrombosis irrespective of phenotype of *JAK2* V617F disease. Rates of thrombosis increase with increasing allele burden and in particular, the risk increases with >50% *JAK2* V617F [93].

Interestingly, higher *JAK2* V617F mutant allele burdens have been found to be responsive to ruxolitinib therapy [94]. Serial measurements can help predict evolution of disease with increasing mutant allele burdens [95], assess effectiveness of therapy and clinical remission, or guide adoptive immunotherapy such as donor lymphocyte infusion [91, 96].

Methods for Detection of JAK2V671F and Its Allele Burden

Detection of the *JAK2* V617F mutation is diagnostic of PV in the appropriate clinicopathological setting. Limited information is available on the clinical significance of a specific quantitative allele burden. The majority of research studies examining *JAK2* in PV patient specimens have utilized various DNA sequencing platforms to identify the mutated allele in the purified granulocyte fraction [18–21]. DNA sequencing is routinely and successfully used in many labs, and is considered the "gold standard" [97]. Direct sequencing has low analytical sensitivity (~20%), is time-consuming, and technically demanding [18, 97, 98]. High throughput techniques for targeted massive parallel/next generation sequencing (MPS/NGS) are preferred as they can detect all *JAK2* mutations, and are not limited to V617F and non-driver mutations affecting prognosis [2]. Most molecular diagnostic laboratories have instrumentation and technical expertise readily available to perform real-time polymerase chain reaction (RT-PCR) [95].

It is important to consider pre-analytical variables of the specimen before choosing an assay [79]. Several PCR-based assays for rapid detection of *JAK2* V617F in peripheral blood, bone marrow aspirate, and paraffin-embedded tissue specimens have been studied and compared [95, 99, 100]. A small amount of peripheral blood (2–10 ml) in Ethylenediaminetetraacetic acid (EDTA) is adequate for genomic DNA extraction, and can be frozen for further analysis. Bone marrow aspirate is acceptable [101] and unstained (preferred) unfixed slides [102] can be used for this purpose. Purification of granulocytes for DNA extraction by Ficoll gradient centrifugation and the amount of nucleic acid template depend on the sensitivity and type of assay, respectively [79].

The JAK2 Mutation Working Group of the Association for Molecular Pathology (AMP) and the MPNs and MPN-related congenital disease (MPNr) European Network (MPN and MPNr-EuroNet) suggest that suitable assays should have a low

limit of detection (at least 1% for diagnosis and 0.1% for residual disease monitoring), nearly 100% specificity at the above detection levels, high reproducibility, and transferability between laboratories [91, 103]. Low levels of *JAK2* V617F allele identification should be interpreted carefully. Less than 1% mutant allele burden and very low levels (<0.1%) have been found in ET and in healthy individuals, respectively. Repeated testing after 3–6 months is warranted in these cases [79, 91].

Studies have shown that various PCR assays to detect the *JAK2* V617F mutation are in agreement when the mutant allele load is higher [97, 104]. Overall, allele-specific quantitative PCR (qAS-PCR) or the amplification refractory mutation system PCR (ARMS-PCR) are the most reliable and sensitive assays (~ analytical sensitivity 0.1–5%) [103, 105, 106]. A clinically significant and pathogenic mutant allele burden is found to be in the range 1–3% [79, 107]. Other methods such as high-resolution melting PCR (HRM-PCR) and melting curve analysis display 1–5% analytical sensitivity [79], which is equivalent to that of ARMS-PCR. However, HRM-PCR is easy to perform because it involves a single amplification step versus ARMS-PCR, which amplifies WT and mutant alleles in two separate consecutive reactions [97]. Real time allele-specific PCR with sensitivity of 0.01–1% can be used to assess minimal residual disease and response to JAK2 inhibitor therapy [79, 96, 106, 108]. Similar to direct sequencing, pyrosequencing lacks optimal sensitivity [103]. Digital PCR, which is technically less demanding, has been found to have analytical sensitivity equivalent to that of real time AS-qPCR [109]. Point of care microchips with visual assays based on microfluidics have also been developed and are potentially helpful in limited resource settings [110].

