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ORIGINAL STUDY

Diagnostic Approach and Risk Stratification of Thrombocytosis: Integrating Morphological, Cytogenetic Features and MPL Gene Mutation

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Abstract

Objectives: **To evaluate the integration of clinical, morphological, cytogenetic features and MPL gene mutation analysis in Egyptian patients with clonal thrombocytosis and correlate these findings to patient's outcome.**

Background: **Thrombocytosis can be caused by either reactive or autonomous processes as a result of genetic alterations that affect signaling pathway through gain−of−function activity of MPL (myeloproliferative leukemia virus), Janus kinase 2 (JAK 2), calreticulin (CALR).**

Methods: **This study was conducted on 60 Egyptian patients having clonal thrombocytosis attending to the clinical pathology department, Ain Shams University Hospitals during the period from August 2020 to December 2021. Complete blood count, LDH level and MPL gene mutation by Real-time PCR were measured.**

Results: **JAK2 gene mutation was more prevalent than MPL gene mutation in our study (40% and 8.3%, respectively). MPL mutation has an aggressive nature than JAK2 mutation as evident by; older age (68.80 ± 2.28 vs. 55.5 ± 8.28 accordingly), more thrombotic manifestation (100% vs. 58.3%), higher platelets count (1221.4 ± 309.70 vs. 879.38 ± 215.98) and poor response to therapy (0% of MPL positive patients achieved complete response to therapy vs. 41.7% for JAK2 positive patients) but with no marked morphological difference regarding megakaryocytes morphology.**

Conclusion: **MPL W515 L/K mutations present in 10.3% in essential thrombocythemia Egyptian patients, included in our current study. MPL gene mutation has a bad prognostic effect. Moreover, the presence of coexisting JAK2 & MPL mutation has a synergistic bad effect to be evaluated in further larger studies.**

Keywords: Calreticulin, Clonal thrombocytosis, Janus kinase 2 gene, Myeloproliferative leukemia virus oncogene, Polymerase chain reaction

1. Introduction

The homeostatic production of platelets is mainly regulated and controlled by thrombopoietin (TPO) and the TPO receptor (MPL)/JAK2 axis [1].

The exact definition of thrombocytosis varies in literatures but is generally considered when platelet counts $\geq 450 \times 10^9$/L, 2016 Revised World Health Organization (WHO) defined. The vast majority (>90%) of cases of thrombocytosis are reactive or secondary in nature [2].

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders characterized by aberrant proliferation and an increased tendency toward leukemic transformation. The genes JAK2, MPL, and CALR are frequently altered in these syndromes, and their mutations are often a strong argument for diagnosis [3].

According to the 2016 Revised, WHO classification of hematologic tumors, the Philadelphia-negative MPNs include polycythemia Vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF) [4,5].

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Our study aimed to evaluate the significance of integrating clinical, morphological, cytogenetic features and MPL gene mutation analysis in patients with clonal thrombocytosis and correlate these finding to patient's outcome.

2. Methods

This cross-sectional study was conducted on 60 Egyptian patients with clonal thrombocytosis; platelet counts $\geq 450 \times 10^9$/L, 2016 Revised WHO defined, attending to the cancer molecular cytogenetic lab, clinical pathology department- Hematology unit, Ain Shams University Hospitals during the period from August 2020 to December 2021.

A written consent was taken from all the participants, and the study was approved from the ethics committee, National Liver Institute- Menoufia University NLI IRB 00003413 FWA0000227. All patients were subjected to the following: Full history taking and thorough clinical examination, complete blood picture with peripheral blood smear examination. Bone marrow aspiration and biopsy were performed whenever available, Fluorescence In Situ Hybridization analysis & assessment of JAK2 mutation analysis were taken from patients' records [6].

Cases of reactive thrombocytosis, as anemia, infection, malignancy, post splenectomy, rheumatologic condition, trauma & reaction to medications, were excluded from our study. Other exclusion criteria were patients <18 years old & BCR/ABL positive myeloproliferative cases.

Currently there are three major risk factors for thrombosis (history of thrombosis, JAK2/MPL mutations, and advanced age), in order to group ET patients into four risk categories: "very low risk" (absence of all three risk factors); "low risk" (presence of JAK2/MPL mutations); "intermediate-risk" (presence of advanced age); and "high-risk" (presence of thrombosis history or presence of both JAK2/MPL mutations and advanced age). The patients in our study were treated with a risk-adapted treatment algorithm [7].

Regarding clinical outcome of our patients, they were categorized according to clinicohematologic response criteria as following [8]:

Platelet's count $\leq 400 \times 10^9$, no disease related symptoms: micro vascular disturbance, pruritus & headache, normal spleen size on imaging & white blood count $\leq 10 \times 10^9$/L were considered as complete response criteria.

In patients who do not fulfill the criteria for complete response, platelet count $\leq 600 \times 10^9$/L, or decrease >50% from baseline, they were considered as having partial response.

