Review Article

Genomic diversity in myeloproliferative neoplasms: focus on myelofibrosis

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Abstract: The classical myeloproliferative neoplasms (MPNs) are a group of clonal diseases comprising essential thrombocythaemia (ET), polycythaemia vera (PV) and primary myelofibrosis (PMF). PMF is the rarest disease sub type and has been challenging to address due to the lack of a specific genetic marker, inadequate risk identification models and a highly variable clinical course. Continuous efforts have over time, seen the inclusion of cytogenetic information in prognostic scoring models that have resulted in improved risk stratification models providing further rationale for therapeutic management. Technological advances using single nucleotide polymorphism arrays increased the detection of known and novel MPN related changes and variant detection by massively parallel sequencing provided a large scale screening tool for the multitude of somatic gene mutations that have more recently been described in MPN. Some of these mutations show an association with specific cytogenetic changes or phenotypes. While PMF occurs mainly in adults, it has also been described in paediatric cases and shows distinct histopathological, genetic and clinical features in comparison. This review provides an overview of the genomics landscape of PMF and current developments in MPN therapy.

Keywords: Myeloproliferative neoplasm (MPN); primary myelofibrosis (PMF); gene mutations

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The chronic myeloproliferative neoplasms (MPNs)

The MPNs are a group of clonal haematological disorders that arise from transformation of a multipotent haematopoietic stem cell (1). Chronic myeloid leukaemia (CML) is the best characterised MPN sub category and is characterised by the Philadelphia (Ph) translocation t(9;22) leading to the *BCR/ABL* gene fusion associated with abnormal tyrosine kinase activation involved in the causative pathophysiology of CML. The Ph negative, *BCR/ABL* negative MPNs encompass three distinct clinical sub sets, namely polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) that make up the classical MPNs (2). In contrast to CML, disease specific genetic abnormalities have not been detected that distinguish PV, ET and PMF. PMF is the rarest and most complex of the MPNs. Despite this, much advancement in our understanding of the underlying genetic changes in PMF has occurred in recent years that impact patient management.

Classification of primary myelofibrosis (PMF) (*Table 1*)

Disease acceleration is indicated in those patients showing 10-19% blasts, an increased CD34 count with clustering and/or endosteal location on the bone marrow (BM) histology. A blast count above 20% is indicative of transformation to blast phase leukaemia. Patients with PMF may rarely present initially in the accelerated or acute phase (3).

Pre fibrotic/early myelofibrosis (MF)

The World Health Organization (WHO) classification

Table 1 In 2008 the World Health Organisation (WHO) provided guidelines for the classification of the classic MPNs. All major criteria and two minor criteria are required for diagnosis of PMF (fibrotic stage) (3)

Major criteria	Megakaryocyte proliferation and marked dysplasia with either reticulin and/or collagen fibrosis; WHO exclusion criteria for CML, PV, MDS or other myeloid neoplasm; Presence of *JAK2*V617F or other clonal marker or absence of reactive marrow fibrosis
Minor criteria	Leukoerythroblastosis; Increased serum LDH; Anaemia; Palpable splenomegaly

MPNs, myeloproliferative neoplasms; CML, chronic myeloid leukaemia; PV, polycythaemia vera; MDS, myelodysplastic syndrome.

requires, in the absence of reticulin fibrosis, that the megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation and decreased erythropoiesis.

Epidemiology of MF

PMF occurs at a frequency of 0.5 to 1.5 per 100,000 (4). It occurs at equal frequency in males and female and the age of onset is usually after the 6th decade. Median reported survival in various studies range between 3.5 to 5 years (4-6). Post MF is found to develop in 25-50% of PV and 2-3% of ET after approximately 15-20 years of follow up (7,8). PMF has been reported rarely in infants and in younger patients with or without a family history of PMF and these cases appear to have a better outcome than adults (4).

