Mutation profile in BCR-ABL1-negative myeloproliferative neoplasms: A single-center experience from India

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Abstract

Objective/Background—Recurrent somatic mutations in the JAK2, calreticulin (CALR), and the MPL genes are described as drivers of BCR-ABL1-negative myeloproliferative neoplasms (MPN) that includes polycythemia vera (PV), essential thrombocytosis (ET), primary myelofibrosis (PMF), and MPN unclassified (MPN-U).

Methods—We describe the mutation profile and clinical features of MPN cases diagnosed at a tertiary care center. JAK2V617F and MPL (S505/W515) mutations were screened by allele-specific polymerase chain reaction, while CALR exon 9 and JAK2 exon 12 mutations were screened by fragment analysis/Sanger sequencing. Among the 1,570 patients tested for these mutations during the study period, 407 were classified as MPN with a diagnosis of PV, ET, PMF, and MPN-U seen in 30%, 17%, 36%, and 17%, respectively, screened.

Results—Similar to previous reports from Asian countries, the incidence of PMF was the highest among the classic MPN. JAK2V617F mutation was detected in 90% of PV, 38% of ET, 48% of PMF, and 65% of MPN-U. JAK2 exon 12 mutations were seen in 5.7% of PV and 1.4% of PMF. CALR exon 9 mutations were seen in 33% of ET, 33% of PMF, and 12% of MPN-U. MPL mutations were detected in 2.8%, 2.7%, and 2.9% of ET, PMF, and MPN-U, respectively. Some 15% of PMF, 26% of ET, and 22% of MPN-U were triple negative.

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Author contributions
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Declaration of Competing Interest
The authors declare that they have no competing interest.

Ethical approval
The study was approved by the institutional research board (IRB Min No: 11,800 dated January 30, 2019). All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research and ethics committee. The study and the laboratory tests have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).
Conclusion—There was a significantly higher incidence of CALR mutation in PMF and ET cases. Our study highlights the challenges in the diagnosis of JAK2-negative PV and the need for harmonization of criteria for the same.

Keywords

CALR; India; JAK2; MPL; Myeloproliferative neoplasm; Mutations

Introduction

The classical myeloproliferative neoplasms (MPNs), also called the BCR-ABL1-negative MPNs, are a group of related clonal hematologic disorders characterized by excess accumulation of one or more myeloid cell lineages. Polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF) are categorized as classic MPN [1]. They are clinically characterized by nonspecific symptoms such as fatigability, pruritus, early satiety due to splenomegaly, increased risk of infections, and thrombotic events [2]. Disease morbidity is associated with thromboembolic and hemorrhagic events. The complication of the disease by transformation to myelofibrosis is seen to a greater extent with PV than ET and to secondary AML with PMF than with PV or ET [2,3].

In the vast majority of MPN cases, a mutation in codon 617 of JAK2, resulting in the replacement of the amino acid valine with phenylalanine [V617F, JAK2 NM_004972.3: c.1849G >T (p.Val617Phe)] is found. This gain of function mutation is present in approximately 96% of PV, 55% of ET, and 65% of PMF [4]. JAK2 exon 12 mutations are described in 5% of cases of PV [4] and in PMF [5]. Point mutations in codon 515 of the thrombopoietin receptor gene (MPL) have been reported in 5—10% of cases of JAK2-negative ET and PMF. Mutations in the calreticulin (CALR) gene have been reported in ~20—25% of ET and PMF [6].

Dysregulated JAK2 signaling is described as the central phenotypic driver of BCR-ABL1—negative MPNs. Further more, MPNs exhibit unexpected levels of genetic complexity, with multiple abnormalities associated with disease progression, interactions between hereditary factors and driver mutations, and effects related to the order in which the mutations are acquired during the disease process [1,6,7].

Although morphology and clinical laboratory analysis continue to play an important role in defining these conditions, emphasis on molecular testing in MPN is made by the World Health Organization (WHO) by including JAK2, MPL, and CALR mutations as one of the diagnostic criteria in the 2016 update on the classification of myeloid neoplasms [8]. Genomic analysis is being increasingly recognized to be of significance in prognostication in addition to diagnosis.

Most of the available data [9-11] regarding the incidence, prevalence, distribution of subtypes, molecular patterns, and natural history come from the developed world. There is limited data from low- and middle-income countries where challenges remain in diagnosing these conditions. The population pyramid is skewed to a younger age; therapeutic interventions are inadequate and often delayed. The spectrum and profile of classical MPNs
presenting to a tertiary center in such low- and middle-income settings are likely to be significantly different than is conventionally expected [9]. There is a paucity of large data sets describing the mutation spectrum of MPN from tertiary care centers in India. Here, we describe the pattern of mutations and clinical features in patients with MPN at our center.

