The Potential Role of CAL2 and EZH2 Immunohistochemical Stainings as Diagnostic and Prognostic Surrogate Markers in Classical BCR/ABL1-negative Myeloproliferative Neoplasms

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ABSTRACT

Classical BCR/ABL-1-negative myeloproliferative neoplasms (MPN) are hematopoietic stem cell disorders characterized by clonal proliferation of more than one mature myeloid cell lineage. The most common etiology is the constitutive activation of the JAK/STAT signalling pathway, caused by three main driver mutations: JAK2V617F, CALR, and MPL. In this study, we analyzed the distribution of these driver mutations in our series of classical BCR/AB1-negative MPN patients and their correlation with clinical symptoms, bone marrow, and laboratory findings. We also explored the efficacy of using the CAL2 antibody to identify CALR mutations and studied the potential of using EZH2 antibody expression levels as a prognostic indicator. Our study of 78 BCR/ABL1-negative MPN patients found JAK2V617 mutation in 57.7%, CALR mutation in 11.5%, and MPL mutation in 1.3% of cases. Thrombosis was the most common initial symptom, observed in 25% of patients, predominantly in those with JAK2V617F mutation (p= 0.02). CAL2 was positive in nearly all megakaryocytes of CALR mutant cases (7/9). EZH2 H-scores in megakaryocytes were lower in patients with a higher reticulin fibrosis score (p= 0.013), and thrombotic events were more frequently observed in these patients (p= 0.081). Our findings suggest that CAL2 and EZH2 immunohistochemical staining have potential as diagnostic and prognostic surrogate markers for MPN. Nevertheless, presently, the most crucial components in the diagnosis and prognosis of MPN are comprehensive molecular profiling and its alignment with other diagnostic tools.

Keywords: Myeloproliferative neoplasms, EZH2, CAL2, Immunohistochemistry

INTRODUCTION

Classical BCR/ABL-1-negative myeloproliferative neoplasms (MPN), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are hematopoietic stem cell disorders characterized by clonal proliferation of more than one mature myeloid cell lineage. The most common aetiology is the constitutive activation of the JAK/STAT signalling pathway. The principal and mutually exclusive driver mutations responsible for this activation in MPN are JAK2, MPL and CALR mutations. Exon 14 JAK2 V617F mutation is detected in 95% of patients with PV and present in approximately 50-60% of ET and PMF.[1,2] Less frequently (2–3%), JAK2 exon 12 mutations can also be found in patients with PV.[3] CALR and MPL are mutated in most of the remaining patients with ET and PMF. CALR mutations are located on exon 9 and have two types (type 1 and type 2). They are present in 20-25% of ET and 25-30% of PMF patients.[4,5] MPL mutations located on chromosome 1p34 are present in 1–3% of ET cases and 5% of MF. The two most frequent types of MPL mutation are W515L and W515K, which occur within exon 10.[6] While “triple negative” patients make up a small part of ET and PMF cases.[7]

World Health Organisation (WHO) 2016 classification included MPL and CALR gene mutations in the diagnostic algorithms in addition to JAK2, which was initially defined as a diagnostic criterion in the WHO 2008 classification.[8,9] According to recent classifications of both WHO (2022)[10] and the International Classification Consensus (ICC)[11], diagnostic criteria mostly remain the same for this group of MPN. Along with being a major criterion of diagnosis, the presence of these driver mutations also carries an important role in determining the prognosis. For example, while the presence of JAK2V617F mutation and high variant allele frequency of this mutation is generally a high-risk factor[12], CALR mutations usually refer to a lower risk of thrombosis.[7] In routine practice, these mutations are detected with Polymerase Chain Reaction (PCR) based molecular techniques from peripheral blood. However, recent studies show that CALR mutation can also be demonstrated accurately by immunohistochemical staining.[13] Although some cases were diagnosed and followed up with only clinical/laboratory and molecular testing, evaluating bone marrow morphology in the definitive differential diagnosis of cases is still necessary, especially when all three mutations are negative (triple negative).

