Original Article

JAK2, *CALR*, *MPL*, and *ASXL1* Mutations in 136 Thai Patients with Philadelphia-Negative Myeloproliferative Neoplasms and Their Correlations with Clinical Outcomes

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Background: Philadelphia chromosome (Ph)-negative myeloproliferative neoplasms (MPN) are a group of hematological malignancies, including polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF). Mutations of *JAK2*, *CALR*, *MPL*, and *ASXL1* are associated with carcinogenesis and clinical characteristics of Ph-negative MPN. However, the availability of the data regarding these mutations is relatively limited in Thai population.

Objective: To investigate these mutations in Thai Ph-negative MPN patients.

Materials and Methods: One hundred thirty-six MPN (48 PV, 72 ET, and 16 PMF) cases were enrolled. Mutations of *JAK2* V617F and *MPL* W515L/K mutations were investigated using allele-specific PCR (AS-PCR) and confirmed by sequencing. *CALR* and *ASXL1* mutations were investigated using Sanger sequencing.

Results: The *JAK2* V617F mutation was detected in 83.3% of PV, 66.6% of ET, and 50.0% of PMF, and correlated with higher RBC, WBC, and PLT counts in PV. *CALR* mutations were detected in 16.7% of ET and 12.5% of PMF and associated with a higher PLT count in ET. The *MPL* W515L mutation was detected in one PMF patient. *ASXL1* mutations were detected in 6.3% of PV, 8.3% of ET, and 12.4% of PMF, with c.1954G>A being the preponderant mutational form. *ASXL1* mutations increased the risk (RR 27.6) and accelerated the onset of AML transformation.

Conclusion: The present study provided the prevalence and clinical correlation of *JAK2*, *CALR*, *MPL*, and *ASXL1* mutations among Thai Ph-negative MPN patients. The association of *ASXL1* mutations with adverse clinical outcomes suggested the potential usefulness of these mutations as a prognostic marker for Ph-negative MPN patients.

Keywords: *JAK2*, *MPL*, *CALR*, *ASXL1*, Thai, Philadelphia-negative myeloproliferative neoplasm (MPN)

Received 22 December 2020 | Revised 11 March 2021 | Accepted 12 March 2021

J Med Assoc Thai 2021;104(5):834-45

Website: http://www.jmatonline.com

Philadelphia chromosome (Ph)-negative myeloproliferative neoplasms (MPN) are a group of hematological malignancies, classically including polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF)[1-4]. The diagnosis of Ph-negative MPN has greatly been improved after the discovery of Janus kinase 2 (*JAK2*) mutations[5,6]. The *JAK2* V617F mutation, the most common form of *JAK2* mutations in Ph-negative MPN, can be detected in 70% to 90% of PV patients and 50% to 60% of ET and PMF patients[7-11]. The structural change caused by this mutation results in constitutive activation of the JAK or signal transducer and activator of transcription (STAT) signaling pathway, contributing to an increase in hematopoiesis[12-14]. The exon 12 mutation of *JAK2* mutation is also identified in 2% to 3% of PV patients and 30% of *JAK2* V617F-negative PV patients[15-17].

Mutations of the myeloproliferative leukemia virus (*MPL*) gene, which normally encodes the thrombopoietin (TPO) receptor, have been reported in up to 10% of *JAK2* V617F-negative ET and PMF patients[18,19]. In addition, somatic mutations of the calreticulin (*CALR*) gene, which encodes a multi-functional protein involved in chaperone activity and calcium homeostasis, have been identified in 20% to 30 of ET and PMF patients without *JAK2* and *MPL* mutations, but not found in PV patients[20-22]. These *CALR* mutations are now included in the 2016 revision to the World Health Organization (WHO)

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How to cite this article:

Apipongrat D, Numbenjapon T, Nimmanon T, Arnutti P. *JAK2*, *CALR*, *MPL*, and *ASXL1* Mutations in 136 Thai Patients with Philadelphia-Negative Myeloproliferative Neoplasms and Their Correlations with Clinical Outcomes. J Med Assoc Thai 2021;104:834-45.

doi.org/10.35755/jmedassocthai.2021.05.12344

classification as the driver mutations for MPN[23]. However, approximately 10% to 15% of PMF or ET patients do not express any diver mutations and are referred to as being "triple negative"[20-22].

Somatic mutations of different epigenetic regulator genes have also been reported, including the mutations of ten-eleven translocation 2 (*TET2*), DNA methyltransferase 3 (*DNMT3*), isocitrate dehydrogenase 1 and 2 (*IDH1/2*), and additional sex comb like 1 (*ASXL1*), which are detected in 10% to 20%[24], 5% to 10%[25,26], 1% to 5%[27], and 10% to 25%[28,29] of MPN cases, respectively[28-30]. Amongst these mutations, *ASXL1* mutations have been consistently reported as a poor prognostic factor in MPN[31,32]. *ASXL1* mutations are found in 2% to 5% of PV and ET and 13% to 23% of PMF[31-34]. Two common forms of *ASXL1* mutations, which result in impaired chromatin modification and dysregulation of cell proliferation, are duplication of guanine (G) in codon 1934 (c.1934dupG) and substitution of G to adenosine (A) in codon 1954 (c.1954G>A)[35].

Many studies have reported the relationship between particular mutations of the *JAK2*, *CALR*, *MPL*, and *ASXL1* genes and clinical characteristics of MPN[36-45]. In ET patients, the *JAK2* V617F mutation is related to older ages, higher hemoglobin (Hb) levels, hematocrits (Hct), red blood cell (RBC) counts, and white blood cell (WBC) counts, with an increased risk of thrombosis[36,41,43]. On the contrary, *CALR* mutations are associated with younger ages, higher platelet (PLT) counts, and a lower risk of thrombosis in both ET and PMF patients[36,38-43]. In addition, the *ASXL1* mutations have been associated with leukemic transformation of MPN and the progression of PV and ET to secondary myelofibrosis (post PV/ET-MF)[31-34]. Frequencies of these mutations and their association with clinical characteristics and prognosis in MPN may vary in different populations, and only few studies have investigated *ASXL1* mutations in Thai MPN patients. Therefore, present study aimed to determine the prevalence of *JAK2*, *CALR*, *MPL*, and *ASXL1* mutations and their clinical correlation in Thai Ph-negative MPN patients.