JAK2 Exon 12 Mutations

JAK2 exon 12 mutations are present in approximately 3% of PV patients; they occur across multiple codons – from 533 to 547 – as substitutions, deletions, duplications or insertions, near the pseudokinase domain, in the linker region between the pseudokinase and SH2 domains. The most common mutation in *JAK2* exon 12 (23–30%) is an in-frame deletion of six nucleotides at codons 542 and 543 (N542-E543del). Other mutations encountered in exon 12 are R541-E543delinsK, E543_D544del, F537_K539delinsL, and K539 L. Each mutation occurs with an approximate frequency 10% [91, 103, 111]. Exon 12 mutations result in erythrocytosis phenotype at a younger age (~50 years of age) involving higher hemoglobin and hematocrit *vis-a -vis JAK2* V617F type, normal WBC and platelet counts with low serum erythropoietin, erythroid hyperplasia in bone marrow with rare trilineage expansion, variable megakaryocytic morphology (ranging from small to medium or large megakaryocytes with mono or hyperlobated nuclei). Transformation to leukemia or MF is a rare occurrence, and patients with *JAK2* exon 12 mutated PV have a good prognosis with nearly normal life expectancy [91, 92, 103, 112]. The molecular mechanisms of *JAK2* exon 12 mutations are similar to that of mutation *JAK2*

V617F, with cytokine independent proliferation, and JAK2 inhibitors capable of targeting the pathway. The consequences of these founding mutations on the signaling cascade downstream of JAK2 (increased ERK1 and ERK2) are distinct from those with *JAK2* V617F [111]. An improved course of the disease is expected with higher proportions of stable heterozygous colonies and small amounts or absence of homozygous clones over time [92, 111, 113]. However, in a multicenter study, it was found that PV due to *JAK2* exon 12 mutations initially presents with isolated erythrocytosis, but its subsequent behavior, risk stratification and prognosis are similar to PV due to the *JAK2* V617F mutation [114]. Direct sequencing has limited diagnostic importance if peripheral blood with low mutant allele burden is used. Several *JAK2* exon 12 mutations exist (at least 27 are known), precluding the choice of targeted AS-PCR despite its superior detection sensitivity (10%) [115]. HRM analysis, locked nucleic acid (LNA)-clamped fragment analysis, melting curve assay, dHPLC (denaturing high-pressure liquid chromatography) are used to screen for this mutation. AS-qPCR has a sensitivity of 0.01% for 10 of the most frequent *JAK2* exon mutant alleles (overall 80% for *JAK2* exon 12) [79, 103].

Latest Somatic Mutations

Molecular analyses have unraveled numerous somatic mutations in MPN with *JAK2*, *MPL*, and *CALR* established as driver mutations (Table 6.2). Additional somatic mutations are "nondriver" mutations, nonspecific for MPNs as they can be found in myeloid malignancies and normal elderly individuals [68]. It has been found that, as a group MPNs have a low frequency of somatic mutations, namely ~0.2 somatic mutations per Mb or 1 somatic mutation per 45 patient years, with most mutations being present early on when the disease is detected [116]. These mutations provide prognostic rather than diagnostic information and some of them occur synergistically or together with the driver mutation, thus providing fertile ground for disease initiation or progression. The number of somatic mutations is a predictor of overall survival and leukemic transformation [2, 116]. The median number of somatic mutations in PV is less than that in PMF (6.5 vs 13 per patient) [117]. Rarely *CALR* mutations have been described in *JAK2* negative PV [118]. The typical triple negative (TN) mutation profile encountered in ET and PMF (overall 10–15%) is essentially not seen in PV; in theory, this could be due to undetected *JAK2*, especially *JAK2* exon 12 variants, or other mutations leading to erythrocytosis. Recent findings on selected mutations are detailed in the next paragraph.

In PV, *TET2* mutations are predominantly found to occur as pre *JAK2* V617F event providing self-renewal and survival advantage to the *JAK2* mutated clone for myeloproliferation with no apparent effect on transformation [116]. Dual concomitant or secondary *TET2* mutations in a disease with *JAK2* V617F mutation are less frequent [116]; but are connected with lower overall survival due to increasing intensity of myeloproliferation and extra-medullary hematopoiesis, and are often acquired at the time of leukemic transformation [119]. TET2 loss may limit

therapeutic efficacy of interferon (IFN)-α as IFN-α acts by depleting the *JAK2* V617F mutated HSCs [120].