Meanwhile, with evolving New Generation Sequencing (NGS) techniques and more common utilization of gene panels for myeloid neoplasms in daily practice, there is an increasing emphasis on non-driver somatic mutations associated with epigenetic regulation, messenger RNA splicing, transcriptional mechanism, signal transduction, and DNA repair mechanisms. The 2022 WHO classification emphasizes the presence of these additional mutations (ASXL1, EZH2, IDH1/2, SRSF2 and U2AF1), which are associated with poor outcomes and call them "High-Risk Mutations". Among these genes, Enhancer of Zeste Homolog 2 (EZH2), which is also in a close relationship with ASXL1, is a catalytic component of the polycomb repressive complex 2 (PRC2) and takes part in the regulation of chromatin. EZH2 mutations can frequently effect patients with myelodysplastic/myeloproliferative neoplasms (MDS/MPN), myelodysplastic syndrome (MDS), and myelofibrosis.[14] In PMF patients, EZH2 mutations are independently associated with shorter survival.[15] Studies show that immunohistochemical loss of expression of EZH2 protein correlates with mutational status and, as a consequence, with prognosis in myeloid neoplasias.[15-17] However, in classical MPN patients, immunohistochemical evaluation of EZH2 has yet to be studied.

Even in light of the accumulated data, diagnosis and subclassification of these diseases can still be challenging in daily routine. As this disease group is heterogeneous and actually represents a continuum, the boundaries between entities are thin. Hence, diagnostic algorithms that are updated over time are being used for daily practice for the integration of clinical, laboratory and bone marrow findings with molecular reflex testing.[18]

Herein, we describe the distribution of three major driver mutations (JAK2V617F, CALR, MPL) with PCR-based molecular reflex tests from peripheral blood and the correlation of these mutations with clinical symptoms, bone marrow and laboratory findings in our series of classical BCR/AB1-negative MPN patients as a tertiary centre. In addition to our retrospective analysis, we also explored the efficacy of using the CAL2 antibody to identify CALR mutations and studied the potential of using EZH2 protein expression levels as a prognostic indicator by examining correlations with clinical, laboratory and bone marrow data.

PATIENTS AND METHODS

Patients

A total number of 78 patients who were diagnosed and followed up as classical BCR/ABL1-negative MPN by the Hematology Department of our institution between 2016 and 2019 with an initial bone marrow biopsy in the Pathology department were included in this study.

Clinical and Laboratory Findings

Clinical and laboratory findings at diagnosis were documented by reviewing our institution's electronic records. Constitutional symptoms, thromboembolic and hemorrhagic events and the presence of splenomegaly are noted as initial clinical findings. Haemoglobin (Hb), leukocyte and platelet

levels, along with Lactate Dehydrogenase (LDH) levels, were included in laboratory findings.

Bone Marrow Biopsy Findings

Bone marrow trephine biopsy and aspiration slides were retrieved from the archive and reviewed retrospectively. Cellularity, megakaryocyte size, morphology, distribution and reticulin stains were noted. Defined dysplasia criteria for megakaryocytes (nuclear lobulation, nuclear shape, hyperchromasia, decreased nucleocytoplasmic ratio, maturation defects) were considered when evaluating the morphology and were graded as “mild”, “moderate”, or “prominent” according to severity. While evaluating the size of megakaryocytes, the presence of different sizes or dominancy of large forms was taken into account. The aggregates of more than five megakaryocytes without any other hematopoietic element in between were considered dense aggregations. Semiquantitative bone marrow myelofibrosis (MF) scoring was taken into consideration in reticulin fibrosis scoring.[19]

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections were stained in the automated immunostainer in the pathology laboratory with CAL2 antibody (clone CAL2, catalogue DIA-CAL250; Dianova, Germany) at a dilution of 1:100 and EZH2 (D2C9 XP RB mAB 5246; Cell Signaling) at a dilution of 1:250 in the Leica Band Max automatic immunostainer, keeping in accordance with the steps mentioned as per the protocol. Colon, tonsil, and lymph node tissues were used as positive controls.

The investigator was blinded to the CALR mutation status when examining the CAL2 stained slides. Positive immunohistochemical staining of CAL2 was defined by the presence of any intensity of cytoplasmic staining of megakaryocytes.

Nuclear EZH2 staining was scored by using a semiquantitative approach. The percentage of positive megakaryocytes and the other hematopoietic cells were separately scored as 0 (no staining, 0%), 1 (1–25%), 2 (25-75%) and 3 (75-100%). The intensity of staining was scored qualitatively from 0 to 3. A multiplicative staining score (H-score) was obtained by multiplying the percentage of positive cells and staining intensity, giving a range of 0–9. The mutational status of EZH2 was unknown during the evaluation of immunohistochemical staining, as molecular studies were not performed.