Clinical features and diagnosis

Signs and symptoms

Patients usually present with splenomegaly or hepatomegaly as the main physical sign at diagnosis. Profound constitutional symptoms such as fatigue, weight loss, night sweats and fever are common. Thirty percent of patients are asymptomatic at presentation and are detected by an incidental abnormal blood picture or enlarged spleen (9). The disease gradually evolves from the early prefibrotic phase to the fibrotic phase with increasing BM failure.

Laboratory indicators

A large proportion of patients present with anaemia and a haemoglobin level below 100 g/L requiring blood transfusions. Other laboratory findings include leucocytosis or leukopenia, thrombocytosis or thrombocytopenia and circulating myeloblasts, increased serum lactate dehydrogenase and low cholesterol are also observed. Abnormalities on blood film examination at diagnosis may include a leukoerythroblastic blood film, left shifted granulocytes and red cell anisopoikilocytosis. Extra medullary haematopoiesis (EMH) is a striking feature and refers to the presence of proliferating haematopoietic stem or progenitor blood cells outside of the BM (myeloid metaplasia). The most common sites are in the spleen or liver but may arise in the skin, lymph nodes, serosal surfaces, the lungs and spine giving rise to lymphadenopathy, pleural effusion, pneumonia like symptoms or compression of the spinal cord and nerve roots (8,10). A vastly increased number of CD34+ cells are also present in the peripheral blood relative to both normal individuals and to other MPN. An increased number of circulating endothelial progenitors (EPCs) and increased vasculature although not specific to PMF, is a notable feature.

Paediatric PMF

A recent review of PMF in the largest series to date comprising 19 paediatric cases studied over a 30 year period, showed some distinct features compared to that of adult PMF and did not support previous findings that paediatric cases largely resolved spontaneously (11). Paediatric cases albeit rare were infrequently associated with a leukoerythroblastic blood film and 50% of cases showed marked BM eosinophilia not observed in adults. All but one showed one or more cytopaenias and moderate to severe reticulin fibrosis was common together with mild collagen fibrosis in some cases. No osteosclerosis was observed and megakaryocyte dysplasia consisted of separation of nuclear lobes, hypolobation and micromegakaryocytes as opposed to the "cloud like" megakaryocyte nuclei typically observed in adults. No increase in blasts or CD34 count or increased vasculature was observed. All patients required supportive care involving blood or platelet transfusions. Four of nine cases who underwent hematopoietic stem cell transplant (HSCT) died. A further four died of infection. Five cases resolved spontaneously. None transformed to acute phase.

Genetic studies in the above series showed no mutations of the Janus kinase 2 (*JAK2* c. 1849G>T) (p.V617F) or myeloproliferative leukaemia viral oncogene [(*MPL*) c.1544G>T, 1543_1544TG>AA] (p.W515L, p.W515K) usually present in adults. An autosomal recessive inheritance was suspected in some families due to the involvement of siblings and/or consanguinity. The outcome of patients in this series was generally poor and further studies are required to distinguish the pathobiology of spontaneously resolving paediatric cases from those requiring therapy. A subsequent study on calreticulin (*CALR)* mutations in 14 paediatric cases showed the type 2 *CALR* c. 1154_1155insTTGTC (p.K385fs*47) mutation to be present in 50% of cases (12) (see section on gene mutations below). These patients showed higher scores for the dynamic international prognostic scoring system plus (DIPSS+) and the international prognostic scoring system (IPSS) models, lower haemoglobin, platelet and total white cell count as well as a lack of splenomegaly and erythroblastosis. No increased eosinophilia was noted and *CALR*+ cases were all older than 6 years of age. The outcome of the whole cohort was poor with 50% of patients transforming to acute myeloid leukaemia (AML). None had accepted HSCT. The type 1, 52 base pair deletion *CALR* c.1099_1150del (p.L367fs*46) mutation was not observed in this series and the prognostic significance of *CALR* mutations in paediatric patients is subject to independent verification.

