Mutation Profile of Myelofibrosis

Subjects: Oncology | Hematology

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Myelofibrosis refers to fibrosis in the bone marrow associated with certain bone marrow cancers. It is a characteristic of primary myelofibrosis and may develop later in other bone marrow cancers with overproduction of blood cells, such as polycythemia vera and essential thrombocythemia. It has been confirmed that mutations in three key genes, Janus kinase 2 (*JAK2*), calreticulin (*CALR*), and myeloproliferative leukemia oncogene (*MPL*), can increase the activity of blood-producing cells, make them grow more actively, and are associated with the development of myelofibrosis. Approximately 80% of myelofibrosis cases carry additional mutations that often involve proteins that control how genes are turned on and off. The presence of mutations provides evidence of a cancerous process. The order in which these mutations occur can influence how the disease manifests. Studies have shown that fibrosis is secondary to the cancerous process and is closely linked to abnormal cell growth driven by mutations.

myelofibrosis driver mutations additional mutations myeloid neoplasms JAK2 CALR MPL

1. Introduction

Myeloproliferative neoplasms (MPNs) are a group of myeloid neoplasms characterized by bone marrow hyperplasia and overproduction of at least one lineage of blood cells. The current subclassification of MPNs is based on changes in blood cell counts, and hematopoietic lineages in the bone marrow that display hyperplasia and dysplasia. Primary myelofibrosis (PMF) is a subtype of *BCR::ABL1*-negative classic MPN, which also includes polycythemia vera (PV) and essential thrombocythemia (ET). The proliferation of abnormal megakaryocytes and varying degrees of fibrosis are defining features of PMF. PMF also typically presents with splenomegaly due to granulocytic proliferation and extramedullary hematopoiesis, and many patients show constitutional symptoms of a hypermetabolic state due to changes in inflammatory cytokines. Recent updates of the 5th edition of the World Health Organization (WHO) Classification of Hematolymphoid Tumors (WHO-HAEM5) [1] and the International Consensus Classification (ICC) [2] have further refined PMF into early, prefibrotic, and overt fibrotic stages. Secondary myelofibrosis (SMF) can present in the later stages of other myeloid neoplasms, particularly other MPNs (post-ET and post-PV MF) and myelodysplastic/myeloproliferative neoplasms (MDS/MPN). It is necessary to differentiate between ET and PV with mild MF and prefibrotic PMF [1]. However, post-PV and post-ET SMF [3][4] can be indistinguishable from PMF when no clear clinical history of PV or ET is documented in patients presenting with myelofibrosis (**Figure 1**). PMF and SMF are frequently studied together and are clinically managed similarly. Bone marrow fibrosis can also occur in reactive conditions, such as infections, autoimmune disorders, and other

malignancies. In the published literature, the term MF is usually reserved for bone marrow fibrosis related to myeloid neoplasms; bone marrow fibrosis is a general term used for other secondary fibrosis [5].