Molecular Tests

Results of PCR-based reflex molecular testing performed in our Basic Oncology laboratory from peripheral blood samples of patients were noted from the hospital’s electronic record system. In our institution’s daily practice, JAK2V617F mutation analysis is performed as the initial step in patients with a suspicion of BCR/ABL-1 negative MPN. In cases where the JAK2V617F mutation can not be detected, CALR and MPL mutations are investigated as the second step. Mutational analysis for the EZH2 gene was not studied.

Statistical Analysis

All statistical analyses were performed by using The Statistical Package for the Social Sciences [SPSS] version 21.0 for Windows (IBM Corp.; Armonk, NY, USA). Chi-square test, T-test, ANOVA, Kruskall Wallis and Mann Whitney U tests were employed. Two-sided p-values of less than 0.05 were considered statistically significant.

The study was performed in agreement with the clinical standards laid down in the 1975 Declaration of Helsinki and its revision in 2004 and was approved by the Research Ethics Committee of Hacettepe University. This study is funded by Hacettepe University Scientific Research Projects Coordination Unit.

RESULTS

A total of 78 patients diagnosed with a BCR/ABL1-negative MPN diagnosis who had bone marrow trephine biopsies were included in the study. One of the cases did not have any electronic record of laboratory findings, except molecular study reports and initial clinical symptoms, and in 5 cases, we did not have any information about initial clinical symptoms. There were 31 male and 47 female patients, aged between 5-90 years.

Table 1. Demographic information and distribution of laboratory findings according to mutational status

Mutational status	Demographics: Total number of cases	Demographics: Female/ Male ratio	Demographics: Median age (years)	Laboratory Findings: Mean Hb level (gr/dL)	Laboratory Findings: Mean platelet level (x10³/µL)	Laboratory Findings: Mean leukocyte level (x10³/µL)	Laboratory Findings: Mean LDH level (U/L)
JAK2V617F mutant	45 (57.7%)	31/16	59	13.5	596.9	13.4	320.9
CALR mutant	9 (11.5%)	3/6	46	14	615.6	9.3	272.8
MPL mutant	1 (1.3%)	1/0	56	9	556	5.3	170
Triple-negative	7 (9%)	2/5	46	13.5	1041.8	11	356.4
Incomplete profile	16 (20.5%)	10/6	44.5	12.9	825.8	12	405.7

Molecular test results showed 45/78 (57.7%) patients with JAK2V617 mutation, 9/78 (11.5%) CALR and a single patient (1.3%) with an MPL mutation. Patients, where none of these mutations were detected with reflex tests [7/78 (9%)], were considered as “triple negative”. In 16/78 (20.5%) patients, further molecular profiling studies were not performed, although the first-line JAK2V617F mutation was negative, so they were accepted as an “incomplete profile”. Out of 9 CALR mutant patients, 6 were type 1, and one harboured type 2 mutation, while 2 of them were not subtyped.

The JAK2V617F mutation was related to older age (p= 0.024) and was more common in females (p= 0.069) compared to other mutational profiles. No statistically significant difference in laboratory values (LDH, Hb or platelet levels) was found between mutational profiles. Although not statistically significant, mean platelet levels were higher in CALR mutant (615.6 x $10^3/\mu$L) and incomplete profile (825.8 x $10^3/\mu$L) groups. There was only one case with MPL mutation, who presented with mild thrombocytosis (556x$10^3/\mu$L). Demographics and laboratory findings according to the type of mutation are described in Table 1.

Out of 72 patients in which we could reach initial clinical findings, 39 were asymptomatic, and 33 had one or more symptoms. Symptomatic patients most frequently (57.8%) harboured JAK2V617F mutation among all profiles (p= 0.003) and vice versa JAK2V617F mutant cases, with available clinical data, 26/41 (63.4%) were mostly symptomatic (p= 0.01). In general, the most common initial symptom was thrombosis [18/72 (25%)]. Among the thrombotic cases (16/18), w/wo any other symptom, the most common mutational status was JAK2V617F mutation (p= 0.02) compared to other mutational profiles. Four of these thrombotic cases also had constitutional symptoms, and one had a hemorrhagic event in addition. Similarly, although not statistically significant, cases with constitutional symptoms (14/18), w/wo any other symptom, were mostly JAK2V617F mutant. One of the cases with thrombosis harboured CALR