Materials and Methods

Patients and samples

Ethical approval for the present study was obtained from the Royal Thai Army Institutional Review Board (IRBRTA 692/2560). One hundred thrity-six cases (75 males and 61 females) of Ph-negative MPN, consisting of 48 PV cases, 72 ET cases, and 16 PMF cases, were enrolled from the Hematology Unit, Department of Medicine, Phramongkutklao Hospital, after the informed consent process had been performed. The diagnosis was established according to the 2008 WHO criteria or the 2016 revision to the WHO Classification Criteria for Newly Diagnosed Patients. Morphologic diagnoses were made by a hematopathologist and discussed with hematologists-oncologists before the final diagnoses were made. Patients' demographic data, including age, gender, weight, height, body mass index, and clinical events such as bleeding, thrombosis, organomegaly, acute myeloid leukemia, and myelofibrosis transformation were collected by retrospective record reviews. The present study was approved by the Committee of Institutional Review Board, Royal Thai Army Medical Department, Bangkok, Thailand. The genomic DNA was extracted from EDTA blood samples using a genomic DNA extraction kit (FavorPrep™ Blood genomic DNA Extraction Mini Kit, Favorgen, Taiwan) and stored at –20°C until used.

Analysis of *JAK2*, *CALR*, and *MPL* mutations

The *JAK2* V617F mutation was investigated using an allele-specific polymerase chain reaction (AS-PCR) technique[45]. The PCR was performed in a 10.0 µL PCR reaction mixture that contained 1.0 µL DNA template (50 to 100 ng/ µL), 5.0 µL KAPA2G ReadyMix® (Sigma-Aldrich, USA), 0.5 µL JAK2V617F-wild-type forward primer (JAK2-WF), 0.5 µL JAK2V617F-mutant forward primer (JAK2-MF), 0.5 µL JAK2-reverse primer (JAK2-R), and 2.5 µL distilled water (DW), with a Mastercycler® Pro-series thermocycler (Eppendorf, New York, USA). All reactions were performed using the following parameters: an initial denaturation step at 95°C for one minute, 30 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds, and then a final extension step at 72°C for ten minutes. The mutant allele had two bands at 364 bp and 203 bp, whereas the wild-type allele exhibited only one band at 364 bp. *JAK2* exon 12 mutations were investigated using Sanger sequencing in PV cases without a *JAK2* V617F mutation.

CALR mutations were investigated using a Sanger sequencing method. The specific primers for PCR reactions were previously described[45]. The genomic region of interest, exon 9, was amplified by PCR. The PCR was performed in a 10.0 µL PCR reaction mixture that contained 1.0 µL DNA template (50-100 ng/ µL), 5.0 µL KAPA2G ReadyMix® (Sigma-Aldrich, USA), 1.0 µL CALR forward primer

(CALR-F), 1.0 µL CALR reverse primer (CALR-R), and 2.0 µL DW, with a Mastercycler® Pro-series thermocycler (Eppendorf, New York, USA). All reactions were performed using the following parameters: an initial denaturation step at 95°C for three minutes, 30 cycles of 95°C for 15 seconds, 65°C for 15 seconds, and 72°C for 30 seconds, and then a final extension step at 72°C for ten minutes. The PCR product size of *CALR* exon 9 was 386 bp. The products were purified and analyzed for *CALR* mutations by DNA sequencing.

MPL mutations, W515L and W515K, were detected using an AS-PCR technique. The specific amplicons were obtained using specific primers for each variant[46]. The PCR was performed in a 10 µL reaction mixture that contained 1.0 µL DNA template (50 to 100 ng/ µL), 5.0 µL KAPA2G ReadyMix® (Sigma-Aldrich, USA), 0.4 µL forward internal control primer (MPL-Fo), 0.4 µL reverse internal control primer (MPL-Ro), 0.8 µL reverse wild-type primer (MPL-Riwt), 1.6 µL forward mutant L primer (MPL-FiL), and 0.8 µL DW, in a Mastercycler® Pro-series thermocycler (Eppendorf, New York, USA). All reactions were performed using the following parameters: an initial denaturation step at 95°C for two minutes, 30 cycles of 95°C for 15 seconds, 62°C for 15 seconds, and 72°C for 30 seconds, and then a final extension step at 72°C for ten minutes. The MPL-Fo and -Ro primers generated a common fragment of 264 bp. The MPL-Fo and -Riwt primers generated a 98 bp specific fragment for wild-type *MPL*. The MPL-FiL and -Ro primers generated a 188 bp specific fragment for the *MPL* W515L mutation. In the *MPL* W515K reaction, the forward mutant K primer (MPL-FiK) was included instead of MPL-FiL to amplify a 188 bp *MPL* W515L-specific fragment. DNA sequencing was performed to confirm positive products.

Analysis of *ASXL1* mutations

ASXL1 mutations were investigated using a Sanger sequencing method. The specific primers for PCR reactions were previously described[32]. The genomic region of interest, exon 12, was amplified by PCR. The PCR was performed in a 10.0 µL PCR reaction mixture that contained 1.0 µL DNA template, 5.0 µL KAPA2G ReadyMix® (Sigma-Aldrich, USA), 1.0 µL ASXL1 forward primer (ASXL1-F), 1.0 µL ASXL1 reverse primer (ASXL1-R), and 2.0 µL DW, with a Mastercycler® Pro-series thermocycler (Eppendorf, New York, USA). All reactions were performed using the following parameters: an initial denaturation step at 95°C for two minutes, 30 cycles of 95°C for 15 seconds, 58°C for 15 seconds, 72°C for 30 seconds, and a final extension step at 72°C for one minute. The size of PCR products for *ASXL1* exon 12 was 339 bp. The products were purified and analyzed for *ASXL1* mutations by DNA sequencing.