Prognosis

The median survival in adult PMF is 3.5-5 years but is highly variable and can be estimated using defined prognostic criteria. Younger adult patients with low risk may survive more than a decade while survival is less than 3 months in those that transform to blast phase (13). The current DIPSS+ prognostic model was formulated in 2009 by an international working party (14). The major inclusion in the DIPSS+ model was formal incorporation of cytogenetic information and transfusion dependency that were not previously included to inform risk. In this model, high risk karyotypic results such as complex karyotypes (>3 karyotypic aberrations), or those with sole or 2 of the following: –5/del5q, –7/del7q, +8, inversion 3q, 11q23 abnormalities or del(12p) as well as transfusion dependency and a platelet count less that 100×10^9/L were also assigned a score. The DIPSS+ study investigators determined that a proportion of patients in each risk group showed adverse cytogenetics, transfusion dependency or low platelets. Due to the inclusion of these latter criteria, further risk stratification of patients is now possible especially in the intermediate risk groups (15).

Cytogenetics of PMF

Clinical management of PMF is confounded by the extreme variability in disease course. Inter-patient variability also exists with regard to the extent of clonal involvement of the haematopoietic lineages. No monoclonality has however, been demonstrated in the BM fibroblasts (5,16). Clonal chromosome aberrations have been demonstrated in 35-60% of PMF, 15-20% of PV and less than 5% of ET (17,18). The most common changes are del(13q),del(20q),+8,+9,+1q and the pattern of abnormalities detected is common to all three MPNs. The complexity of the karyotype tends to increase over time and there is no difference in the type of chromosomal rearrangements observed in PMF and post MF MPN. Multivariate analysis showed +8 and 12p– to be independent poor prognostic markers while 13q– and 20q– showed no adverse effect on survival in multiple studies (19). More recent studies have indicated a monosomal karyotype, –5/del(5q), –7/del(7q), 11q23 abnormalities or 3q abnormalities to confer a dismal outcome reflecting the importance of karyotype analysis in clinical management of patients (15).

Single case abnormalities are not uncommon in MPN and the further characterization of these seemingly obscure abnormalities have led to the discovery of highly relevant gene rearrangements such as the ten eleven translocation member 2 (*TET2*) gene mutation on 4q24, FIP1-like-1/platelet-derived growth factor alpha (*FIP1L1/PDGFRA*) fusion on 4q12 and also a novel mechanism involving inactivation of the Casitas B-lineage lymphoma *(CBL*) gene on 11q23 that has proven to be biologically significant in several myeloid diseases with implications for patient therapy (2,20,21). Recent cytogenetics studies reported the association of 1q gain with polyploidy and a trend towards advancing disease in a patient series (22). Patients that transform to acute phase usually show complex karyotypes at transformation and a significantly decreased median survival (23). The role of specific chromosome abnormalities in chronic phase and leukaemic transformation are unknown. Preliminary correlations between the karyotype and specific gene mutations showed a significant association between a normal karyotype and the presence of additional sex combs like 1 (*ASXL1*) and U2 small nuclear RNA auxiliary factor 1 (*U2AF1*) mutations (24). A strong correlation was also

observed between the del(20q)/serine-arginine-rich splicing factor 2 (*SRSF2*) mutation, del(13q)/*CALR* mutation and +9/JAK2 mutation.

The use of SNPa added a powerful new dimension to the spectrum of cytogenetic abnormalities that could be detected. The major advantage with SNPa is the ability to provide independent but simultaneous genotype calls and copy number changes. This allows the detection of somatic mosaicism and copy neutral loss of heterozygosity (CNLOH) in a single analysis. The majority of CNLOH involved the 9p arm containing the *JAK2* gene, however, other regions were also recurrently altered such as 1p, 4q22, 11q, 14q, 15q and 17q (22,25-27). Non random copy number changes affected the 6p, 8p, 17q11.2 and 22q regions. The detection of copy number abnormalities known to be associated with MPN such as Neurofibromatosis 1 *(NF1)*, 20q and 13q deletions among others were significantly enhanced by SNPa. Novel copy number changes described at leukaemic transformation involved 7q, 16q, 19p, and 21q (28). Failure to detect balanced rearrangements, polyploidy or minor sub clones were the major limitations encountered with SNPa in the absence of a karyotype.