Patients and methods

Patients

All patients tested in our laboratory for JAK2 V617F, JAK2 exon 12, CALR, and MPL hotspot mutations (MPN panel) between January 2016 and March 2020 were included in this study. Clinical and laboratory characteristics at diagnosis or referral were documented by reviewing the patients’ electronic records. A multidisciplinary team reviewed the available clinical, laboratory, and histopathological information to make the diagnosis, and they were subsequently classified as MPN based on the WHO 2016 criteria. Cases that did not meet these criteria and those with inadequate data were excluded from further analysis. Chronic neutrophilic leukemia, chronic eosinophilic leukemia, and myelodysplastic syndrome/MPN were also excluded from this analysis. Stringent criteria (British Society for Haematology [BSH] 2019) [12] were applied to classify cases of JAK2-negative PV (Supplementary Table S1).

Screening of JAK2, CALR, and MPL mutations

DNA was extracted from whole blood by Gentra Puregene, blood DNA kit (Qiagen, Hilden, Germany). DNA quality and quantity of these samples were assessed by Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). JAK2 V617F, CALR, MPL, and JAK2 exon 12 mutations were screened in all the samples that were referred for MPN panel analysis. JAK2 V617F mutation was screened by an allele-specific polymerase chain reaction assay (assay sensitivity 1%), as reported previously [4]. JAK2 exon12 mutations was screened by Sanger sequencing, while mutations in CALR exon 9 were screened using capillary electrophoresis-based fragment length analysis as reported previously [4,13]. The type of CALR mutation was identified by Sanger sequencing. Four common mutations in MPL were screened using an allele-specific oligonucleotide polymerase chain reaction assay as reported previously [14].

Statistical analysis

The chi-square test and Mann—Whitney U-test were used to compare variables. For all the tests, a two-sided p value < 0.05 was considered statistically significant. The analysis was done using IBM SPSS statistics version 24.0 software (SPSS, Chicago, IL, USA).

Results

During the study period, 1570 samples were screened for MPN panel (JAK2V617F, JAK2 exon 12, MPL, and CALR mutations) with a suspected diagnosis of MPN (Fig. 1). Of these, 407 cases fulfilled the WHO 2016 criteria for MPN and were further analyzed. The demographics of the patients are listed in Supplementary Table S2. The median age of this cohort was 50 years (range: 1—80 years) with a male predominance (M:F = 2.1:1). All
MPN cases were subcategorized further into PV (n = 123), ET (n = 69), PMF (n = 146), and MPN unclassified (MPN-U) (n = 69). Laboratory features of individual subtypes are listed (Supplementary Table S2). JAK2, MPL, and CALR mutations were mutually exclusive of each other.

**Mutation spectrum in PV**

Among the 123 patients with PV, 118 were JAK2 mutation positive (V617F in 94% [n = 111] and exon 12 mutations 6% [n = 7]) (Fig. 2A). Of the seven patients with JAK2 exon 12 mutations, one was a missense mutation and six were indels. These patients presented with absolute erythrocytosis, which is characteristic of JAK2 exon 12 mutations. However, bone marrow morphology was similar to that of JAK2V617F-mutated PV with no isolated erythroid hyperplasia. Thrombotic events such as thrombosis of the portal vein, central vein, superior mesenteric vein, deep veins of the lower limbs and cardiovascular events (STEMI/ischemia) were recorded in 19.5% (24 of the 123 cases), while splenomegaly was seen in 34% (42 of the 123) cases.

**Mutation spectrum in ET**

The median age at diagnosis of the patients classified as having ET (n = 69) was 36 years (range: 1—78). Mutations identified in the driver genes in this group were: JAK2V617F (37.6%), CALR (33.3%), and the MPL (2.8%) (Fig. 2B). Among the JAK2 wt/MPL wt CALR-mutated cases, type 2 was more common (52%; n = 12), followed by type 1 (44%; n = 10) and a 34 base pair deletion was classified as type 1 like (c.1092_1125del; p.E364Dfs*55) (4%) (Table 1). No mutation in any of the driver genes was identified in 26% (n = 18) cases.