Statistical analysis

Continuous variables were presented as mean, median, and standard deviation and compared using Student's t, Mann-Whitney U, and ANOVA tests. Categorical variables were described as frequency and percentage. Clinical data and laboratory parameters were compared among different mutational statuses using chi-square (χ^2) and Fisher's exact tests. Univariate and multivariate analyses were performed to analyze the association between the mutational statuses and the patients' outcomes. For multivariate analysis, a Cox proportional hazards model was constructed for time to AML transformation and was adjusted for potential confounding covariates. Relative risk (RR) and 95% confidence interval (CI) were calculated to demonstrate the risk association. A Kaplan-Meier curve for time to event-free survival (EFS) and overall survival (OS) in different mutational statuses was plotted and compared using a log-rank test. Data were analyzed with IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA). A p-value of less than 0.05 was considered statistically significant.

Results

Clinical characteristics of 136 Thai Ph-negative MPN patients

One hundred thirty-six patients, consisting of 75 males and 61 females, were subjected to analysis. The laboratory testing and clinical manifestations in 136 Thai Ph-negative MPN patients were shown in Table 1. No difference in gender, age, and body mass index (BMI) was observed among patients with PV, ET, and PMF. Consistent with the salient clinical picture for each MPN group, the RBC count, Hb, and Hct were higher in PV patients ($p<0.001$), whereas the PLT count was higher in ET patients ($p<0.001$). The most common complication observed was thrombosis, either arterial or venous, which affected up to 28.7% (39/136) of the patients. Of these, 27.0% (13/48), 29.1% (21/72), and 31.1% (5/16) were observed in PV, ET and PMF patients, respectively. Organomegaly (hepatomegaly or splenomegaly) was observed in 6.6% (9/136) of the patients with the highest frequency seen among 25.0% (4/16) of PMF patients, whereas bleeding complications were found in 2.9%

Table 1. Laboratory testing and clinical manifestations in 136 Thai Ph-negative MPN patients

Characteristics	MPN patients; n (%)			Total (n=136); n (%)	p-value
	PV (n=48)	ET (n=72)	PMF (n=16)		
Sex					0.142
Male	31 (64.6)	34 (47.2)	10 (62.5)	75 (55.1)	
Female	17 (35.4)	38 (52.8)	6 (37.5)	61 (44.9)	
Age (year); mean±SD	59.9±14.9	61.3±14.2	53.5±20.2	59.9±15.2	0.201
BMI (kg/m^2); mean±SD	23.4±4.3	24.3±6.7	23.3±2.7	23.9±5.6	0.593
Laboratory testing; mean±SD					
Hb (g/dL)	17.1±3.1	13.0±1.9	11.6±4.5	14.3±3.4	<0.001*
Hct (%)	53.3±9.2	40.0±7.8	34.9±11.7	44.2±11.2	<0.001*
RBC (×10^6/L)	6.4±1.6	4.6±0.9	4.3±1.4	5.2±1.5	<0.001*
WBC (×10^3/L)	16.4±12.1	13.2±16.3	10.3±8.6	14.0±14.3	0.238
PLT (×10^9/L)	489.1±317.2	929.6±571.8	440.2±442.6	718.6±529.6	<0.001*
MPV (fL)	8.6±1.4	9.5±8.1	8.8±1.3	9.1±6.0	0.746
Clinical manifestations					
Bleeding	2 (4.2)	1 (1.4)	1 (6.3)	4 (2.9)	0.479
Thrombosis	13 (27.0)	21 (29.1)	5 (31.3)	39 (28.7)	0.942
Organomegaly	3 (6.3)	2 (2.8)	4 (25.0)	9 (6.6)	0.050*
PMF transformation	6 (12.5)	4 (5.5)	N/A	10 (7.4)	0.176
AML transformation	0 (0.0)	3 (4.2)	0 (0.0)	3 (2.2)	0.261
Death	6 (12.5)	7 (9.7)	5 (31.3)	18 (13.2)	0.070

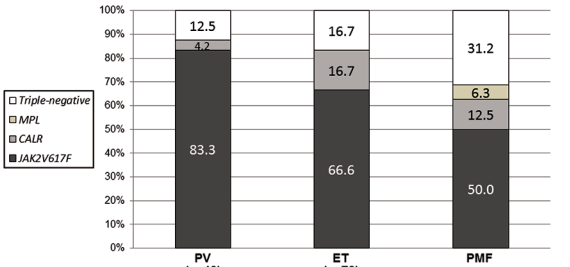
AML=acute myeloid leukemia; BMI=body mass index; ET=essential thrombocytosis; Hb=hemoglobin; Hct=hematocrit; MPN=myeloproliferative neoplasms; MPV=mean platelet volume; N/A=not available; PLT=platelet; PMF=primary myelofibrosis; PV=polycythemia vera; RBC=red blood cell; SD=standard deviation; WBC=white blood cell

* Statistically significant when compared to other groups (p<0.05)

(4/136) of the patients. With the follow-up time of 72 months, transformation into myelofibrosis was seen in 12.5% (6/48) of PV and 5.5% (4/72) of ET cases, transformation into AML was seen in two ET patients, and death was the outcome of 12.5% (6/48) of PV, 9.7% (7/72) of ET, and 31.1% (5/16) of PMF cases.