While there was not any response that did not satisfy partial or complete response, it was categorized as no response.

Regarding sampling; total volume of seven ml divided into three tubes; two tubes containing 2 ml in EDTA tube for complete blood counting and molecular analysis and the third tube containing 3 ml in plain vacutainer tube for LDH analysis.

Complete blood picture using hydrodynamic focusing and flow cytometry on Sysmex XS- 1800i hematology analyzer, Japan) with peripheral blood smear examination. Bone marrow aspiration and biopsy was performed whenever available. LDH was done by Cobas 6000 analyzer (Roche diagnostics, Rotkreuz, Schweiz).

The Fluorescence In Situ Hybridization using the following panel of chronic myeloproliferative cases including; LSI BCR/ABL ES probe for detection of t (9; 22), LSI 13q14 probe for detection of del 13q14, CEP 8 probe for detection of Trisomy 8, LSI 17p13(p53) for detection of −17, +17 or del 17p13, LSI 5q33 probe for deletion 5q33, CEP 7 for monosomy 7, using probes supplied by Vysis, Abbott, USA [9] and molecular assay for JAK2 V617F, using the TaqMan allelic discrimination kit supplied by Thermo Fisher Scientific, USA, were taken from patients' records.

Regarding DNA extraction; total DNA was extracted from EDTA anticoagulated whole blood using Gene JET Whole Blood Genomic DNA Purification Mini Kit supplied by Thermo Fisher Scientific, USA.

Regarding MPL W515 L/K TaqMan genotyping assay; Genotyping was done using the ABI TaqMan allelic discrimination kit (catalog # NP-412-100) (Scientific and Production Company SYNTOL, RUSSIA).

The PCR reaction was set as the following; DNA amplification was implemented, including10 μL of PCR Reaction Mix 2.5 × (SYNTOL, RUSSIA), 10 μL of 2.5x Diluent, 0.5 μL of Taq DNA polymerase, and 5 μL of template DNA.

The PCR cycling conditions was set as the following; An initial denaturation step at 95 °C for 3 min, followed by 10 cycles of 95 °C for 15 s and 10 cycles 63 °C for 40 s. Then final extension 30 cycles of 95 °C for 15 s and 30 cycles 63 °C for 40 s were carried out. The fluorescence profile of each well was measured by the Real-Time PCR Rotor gene Q System (Qiagen GmbH, Hilden, Germany). Fig. 1 showed the result of the Allelic Discrimination Assay for MPL W515 L/K gene mutation G/T as MPL W515L (G1544T) or W515K (TG1543_1544AA) mutation.

Results were interpretated as following: FAM channel was designed for MPL wild type, HEX channel for MPL mutant L & ROX channel for MPL mutant K type. Negative control had no reaction in any channel. Positive 1 control Wild type had a

reaction in Cycling A Green. Positive 2 control Mutant L type had a reaction in Cycling A Yellow. Positive 3 control Mutant K type had a reaction in Cycling A Orange (Fig. 1).

2.1. Statistical analysis

Statistical computations were analyzed by SPSS-PC software (IBM SSPS 20, Inc., Chicago, USA). Quantitative data was shown as mean, SD, and range and expressed as frequency and percent. Comparisons between groups were made using Student's t-test, and Mann–Whitney test for the quantitative variables, Chi-square (χ^2) test was used for the qualitative variables. Student t-test was used to compare mean and SD of 2 sets of quantitative normally distributed data, while Mann–Whitney test was used when this data is not normally distributed. P-value was considered statistically significant when it is less than 0.05.

3. Results

Forty-two cases were diagnosed as having MPN according to the 2016 Revised, WHO criteria (39 ET, 3 Prefibrotic-PMF). The 18 remaining cases did not display enough criteria for a specific MPN sub-type and were classified as MPN-U.

A total of 24 harbored JAK2 V617F mutation and 5 patients were MPL W515 L/K positive (4 patients with MPL W515L while only one patient with MPL W515K). In our study, JAK2 V617F mutation was associated with MPL exon 10 in three cases (all were MPL W515L) and they were all diagnosed as ET (Table 1).

Regarding clinical outcome of our patients, it was found that 39 (65%) patients had complete response, 18 (30%) patients had partial response and 3 (5%) patients had no response to therapy.

There was a highly statistically significant increase in platelets counts among the JAK2 mutated group compared to non-mutated one (P < 0.001) (Table 2).

Regarding the relation between the laboratory data & MPL gene mutation, there was a statistically significant increase in TLC, Platelets, MPV & LDH in the MPL-mutated group when compared to non-mutated group. There was a statistically significant decrease in Hb level & achievement of therapeutic response in the MPL-mutated group compared to the non-mutated group ($P < 0.001$ & $P = 0.005$, respectively) (Table 2).

It is important to note that 53.8% of patients harboring JAK2 mutation had thrombotic complications and this percent increased to 61.5% when these patients harbor MPL mutation too. We also observed that all MPL positive cases had thrombotic complication (Table 3).