Comparison of demographics in patients with ET having different mutations (Table 2) showed a significantly higher platelet count and a lower total leucocyte count in the CALR-mutated ET than the JAK2-mutated ET. Patients who were categorized as triple-negative ET were significantly younger with lower hemoglobin and higher platelet counts when compared with patients with ET having a mutation in any of the three driver genes.

Thrombotic events were recorded in 13% (n = 9) among the 69 cases of ET in this study. These included occlusions in the retinal artery, the coronary artery leading to ischemia, thrombosis of major cerebral vessels, deep vein thrombosis, and one case of extrahepatic portal vein obstruction. Splenomegaly was seen in 17.3% (n = 12).

**Mutation spectrum in PMF**

Among the 146 cases classified as PMF, JAK2V617F, CALR, and the MPL mutations were seen in 48.3%, 32.9%, and 2.7% of them, respectively (Fig. 2C). Of the JAK2 wt/MPL wt cases, six different types of CALR mutations were identified. The majority of these were type 1, (75%; n = 36), 17% (n = 8) were type 2, 6% (n = 3) were type 1-like, and one was classified as other type (2%) (Table 1). A comparison of demographics in patients with PMF having different mutations (Table 2) showed that patients with CALR-mutated PMF had higher platelet counts than JAK2-mutated PMF. Patients who had no mutation in any of the
driver genes were younger and had lower platelet counts and LDH values when compared with PMF with mutation in any of the driver genes.

A \textit{CALR} mutation was detected in one case (c.1099_1152delinsAG; p.L367Rfs*46) which was novel in terms of a genomic change where there was an indel (complex deletion insertion) involving an insertion of two nucleotides AG in the alternate allele along with a 54 base pair deletion. This molecular event, however, resulted in a protein change similar to the type 1 deletion except the first amino acid change is arginine instead of threonine. Hence, this was classified as type 1-like based on the predicted effect on three different stretches of negatively charged amino acids of the wild-type \textit{CALR} sequence (Fig. 3). Tryptophan was replaced by leucine at codon 515 (c.1544G > T; p.W515L) in 75\% (n = 3) of the MPL-mutated cases and in one case the substituted amino acid was lysine (MPL:p.W515K). No mutation in any of the driver genes was identified in 15\% (n = 22) of cases.

Thrombotic events were recorded in only five patients with PMF. Splenomegaly ranging from tip palpable to massive splenomegaly (2—28 cm) was observed in 111 of 146 cases. Two cases underwent a splenectomy at the time of diagnosis.

\textbf{MPN-U}

Patients with overlapping features that could not be further categorized as any particular classical MPN (n = 69) were placed under the MPN-U entity. \textit{JAK2}V617F mutations were identified in 65\% (n = 44), \textit{CALR} mutations in 11.6\% (n = 8) (seven with type 1 and one with type 2), and \textit{MPL} mutation in 2.9\% (n = 2) (p.W515K) of these patients (Fig. 2D). No mutations in any of the driver genes were identified in 21.7\% (n = 15) of these cases. Both arterial and venous thrombotic events were recorded in 24.6\% (n = 17) cases. Splenomegaly was observed in 49\% (34/69) cases.

\textbf{Discussion}

The discovery of mutations in the three driver genes (\textit{JAK2}, \textit{CALR}, and \textit{MPL}) has significantly contributed to the understanding of the classical \textit{BCR-ABL1}-negative MPN. The introduction of mutations in one of the driver genes as a major WHO criterion highlights the importance of testing for these markers in the diagnosis, classification, and prognosis of various MPN subtypes. Our study is one of the largest single-center studies on MPN from India, where we report the proportion of MPN patients with PV, ET, PMF, and MPN-U of 30.2\%, 17\%, 35.8\%, and 17\%, respectively. When compared with the registry data from other Asian countries, the frequency of PMF was higher and ET was lower in our study group [9].

\textit{JAK2}V617F mutation accounts for \textasciitilde95\% of PV and mutation in exon 12 contributes to a further 2—3\% of these cases. Classification of cases presenting with persistent and significant erythrocytosis which are \textit{JAK2}-negative is difficult and are classified as ‘\textit{JAK2}-negative PV’ only after excluding all possible causes of secondary erythrocytosis. Stringent criteria for this entity, as laid out by the BSH 2019 guidelines, aid in differentiating this rare entity from idiopathic and secondary erythrocytosis. In this study, \textit{JAK2}V617F accounted for 90.3\% and exon 12 mutations, 5.7\% of PV.
Confirming the diagnosis of JAK2-negative PV has been a challenge. Although 52 cases in this study were categorized as JAK2-negative PV based on the WHO 2016 criteria for PV, only five of these cases met the BSH 2019 criteria. The WHO criteria for the diagnosis of PV are less stringent and lack a separate criterion for labeling JAK2-negative PV, while the BSH criteria are more stringent in terms of different hematocrit requirements and other additional criteria like splenomegaly and WBC counts. The frequency of JAK2V617F mutation in the present study in PV cases is less than in published studies [15-17]. Further research focusing on the identification of a possible genetic etiology for these JAK2-negative PV cases is warranted.