Prevalence of *JAK2* V617F, *CALR*, and *MPL* mutations in Ph-negative MPN patients

The *JAK2* V617F mutation was detected in 70.6% (96/136) of all MPN cases, which include 83.3% (40/48) of PV, 66.6% (48/72) of ET, and 50.0% (8/16) of PMF cases (Figure 1). All the PV patients without the *JAK2* V617F mutation were confirmed to have no mutation on exon 12 by sequencing. The *CALR* mutations were detected in 11.8% (16/136) of all MPN cases, which include 4.2% (2/48) of PV, 16.7% (12/72) of ET, and 12.5% (2/16) of PMF cases, with type 1 (52-bp deletion) and type 2 (5-bp insertion) mutations being detected in 62.5% (10/16) and 37.5% (6/16) of the *CALR*-positive cases, respectively (Figure 1). The *MPL* W515L mutation was detected only in a PMF case (Figure 1). Noteworthy, co-existence of *JAK2* V617F and either *CALR* or *MPL* mutations was not observed in the present study. The authors unexpectedly discovered *CALR* mutations in two PV cases, one with the type 1 mutation and the



Mutation, n (%)	PV (n=48)	ET (n=72)	PMF (n=16)
JAK2V617F	40 (83.30)	48 (66.6)	8 (50.0)
CALR	2 (4.2)	12 (16.7)	2 (12.5)
Type 1 (52-bp del)	1 (2.1)	8 (11.1)	1 (6.25)
Type 2 (5-bp ins)	1 (2.1)	4 (5.6)	1 (6.25)
MPL	0 (0.0)	0 (0.0)	1 (6.3)
Triple-negative	6 (12.5)	12 (16.7)	5 (31.2)

Figure 1. Distribution of *JAK2* V617F, *CALR,* and *MPL* mutations in 136 Thai Ph-negative MPN patients.

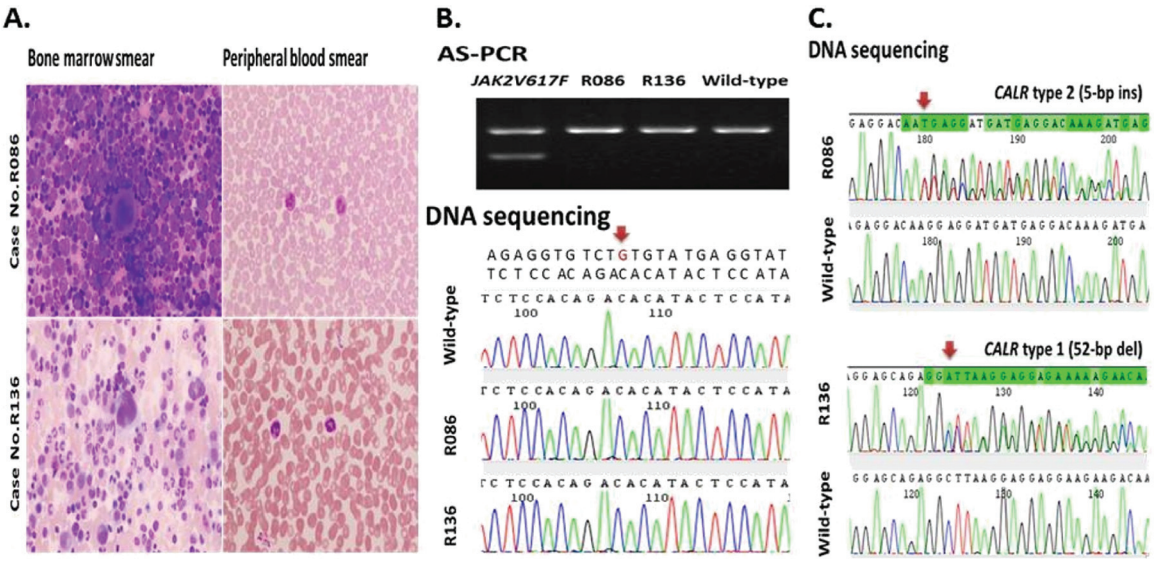


Figure 2. (A) Bone marrow and peripheral blood smears of patients No. R086 and R136 (Wright's stained, 1000x) show a hypercellular bone marrow with erythroid preponderance and normal megakaryocytes and peripheral blood smear with erythocytosis without thrombocytosis. (B) AS-PCR and DNA sequencing demonstrate a negative result for the *JAK2* V617F mutation. (C) DNA sequencing shows a positive result for the *CALR* type 1 (No.R136) and type 2 mutations (No.R086).

other with the type 2 mutation, regardless of a report of these specific *CALR* mutations being absent in a large cohort of *JAK2*-negative PV patients[47]. Both patients presented with high Hb and Hct without thrombocytosis at the time of diagnosis. They also had a normal karyotype and an EPO level within normal limits with a hypercellular bone marrow exhibiting trilineage hematopoiesis, endogenous erythroid colony formation, clusters of polymorphous megakaryocytes, and no excess blasts (Figure 2A). Their clinical data completely fulfilled the 2008 WHO diagnostic criteria for PV. Additional DNA sequencing for the *JAK2* V617F mutation and repeat DNA sequencing for the *CALR* mutations confirmed the negative results for the *JAK2* V617F mutation (Figure 2B) with presence of the specific *CALR* mutations in these cases (Figure 2C), confirming the authors' original AS-PCR and sequencing results.

The PV case with the type 2 *CALR* mutation was a 76-year-old woman who presented with erythropoiesis (Hb 18.2 g per dL and Hct 55.8%), a normal erythropoietin (EPO) level, and a PLT count within normal limits (348×10⁹/L). She was treated with hydroxyurea and bloodletting. Thrombotic events, bleeding events, and transformation into AML were not observed. Interestingly, during a 10-year follow-up period at the authors' department, the PLT count was slowly increasing, reaching the peak of 774×10⁹ /L in the seventh year. In contrast to this case, the other PV case with the type 1 *CALR* mutation was more recently diagnosed, showing no increase in the PLT count during a 2-year follow-up period.