By doing univariate logistic regression analysis, we found that only platelet count and patients who had evidence of genetic mutation of JAK2 and MPL genes (positive for JAK2 and/or MPL mutations) had a significant increased risk for thrombotic complications by 1.004 and 3.84 time respectively (Table 4).

There was a statistically significant increase in LDH level in non-complete response NCR group when compared to complete response CR group (P = 0.026). It is clear to note that no one of MPL positive patients & only 25.6% of JAK2 positive patients achieved complete response, however more than 60% of MPL & JAK2 double negative patients achieved complete response to therapy (Table 5).

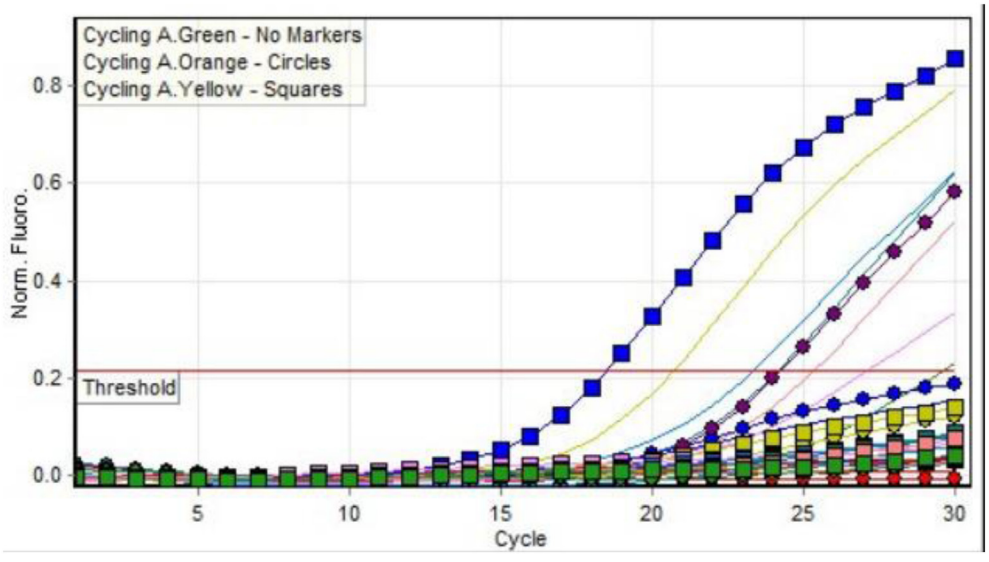

Fig. 1. Allelic discrimination assays for MPL gene.

Table 1. Demographic, clinical, laboratory characteristics and treatment outcome of the studied population.

Demographic and clinical parameters	Cases (n = 60)
Age (years)	
Mean ± SD	54.40 ± 9.88
Median	56.0
Range (min−max)	31.0−72.0
Sex [*n* (%)]	
Male	23 (38.3)
Female	37 (61.7)
Hepatomegaly [*n* (%)]	
No	51 (85.0)
Yes	9 (15.0)
Splenomegaly [*n* (%)]	
No	38 (63.3)
Yes	22 (36.7)
Thrombotic manifestations [*n* (%)]	
Present	**26 (43.3)**
Digital ischemia	9 (34.6)
Transient ischemic attacks	8 (30.7)
Headache	4 (15.4)
Gangrene big toe	2 (7.7)
Hemorrhage	1 (3.8)
Paresthesia	1 (3.8)
Stroke	1 (3.8)
Absent	**34 (56.7)**
Hemoglobin (g/dL)	
Mean ± SD	11.32 ± 1.53
Median	11.10
Range (min−max)	8.10−14.60
TLC (10^3nbsp;cell/μL)	
Mean ± SD	9.75 ± 2.71
Median	9.10
Range (min−max)	5.40−17.00
Platelet's count (10^3nbsp;cell/μL)	
Mean ± SD	737.30 ± 251.97
Median	640.50
Range (min−max)	456.00−1600.00
MPV (fL)	
Mean ± SD	11.27 ± 1.87
Median	11.30
Range (min−max)	7.50−14.20
Morphological findings in the bone marrow examination [*n* (%)]	
Cellularity	
Normocellular	11 (18.3)
Hypercellular	49 (81.7)
Megakaryocytes	
Normal	12 (20)
Increased	48 (80)
Small-large clusters	41 (68.3)
Reticulin fibrosis	
Grade 0-1	3 (5)
LDH (U/L)	
Mean ± SD	587.78 ± 207.54
Median	699.00
Range (min−max)	200.00−913.00
FISH [*n* (%)]	
Normal	56 (93.3)
del 13q	2 (3.3)
Trisomy 8	2 (3.3)
JAK2 [n (%)]	
Negative	36 (60.0)
Positive	24 (40.0)
MPL [*n* (%)]	
Negative	55 (91.7)
Positive MPLW515L	4 (6.7)
Positive MPL W515K	1 (1.6)
JAK2/MPL [*n* (%)]	
JAK2 (−) and MPL (−)	34 (56.7)
JAK2 (+) or MPL (+)	23 (38.3)
JAK2 (+) and MPL (+)	3 (5.0)
Response to therapy [*n* (%)]	
Complete response	39 (65.0)
Partial response	18 (30.0)
No response	3 (5.0)

(*continued on next page*)

Table 1. (continued)

%: percentage within cases population.