In this study group, the proportion of MPN patients with ET is less when compared with registry data from other Asian countries [9]. Comparison with previous studies showed that the mutation profile of ET cases in our study was different. JAK2-mutated ET cases were fewer, while CALR-mutated ET and triple-negative cases were higher (Table 3). CALR-mutated ET cases were younger, had significantly less hemoglobin, and greater platelet counts compared with the JAK2-mutated ET cases (Table 2). Triplenegative ET cases presented at a younger age with a lower hemoglobin and a higher platelet count than JAK2-mutated ET cases. This observation is in concordance with a previous report [18]. A lower overall median age of the ET patients in this study can be explained by the skewing of data due to a larger number of triple-negative ET cases who were younger at presentation. The spectrum of mutations in the driver genes in patients with PMF was different in our study. In comparison to published data, though the frequency of JAK2V617F-mutated cases was comparable (~50%), detection of JAK2 exon 12 mutations adds to the varied molecular spectrum of this group. CALR-mutated cases were higher while the frequencies of MPL-mutated PMF and triple-negative cases were comparable. CALR-mutated PMF cases were significantly younger and had significantly higher platelet counts than those with JAK2-mutated PMF (Table 2). However, there was no significant difference in hemoglobin and total leukocyte count as described in previous studies [19-21]. Among the CALR-mutated MPN, type 1 CALR mutation (52 bp deletion) was more common in PMF than in ET (75% vs. 44%) and type 2 CALR was more common in ET than PMF (52% vs. 17%) in our study. This is in concordance with previously published data [21,22].

In our study, 78.2% of the patients categorized as MPN-U carried a mutation in one of the three driver genes, with JAK2V617F being the most frequently represented (65%) followed by CALR mutations (11.2%). This is comparable to a previous study on MPN-U [23]. A more complete molecular characterization of these cases would not only help in identifying the driver mutations, but also in understanding whether or not these cases represent only a prodromal or advanced phase of the classic BCR-ABL1-negative MPNs.

Previous study using the whole-exome sequencing approach on cases of triple-negative ET and PMF has identified noncanonical mutations in JAK2 and MPL genes [24]. A possible hereditary thrombocytosis, owing to the predominant younger age at presentation (median age 18 years for triple-negative ET) in this group cannot be excluded.

Our study provides a large data set from India on the mutation spectrum and demographics of MPN. Future directions of this study are on the role of next generation sequencing in
cases with features of MPNs where no mutation was identified in any of the driver genes (triple negative) in establishing clonality or excluding reactive conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