Table 2. The distribution of *ASXL1* mutations in 136 Thai Ph-negative MPN patients

Diagnosis	*ASXL1*-mutated; n (%)			Wild-type; n (%)	p-value[a]
	c.G1954A	c.1934dupG	Total		
All 136 patients	9 (6.6)	2 (1.5)	11 (8.1)	125 (91.9)	0.907
PV (n=48)	3 (6.3)	0 (0.0)	3 (6.3)	45 (93.7)	
ET (n=72)	5 (6.9)	1 (1.4)	6 (8.3)	66 (91.7)	
PMF (n=16)	1 (6.2)	1 (6.2)	2 (12.4)	14 (87.6)	

ET=essential thrombocytosis; PMF=primary myelofibrosis; PV=polycythemia vera

[a] p-value from the Fisher's exact test, compared between *ASXL1*-mutated and wild-type groups

Prevalence of *ASXL1* mutations in Ph-negative MPN patients

ASXL1 mutations were detected in 8.1% (11/136) of all cases, which include 6.3% (3/48) of PV, 8.3% (6/72) of ET, and 12.4% (2/16) of PMF cases, with no difference between groups (Table 2). The c.1954G>A mutation was found in 81.8% (9/11), whereas the c.1934dupG mutation was found in 18.2% (2/11) of *ASXL1*-mutated cases (Table 2). These 11 cases consisted of seven triple negative cases, a *JAK2* V617F-positive PV case, a *JAK2* V617F-positive ET case, a *CALR*-mutated ET case, and a *MPL*-mutated

Table 3. Information of the 11 Thai Ph-negative MPN patients with *ASXL1* mutations

Cases No.	Age (year)	Diagnosis	Diver mutation	Clinical events			Transformation to		Status	Following time (months)
				Bleeding	Thrombosis	Organomegaly	PMF	AML		
R014	68	ET	Triple negative	No	No	No	No	Yes	Alive	89
R015	78	ET	Triple negative	No	No	No	No	Yes	Death	78
R020	43	PV	Triple negative	No	No	No	No	No	Alive	295
R028	71	PV	Triple negative	No	No	No	No	No	Alive	77
R043	81	PV	*JAK2* V617F	No	No	No	No	No	Alive	47
R097	46	ET	*JAK2* V617F	No	No	No	No	No	Alive	150
R115	69	PMF	*MPL*	No	Yes	Yes	No	No	Death	27
R116	64	ET	*CALR*	No	No	No	No	No	Alive	30
R118	62	ET	Triple negative	No	Yes	No	No	No	Alive	19
R136	67	PMF	Triple negative	No	No	No	No	No	Alive	21
R146	68	ET	Triple negative	No	No	No	No	No	Alive	18

AML=acute myeloid leukemia; ET=essential thrombocytosis; PMF=primary myelofibrosis; PV=polycythemia vera

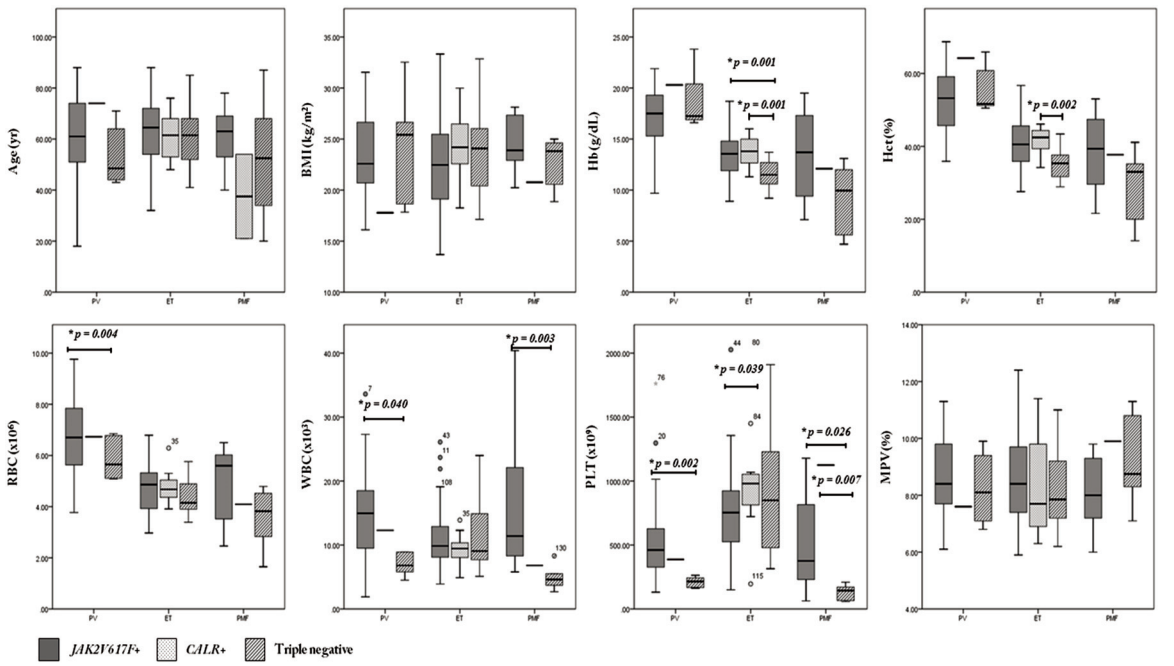


Figure 3. Comparison of patients' laboratory data between different diver mution groups in 136 Thai Ph-negative MPN patients.

PMF case (Table 3).