Table 2. Distribution of clinical findings according to mutational status

	Clinical findings: Asymptomatic-	Clinical findings: Constitutional symptoms (CS)	Clinical findings: Thrombotic event (TE)	Clinical findings: Hemorraghic event (HE)	Clinical findings: CS+TE	Clinical findings: TE+HE	Clinical findings: N/A*	Splenomegaly**
JAK2V617Fmutant (n=45)	15	10	12	0	3	1	4	16/45
CALR mutant (n=9)	6	2	1	0	0	0	0	3/9
MPLmutant (n=1)	0	0	0	1	0	0	0	N/A*
Triple negative (n=7)	5	2	0	0	0	0	0	2/7
Incomplete profile (n=16)	13	0	1	0	0	0	2	5/16

** N/A: No data about the clinical presentation was available in the electronic record system*

*** One of the patients was splenectomized, and 31 of the patients did not have records about spleen size in the electronic system.*

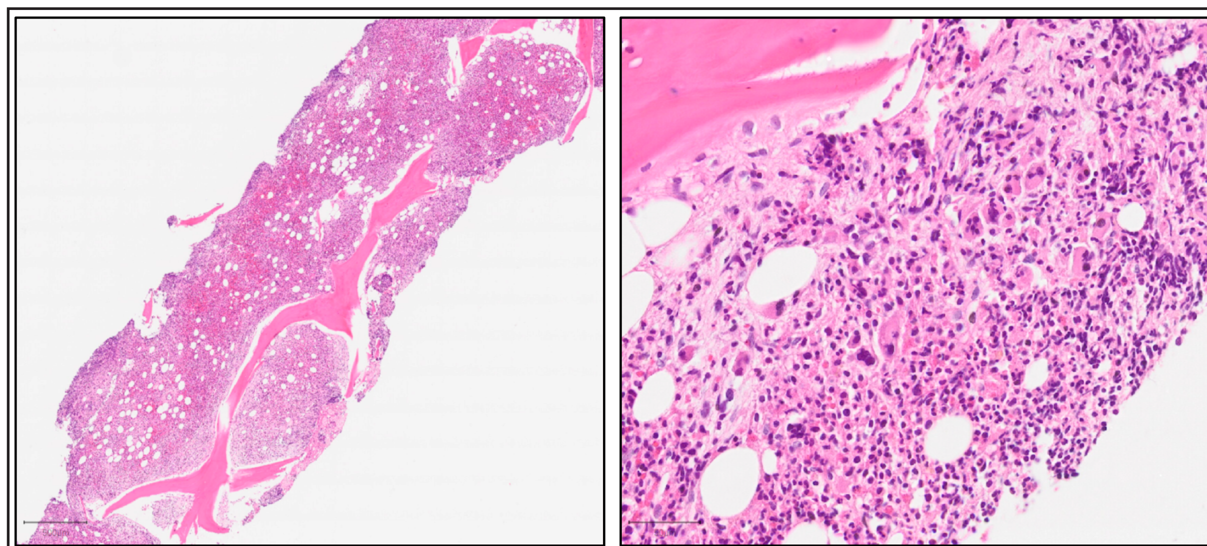

Figure 1: A hypercellular bone marrow of a 61-year-old patient with JAK2V617F mutation. Megakaryocytes show various sizes.

type-1 mutation, and the other was a JAK2V617F negative but "incomplete profile" case with negative CAL2 immunostaining. While most of the cases with CALR mutation were asymptomatic (6/9), the single MPL mutant case presented with a hemorrhagic event (Table 2). There was no statistically significant difference between molecular groups regarding the hemorrhagic events or the presence of splenomegaly.

All thrombotic patients had hypercellular bone marrows (14/18) (p= 0.07), and megakaryocytes showed paratrabecular localization (12/18) (p= 0.2). JAK2V617F mutant cases had significantly (p= 0.001) hypercellular bone marrows (Figure 1) and predominantly had megakaryocyte forms in various sizes (p= 0.023). Meanwhile, CALR mutant cases mostly had either "many large megakaryocytes" (6/9) or "large megakaryocyte dominancy" (1/9). No statistically significant difference in megakaryocyte dysplasia level or reticulin fibrosis between mutational profiles was found (Table 3).