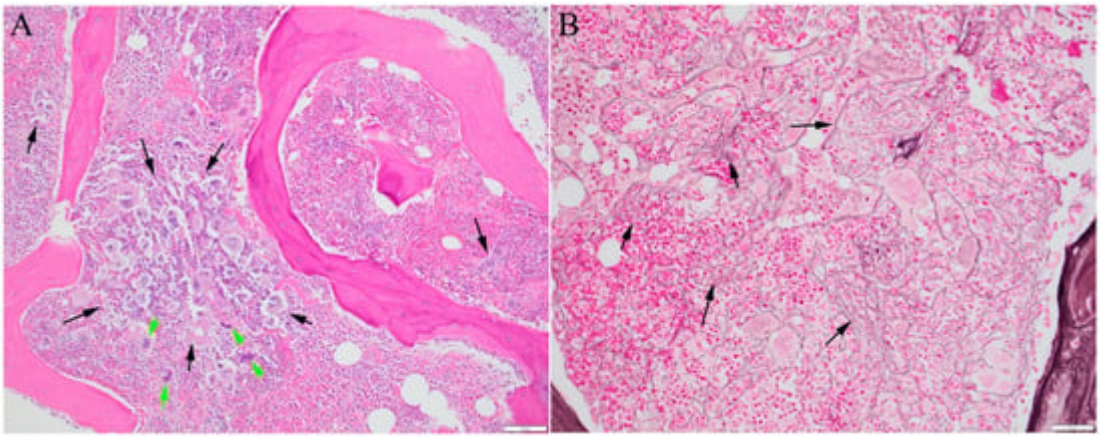

Figure 1. Myelofibrosis (MF, case and images by L.Z.). Bone marrow biopsy images are from a 64-year-old woman diagnosed with essential thrombocythemia (ET) 15 years ago and on intermittent hydroxyurea therapy. (**A**) The hypercellular bone marrow shows frequent atypical megakaryocytes, some displaying hyperchromatic nuclei (green arrows) and forming clusters (black arrows) (H&E stain, 100×, scale: 100 μm). (**B**) Reticulin stain (200×, scale: 50 μm) reveals moderate myelofibrosis (MF grade 2 of 3, representative areas with increased reticulin fiber forming meshwork are indicated by black arrows). Next-generation sequencing of 75 genes associated with myeloid neoplasms revealed *JAK2* V617F at 34.5% and *DNMT3A* R635W at 18.9%. The difference in the variant allele frequency suggests that either the *DNMT3A* mutation is subclonal or the *JAK2* mutation is homozygous. At this stage, the morphologic features and mutation profile of post-ET MF are indistinguishable from those of primary myelofibrosis (PMF).

MF is a distinctive entity among MPNs, signified by a higher risk of transformation to acute myeloid leukemia (AML). Disease progression of MF can also present as refractory cytopenia, progressive leukocytosis, or refractory progression with an increasing fibrotic burden [6]. With the availability of molecular testing, especially next-generation sequencing (NGS) in clinical laboratories, mutational profiling has transformed the diagnostic and classification paradigms for myeloid neoplasms. Detecting the genetic alterations of MF is not only required for diagnosis as clonal evidence but also provides crucial information to help understand its pathobiology in relation to other myeloid neoplasms. Reflecting the expanding utilization of molecular testing and NGS in clinical laboratories, a bibliometric analysis of publications on MPN from 2001 to 2022 indicated that “gene mutations” has been the top keyword for published studies over the past two decades [7]. However, several aspects of MF remain poorly understood, including the biologic and molecular basis of fibrosis as a distinct feature of PMF, potential biologic distinctions between PMF and post-PV or post-ET MF, and differences between proliferative and dysplastic/cytopenic forms of MF.

2. The Driver Mutations

The discovery of recurrent mutations in Janus kinase 2 (*JAK2*), calreticulin (*CALR*), and myeloproliferative leukemia oncogene (*MPL*) as driver mutations has transformed the diagnostic approach of MPN, as evident in the revisions of WHO classifications [1][8][9]. Clonal evidence, supported by the presence of a driver or other mutations commonly associated with various myeloid neoplasms, is crucial for definitive diagnosis. Both *JAK2* and *MPL* encode proteins that activate the JAK/STAT signaling pathway, which is essential for signal transduction from erythropoietin (EPO), thrombopoietin (TPO), and granulocyte colony-stimulating factor (G-CSF) receptors. The pathobiology and diagnostic relevance of activating mutations in *JAK2*, *CALR*, and *MPL* have been extensively investigated in the clinical setting. *JAK2* V617F mutation is associated with an increased risk of thrombosis, and a high allele burden is associated with disease progression [10]. *MPL* encodes the TPO receptor, and mutations, usually at codon W515, lead to constitutively active signaling independent of ligand binding. The interaction between MPL and altered calreticulin encoded by mutant *CALR* results in MPL hyperactivity [11]. *CALR* and *MPL* mutations are typically exclusive to ET and PMF and very rarely occur in PV [10]; however, *JAK2* V617F mutation remains the most common driver mutation in PMF, reported in 50–60% of cases, followed by *CALR* mutations in 25–35% and *MPL* mutations in 5–10% cases [12][13]. Interestingly, *JAK2* exon 12 mutations [14], which are also activating mutations, have not been documented in ET or PMF. All oncogenic *CALR* mutations are frame-shifting insertions or deletions (indels) that alter the C-terminal end of calreticulin from negatively charged acidic amino acids, aspartic acid (D)- and glutamic acid (E)-rich, to positively charged basic amino acids, arginine (R)- and lysine (K)-rich, removing the endoplasmic reticulin retention signal KDEL. Mutant calreticulin can be secreted and functions as a cytokine, retaining its ability to bind to MPL in the *CALR*-mutated clone [15]. In PMF, type 1 *CALR* mutations (51 bp deletion, L367Tfs*46) are approximately three times more prevalent than type 2 (5 bp insertion, K385Nfs*47) [13], with phenotypic variations observed among *CALR* mutation types [16]. In PMF, type 1 *CALR* mutations correlate with lower leukocytosis, lower bone marrow cellularity, and an increased number of megakaryocytes [13], while type 2 mutations align more closely with the phenotype of cases harboring *JAK2* V617F [17].