Association of *JAK2* V617F, *CALR*, and *MPL* mutations with clinical outcomes

The PV patients with the *JAK2* V617F mutation had significantly higher RBC (p=0.004), WBC (p=0.040), and PLT (p=0.002) counts than those with wild-type *JAK2* (Figure 3). Thrombotic events were observed in 12 PV cases with no statistical difference between those with and without the *JAK2* V617F mutation (p=0.418). The ET patients a *CALR* mutation had a higher PLT count than the other groups (p=0.039), whereas the triple-negative cases had lower Hb and Hct levels than the other groups (Figure 3). The *JAK2* V617F mutation was not associated with thrombotic events in the ET patients in the present study. Additionally, the PMF patients with a *CALR* mutation had an increased PLT count compared to the triple-negative (p=0.007) and *JAK2* V617F-mutated groups (p=0.026) (Figure 3). A PMF

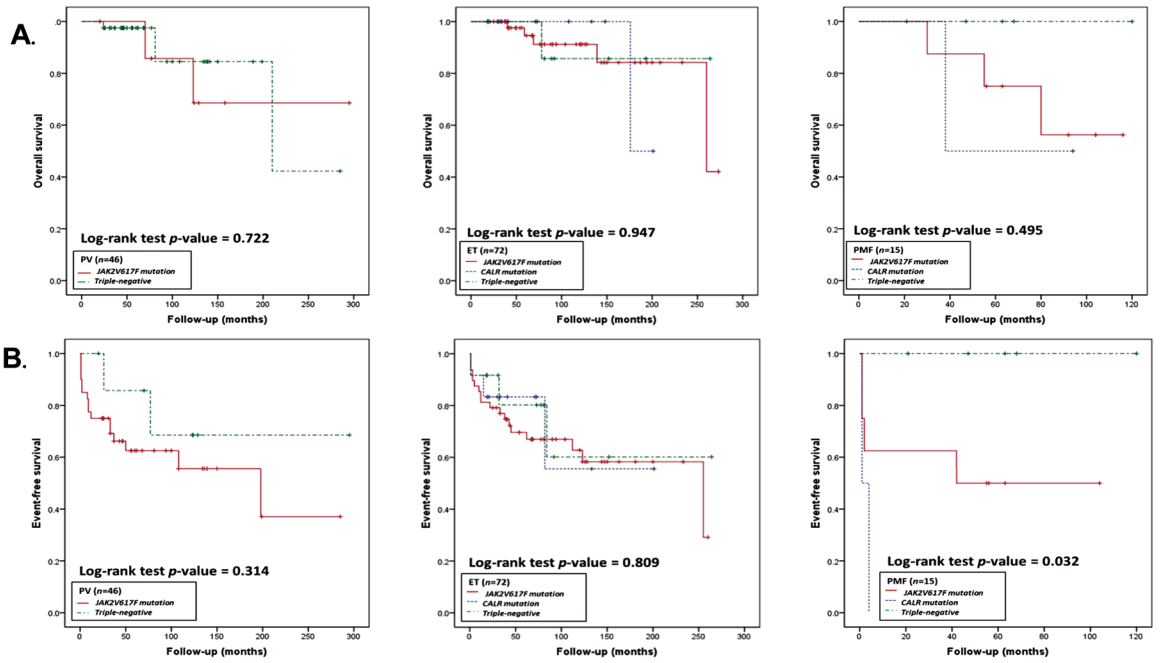


Figure 4. Kaplan-Meier curves demonstrating overall survival (OS) (A) and event-free survival (EFS) (B) in patients with PV, ET, and PMF.

case with the *MPL* W515L mutation was excluded from this analysis due to small population.

The median follow-up time of the present study was 68.5 months (20 to 295 months) for PV cases, 79.5 months (18 to 273 months) for ET cases, and 63.0 months (21 to 120 months) for PMF cases. The two PV cases with a *CALR* mutation and the PMF case with an *MPL* mutation were excluded from the present analysis. Thrombotic events were seen in 28.7% of all cases with no association between different mutational statuses (p=0.068; 95% CI 0.456 to 1.029). The Kaplan-Meier estimate was not able to detect any difference in the overall survival between different mutational statuses among PV cases (p=0.722), ET cases (p=0.947), and PMF cases (p-value=0.495). The patients who had no complications including bleeding, thrombosis, organomegaly, and leukemic transformation were analyzed for the event-free survival (EFS). The Kaplan-Meier estimate revealed no effect of mutational statuses on the EFS among PV (p=0.314) and ET cases (p=0.809) (Figure 4A). However, the *CALR* mutations were shown to increase the risk of developing complications among PMF cases (p=0.032) (Figure 4B).

Association of *ASXL1* mutations with clinical outcomes

ASXL1 mutations were associated with a lower PLT count in ET cases (p=0.008) (Figure 5) and lower mean platelet volume (MPV) in PMF cases (p=0.039) (Figure 5). No differences in age, BMI, Hb, Hct, RBC, and WBC count were observed among PV, ET, and PMF cases. Information of the 11 cases with *ASXL1* mutations is shown in Table 3. Thrombotic events were seen in one triple negative ET case and one *MPL*-mutated PMF case. This PMF case also had hepatosplenomegaly and deceased at 27 months of follow-up, suggesting the possible association of co-existing *MPL* W515L and an *ASXL1* mutation with disease aggressiveness. Two other triple negative ET cases suffered AML transformation, one of which deceased at 89 months of follow-up.

Comparison of clinical manifestations between MPN patients with and without *ASXL1* mutations is shown in Table 4. *ASXL1* mutations increased the risk of AML transformation in these PMN patients (p=0.017; RR 27.6; 95% CI 2.3 to 333.7). This association was also observed when analyzed specifically in the ET group (p=0.014; RR 31.0; 95% CI 2.5 to 379.4). Moreover, Kaplan-Meier analysis demonstrated an earlier onset of AML transformation in *ASXL1*-mutated cases than those without the mutations (log-rank test, p=0.001) (Figure 6), which was further confirmed by Cox-regression analysis (p=0.015; hazard ratio (HR) 22.3; 95% CI 1.8 to 271.1). However, *ASXL1* mutations were not

