RESEARCH ARTICLE

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Study of *CALR, MPL*, and *c-kit* Gene Mutations in Thai Patients with JAK2 V617F Negative Myeloproliferative Neoplasms

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Abstract

Objective: The aim of this study to determine the prevalence of *CALR, MPL* and *c-kit* gene mutations in JAK2 V617F negative-MPN patients. **Methods:** The retrospective study of CALR, MPL and c-kit mutations were analyzed in 113 samples collected from March 2010 to May 2017 and identified as JAK2 V617F–negative MPN Thai patients. The samples were analysis by gel electrophoresis and direct sequencing. **Results:** 28.3% of JAK2 V617F–negative MPN patients showed *CALR* gene mutations. Within the MPN patients with CALR mutation, 46.9% were classified as essential thrombocythemia (ET) and 20.9% were classified as primary myelofibrosis (PMF). Previous studies classified CALR mutations into three types using negatively charged amino acid stretches at the C-terminal domain. Type 1-like mutations were observed in 12 of 49 (24.5%) ET patients and type 2-like mutations were observed in 10 of 49 (20.4%) patients. In addition, 8 of 43 (18.6%) PMF patients showed type 1-like mutations and 1 of 43 (2.3%) showed type 2-like CALR mutation. Interestingly, platelet counts were higher in patients with *CALR* gene mutation than in patients without *CALR* gene mutation. MPL mutations (W515K and W515L) were identified in 2 of 109 (1.8%) MPN patients; the MPL mutations were only found in ET patients, which was consistent with previous studies. We did not detect exon 17 c-kit mutation in JAK2-negative MPN patients but detected intronic single nucleotide polymorphisms at c.74,978 and c.75,255 in these samples. Approximately 66% of patients did not have mutations in *CALR* and *MPL* genes, in addition to lacking *JAK2* gene mutation, and these cases are classified as triple-mutations. **Conclusion:** Our results showed that 66% of cases were triple-negative mutation MPN because they lacked mutations in *JAK2, CALR* and *MPL* genes. The frequencies of CALR and MPL mutation in this study are similar to other CALR and MPL patient data.

Keywords: CALRecticulin (CALR) mutation- myeloproliferative neoplasm (MPN)- Janus kinase 2 (JAK2) mutation

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Introduction

Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) are chronic diseases and characterized by the clonal expansion of differentiated myeloid cells driven by somatic mutations (Dameshek, 1951; Levine and Gilliland, 2008). MPNs include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). These disorders are driven by various gene mutations, including mutations in *JAK2, MPL* and *CALR* genes.

Approximately 50% to 60% of patients with ET and PMF harbor the JAK2 V617F mutation, and this mutation is detected in about 95% of PV patients (Campbell and Green, 2006). Moreover, mutation in JAK2 exon 12 occurs in approximately 3% of PV patients (Campbell et al., 2005), while 5% of ET and PMF patients negative for JAK2 mutation carry myeloproliferative leukemia virus oncogene (MPL) mutation (Scott et al., 2007). Over 50 MPL mutations have been identified, and the most common mutation occurs at W515 in exon 10 (Bennett and Stroncek, 2006; Pardanani et al., 2006; He et al., 2013). MPL is a thrombopoietin receptor that regulates thrombopoiesis and hematopoietic stem cell maintenance (Ihara K et al.,1999). Mutation in the MPL gene provides an additional mechanistic explanation for JAK–STAT

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activation in MPN (Pikman, Y. et al.,2006; Greenfield et al., 2021).

Systemic mastocytosis with an associated hematological neoplasm (SM-AMS) is detected in >90% of patients with myeloid neoplasms including myeloproliferative syndrome (SM-MPS) and myeloproliferative neoplasm (SM-MPN) (Pardanani, 2013). Approximately 90% of patients with SM also harbor mutation in KIT, and the most common mutation occurs at D816 (Gleixner et al., 2011).