Gene mutations

In 2005 four different groups detected an identical gain of function point mutation of the *JAK2* gene (29-32). This mutation causes a valine to phenylalanine substitution (V617F) in exon 14 of the *JAK2* gene located on chromosome 9p24. The *JAK2V617F* gene mutation results in the continuous activation of the JAK-STAT signalling pathway and constitutive expression of STAT3/5 resulting in unchecked myeloproliferation. The incidence of the *JAK2* mutation in the classic MPNs ranges between 35-50% in PMF, 57% in ET and 74-97% in PV but the mutation by itself does not explain the distinctly different phenotypes associated with the three disease sub entities (33).

A second mutation in *JAK2* exon 12 was discovered exclusively in patients with PV (3%) who are negative for the *JAK2V617F* mutation. The exon 12 mutation does not alter the typical PV disease phenotype. A number of new mutations in MPN have been described in recent years that are thought to be co-operating mutations needed for disease initiation and progression (34). *Table 2* indicates the frequency of these gene mutations in the different disease sub types (33,34,52,53).

Activating mutations have been ascribed to mutations in *JAK2* and the thrombopoietin receptor *MPL*. The *MPL* mutation is more specific to PMF and ET and has not been reported in PV (*Table 2*). The level of the *JAK2* mutant allele burden has been shown to be a factor implicated in the MPN sub types. Low levels of *JAK2* mutation in PMF are characterised by BM failure, low cell counts, infections and show an inferior outcome compared to those PMF patients with high mutant load or wild type *JAK2* that show a more classic myeloproliferative phenotype (54-56). This possible biological sub group bears some significance, as these patients show marked cytopaenias and are likely to be excluded from clinical trials and thus make conclusions about generalised outcomes difficult (57). The above studies underscore the importance of serial studies on *JAK2* in these patients in order to elucidate the presence, timing of mutation acquisition and the role of *JAK2* allele burden in PMF. Other genes involved with activation of the JAK-STAT pathway include inactivating mutations in the SH2B adaptor protein 3 (*SH2B3)/LNK* and *NF1* genes while dominant negative mutations occur in *CBL*.

Recently, mutations in the *CALR* gene have been described in 67% of cases with ET and 86% of cases with PMF that are negative for *JAK2* or *MPL* mutations (48,49). *CALR* mutations were found to be mutually exclusive of both *JAK2* and *MPL* mutations. The two most common mutations found comprised a 52 base pair deletion (type 1) or the 5 base pair insertion (type 2) in exon 9 of *CALR*. All mutations resulted in the same one base pair frame shift with the production of an abnormal protein with a novel C terminus. The *CALR* mutation also showed cytokine independent growth of cells due to activation of signal transducer and activator of transcription 5 (STAT5) involved with the JAK/STAT pathway but its exact role in MPN remains to be clarified. Patients with the *CALR* mutation showed an improved survival and lower risk of thrombosis compared to patients carrying the JAK2 mutation. Triple negative (*JAK2*–, *MPL*–, *CALR*–) patients make up approximately one third of PMF cases and show an inferior outcome compared to *CALR*+ cases.

Mutations in splice factor genes *U2AF1*, *SRSF2* and splicing factor 3b, subunit 1 (*SF3B1*) are a common feature in PMF and occur during the early stages of the disease (58). These are rarely described in association with *CALR* mutations but are frequently associated with mutations of *JAK2* or *MPL*. *U2AF1* and *SRSF2* are strongly associated with anaemia and thrombocytopaenia.