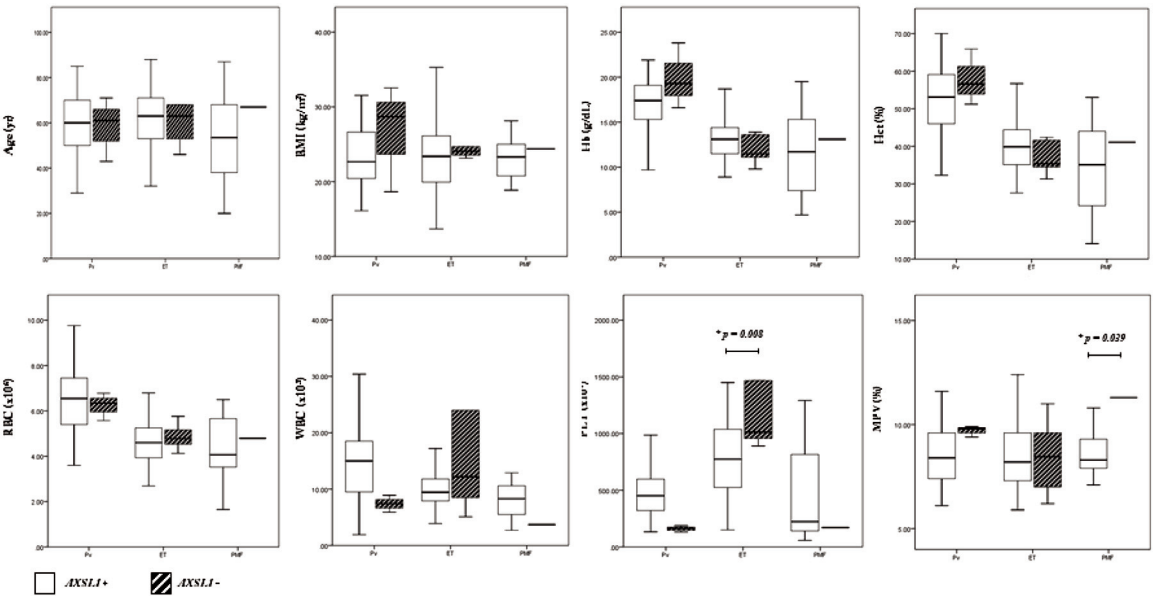


Figure 5. Comparison of laboratory data in 136 Thai Ph-negative MPN patients with and without *ASXL1* mutations.

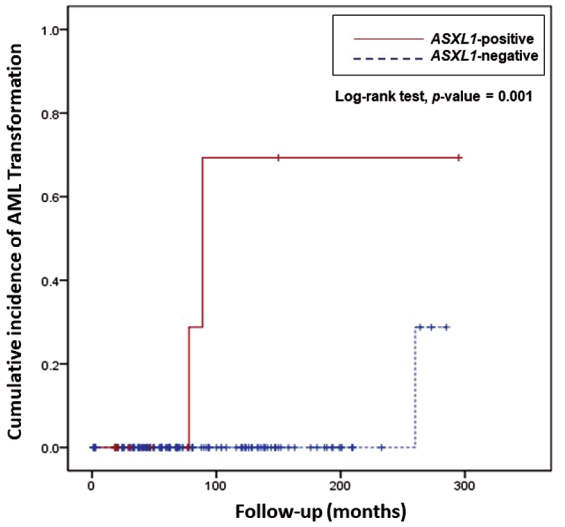


Figure 6. Kaplan-Meier curves demonstrating cumulative incidence of AML tranformation in 136 Thai Ph-negative MPN patients. The *ASXL1*-mutated cases (n=11) have a shorter interval between the time of diagnosis and AML transformation than the *ASXL1*-wild type (n=125) (p=0.001).

associated with either OS (log-rank test, p=0.511) or EFS (log-rank test, p=0.993).

Discussion

Driver mutations involving *JAK2*, *CALR*, and *MPL* genes have been linked to clinical characteristics of Ph-negative MPN in many populations[36-45]. In the present study, 136 Thai Ph-negative MPN patients were screened for these mutations. The *JAK2* V617F mutation was detected in 83.3% of PV cases, 66.6%

Table 4. Comparison of clinical manifestations between Thai Ph-negative MPN patients with and without *ASXL1* mutations

Clinical manifestations	*ASXL1* mutation; n (%)		Total (n=136); n (%)	p-value[a]
	Positive (n=11)	Negative (n=125)		
Bleeding				1.000
Yes	0 (0.0)	4 (3.2)	4 (2.9)	
No	11 (100)	121 (96.8)	132 (97.1)	
Thrombosis				0.282
Yes	3 (27.3)	37 (29.6)	39 (28.7)	
No	8 (72.7)	88 (70.4)	97 (71.3)	
Organomegaly				1.000
Yes	1 (9.1)	8 (6.4)	9 (5.9)	
No	10 (98.9)	117 (93.6)	128 (94.1)	
Transformation to PMF				1.000
Yes	0 (0.0)	10 (8.0)	10 (7.4)	
No	11(100)	115 (92.0)	126 (92.6)	
Transformation to AML				0.017*
Yes	2 (18.2)	1 (4.0)	3 (2.2)	
No	9 (81.8)	124 (96.0)	133 (97.8)	
Death				0.640
Yes	2 (18.2)	16 (12.8)	18 (13.2)	
No	9 (81.8)	109 (87.2)	118 (86.8)	

AML=acute myeloid leukemia; PMF=primary myelofibrosis

* Statistically significant when compared to other groups (p<0.05), [a] p-value from the Fisher's exact test

of ET cases, and 50.0% of PMF cases, consistent with the previously reported prevalence of 70% to 90% for PV and 50% to 60% for ET and PMF in various geographic areas[7-9,36,38,42,45] including Thailand[11,43]. Furthermore, *CALR* mutations, either type 1 or type 2, were detected in 4.2%, 16.7%, and 12.5% of PV, ET, and PMF cases, respectively. The prevalence of *CALR*-mutated cases in the present study was less than that reported in some different populations, which was up to 25% to 33% of ET patients and 26%to 38% of PMF patients[20,21], but comparable to that reported in Japan[41] and Thailand[48] with prevalence of 16.0% and 14.0% for ET, respectively. In addition, the authors observed the *MPL* W515L mutation in one patient with PMF, and no co-existence of *JAK2* V617F and either a *CALR* mutation or an *MPL* mutation found. A proportion of MPN patients (12.5% of PV, 16.7% of ET, and 31.2% of PMF) were seen to be triple-negative with no *JAK2* V617F, *CALR*, or *MPL* mutation. The prevalence of these triple negative patients was higher than other studies[20,21]. This discrepancy may be due to the relatively low number of subjects in the present studies and the methods of mutation detection employed. Noteworthy, to verify the present study results and to decrease the likelihood of false-positive results, the authors performed DNA sequencing in all samples with positive results.