[1]. Zoi K, Cross N. Genomics of myeloproliferative neoplasms. J Clin Oncol. 2017; 35: 947–54. [PubMed: 28297629]
[2]. Iurlo A, Cattaneo D, Gianelli U. Blast transformation in myeloproliferative neoplasms: risk factors, biological findings, and targeted therapeutic Options. Int J Mol Sci. 2019; 20: 1839.
[3]. Szuber N, Mudireddy M, Nicolosi M, Penna D, Vallapureddy RR, Lasho TL, et al. 3023 Mayo clinic patients with myeloproliferative neoplasms: risk-stratified comparison of survival and outcomes data among disease subgroups. Mayo Clin Proc. 2019; 94: 599–610. [PubMed: 30824279]
[4]. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005; 365: 1054–61. [PubMed: 15781101]
[5]. Maddali M, Kulkarni UP, Ravindra N, Jajodia E, Arunachalam AK, Suresh H, et al. JAK2 exon 12 mutations in cases with JAK2V617F-negative polycythemia vera and primary myelofibrosis. Ann Hematol. 2020; 99: 983–9. [PubMed: 32277273]
[6]. Tefferi A. Myeloproliferative neoplasms: A decade of discoveries and treatment advances. Am J Hematol. 2016; 91: 50–8. [PubMed: 26492355]
[7]. Grinfeld J, Nangalia J, Baxter EJ, Wedge DC, Angelopoulos N, Cantrill R, et al. Classification and personalized prognosis in myeloproliferative neoplasms. N Engl J Med. 2018; 379: 1416–30. [PubMed: 30304655]
[8]. Swerdlow, SH, Campo, E, Harris, NL, Jaffe, ES, Pileri, SA, Stein, H, Thiele, J, editors. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th. IARC; Lyon: 2017. Revised
[9]. Yassin MA, Taher A, Mathews V, Hou H-A, Shamsi T, Tuğlular TF, et al. MERGE: A multinational, multicenter observational registry for myeloproliferative neoplasms in Asia, including Middle East, Turkey, and Algeria. Cancer Med. 2020; 9: 4512–26. [PubMed: 32351024]
[10]. Mesa R, Miller C, Thyne C, Mangan J, Goldberger S, Fazal S, et al. Myeloproliferative neoplasms (MPNs) have a significant impact on patients’ overall health and productivity: the MPN Landmark survey. BMC Cancer. 2016; 16: 167. [PubMed: 26922064]
[11]. Harrison CN, Koschmieder S, Foltz L, Guglielmelli P, Flindt T, Koehler M, et al. The impact of myeloproliferative neoplasms (MPNs) on patient quality of life and productivity: results from the international MPN Landmark survey. Ann Hematol. 2017; 96: 1653–65. [PubMed: 28780729]
[12]. McMullin MF, Harrison CN, Ali S, Cargo C, Chen F, Ewing J, et al. A guideline for the diagnosis and management of polycythaemia vera. A British Society for Haematology Guideline. Br J Haematol. 2019; 184: 176–91. [PubMed: 30478826]
[13]. Klampfl T, Gisslinger H, Harutyunyan A, Nivarthi H, Rumi E, Milosevic J, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med. 2013; 369: 2379–90. [PubMed: 24325356]

[14]. Arunachalam AK, Suresh H, Mathews V, Balasubramanian P. Allele specific PCR: a cost effective screening method for MPL mutations in myeloproliferative neoplasms. Indian J Hematol Blood Transfus. 2018; 34: 765–7. [PubMed: 30369762]

[15]. Passamonti F, Rumi E, Pietra D, Elena C, Boveri E, Arcaini L, et al. A prospective study of 338 patients with polycythemia vera: the impact of JAK2 (V617F) allele burden and leukocytosis on fibrotic or leukemic disease transformation and vascular complications. Leukemia. 2010; 24: 1574–9. [PubMed: 20631743]

[16]. Tefferi A, Pardanani A. Myeloproliferative neoplasms: a contemporary review. JAMA Oncol. 2015; 1: 97–105. [PubMed: 26182311]

[17]. Nangalia J, Green AR. Myeloproliferative neoplasms: from origins to outcomes. Blood. 2017; 130: 2475–83. [PubMed: 29212804]

[18]. Tefferi A, Wassie EA, Lasho TL, Finke C, Belachew AA, Ketterling RP, et al. Calreticulin mutations and long-term survival in essential thrombocythemia. Leukemia. 2014; 28: 2300–3. [PubMed: 24791854]

[19]. Tefferi A, Lasho TL, Finke CM, Knudson RA, Ketterling R, Hansson CH, et al. CALR vs JAK2 vs MPL-mutated or triple-negative myelofibrosis: clinical, cytogenetic and molecular comparisons. Leukemia. 2014; 28: 1472–7. [PubMed: 24402162]

[20]. Pietra D, Rumi E, Ferretti VV, Buduo CAD, Milanesi C, Cavalloni C, et al. Differential clinical effects of different mutation subtypes in CALR -mutant myeloproliferative neoplasms. Leukemia. 2016; 30: 431–8. [PubMed: 26449662]

[21]. Tefferi A, Lasho TL, Finke C, Belachew AA, Wassie EA, Ketterling RP, et al. Type 1 vs type 2 calreticulin mutations in primary myelofibrosis: differences in phenotype and prognostic impact. Leukemia. 2014; 28: 1568–70. [PubMed: 24569778]

[22]. Tefferi A, Wassie EA, Guglielmelli P, Gangat N, Belachew AA, Lasho TL, et al. Type 1 versus Type 2 calreticulin mutations in essential thrombocythemia: a collaborative study of 1027 patients. Am J Hematol. 2014; 89: E121–4. [PubMed: 24753125]

[23]. Gianelli U, Cattaneo D, Bossi A, Cortinovis I, Boiocchi L, Liu Y-C, et al. The myeloproliferative neoplasms, unclassifiable: clinical and pathological considerations. Mod Pathol. 2017; 30: 169–79. [PubMed: 27739437]