In 2/9 of CALR mutant cases, CAL2 immunostaining was not successful because of technical problems, but in the rest of the cases (7/9), CAL2 was positive in nearly all megakaryocytes (Figure 2). None of the JAK2617F and MPL mutant or triple-negative cases showed positivity with CAL2 stain, while 5/16 (31%) of cases with an incomplete molecular profile showed positive staining. These five patients showed higher mean platelet levels ($1199.8x10^3/\mu L$) compared to other "incomplete profile" group patients ($638.9x10^3/\mu L$), similar to CALR positive profile ($615.6x10^3/\mu L$). However, demographics, other laboratory results, and clinical or bone marrow findings showed no overt difference between the CAL2 positive and negative groups in the "incomplete profile" group.

In immunohistochemical staining of EZH2, H-scores in megakaryocytes were lower in patients with a higher reticulin fibrosis score (p= 0.013) (Figure 3), and also, although not statistically significant (p= 0.081) thrombotic events were more frequently observed in these patients as well. However, EZH2 staining levels did not show any difference between mutational profiles.

Diagnoses based on the pathologic findings in the bone marrow samples, as well as laboratory and clinical findings of patients, were distributed as 18 PV, 37 PMF and 9 ET. Eleven patients could not be definitely subclassified but were decided to be followed as MPN because either molecular findings or laboratory/clinical symptoms were compatible with MPN. Three of the patients did not have bone marrow biopsies, so no definite subclassification according to bone marrow findings could be done. In bone marrow findings, it was only noticeable that PMF patients had higher (2/3) median reticulin fibrosis score than other diagnostic groups (1/3).

Table 3. Bone marrow findings in mutational profiles

	JAK2V617F mutant (n=45)	CALR mutant (n= 9)	MPL mutant (n=1)	Triple-negative (n=7)	Incomplete profile * (n=16)
Histopathology					
Hypercellularity	37 (82%)	3 (30%)	0	2 (%28.5)	4** (30.7%)
Megakaryocyte dysplasia					
mild	9	2	–	–	5
moderate	20	3	1	5/7	5
prominent	15	4	–	2/7	4
N/A*	1	–	–	–	1
Megakaryocytes in various sizes	28	2	1	4/7	7*
Many large megakaryocytes	17	6	–	3/7	7*
Large megakaryocyte forms dominant	–	1	–	–	–
Dense aggregates	32/44*	7	1	5/7	9/14*
Paratrabecular localization	26/44*	6	0	4/7	9/14*
Reticulin fibrosis					
0/3	10	0	0	2	4
1/3	16	5	1	3	4
2/3	13	3	0	1	2
3/3	6	1	0	1	3

** Histopathological evaluation was not possible because of extensive fibrosis or suboptimal biopsy material in some cases*
*** In 3 of the cases, bone marrow cellularity could not be assessed either because of extensive fibrosis or suboptimal biopsy material.*

DISCUSSION

Diagnosis of BCR-ABL1-negative classical MPNs (ET, PV, and PMF) can be challenging due to overlapping morphological and molecular features and complex criteria. Both the World Health Organization and ICC suggest the integration of clinical, laboratory, histopathological, and molecular data for this diagnostic category.[4,5] We examined the general distribution of molecular reflex test results, their relationship with other diagnostic tools, and the possible contributions of CAL2 and EZH2 immunohistochemical stainings in our series of classic MPN patients diagnosed by bone marrow biopsy over a 3-year period.