The authors also discovered the specific *CALR* mutations, confirmed by DNA sequencing, in two *JAK2*-negative PV cases, whose clinical data and laboratory findings completely matched the WHO 2008 diagnostic criteria for the disease. It has been widely perceived that *CALR* mutations are exclusively detected in a proportion of Ph-negative MPN, particularly ET or PMF, with a virtual impossibility to observe a *CALR* mutation, either type 1 or type 2, in PV cases according to large cohorts of *JAK2*-non-mutated patients[20,47]. This exclusiveness could be explained by the direct mechanistic link of these mutations specifically to thrombocytosis, not erythropoiesis, through the interaction of *CALR* with *MPL*, a TPO receptor, with consequential activation of a JAK or STAT signaling cascade without a need for TPO attachment[21,49]. The present study discovery of *CALR* mutations in the PV cases was unexpected. In fact, detectable *CALR* mutations have previously been described in at least three PV patients according to published reports[50,51]. However, based on the revised WHO 2016 diagnostic criteria and evidence of genetic mutations, the authors' two cases did not fulfil the criteria for PV and should instead be diagnosed as unclassifiable MPN (MPN-U)[23]. Therefore, the present study findings supported the potential usefulness of *CALR* mutation testing in *JAK2* V617F-negative PV cases, which might help precisely differentiate PV cases from MPN-U cases. Further studies are required to verify the usefulness and clinical relevance of *CALR* mutation testing in this group of PV patients.

Similar to previous studies, the *JAK2* V617F mutation was associated with an increase in the blood cell count in PV, the PLT count in ET and PMF, and the risk of thrombosis compared to those without this mutation[36,41,43]. However, in contrast to those studies, the authors did not observe its correlation with older age. Compared to the *JAK2* V617F-mutated ET and PMF patients, the present study demonstrated that *CALR*-mutated patients had an increase in the PLT count and a decrease in the risk of thrombotic events, even though these were seen to be statistically significant only for ET cases. While previous reports demonstrated the protective effect of the *CALR* mutations on the development of thrombotic events[20,40], this correlation was not observed in the present study. In addition, the authors also found the PMF patients harboring the *CALR* mutations had significantly reduced time to complication events. To validate this protective effect of the *CALR* mutations, further studies with larger sample sizes are needed.

The prevalence of *ASXL1* mutations observed in the present study was 8.1%, which was lower than the previous reports in which these mutations were detected in 10% to 25% of MPN patients[28,29]. These mutations were more frequently detected in PMF patients than in the other MPN groups, supporting the previous studies[33,34]. The present study finding of c.1954G>A preponderance was in accordance with a report in Chinese ET patients in which only this mutational form was seen[52], in contrast to studies conducted in other populations that observed c.1934dupG as the most common form[32,35]. It is noteworthy that there is conflicting data whether c.1934dupG-related frameshift mutation is a true somatic mutation. There is evidence supporting it as a genuine somatic alteration, whereas its presence in 25% of healthy volunteers suggests this mutational form as a potential PCR amplification artifact[53].

ASXL1 mutations have been associated with an increased risk of AML transformation and poor prognosis in MPN patients[54,55] and linked to development of myelofibrosis in PMF patients[33]. Consistent with the previous studies, the authors observed the association of *ASXL1* mutations with

an increased risk and an earlier onset of AML transformation. Furthermore, *ASXL1* mutations were detected in five *CALR*-negative ET patients and two *CALR*-negative PMF patients. Therefore these patients belonged to the high risk group with the lowest 2-year median survival according to risk stratification based on *ASXL1* and *CALR* mutational statuses as proposed in a previously study[56]. Importantly, two of these *ASXL1*-mutated ET patients developed AML transformation, and one of these PMF patients also had an *MPL* mutation that was associated with adverse clinical events (thrombosis and splenomegaly) and eventually died, 27 months after the diagnosis. Altogether, the present study findings further support the adverse effects of *ASXL1* mutations on the clinical outcomes in MPN patients. Because of the limited number of patients in the present study, further studies are yet to be performed to firmly establish the effects of these mutations on the prognosis of Ph-negative MPN patients.

Conclusion

Not only did the present study provide the prevalence of *JAK2* V617F, *CALR*, *MPL*, and *ASXL1* mutations among Thai Ph-negative MPN patients, but it also revealed clinical correlation of these mutations, confirming previous studies conducted in different populations. The present study also confirmed the association between *ASXL1* mutations and the adverse clinical outcomes in MPN, suggesting the potential usefulness of these mutations as a prognostic marker for MPN patients.

What is already known on this topic?

Mutations of *JAK2*, *CALR*, *MPL*, and *ASXL1* are associated with carcinogenesis, clinical characteristics, and prognosis of Ph-negative MPN.

What this study adds?

Prevalence of *JAK2*, *CALR*, *MPL*, and *ASXL1* mutations and association of these mutations with clinical characteristics and prognosis specifically in Thai Ph-negative MPN patients.

Funding disclosure

The present research and publication were supported by the Phramongkutklao College of Medicine.

Conflicts of interest

The authors have no conflicts of interest to declare.

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