4. Discussion

The study included a total of 60 patients, 37 women and 23 men, with a median age at diagnosis of 56 years (range 31−72), which agreed with Accurso et al. [10], who stated that most of clonal thrombocytosis cases were of the elderly with a higher incidence in female with an approximate ratio of 2:1.

In our study, the bone marrow microscopic examination of patients with ET revealed hypercellular bone marrow in more than 80% with clustered enlarged megakaryocytes, most of them showed staghorn megakaryocytes, with matured cytoplasm containing multilobulated nuclei, rather than bizarre highly atypical megakaryocytes, similar to the Quatrain study by Yassin et al. [11].

In the case of our patients with pre-PMF, bone marrow biopsy showed megakaryocytic abnormalities (including extensive clustering, megakaryocytic atypia, and abnormal chromatin clumping with hyper-chromatic nuclei) which are the key findings in diagnosing PMF and its distinctive form from other MPN, including ET, similar finding was reported by Schino et al. [12].

Cytogenetic studies among our 60 patients with clonal thrombocytosis, revealed normal Karyotype in 56 (93.3%). In 2022, the American study done by Gangat et al. [13], found similar distribution of cytogenetic abnormalities.

The JAK2V617F mutation plays a decisive role as a screening tool for the diagnosis of MPN, particularly for PV, additional mutations such as CALR and MPL mutations are also useful to confirm Philadelphia-negative MPN.

In our study, JAK2V617F was more prevalent than MPL mutation in MPN, accounting for 40% of our population.

A total of 24 patients (40%) harbored JAK2 V617F mutation and 5 patients were MPL W515 L/K positive (8.3%) (4 patients with MPL W515L while only

Table 2. Comparison of demographic, clinical, laboratory characteristics and treatment outcome regarding JAK2 and MPL mutations among the studied groups.

Biochemical Parameters	JAK2 mutation		MPL mutation		JAK2 & MPL mutations		*P* value P1:JAK2 + vs. JAK2− P2: MPL + vs. MPL− P3:JAK2 &/or MPL + vs. double JAK2 & MPL -
	JAK2 + (No = 24)	JAK2 − (No = 36)	MPL + (No = 5)	MPL − (No = 55)	JAK2&/or MPL + (No = 26)	Double JAK2& MPL − (No = 34)	
Age (years)							P1 = 0.747 **P2<0.001**[a] P3 = 0.205
Mean ± SD	55.5 ± 8.28	53.6 ± 10.87	68.80 ± 2.28	53.0 ± 9.24	55.88 ± 8.43	52.68 ± 10.39	
Sex [*n* (%)]							P1 = 0.470 P2 = 1.00 P3 = 0.832
Male	9 (37.5)	15 (41.7)	2 (40.0)	22 (40.0)	10 (38.5)	14 (41.2)	
Female	15 (62.5)	21 (58.3)	3 (60.0)	33 (60.0)	16 (61.5)	20 (58.8)	
Thrombotic manifestations [*n* (%)]							P1 = 0.053 **P2<0.001**[a] **P3=0.013**[a]
Present	14 (58.3)	12 (33.3)	5 (100.0)	23 (41.8)	16 (61.5)	10 (29.4)	
Absent	10 (41.7)	24 (66.7)	0 (0.0)	32 (58.2)	10 (38.5)	24 (70.6)	
Hepatomegaly [*n* (%)]							P1 = 0.302 P2 = 0.744 P3 = 1.000
Yes	5 (20.8)	4 (11.1)	1 (20)	8 (14.5)	5 (19.2)	6 (17.6)	
No	19 (79.2)	32 (88.9)	4 (80)	47 (85.5)	21 (80.8)	28 (82.4)	
Splenomegaly [*n* (%)]							P1 = 0.080 **P2=0.036**[a] P3 = 0.944
Yes	12 (50.0)	10 (27.8)	4 (80.0)	18 (32.7)	14 (53.8)	18 (52.9)	
No	12 (50.0)	26 (72.2)	1 (20.0)	37 (67.3)	12 (46.2)	16 (47.1)	
TLC (10^3nbsp;cell/μL)							P1 = 0.123 **P2=0.004**[a] **P3=0.022**[a]
Mean ± SD	10.60 ± 3.47	9.19 ± 1.90	14.0 ± 2.7	9.37 ± 2.37	10.76 ± 3.49	8.99 ± 1.57	
Hb (g/dL)							P1 = 0.169 **P2<0.001**[a] P3 = 0.221
Mean ± SD	11.65 ± 1.61	11.09 ± 1.45	8.44 ± 1.30	11.58 ± 1.3	11.6 ± 1.3	11.2 ± 1.40	
Plat (10^3nbsp;cell/μL)							**P1<0.001**[a] **P2<0.001**[a] **P3<0.001**[a]
Mean ± SD	879.38 ± 215.98	642.58 ± 230.88	1221.4 ± 309.70	693.29 ± 196.45	821.43 ± 257.41	541.00 ± 147.57	
MPV (fL)							P1 = 0.052 P2 = 0.052 **P3=0.028**[a]
Mean ± SD	11.78 ± 1.81	10.92 ± 1.87	12.70 ± 1.03	11.13 ± 1.87	11.46 ± 1.81	10.81 ± 1.97	
Morphological findings in the bone marrow examination [n (%)]							P1 = 0.907 P2 = 0.699 P3 = 0.491
Cellularity							
Normocellular	3 (12.5)	6 (17)	1 (20)	15 (27)	4 (15)	5 (14.7)	
Hypercellular	21 (87.5)	30 (83)	4 (80)	40 (73)	22 (85)	29 (85.3)	
Megakaryocytes							
Normal	4 (16.7)	5 (14)	1 (20)	15 (27)	4 (15)	7 (21)	
Increased	20 (83.3)	31 (86)	4 (80)	40 (73)	22 (85)	27 (79)	
Small-large clusters	15 (41.7)	25 (69.4)	2 (40)	35 (63.6)	15 (57.7)	25 (73.5)	
Reticulin fibrosis							
Grade 0-1	0 (0.0)	0 (0.0)	1 (20)	0 (0.0)	1 (3.8)	2 (5.9)	
LDH (U/L)							**P1=0.014**[a] **P2=0.014**[a] **P3<0.001**[a]
Mean ± SD	700.29 ± 88.18	512.78 ± 230.29	726.20 ± 99.73	575.20 ± 210.7	707.31 ± 107.68	308.89 ± 62.23	
FISH [*n* (%)]							P1 = 0.915 P2 = 0.823 P3 = 0.823
No	22 (91.7)	34 (94.4)	5 (100.0)	51 (92.7)	24 (92.3)	32 (94.1)	
Trisomy 8	1 (4.2)	1 (2.8)	0 (0.0)	2 (3.6)	1 (3.8)	1 (2.9)	
del 13q	1 (4.2)	1 (2.8)	0 (0.0)	2 (3.6)	1 (3.8)	1 (2.9)	
Response to therapy [*n* (%)]							**P1=0.005**[a] **P2=0.005**[a] **P3=0.005**[a]