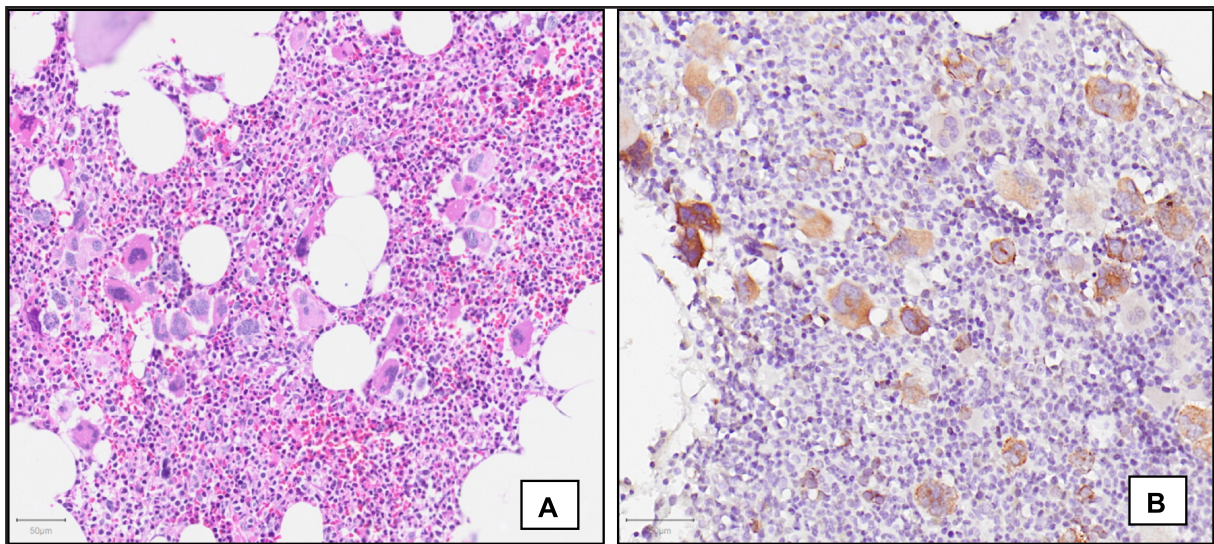

Figure 2. CALR mutant case with increased megakaryocytes and cellularity. CAL2 immunostaining is positive in practically all megakaryocytes. (A, B)