ASXL1 mutations have a relatively low frequency in PV (~2–10%) versus ET (5–10%) and PMF (13–26%), and post PV/ET MF (22–39%) [121]. A study showed that patients with ASXL1 loss of function had significantly lower hemoglobin levels than those without the mutation [116]. The same study noted that despite no specific temporal relation with JAK2 mutation, *ASXL1* mutations were often acquired as a pre *JAK2* event [116]. Loss of ASXL1 is an independent negative prognostic marker in PMF, MDS, CMML [122, 123], and may indicate fibrotic transformation in PV [2]. It was recently shown that ASXL1 has a role in effective erythropoiesis and effective maturation of erythroids; its loss leads to anemia, and anemia confers poor prognosis in MDS, CMML, and PMF [122, 124].

The *EZH2* gene is involved in DNA methylation [116], and its mutations are thought to act synergistically with *JAK2* V617F and result in MPNs [125]. Murine experiments show that heterozygous loss in conjunction with *JAK2* V617F leads to PV, while homozygous mutations result in MF with higher platelet and WBC counts without intermediate erythrocytosis. Experimentally deletion of *EZH2* in mice without *JAK2* V617F mutation resulted in thrombocytosis. Additional studies are necessary to determine the efficacy and suitability of EZH2 inhibitors in MPN, as it is thought to have a tumor suppressor effect.

DNMT3A loss is more frequently associated with AML and leukemic transformation in MPN, possibly by upregulating genes such as *RUNX1* and *GATA3* [126]. *DNMT3A* mutations by inhibiting differentiation may provide the circumstance for HSC proliferation in the presence of the *JAK2* mutation with limited self-renewal [127]. The *DNMT3A* R882 mutation has been found to induce leukemia in animal models in presence of NRAS mutation by affecting DNA methylation apparatus [128].

IDH1/2 are enzymes in the tricarboxylic acid (TCA) cycle and fatty acid synthesis producing metabolites involved in epigenetic regulation of progenitor cell differentiation. Although they are less frequently mutated in PV as compared to other MPNs, one study has shown that leukemia-free survival was adversely affected by *IDH2* mutations in PV patients [2]. In the same study, *SRSF2* mutation was found to affect OS, *RUNX1* mutation affected OS and leukemia-free survival among PV patients.

Clinical Management of PV

Contemporary management of PV is driven by the presence of major risk factors of thrombosis and a history of thrombosis in PV patients [129, 130]. Thrombosis prevention with low-dose aspirin, and management of cardiovascular risk factors is recommended for all PV patients. Phlebotomy remains a key intervention with a target hematocrit less than 45% in patients with PV. Chemotherapy with hydroxyurea should be considered as a first line of therapy depending on the history of

thrombosis, and for patients over 60 years of age. Interferon-α can be used in younger patients as the first line of therapy due to the absence of leukemogenic effect when compared to hydroxyurea, and its ability to induce clinical, hematological and molecular responses with persistence of response after therapy cessation [130]. However, additional mutations such as loss of *TET2* may limit therapeutic efficacy of IFN-α. Failure to respond to the first line of therapy, which is seen in ~16–24% of patients with progressive splenomegaly, leukocytosis, or thrombocytosis may warrant use of ruxolitinib, a JAK2 inhibitor. Although ruxolitinib reduces spleen volume, and alleviates constitutional symptoms, it does not appear to alter the natural course of the disease. Prognostic models developed for PMF cannot be accurately applied to post-PV-MF [130]. However, bone marrow transplantation can be considered for younger patients who rapidly progress to MF or leukemia [129, 130].

Conclusions

Because the identification of *JAK2* V617F as its driver mutation in 2005, tremendous strides have been made by the scientific community in understanding the pathogenesis of PV. The 2016 WHO classification has updated disease diagnostic criteria, which significantly improves sensitivity to identify patients with PV and distinguish them from patients with other MPNs, thus offering benefit of specific treatment to reducing morbidity and mortality. In addition, molecular dynamic simulations are revealing pathogenic mechanisms involved in PV development, helping to identify new therapeutic targets for precision medicine that will have no or less adverse effects in patients. Moreover, new somatic mutations in PV are being identified through modern sequencing techniques. These mutations have an interesting interplay with *JAK2* V617, providing prognostication of disease evolution and transformation, and thus guiding personalized therapy in patients with PV.

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