Inactivating mutations have been detected in genes involved with transcriptional regulation through methylation repression/de repression of genes. These

Table 2 Genes mutated, chromosome location, gene function, mutator type and frequency in MPN

Gene	Chromosome position	Gene function	Mutator type	Frequency (%)
JAK2	9p24	Tyrosine kinase, signalling	Gain of function	PMF (40-50%), PV (95-99%), ET (50-70%) (29,30,32,35)
MPL	1p34	Receptor, signalling	Gain of function	PMF (11%), ET (4%) (36-38)
SH2B3	12q24	Adaptor, signal regulation	Loss of function	PMF (<5%), ET (<5%), *JAK2* mutation-negative erythrocytosis (25%), post-MPN AML (13%) (39)
CBL	11q23	Adaptor, E3 ubiquitin ligase, signal regulation	Dominant negative	PMF (6%), post-MPN AML (20)
NF1	17q11.2	RAS signalling regulation	Loss of function	PMF (0-6%), post ET/PV MF (14%) (25,27)
TET2	4q24	DNA hydroxymethylation	Loss of function	PMF (19%), PV (15%), ET (4-11%), post-MPN AML (26%) (21)
ASXL1	20q11.21	Chromatin modifications	Loss of function	PMF (19-40%), PV and ET (<7%), post-MPN AML (19%) (33)
EZH2	7q35	Chromatin methylation	Loss of function	PMF (13%), PV (3%) (40,41)
IKZF1	7p12	Transcription factor, lymphopoiesis	Dominant negative	post-MPN AML (21%)
RUNX1	21q22.3	Transcription factor, haematopoiesis	Loss of function	post-MPN AML (37%) (37,42)
RB	13q14	Cell cycle, apoptosis	Loss of function	PMF (19%) (27)
TP53	17p13.1	Cell cycle, apoptosis	Loss of function	post-MPN AML (20%) (43)
IDH1	2q33.3	Metabolism	Neomorphic enzyme	PMF (2%), post-MPN AML (8%) (44)
IDH2	15q26.1	Metabolism	Neomorphic enzyme	PMF (2%), post-MPN AML (18%) (44)
DNMT3A	2p23	Chromatin methylation	Loss of function	PMF (10%), AML (4-22%) (45)
SRSF2	17q25.1	Splice factor	Dominant negative	PMF (17%), post MPN (19%) (46)
NFE2	12q13	Transcription factor	Gain of function	PMF (3%) (47)
CALR	19p13.2	Calcium binding chaperone in ER	Loss of function	PMF (86%) ET (67%), MDS (8%) (48,49) (in unmutated JAK2/MPL)
U2AF1	21q22.3	Splice factor	Dominant negative	PMF (16%) (50) MDS (12%), CMML (8%) (51)
SF3B1	2q33.1	Splice factor	Dominant negative	PMF (6%), MDS (75%) (51)

Adapted from Vainchenker *et al*. (34). MPN, myeloproliferative neoplasm; PMF, primary myelofibrosis; PV, polycythaemia vera; ET, essential thrombocythaemia; AML, acute myeloid leukaemia; post-MPN, post myeloproliferative disease; MDS, myelodysplastic syndrome; CBL, Casitas B-lineage lymphoma; NF1, Neurofibromatosis 1; TET2, ten eleven translocation member 2; ASXL1, additional sex combs like 1; EZH2, enhancer of zeste homolog 2; IKZF1, IKAROS zinc finger protein 1; RUNX1, Runt-related transcription factor 1; TP53, tumour protein 53; IDH1/2, isocitrate dehrogenase 1/2; DNMT3A, DNA (cytosine-5-)-methyltransferase 3 alpha; U2AF1, U2 small nuclear RNA auxiliary factor 1.

include *ASXL1*, enhancer of zeste homolog 2 (*EZH2*), DNA (cytosine-5) methyltransferase 3 alpha (*DNMT3A*) and *TET2*. It has been suggested that these mutations may predate the acquisition of the *JAK2* mutation. Low levels of *JAK2* mutation in cases with a high disease load determined by other markers of clonality such as X inactivation or cytogenetic abnormalities is further supporting evidence of a pre *JAK2* mutation clone (34). Alternatively, the *JAK2* mutation may be acquired prior to these epigenetic mutations or may occur concurrently with one or several mutations in PMF as opposed to PV or ET that usually show only one mutation.