(*continued on next page*)

Table 2. (continued)

Biochemical Parameters	JAK2 mutation		MPL mutation		JAK2 & MPL mutations		*P* value P1:JAK2 + vs. JAK2− P2: MPL + vs. MPL− P3:JAK2 &/or MPL + vs. double JAK2 & MPL -
	JAK2 + (No = 24)	JAK2 − (No = 36)	MPL + (No = 5)	MPL − (No = 55)	JAK2&/or MPL + (No = 26)	Double JAK2& MPL − (No = 34)	
Complete response	10 (41.7)	29 (80.6)	0 (0.0)	39 (71.0)	10 (38.5)	29 (85.3)	
Partial response	13 (54.2)	5 (13.9)	2 (40.0)	16 (29.0)	13 (50.0)	5 (14.7)	
No response	1 (4.1)	2 (5.5)	3 (60.0)	0 (0.0)	3 (11.5)	0 (0.0)	

%: percentage within genetic marker subgroup.
P1: JAK2 + Vs JAK2 −.
P2: MPL + Vs MPL −.
P3: JAK2 and/or MPL + Vs double JAK2 & MPL -.
Comparison between groups done by Student t-test, Pearson's Chi-square test, Fisher's Exact test & Mann−Whitney U test.
[a] Significant at P value < 0.05.

one patient with MPL W515K). In our study, JAK2 V617F mutation was associated with MPL exon 10 in three cases (all were MPL W515L) and they were all diagnosed as ET.

The Polish study done by Lewandowski et al. [14] on 162 ET patients, including 30 diagnosed with post-ET-MF, also found the JAK2V617F, CALR, and MPL mutations in 59.3%, 19.1%, and 1.2% of patients, respectively, which is located within a walking distance of ours.

The frequency of JAK2 V617F mutation in our study agreed with previous reports from China (57.79%) done by Ji et al. [15] Korea (57.1%) done by Kim et al. [16] and Italy (57%) published by Antonioli et al. [17]. While other studies reported a higher frequency such as studies from Argentine (61.2%) done by Ojeda et al. [18] India (92.3%) published by Syeed [19] which may be due to diverse genetic background in different races.

In our study while comparing JAK2 V617F mutation, we found that JAK2 positive patients age ranged from 42 to 71 years old with a mean of 55.54 ± 8.28 years, which agreed with Al Assaf et al. [20] which may reflect that the mutation was acquired throughout the aging process.