[24]. Milosevic Feenstra JD, Nivarthi H, Gisslinger H, Leroy E, Rumi E, Chachoua I, et al. Whole-exome sequencing identifies novel MPL and JAK2 mutations in triple-negative myeloproliferative neoplasms. Blood. 2016; 127: 325–32. [PubMed: 26423830]

[25]. Kim SY, Im K, Park SN, Kwon J, Kim J-A, Lee DS. CALR, JAK2, and MPL mutation profiles in patients with four different subtypes of myeloproliferative neoplasms. Am J Clin Pathol. 2015; 143: 635–44. [PubMed: 25873496]

[26]. Pardanani A, Lasho TL, Finke C, Hansson CA, Tefferi A. Prevalence and clinicopathologic correlates of JAK2 exon 12 mutations in JAK2 V617F-negative polycythemia vera. Leukemia. 2007; 21: 1960–3. [PubMed: 17597810]

[27]. Ancochea À, Alvarez-Larrán A, Morales-Indiano C, García-Pallarols F, Martínez-Avilés L, Angona A, et al. The role of serum erythropoietin level and jak2 v617f allele burden in the diagnosis of polycythemia vera. Br J Haematol. 2014; 167: 411–7. [PubMed: 25040297]

[28]. Rabade N, Subramanian PG, Kodgule R, Raval G, Joshi S, Chaudhary S, et al. Molecular genetics of BCR-ABL1 negative myeloproliferative neoplasms in India. Indian J Pathol Microbiol. 2018; 61: 209. [PubMed: 29676359]

[29]. Rotunno G, Mannarelli C, Guglielmelli P, Pacilli A, Pancrazzi A, Pieri L, et al. Impact of calreticulin mutations on clinical and hematological phenotype and outcome in essential thrombo-cythemia. Blood. 2014; 123: 1552–5. [PubMed: 24371211]

[30]. Sazawal S, Singh N, Mahapatra M, Saxena R. Calreticulin mutation profile in Indian patients with primary myelofibrosis. Hematology. 2015; 20: 567–70. [PubMed: 25959795]
Fig. 1.
Representation of MPN categorization among the cases analyzed. ET = essential thrombocytosis; MPN = myeloproliferative neoplasm; MPN-U = myeloproliferative neoplasm unclassified; PMF = primary myelofibrosis; PV = polycythemia vera.
Fig. 2. Mutation profile of polycythemia vera.
(A) Essential thrombocytosis; (B) primary myelofibrosis; (C) MPN-U; (D) in the present study. Note. CALR = calreticulin; MPL = ; MPN-U = myeloproliferative neoplasm unclassified.
Fig. 3. Alignment of C-domain in wild-type and mutant calreticulin (CALR) proteins.
The reference amino acid sequence starts from codon A361: acidic, basic, and neutral residues are in red, blue, and green, respectively. All the variants involved three different stretches of negatively charged amino acids, here defined as I, II, and III, and highlighted in red in the wild-type sequence. Type 1-like mutations predict deletion of stretches II and III (as happens with the L367fs*46 or type 1 mutation), while type 2-like mutations predict conservation of all three stretches (as happens with the K385fs*47 or type 2 mutation); other types involve deletion of stretches III exclusively.
Table 1
Spectrum of Calreticulin (CALR) mutations identified in PMF, ET, and MPN-U in our study.

| Mutation (CDS); amino acid<sup>a</sup> | Type of mutation | COSMIC mutation ID | Number of cases |
|----------------------------------------|------------------|--------------------|-----------------|
|                                        |                  | PMF (N = 48), % (n) | ET (N = 23), % (n) | MPN-U (N = 8), % (n) |
| c.1099_1150del; p.L367Tfs*46           | Type 1; 52 bp deletion | COSMI 738055       | 75 (36)         | 44 (10)         | 87.5 (7) |
| c.1154_1155insTTGTC; p.K355Nfs*47      | Type 2; 5 bp insertion | COSMI 738056       | 17 (8)          | 52 (12)         | 12.5 (1) |
| c.1097_1130del; p.R366Kfs*53           | Type 1-like; 34 bp deletion | COSMI 738357       | 6 (3)           | -               | -       |
| c.1100_1145del; p.L367Qfs*48           | Type 1-like; 46 bp deletion | COSMI 738150       | -               | -               | -       |
| c.1099_1152delinsAG; p.L367Rfs*46      | Type 1-like Novel | Novel              |                 |                 |         |
| c.1120_1125delinsTGCGT; p.K374Cfs*56   | Other             | COSMI3355758       | 2 (1)           | -               | -       |
| c.1092_1125del; p.E364Dfs*55           | Type 1-like; 34 bp deletion | COSMI 738333       | -               | 4(1)            | -       |

Note: ET = essential thrombocytosis; MPN-U = myeloproliferative neoplasm unclassified; PMF = primary myelofibrosis.