In studies of patients with *JAK2* positive MPN half the cases that transformed to blast phase did not retain the *JAK2* mutation after transformation (59). A possible explanation is the presence of two different haematopoietic stem cell clones that subsequently acquired a *JAK2* mutation in one clone and an AML inducing mutation in the other (60,61).

Several mutations have been associated with blast phase MPN that are rare in chronic phase (28). This would provide indicators for an increased risk of disease transformation that may necessitate more aggressive therapy or provide the option of BM transplantation in suitable candidates. Genes in blast phase MPN involve Runt-related transcription factor 1 *(RUNX1*), tumour protein 53 (*TP53*), *IKAROS* zinc finger protein 1 (*IKZF1*), *CBL*, Wilms tumour 1 *(WT1*), Kirsten rat sarcoma viral oncogene (*KRAS*) and *NF1*. In addition, isocitrate dehrogenase 1/2 (*IDH1/2*) are also implicated in disease progression. *SRSF2* mutations appear to be most strongly associated with post MPN AML and show a reduced overall survival. The highest risk mutation profile associated with PMF is *CALR* negative and *ASXL1* positive. The inclusion of the gene mutation profile in the DIPSS+ prognostic model is thus becoming increasingly important to identify risk factors for disease transformation and survival in PMF.

Genetic predisposition to MPN

Several lines of evidence have been put forward to support a hypothesis for the genetic predisposition to the development of MPN. This is validated by the markedly increased risk for MPN in first degree relatives of MPN patients that occurs in 5-10% of MPN as well as the bi clonal nature of the disease in some cases (43). Polymorphic SNP variants have been described in the *JAK2* gene itself. The 46/1 variant (rs1327494) results in a 3 fold increase in the risk of developing *JAK2* positive MPN and a 1.4 fold risk for *MPL* mutations. The SNP rs2853677 A/G transition in the telomerase reverse transcriptase (*TERT)* gene is also a strong contender for disease predisposition to MPN.

Patient therapy

Haematopoietic stem cell transplantation is the only curative therapeutic option currently available for PMF. However, this is a high risk procedure and the mortality associated with HSCT is extremely high in patients above 70 years of age making this an unsuitable option for many patients with PMF (62). The majority of therapeutic regimes in clinical practice have been largely empirically derived and are considered mostly palliative (63).

Since the discovery of the *JAK2* mutation, emphasis has since shifted to the therapeutic targeting of the abnormal clone (64). Several *JAK* inhibitors have undergone clinical trials. Of theses the COMFORT (controlled MF study with oral *JAK2* treatment) 1 and 11 trials using ruxolitinib has shown superior results in patients with intermediate and high risk MF (65). Participants showed a major relief of constitutional symptoms, a marked decrease (35%) in spleen size and an increase in overall survival. Ruxolitinib does not eradicate the malignant clone but provides a superior palliative care option resulting in an increase in quality of life. The main side effects associated with ruxolitinib include anaemia, thrombocytopaenia and neutropaenia. Selection criteria for treatment may thus exclude some patient subsets such as those with significant BM failure or transfusion dependency. In addition, adverse events such as myelosupression, failure to respond and disease transformation have also been described (66). In addition, availability of the drug to patients on a worldwide scale may be limited due to cost. As an alternative approach, the recent application of gene editing using the CRISPR/cas9 system to precisely target the *JAK2V617F* mutation in induced pluripotent stem cells, demonstrated a strategy to replace the mutant *JAK2* with the normal wild type copy by using the double stranded DNA repair mechanism of the cell (67,68). This represents a major step forward in the quest for more effective therapy in PMF with relevant known mutations and highlights the need for comprehensive genetic screening of patients as well as continued research on the causative molecular defects in MPN.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare

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