In the vast majority of MPN cases, driver mutations in *JAK2*, *CALR*, and *MPL* are mutually exclusive. However, there have been occasional reports of cases exhibiting coexistence of *JAK2* V617F, *MPL*, and/or *CALR* mutations [18][19]. Such cases likely involve distinct subclones of neoplastic cells harboring different driver mutations, as demonstrated by a single-cell sequencing study [20], although instances of dual mutations in a single clone have also been documented [21]. Approximately 10% of MPN cases lack detectable canonical mutations in *JAK2*, *CALR*, or *MPL*, categorizing them as triple-negative (TN) MPNs. A small subset of these cases may not truly be TN, as other rare gain-of-function mutations in one of these three genes, particularly *MPL*, have been reported [22][23][24][25][26]. True TN cases often harbor mutations outside of these three genes, confirming clonal hematopoiesis. However, these mutations, which are also prevalent in other myeloid neoplasms, are not considered driver mutations of MPNs. Despite the availability of NGS tests for clinical analysis, the driver mutations of TN cases have not yet been determined, even with comprehensive whole-exome sequencing (WES) studies. One candidate driver, *SH2B3* mutation, has been identified in a subset of TN MPNs [27]. However, *SH2B3* mutations and other driver mutations are not mutually exclusive. The pathogenic drivers of TN MPNs are either heterogeneous non-recurrent mutations, more complicated alterations that evade ready identification by currently available methods, or

with mechanisms not yet recognized. Further exploration to understand the regulatory sequences within the non-coding regions of the human genome may shed light on the drivers and molecular pathogenesis of TN MPNs.

3. Additional Mutations

With the accumulation of mutation profiling data from clinical studies, it is now clear that over 50% patients with MPNs harbor mutations in addition to driver mutations. Among the classic MPNs, PMF has the highest prevalence of additional mutations. With targeted sequencing of myeloid neoplasm-related genes, additional mutations have been reported in approximately 50% of PV and ET cases, and as high as 80% of PMF cases [28][29][30]. PMF also harbors a higher number of mutations than PV or ET [28][29][31] (**Figure 2**). Although additional mutations are not considered driver mutations of MPNs, they help establish the clonal nature of TN patients and have been integrated into the major diagnostic criteria of MPNs [1][2]. A query of the American Association for Cancer Research (AACR) Project GENIE public database in cBioportal [32] found 299 samples from 202 cases documented as PMF (https://genie.cbioportal.org/study?id=6562046bb01fff74fbb6c576 (accessed on 25 November 2023)). In these 299 samples, in addition to *JAK2* (44.8%), *CALR* (14.7%), and *MPL* (9.4%) mutations, the prevalence of other mutations is similar to those reported by other studies [29][31][33][34]. **Table 1** lists the prevalence of relatively frequent non-driver mutations and the most common mutations or mutation types cataloged in the GENIE database. In addition to the mutations detected in sequencing studies, cytogenetic abnormalities have been reported in 30–57% of PMF cases. However, none of the abnormal karyotypes are specific to PMF [35].