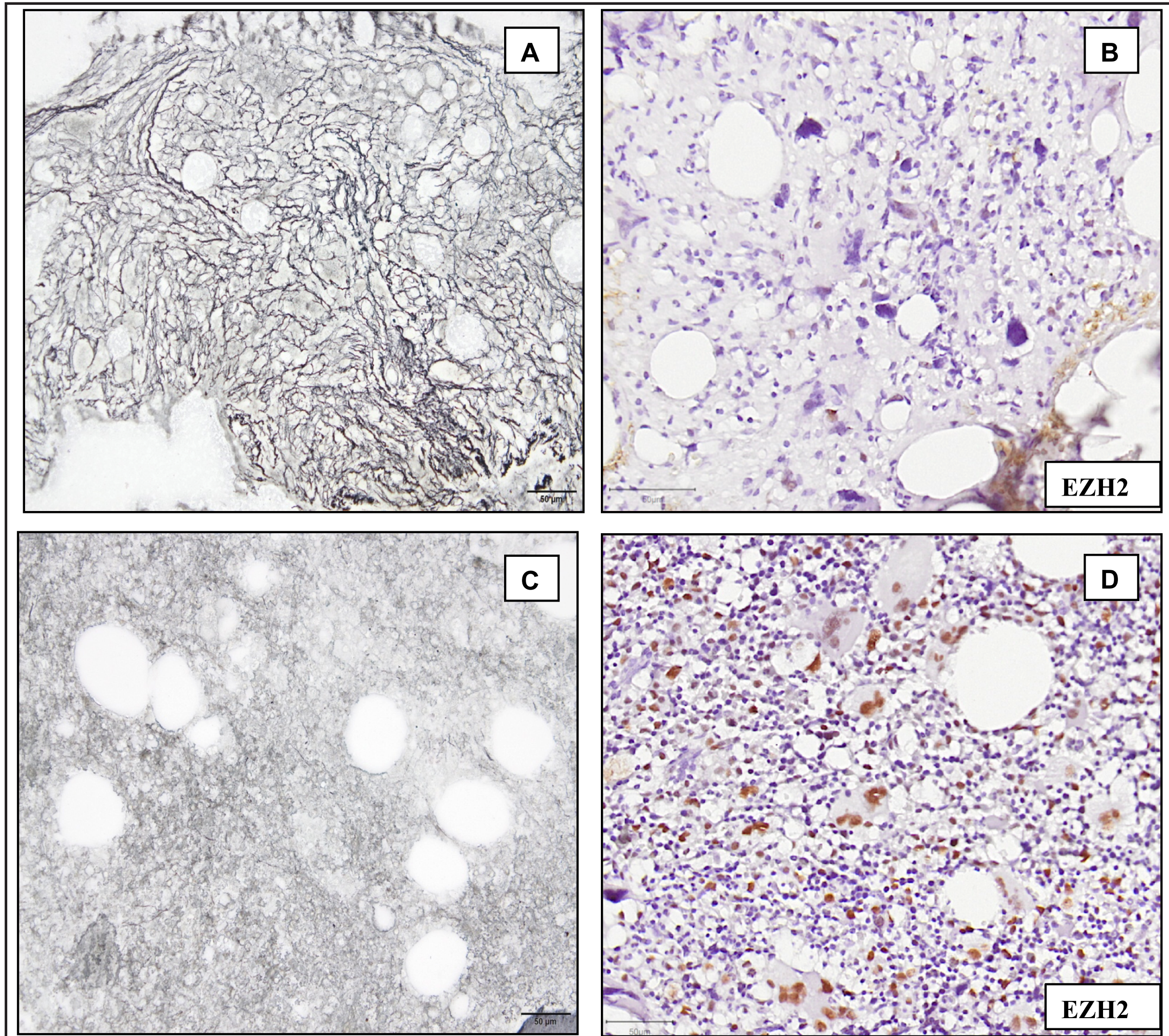

Figure 3. Reticulin stain of a case with increased reticulin fibres showing many crossings, with a fibrosis score of 3/3. (A) While weak staining can be observed in some megakaryocytes and hematopoietic cells, complete loss of staining with EZH2 is observed in many megakaryocytes and marrow cells (B). In a case with a reticulin fibrosis score of 0/3 (C), most of the hematopoietic cells, including megakaryocytes, are positive with EZH2 stain showing no loss of staining. (D).

Up to 85% of MPN patients harbour a canonical driver mutation in one of the JAK2, CALR or MPL genes, and the most common mutation seen is JAK2V617F.[15] Consistent with the literature, the most common mutation in our series was JAK2V617F (57.6%), and this mutation was significantly related to female gender and older age (p= 0.056). CALR mutant cases were relatively low (11.5%), and only one patient had an MPL mutation. The most common reason for this limitation is that tests for CALR or MPL status in JAK2V617F-negative cases sometimes were not performed in our institution and, as a result, were not recorded in our institution's electronic system. Therefore, our "incomplete profile" case group also includes CALR, MPL mutant, or "triple negative" cases with clinical, laboratory, and bone findings compatible with MPN. In fact, there were five (31%) cases with CAL2 positivity in this "incomplete profile" group, suggesting CALR mutation. Five cases that tested positive for CAL2 also showed higher platelet counts compared to other patients with incomplete profiles. Interestingly, the incomplete profile group had similar or slightly higher plate-

Table 4. The distribution of clinical and laboratory findings regarding diagnostic groups

	Asympto-matic	Constitutional sympoms (CS)	Thrombotic event (TE)	Hemorraghic event (HE)	Thrombotic event + (CS) or (HE)	N/A*	Mean Hb level (gr/dL)	Mean platelet level (x10^3/µL)	Mean leuko-cyte level (x10^3/µL)	Mean LDH level (U/L)
PV (n=18)	6	3	4	0	2	3	15.23	623.7	12.3	281
PMF (n=37)	17	7	8	1	2	2	12.7	682.2	12.5	353
ET (n=9)	6	2	0	0	0	1	13.6	819	10.7	531.6
MPN (can not be further subclassified) (n=11)	9	1	1	0	0	0	13.6	805	10	286.5
N/A (no bone marrow biopsy available for classification) (n=3)	1	1	0	0	1	0	10.7	1156	18.3	360

** N/A: No data about the clinical presentation was available in the electronic record system*

let levels than the CALR mutant group. However, as mentioned before, since the incomplete profile group was only evaluated for JAK2V617F mutation, these cases may have an MPL or CALR mutation. Moreover, other genetic alterations related to myeloid neoplasia, such as ASXL1, TET2, etc, may also contribute to the final outcome in clinical and laboratory findings. A more detailed molecular study of these cases may help explaining the difference in platelet counts among these groups.

It is also noteworthy that CAL2 was positive in all -technically evaluable- CALR mutant cases, while no CAL2 staining was observed in any of the cases in the "triple negative," JAK2V617F or MPL mutant groups. Although not yet included in diagnostic algorithms, the CAL2 antibody could be a cost-effective, sensitive, and specific potential method for testing CALR mutations[8] or at least a diagnostic surrogate marker. These limitations exemplify the difficulty of fully implementing the molecular diagnostic algorithm steps in daily practice owing to the disruptions and difficulties in the diagnosis and follow-up processes of this group of chronic patients.