<sup>a</sup> Transcript ID: CALR-201 ENST00000316448.10; RefSeq: NM_004343.4 for all the mutations in the CALR gene described in this study.
## Table 2
Laboratory characteristics of essential thrombocytosis (n = 69) and primary myelofibrosis (n = 146) patients according to mutation profiles in this study.

|                  | N (%) | Age (yr) | M:F (% males) | Hemoglobin (g/dL) | Hematocrit | Total WBC count (x10⁹/L) | Platelet count (x10⁹/L) | LDH (U/L) |
|------------------|-------|----------|----------------|-------------------|------------|--------------------------|------------------------|-----------|
| **ET (mutation-positive)** |       |          |                |                   |            |                          |                        |           |
| ET (mutation-positive) JAK2 | 51 (74) | 42 (12-78) | 24:27 (47) | 12.9 (8.9-16) | 39.6 (27.5-47.5) | 10.2 (5.2-55.2) | 875 (462-2,368) | 622 (338-3820) |
| ET (mutation-positive) CALR | 26 (37.6) | 46 (12-78) | 11:15 (42.3) | 12.8 (10.1-15.7) | 39.8 (31.3-47.5) | 11.3 (5.2-36.3) | 779 (462-1,856) | 601.5 (401-3820) |
| ET (mutation-positive) MPL | 23 (33.3) | 37 (24-65) | 12:11 (52) | 12.9 (8.9-16) | 39.2 (27.5-46.4) | 9.6 (5.8-55) | 1,045 (657-2,368) | 629 (423-1589) |
| **PMF (mutation-positive)** |       |          |                |                   |            |                          |                        |           |
| PMF (mutation-positive) JAK2 | 124 (85) | 54 (31-80) | 87:38 (69.6) | 9.6 (3.7-15.8) | 29.9 (11.6-54.2) | 8.7 (1.8-197.4) | 243 (7-1664) | 1,162 (403-3,896) |
| PMF (mutation-positive) CALR | 48 (32.8) | 55 (36-80) | 47:33 (67) | 9.85 (3.8-15.8) | 31.3 (11.6-54.2) | 11.5 (1.8-152.5) | 211.5 (7-1664) | 1,144 (403-3,687) |
| PMF (mutation-positive) MPL | 4 (2.7) | 61 (51-74) | 36:12 (75) | 10.1 (3.7-14.7) | 29.9 (11.7-44.4) | 7.85 (2.5-197.4) | 295.5 (9-1565) | 1,235 (562-3,896) |
| **Triple-negative** |       |          |                |                   |            |                          |                        |           |
| Triple-negative ET | 18 (26) | 18 (1-60) | 7:11 (38.8) | 11.9 (9.5-14.3) | 35.6 (29.5-43.5) | 10.2 (4.4-15.4) | 1,190 (622-2,708) | 618 (338-1,117) |
| (Mutated vs. triple-negative) p value * | - | 0.002 | 0.54 | 0.001 | 0.0002 | 0.58 | 0.02 | 0.8 |
| (JAK2V617F vs. CALR) p value * | - | 0.42 | 0.48 | 0.91 | 0.25 | 0.02 | 0.01 | 0.56 |
| (CALR vs. triple-negative) p value * | - | 0.001 | 0.54 | 0.01 | 0.009 | 0.09 | 0.32 | 0.8 |
| (JAK2 vs. triple-negative) p value * | - | 0.001 | 0.82 | 0.004 | 0.0001 | 0.36 | 0.004 | 0.98 |
| **PMF (mutation-negative)** |       |          |                |                   |            |                          |                        |           |
| PMF (mutation-negative) JAK2 | 70 + 2b (47.9 + 1.4) | 55 (36-80) | 47:33 (67) | 9.85 (3.8-15.8) | 31.3 (11.6-54.2) | 11.5 (1.8-152.5) | 211.5 (7-1664) | 1,144 (403-3,687) |
| PMF (mutation-negative) CALR | 48 (32.8) | 50 (31-76) | 36:12 (75) | 10.1 (3.7-14.7) | 29.9 (11.7-44.4) | 7.85 (2.5-197.4) | 295.5 (9-1565) | 1,235 (562-3,896) |
| PMF (mutation-negative) MPL | 4 (2.7) | 61 (51-74) | 36:12 (75) | 10.1 (3.7-14.7) | 29.9 (11.7-44.4) | 7.85 (2.5-197.4) | 295.5 (9-1565) | 1,235 (562-3,896) |
| **Triple-negative PMF** |       |          |                |                   |            |                          |                        |           |
| Triple-negative PMF | 22 (15) | 42 (14-64) | 16.6 (72.7) | 8.4 (5.9-14.7) | 27.9 (18.3-42.5) | 7 (1.3-66.5) | 87.5 (7-1371) | 723.5 (241-1803) |
| (Mutated vs. triple-negative) p value * | - | 0.002 | 0.76 | 0.07 | 0.17 | 0.41 | 0.02 | 0.001 |
| (JAK2V617F vs. CALR) p value * | - | 0.013 | 0.84 | 0.81 | 0.66 | 0.07 | 0.01 | 0.24 |
| (CALR vs. triple-negative) p value * | - | 0.026 | 0.83 | 0.95 | 0.94 | 0.42 | 0.68 | 0.002 |
| (JAK2 vs. triple-negative) p value * | - | <0.00001 | 0.62 | 0.09 | 0.17 | 0.16 | 0.1 | 0.006 |