Regarding the relation between JAK2 mutation and laboratory data, there was a highly statistically significant increase in platelets count in the mutated group compared to non-mutated group, similarly to the study from Belgium done by Al Assaf et al. [20].

The majority of JAK2V617F-mutated patients in this study had very high serum LDH compared to JAK2 negative patients. The utility of serum LDH is not only limited to the diagnosis of MPN, but it can also be used as one of the prognostic markers. A study by Zulkeflee et al. [21] proposed that marked elevation of serum LDH ≥1000 U/L may predict a shorter survival outcome based on rapid cell turnover, ineffective hematopoiesis and hemolytic processes occurring in the spleen.

In this study, we found five cases with mutations at codon 515 of the MPL gene. About MPL mutation frequency in Egyptian patients, our study found that this mutation was reported in 8.3% of ET and pre-PMF individuals; 10.3% of ET patients, which is within range of earlier records that ranged from 1% to 14% in Jordanian patients by Jaradat et al. [22] from Italy published by Lussana et al. [23], & in Munich Leukemia Laboratory by Schnittger et al. [24].

On the other hand, Zulkeflee et al. [21], reported that MPL W515 L/K could not be found in 88 Taiwanese patients with MPN.

This variation in the frequency of the driver MPL mutation could be related to different characteristics

Table 3. Relation between thrombotic manifestations and demographic, Clinico-pathological parameters.

Demographic & Clinico-pathological Parameters:	Thrombotic manifestations		Significance test	*P* value
	Present ($n = 26$)	Absent ($n = 34$)		
Age (years)			$t = 1.03$[a]	0.306
Mean ± SD	55.54 ± 9.34	52.94 ± 9.87		
Range (min–max)	33.00–71.00	31.00–72.00		
Sex [*n* (%)]			$\chi^2 = 0.55$[b]	0.457
Male	9 (34.6)	15 (44.1)		
Female	17 (65.4)	19 (55.9)		
TLC (10^3nbsp;cell/μL)			z = 1.72[d]	0.085
Mean ± SD	9.76 ± 1.58	8.99 ± 1.07		
Range (min–max)	5.80–17.00	5.40–14.90		
Hb (g/dL)			z = 0.22[d]	0.829
Mean ± SD	11.1 ± 2.60	11.2 ± 2.40		
Range (min–max)	8.10–13.90	9.80–14.60		
Plat (10^3 cell/μL)			z = 3.2[d]	<0.001[e]
Mean ± SD	721.43 ± 375.41	504.00 ± 209.57		
Range (min–max)	459.00–1600.00	456.00–1000.00		
MPV (fL)			z = 1.99[d]	0.046[e]
Mean ± SD	11.60 ± 1.81	10.96 ± 1.07		
Range (min–max)	7.50–14.00	7.50–14.20		
LDH (U/L)			z = 0.16[d]	0.876
Mean ± SD	698.31 ± 129.68	402.89 ± 159.96		
Range (min–max)	215.00–871.00	200.00–913.00		
Morphological findings in the bone marrow examination [n (%)] Cellularity				
Normocellular	**3 (11.6)**	**5 (14.7)**		
Hypercellular	**23 (88.4)**	**29 (85.3)**		
Megakaryocytes			$\chi^2 = 0.39$[c]	0.563
Normal	**4 (15.3)**	**6 (17.5)**		
Increased	**22 (84.7)**	**28 (82.5)**		
Small-large clusters	**15 (57.7)**	**25 (73.5)**		
Reticulin fibrosis				
Grade 0-1	**1(3.8)**	**2 (5.9)**		
JAK2 [*n* (%)]			$\chi^2 = 3.67$[b]	0.056
Positive	14 (53.8)	10 (29.4)		
Negative	12 (46.2)	24 (70.6)		
MPL [*n* (%)]			$\chi^2 = 7.13$[c]	0.012[e]
Positive	5 (19.2)	0 (0.0)		
Negative	21 (80.8)	34 (100.0)		
Double mutations [*n* (%)]			$\chi^2 = 6.19$[b]	0.013[e]
JAK2 (+) and/or MPL (+)	16 (61.5)	10 (38.5)		
Double JAK2 & MPL negative	10 (29.4)	24 (70.6)		
FISH [*n* (%)]			$\chi^2 = 0.59$[c]	1.000
No	24 (92.3)	32 (94.1)		
Trisomy 8	1 (3.8)	1 (2.9)		
del 13q	1 (3.8)	1 (2.9)		
Hepatomegaly [*n* (%)]			$\chi^2 = 0.03$[c]	1.000
Yes	5 (19.2)	6 (17.6)		
No	21 (80.8)	28 (82.4)		
Splenomegaly [*n* (%)]			$\chi^2 = 0.35$[b]	0.554
Yes	15 (57.7)	17 (50.0)		
No	11 (42.3)	17 (50.0)		
Response to therapy [*n* (%)]			$\chi^2 = 4.69$[c]	0.079
Complete response	14 (53.8)	25 (73.5)		
Partial response	9 (34.6)	9 (26.5)		
No response	3 (11.5)	0 (0.0)		

%: percentage within thrombotic manifestations subgroup.
[a] Student t-test.
[b] Pearson's Chi-square test.
[c] Fisher's Exact test.
[d] Mann–Whitney U test.
[e] Significant at P value < 0.05.