A study in 2013 using next-generation sequencing revealed a mutation in exon 9 of the *CALR* gene in many ET or PMF cases negative for JAK2 mutation. Over 36 CALR mutation types have been identified in MPN patients (Klampfl et al., 2013; Nangalia et al., 2013). The majority of CALR mutations are 5 bp insertion and 52 bp deletion. Both types are classified as type I and type II mutations. CALR functions as a chaperone protein and maintains Ca^{2+} homeostasis. The interaction between mutant CALR and MPL protein contributes to the induction of cellular transformation (Araki and Komatsu, 2017). Moreover 7.3% of JAK2 V617F mutation patients were also identified CALR mutation while the major of co-mutation patients represented low alleles burden of JAK2 V617F (< 1% allele) (Makarik et al., 2021). In one report, triple-negative JAK2, CALR and MPL mutations were found in ET and PMF patients. Moreover, the triple-negative mutations were found in younger patients compared with cases with those mutations (Shirane et al., 2015). The overall survival and leukemia-free survival in PMF patients are worse than PMF patients with JAK2, CALR or MPL mutation (Tefferi et al., 2014).

In this study, we determined the frequencies of CALR, MPL and c-kit mutations in MPN patients and compared these results with clinical data from MPN patients without JAK2 V617F mutation at our institution.

Materials and Methods

Patients

Blood and bone marrow samples from 113 MPN patients negative for JAK2 V617F mutation were collected from March 2010 to May 2017. This study was approved by the Ethics Committee of Human Research of Chulabhorn Research Institute (EC No.005/2558). Because of low quality DNA in the collection, CALR exon 9, c-kit exon 17 and MPL exon 10 gene mutations were analyzed form 113, 109 and 90 MPL patients respectively by PCR and direct sequencing at the Cancer Molecular Diagnostics Laboratory, Chulabhorn Hospital, Bangkok, Thailand.

PCR for CALR exon 9, MPL exon 10, and c-kit exon 17

Exon 9 of CALR was amplified by PCR (Applied Biosystems, Thermo Fisher Scientific®, MA USA) using CALR Ex9-F (5′GTAACATCCACCCAGATCACTG3') and CALR Ex9-R (5'GCCAGACATGAGAAAAGGTGG3') primers. PCR reactions were as follows: denaturation at 94˚C for 4 min, followed by 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec, and extension at 72˚C for 5 min. PCR amplification of exon 17 of the *c-kit* gene was performed using nck17-F (5'TGTGAACATCATTCAAGGCG3') and nck17-R (5'TCCTGCTGTGACCTTCAATG3') primers. PCR reactions were as follows: denaturation at 95˚C for 7 min, followed by 30 cycles of 94˚C for 30 sec, 64˚C for 30 sec and 72˚C for 1 min, and extension at 72˚C for 5 min. PCR amplification of exon 10 of *MPL* gene was performed using MPL10-F (5'TAGCCTGGATCTCCTTGGT3') and MPL10-R (5'AGAGGTGACGTGCAGGAAGT3') primers. PCR reactions were as follows: denaturation at 95˚C for 2 min, followed by 36 cycles of 94˚C for 30 sec, 64˚C for 30 sec and 72˚C for 30 sec, and extension 72˚C for 2 min. The PCR products were examined by 2% agarose gel electrophoresis. Direct sequencing of PCR products was performed with the ABITM 3500xL Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific®, MA USA).

Sequencing Analysis

Sequencing results were compared with the wild-type CALR sequence (Accession No.:NG_029662.1), MPL sequence (Accession No.: NG_007525.1) and c-kit sequence (Accession No.: NG_007456.1) from the GenBank database. Multiple sequence alignments of nucleic acids were performed using GenalysWin3 (Metrowerks CodeWarrior). The deduced amino acids were archived using NCBITM open reading frames finder (https://www.ncbi.nlm.nih.gov/orffinder/). Amino acid sequence multiple pairwise alignments were analyzed using EMBL-EBITM Clustal Omega (https://www.ebi.ac.uk/ Tools/msa/clustalo/).

Statistical Analysis

Associations between mutations and demographic data were analyzed by Wilcoxon rank-sum (Mann–Whitney) test or T-test.