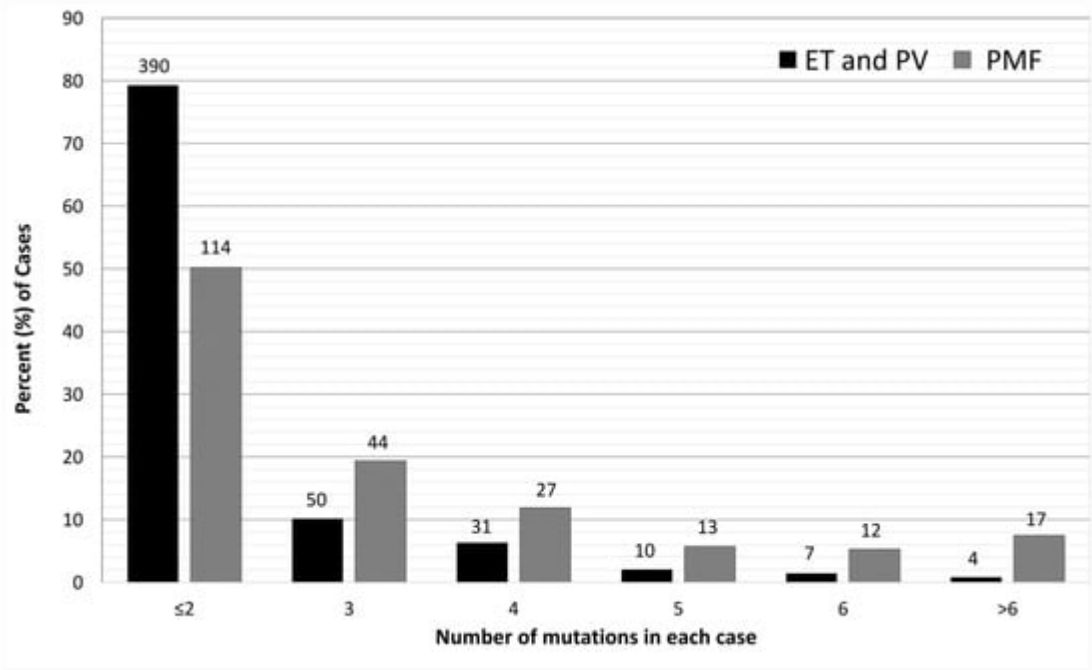

Figure 2. Number of mutations in each sample, essential thrombocythemia and polycythemia vera (ET and PV) versus primary myelofibrosis (PMF). Data source: The AACR GENIE public database [32] (see text for the link to the dataset). ET and PV: 492 samples; PMF: 227 samples. The bar height is displayed as the percentage of samples in each category (Y-axis), and the absolute number of samples in each category is displayed on top of the bar.

There is a significantly higher percentage of PMF cases harboring >2 mutations compared with ET and PV cases (49.78% vs. 20.73%, $p < 0.00001$ by Fisher exact test).

The spectrum of additional mutations detected in PMF did not differ from that detected in PV or ET. However, mutations in genes involved in chromosome modification (*ASXL1* and *EZH2*), DNA methylation (*DNMT3A*), and RNA splicing (*SRSF2*, *ZRSR2*, and *U2AF2*) were more frequently observed in PMF [36][37]. Follow-up studies have shown that most somatic mutations in MPN are present at diagnosis, instead of developing during disease progression [38][39]. The mutation profiles were similar in PMF and SMF. *ASXL1* mutation has the highest prevalence, close to 50% in PMF and 30–40% in SMF in some studies [40][41]. Yan et al. studied 258 consecutive PMF patients with 275 samples by sequencing 27 genes, with 17 patients tested on at least two time points, and found that the variant allele frequency (VAF) of *ASXL1* mutations was relatively stable during the disease process [42]. Luque et al. reported that *ZRSR2* and *NFE2* mutations were more common in SMF [41].

Table 1. Non-driver mutations in primary myelofibrosis.

Gene	Mutation Prevalence (%)	Most Frequent Mutations #	More Frequent in PMF Than Other MPN [34][43]	Clinical Relevance
Epigenetic Regulation (Chromosome Modification and DNA Methylation)				
ASXL1	21	Truncation; E635Rfs	Yes	HMR Prevalence increases with age
DNMT3A	12	R882H/C	Yes	
EZH2	4	Truncation and splice	Yes	HMR
IDH1/2	2	*IDH1* R132C/H, *IDH2* R140Q/W	Yes	HMR Prevalence higher in other studies
TET2	17	Truncation	No	The order of acquiring mutation affects phenotype
RNA splicing				
SF3B1	4	K666N, K700E	No	Associated with ring sideroblasts
SRSF2	8	P95	Yes	HMR
U2AF1	5	Q157, S34	Yes	HMR
ZRSR2	2	Truncation and splice	Yes	More common in SMF [41]
Signal transduction and transcription factors				