Table 4. Univariate logistic regression analysis for the parameters predicting occurrence of thrombotic complications (n = 26 vs. 34).

	Univariate	
	P	OR (LL−UL 95% C.I)
Age	0.301	1.029 (0.974−1.088)
Male	0.457	0.671 (0.234−1.924)
Female	0.457	1.491 (0.520−4.279)
Platelets	0.002[a]	1.004 (1.002−1.007)
MPV	0.052	1.350 (0.998−1.827)
TLC	0.051	1.232 (0.999−1.518)
Hb	0.468	0.881 (0.626−1.240)
LDH	0.555	1.001 (0.998−1.003)
Positivity of JAK2	0.069	2.800 (0.963−8.139)
Positivity of MPL	0.999	−
JAK2 &/or MPL (+)	0.015[a]	3.840 (1.303−11.318)

OR: Odd's ratio.
C.I: Confidence interval; LL: Lower limit; UL: Upper Limit.
[a] Significant at P value < 0.05.

of the studies cases including sample size, disparate sensitivities of the methods used, and ethnicity-based diversity in the genetic backgrounds.

With regard to MPL mutation alone, we observed that patients with MPL W515 L/K mutation were older when compared to those without mutation (68.80 ± 2.28 vs. 53.09 ± 9.24 years) which agreed with Alvarez-Larran A et al. [25].

We observed in our study a significant association between patients with MPL W515 L/K mutation and higher prevalence of splenomegaly 80% compared to negative MPL mutation cases, which reflects the myeloproliferating state occurring in those patients.

Regarding the hematological features, there was a significant increase in TLC & Platelets in the MPL-mutated group when compared to non-mutated group, the same found by the Korean study done by Michiels et al. [26], which is explained by MPL mutation, showing gain−of−function activity with subsequent constitutive activation of the receptor with subsequent thrombocytosis.

Regarding the microscopic examination of the bone marrow, we observed a characteristic megakaryocytes morphology like staghorn megakaryocytes in most of ET patients but some of pre-PMF patients showed megakaryocytes with cloud-like nuclei. Also, the median number of megakaryocytes was similar among the different genetic subgroups; JAK2 mutant and MPL mutant groups, without significant difference in histological features regarding sinusoidal hyperplasia, dense clusters of megakaryocytes and reticulin fibrosis, unlike Alvarez-Larran et al. [25], who stated that there was a higher no. of megakaryocytes in MPL than JAK2V617F genotype, which could be explained by small sample size of patients harboring MPL mutation in our study.

The overall frequency of the simultaneous coexisting mutations JAK2 V617F & MPL W515L in this study was estimated to be 5% (3/60) and all those cases were restricted to ET-diagnosed patients.

Also, in line with our study, the French cohort study done by Usseglio & his followers in 2017 [3] found that, JAK2 V617F mutation was associated with MPL exon 10 in four cases of 164 suspected myeloproliferative neoplasms. All coexisting mutations were detected in ET cases, with a median platelet count of 1120 × 10^9 platelets/L.

These simultaneous double-mutation JAK2 V617F/MPL W515L cases seem to display particular characteristics differentiating them from single-mutation MPNs. Among our three double-mutation cases, two were women. Also, the median platelet count of these patients was 1169 × 10^9 platelets/L, which was higher than that of single-mutation JAK2 V617F or MPL cases, leading to several thrombotic or hemorrhagic complications. None of these 3 cases achieved complete response. These characteristics could point out a particular group of patients. Moreover, it suggests a synergistic bad effect of both concomitant mutations together to be evaluated in larger studies.

MPN is known to have an increased risk of thrombosis. In our study on comparing patients with thrombotic manifestation versus those without any, there was a statistically significant increase in platelets count & MPV in thrombotic patients than asymptomatic ones. And by doing logistic regression analysis, we found a positive relationship between platelets count and thrombotic tendency because as the platelets count increased, the thrombotic tendency also increased. This was agreed by Papageorgiou et al. [27], who stated high blood counts, platelet alterations, presence of JAK2 mutation and possibly of other mutations such as TET2, DNMT3A, and ASXL1, procoagulant microparticles, NETs formation, endothelial activation and neo-angiogenesis are some of the parameters accounting for hypercoagulability in patients with myeloproliferative neoplasms.

We observed that thrombotic events occurred in 58.3% of our JAK2 positive patients. Similar to us, the study done by Abdelghani et al. [28] on Tunisian ET patients found that 6/7 patients experienced thrombotic events carried JAK2V617F mutation. The patho physiology behind the thrombotic risk in JAK2 positive ET patients may be due to increased myeloid production, activation of leukocytes, endothelial dysfunction and biological difference in platelets such as hypersensitive signaling through TPO receptor after stimulation with low concentration of TPO.