Note. CALR = calreticulin; ET = essential thrombocytosis; LDH = lactate dehydrogenase; MPL = ; PMF = primary myelofibrosis; WBC = white blood cell.

* All p values were calculated using the \( \chi^2 \) test for categorical variables and the Mann-Whitney U test for continuous variables in patients with ET and PMF with mutations in either of the driver genes and JAK2-mutated ET or PMF versus CALR-mutated ET or PMF. Significant values are in boldface.
### Table 3
Comparison of frequencies of driver mutations in myeloproliferative neoplasms.

|       | Median age (range), yr | JAK2 V617F (%) | JAK2 Exon 12 (%) | JAK2-negative (%) | CALR (%) | MPL (%) | Triple-negative (%) |
|-------|------------------------|----------------|------------------|-------------------|----------|---------|-------------------|
| **PV**  | Kim et al. (N = 58) [25] | 57.4 (25.8-75.9) | 87.9 | 3.5 | 8.6 | - | - | - |
|       | Pardanani et al. (N = 220) [26] | - | 97 | 2 | 1 | - | - | - |
|       | Passamonti et al. (N = 338) [15] | - | 95 | 4 | 1 | - | - | - |
|       | Ancochea et al. (N = 99) [27] | 69 (20-94) | 94 | 3 | 3 | - | - | - |
|       | Rabade et al. (N = 20) [28] | 50 (31-83) | 100 | - | - | - | - | - |
|       | Present study (N = 123) | 51 (21-75) | 90.3 | 5.7 | 4 | - | - | - |
| **ET**  | Kim et al. (N = 79) [25] | 55.0 (19-84) | 63.3 | - | - | 17.7 | 2.5 | 16.5 |
|       | Tefferi et al. (N = 299) [18] | 56 (15-91) | 53 | - | - | 32 | 3 | 12 |
|       | Rotunno et al. (N = 576) [29] | 58.1 (13-93) | 64.1 | - | - | 15.5 | 4.3 | 16.1 |
|       | Rabade et al. (N = 34) [28] | 46 (21-76) | 61.7 | - | - | 15.1 | 9.1 | 15.2 |
|       | Present study (N = 69) | 36 (1-78) | 37.8 | - | - | 33.3 | 2.9 | 26 |
| **PMF** | Kim et al. (N = 54) [25] | 62 (8-83) | 57.4 | - | - | 14.8 | 9.3 | 20.4 |
|       | Tefferi et al. (N = 254) [19] | 64 (32-87) | 58 | - | - | 25 | 8.3 | 8.7 |
|       | Sazawal et al. (N = 80) [30] | 48 (32-63) | 56.2 | - | - | 11.2 | 0 | 32.6 |
|       | Rabade et al. (N = 59) [28] | 53 (16-81) | 57.6 | - | - | 23.7 | 3.4 | 15.3 |
|       | Present study (N = 146) | 52 (14-80) | 48 | 1.4 | - | 32.9 | 2.7 | 15 |

**Note:** CALR = calreticulin; ET = essential thrombocytosis; MPL = ; PMF = primary myelofibrosis; PV = polycythemia vera.