Gene	Mutation Prevalence (%)	Most Frequent Mutations #	More Frequent in PMF Than Other MPN [34][43]	Clinical Relevance
CBL	6	X366_splice, Y371H	No	Present with other additional mutations [44] Predict poor response to JAK inhibitors [45]
CUX1	3	Truncation	Yes	
NFE2	2–5 *	E261fs	No, related to erythroid differentiation [25]	Associated with higher risk of transformation to AML, shorter OS. More common in SMF [41]
NRAS/KRAS	9	G12	Yes	Relatively specific for MF [25][46]
RUNX1	4	Truncation	Yes	Associated with transformation to AML [42]
SH2B3	1	Truncation	No	May be considered a driver, or promoting JAK2 activity
TP53	2	DNA-binding domain mutations	Yes	Relatively uncommon in MPNs. Associated with higher risk of transformation to AML [39]; however, low VAF in subclone may not increase risk [47]

Abbreviations: HMR: High molecular risk (see the prognostic score section below); MPN: myeloproliferative neoplasm; PMF: primary myelofibrosis; SMF: secondary myelofibrosis, including post-PV and post-ET MF [41]; VAF: variant allele frequency. See footnote for a list of abbreviations for the gene names. Data source: AACR GENIE public database (299 samples from 202 patients; at least 200 samples were studied) [48]. More details can be found at: https://genie.cbioportal.org/study?id=6562046bb01fff74fbb6c576 (need login) (accessed on 25 November 2023). #, The mutations are named using a single-letter amino acid code if the most frequent mutations are documented as amino acid changes. * Documented at 0.5% (1/199) in GENIE; prevalence was adjusted based on other studies [49].

MPNs exhibit considerable phenotypic heterogeneity, characterized by variable changes in blood cell counts, presence or absence of dysplastic features and fibrosis, and diverse disease evolution trajectories. However, the biologic ramifications and phenotypic associations with mutation profiles, particularly the impact of additional mutations in individuals with the same driver mutation, remain poorly understood. Studies have indicated that a higher allelic burden of *JAK2* V617F and type 2 *CALR* mutations is correlated with elevated blood cell counts (reviewed by Chifortides et al. [50]). A study by Grinfeld et al. on 2035 MPN patients suggested that genetic mutations and germline polymorphisms contribute, at least partially, to the determination of the phenotype [25]. *JAK2* V617F mutation in the background of *EZH2*-knockout mice resulted in a shift in differentiation toward megakaryopoiesis and development of myelofibrosis at the expense of erythropoiesis [51]. *ASXL1* mutation was

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associated with a unique methylation signature [52]. Among single-cell subclones with different profiles showed unique transcription signatures [53][54]. However, the biological effects of these signatures have yet to be characterized.

A recent study of 216 patients with PMF versus ET/PV found that *KRAS* and *NRAS* mutations were characteristically present in the MF cohort [46], consistent with earlier findings by Grinfeld et al. [25], indicating a strong association between *NRAS* mutations and the MF phenotype. This is also confirmed by a query of the AACR GENIE public database comparing PMF to PV and ET (https://genie.cbioportal.org/study?id=65622d3b01ff74fab6c5cc (accessed on 25 November 2023)). *KRAS/NRAS* mutations were found in 17 of 202 (8.4%) PMF patients; however, only 3 of 547 (0.55%) PV and ET patients harbored subclonal *KRAS/NRAS* mutations at low VAFs (<10%). *SRSF2* P95H mutation unexpectedly delayed *JAK2* V617F-associated MF in mouse study [55]. These results highlight the potential influence of additional mutations on the phenotype of MPN and development of MF. Notably, the presence of a clone harboring a *JAK2* mutation independent of those harboring non-driver mutations is not uncommon, and blast transformation of *JAK2*-mutated MPN has been documented in *JAK2* wild-type cells [56]. While *TP53* mutations are relatively uncommon in MPN and are recognized as a late event [57], a recent study of 349 patients with MF undergoing hematopoietic stem cell transplantation (HSCT) revealed a significantly higher prevalence of *TP53* mutations at 13% [58]. Multiple subclones carrying different *TP53* mutations can coexist within a single patient, further demonstrating a complicated mutational landscape in the late stage of MF. Other genetic alterations associated with transformation to AML are less investigated. A study on samples from 11 patients with MF that progressed to AML, utilizing gene set enrichment analysis (GSEA), reported that samples progressed to AML had increased *E2F* transcription factors. Moreover, in blast phase MPN samples, microRNA *MIR29B1* was upregulated compared to de novo AML [59].

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