In daily practice, some cases are diagnosed and followed up with only clinical/laboratory findings and molecular testing. However, it still can be necessary to evaluate bone marrow morphology for definitive differential diagnosis, especially in the "triple negative" group.[13] Another benefit of bone marrow biopsy is that it allows further subclassification of MPNs by evaluating megakaryocyte morphology. In our series, we observed that bone marrow histology in the CALR mutant group was generally normocellular and associated with large megakaryocyte morphology, which is suggestive of ET. Meanwhile, the JAK2V617F mutant group had primarily hypercellular bone marrow with megakaryocytes of variable size, but we did not find a statistically significant relationship between JAK2V617F mutation and reticulin fibrosis score. Other bone marrow findings (megakaryocyte dysplasia score, paratrabecular localization or dense aggregation) also showed no difference between molecular groups.

Regarding the diagnostic groups, the majority of patients were asymptomatic. However, most of the thrombotic events (10/25) -with or without other

clinical findings- were observed in PMF patients, which is known to have a worse prognostic course than the other MPNs.

Treatment for classical MPNs is currently mostly focused on reducing the risk of thrombosis as it is the most critical determinant in the course and prognosis of the disease. The most common initial symptom in our series was thrombotic events (25%), and these cases were found to be associated with JAK2V617F mutation, higher platelet levels, bone marrow hypercellularity and paratrabecular localization of megakaryocytes in our patients. Among these findings, the presence and variant allele frequency (VAF) value of JAK2V617F mutation and platelet levels are already included in risk scoring systems of MPNs[16], but only bone marrow finding which is used in risk scoring is reticulin fibrosis. Although we could not find precisely a statistically significant relation between reticulin fibrosis and thrombotic events (p= 0.073) or platelet levels (p= 0.091), we observed that both were increased in cases with higher reticulin fibrosis scores.

While JAK2, CALR, and MPL mutations are considered driver events, mutations in other genes, like TET2, ASXL1, EZH2, and DNMT3A, are found in over half of patients with MPN.[1] These additional mutations are more frequent in PMF compared to PV and ET, and some are known to correlate with a poorer prognosis.[17] Mutation of EZH2 is an independent poor prognostic factor for overall survival[10], and it is found to be mutated in around 10% of MPNs. Studies show that this mutation can be screened by immunohistochemical studies in myelodysplastic syndromes.[11,12] Although, especially in PMF, EZH2 mutations correlate with a higher leukocyte count, blast count, and larger spleen size at diagnosis, we could not detect any statistically significant correlation between EZH2 H-scores and any clinical or laboratory findings. However, remarkably, we found that patients with lower EZH2 H-scores, that is, patients with immunohistochemical EZH2 loss, had significantly higher reticulin fibrosis scores in bone marrow biopsies (p= 0.013). There are studies showing the tumor suppressor role of EZH2 in MPN, and a high number of loss of function EZH2 mutations have been shown to synergize with JAK2V617F in initiating MPN and promoting myelofibrosis.[18,19] Therefore, we think this negative correlation between the EZH2 H-score and the reticulin fibrosis score may indirectly suggest the presence of an EZH2 mutation. Another interesting but not statistically significant finding is the higher incidence of thrombotic events in lower EZH2 H-scores (p= 0.081), which could also be a reflection of poor prognosis in MPN patients. Although we do not know the mutational status of EZH2 in our case group, we think these findings may suggest a loss of function mutation in EZH2; but to further prove this, molecular studies should be performed and correlated with immunohistochemical studies.

The main limitations of our study were the inability to study EZH2 mutational status and the unavailability of clinical, laboratory and molecular data for some patients. Additionally, the inclusion of only one MPL mutant case in the study population may have limited the scope of the investigation.

Conclusion

The landscape of genetic testing is changing rapidly, with broad screening techniques such as large pan-cancer panels, whole-genome sequencing and RNA sequencing beginning to impact routine practice. These newly developing sequencing technologies, investigating all primary driver mutations and other prognostically important mutations in a shorter time and simultaneously with large gene panels, can help overcome the difficulties we face in diagnosis and prediction of prognosis in this heterogeneous group of myeloid neoplasms. As the complex genomic landscape of MPNs is being revealed, along with reducing the thrombosis risk, disease-modifying targeted therapies could be ahead of us as an option in the therapeutic strategies. At this point, immunohistochemical markers may also be cost-effective as diagnostic and/or prognostic surrogate markers in centres that do not have advanced molecular facilities or which have to use them in a limited capacity. Regardless of the method detected, it is undeniable that molecular changes, integrated with clinical, laboratory and bone marrow findings, are now one of the cornerstones in diagnosing, defining the prognosis and the therapeutic management of myeloproliferative neoplasms.

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