Table 5. Comparison of the studied patients regarding achievement of complete response (response to therapy) (n = 60).

Biochemical parameters:	Complete response (CR) (Number = 39)	No complete response: Partial/No response (NCR) (Number = 21)	Significance test	*P* value
Age (years)			t = 2.728	0.008[a]
Mean ± SD	51.97 ± 9.60	58.90 ± 8.97		
Range (min–max)	31.0–70.0	42.0–72.0		
Sex [*n* (%)]			χ^2 = 1.758	0.185
Male	18 (46.2)	6 (28.6)		
Female	21(53.8)	15 (71.4)		
Hb (g/dL)			t = 0.485	0.630
Mean ± SD	9.12 ± 2.03	10.93 ± 3.40		
LDH (U/L)			Z_{MWU} = 2.232	0.026[a]
Mean ± SD	535.87 ± 218.25	684.19 ± 146.81		
Thrombotic manifestations [*n* (%)]			χ^2 = 9.056	0.249
Present	14 (35.9)	12 (57.1)		
Absent	25 (64.1)	9 (42.9)		
Morphological findings in the bone marrow examination [*n* (%)]			χ^2 = 0.319	0.569
Cellularity				
Normocellular	**6 (17)**	**3 (14.3)**		
Hypercellular	**30 (83)**	**18 (85.7)**		
Megakaryocytes				
Normal	**9 (23.1)**	**4 (19.1)**		
Increased	**30 (76.9)**	**17 (80.9)**		
Small-large clusters	**22 (56.4)**	**14 (66.7)**		
Reticulin fibrosis	**2**	**1**		
Grade 0-1	**(5.1)**	**(4.7)**		
FISH [*n* (%)]			χ^2 = 1.287	0.525
No	36 (92.3)	20 (95.2)		
Trisomy 8	1 (2.6)	1 (4.8)		
del 13q	2 (5.1)	0 (0.0)		
Hepatomegaly [*n* (%)]			χ^2 = 1.068	0.302
Yes	7(17.9)	2 (9.5)		
No	32 (82.1)	19 (90.5)		
Splenomegaly [*n* (%)]			χ^2 = 0.028	0.866
Yes	14 (35.9)	8 (38.1)		
No	25 (64.1)	13 (61.9)		
Gene mutation [*n* (%)]				
JAK2 mutation			χ^2 = 9.573	0.002[a]
Yes	10 (25.6)	14 (66.7)		
No	29 (74.4)	7 (33.3)		
MPL mutation			FET	0.001[a]
Yes	0 (0.0)	5 (23.8)		
No	39 (100.0)	16 (76.2)		
Double mutations			χ^2 = 14.49	<0.001[a]
JAK2 &/or MPL +	10 (38.5)	16 (61.5)		
Double negative JAK2 & MPL -	29 (61.5)	5 (38.5)		

%: percentage within therapy response subgroup.
Comparison between groups done by Student t-test, Pearson's Chi-square test, Fisher's Exact test & Mann–Whitney U test.
[a] : Significant at P value < 0.05.

Regarding the relation between MPL mutation and thrombotic manifestation, we observed that all MPL positive cases had thrombotic complication, which could be explained by the increased platelets count with hypersensitivity & thrombogenic tendency in those patients. We also observed that MPL mutations with JAK2 mutation patients had more than 3 folds increased risk for thrombotic tendency.

When we are talking about patients' response to therapy, it is important to note that no one of MPL positive patients & only 25.6% of all JAK2 positive patients achieved complete response, however more than 60% of MPL & JAK2 double negative patients achieved complete response to therapy. In support of this, the new study done by Ramos & Wiita [29] found that MPN cases with known driver mutations had poor response to traditional treatment and hypothesized that the cell surface of MPN revealed a distinct biological signature and therapeutic targets such as specific immunotherapy as anti-C3AR1, a

multi-pass G-protein coupled receptor, as a potential therapeutic target given. This indicates the synergistic bad effect of both mutations which proved by poorer response to therapy.

4.1. Conclusion

JAK2 was more prevalent than MPL mutation in our study. MPL mutation has a bad prognostic effect than JAK2 mutation as evident by; older age, more thrombotic manifestation, higher platelets count, lower Hb level and poor response to therapy with no marked morphological difference regarding megakaryocytes morphology & reticulin fibrosis. Furthermore, presence of MPL W515 L/K mutation in conjunction with JAK2 may predict bad outcome for these patients.

4.2. Recommendations

Larger scale studies to define a discrete clinical phenotype of patients with MPL & JAK2 mutations differentiating them from single gene-mutation MPNs and also for further integration of other mutations as Calreticulin, TET2, DNMT3A, and ASXL1.

Conflicts of interest

There are no conflicts of interest.

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