Results

Blood and bone marrow samples from 113 JAK2 V617F-negative MPN patients were examined for CALR exon 9, MPL exon 10 and c-kit exon 17 mutations (Table 1). The overall patient group included 49 ET patients, 43 PMF patients and 21 PV patients.

In the overall group of JAK2 V617F–negative MPN patients, 32 cases (28.32%) harbored a *CALR* gene mutation. Among the 43 ET patients, 23 ET patients had *CALR* gene mutations; 12 mutations were deletions and 11 mutations were insertions. Among the PMF patients, we found 9 patients with *CALR* gene mutation, which included 8 patients with deletions and 1 patient with an insertion. No PV patients showed mutation in CALR.

We evaluated the predicted amino acid sequences from the identified *CALR* gene mutations. For 32 PMF patients that have CALR mutation, there are 8 CALR mutation types were found in this study. Four mutation types were categorized as type 1-like mutation group, 3 mutation types were types 2-like mutation group from and 1 mutation type were other type group mutations (Figure 1 and 3). We also found the extra-band show as lane 3 in

Table 1. Clinical Parameters of *JAK2 V617F*-negative *MPN* Patients According to Tumor/Patient Characteristics

Characteristic	PV	ET	PMF	ALL
Number of patients	21	49	43	113
Age at onset, years (average±SD)	55±17	57±18	64±12	59±16
WBC count, x10^9/L (average±SD)	7.6±2.4	14.42±14.01	17.84±24.57	14.5±18.0
PLT count, x10^9/L (average±SD)	222±77	1328±1130	182±214	686±940
Hemoglobin, g/dl (average±SD)	18.7±1.2	10.6±2.6	8.4±2.1	11.3±4.3
Hematocrit, L (average±SD)	56.2±4.1	32.8±7.3	35.9±60.5	38.4±37.6
CALR mutation	0/21 (0%)	23/49 (46.9%)	9/43 (20.9%)	32/113 (28.3%)
MPL mutation	0/20 (0%)	2/47 (4.3%)	0/42 (0%)	2/109 (1.8%)
c-kit mutation				
SNP position c.74978				
+ *c-kit* SNPs G/A	44835	14702	13940	33117
+ *c-kit* SNPs G/G	44843	36/40	35/38	81/90
SNP position c.75255				
+ *c-kit* SNPs T/C	44837	18/40	19/38	41/90
+ *c-kit* SNPs T/T	0/10	14642	13881	32933
+ *c-kit* SNPs C/C	44841	20/40	18/38	46/90

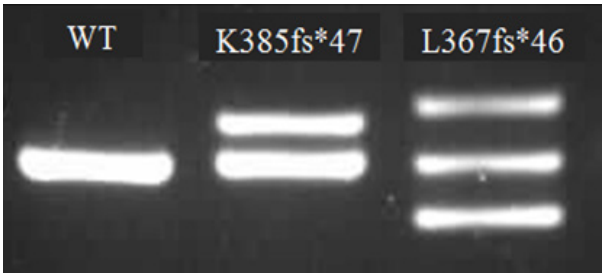


Figure 1. Ethidium Bromide–Stained 2% Agarose Gel for the Results of PCR Analysis of CALR exon 9 mutation by PCR. Lane 1 shows the band for wild-type CALR, lane 2 indicates the mutant with an insertion in CALR exon 9, and lane 3 represents the mutant with a deletion of CALR exon 9

agarose gel of Figure 1, then we analyzed the sample using DNA sequencing. We found that the extra-band contain both 52 bp deletion mutant allele and wild-type allele of *CALR* gene as shown the details in Figure 2.

We found significantly differences in platelet count (Plt) between ET patients with wild-type CALR and ET patients with CALR mutation (808.5x10^9/L vs. 1395x10^9/L, p<0.001) (Table 2). Hemoglobin (Hb), hematocrit (Hct) and white blood cell count (WBC) were similar in the two groups.

We identified two cases (1.83%) with *MPL* gene mutations in the overall 109 MPN patients. Both cases with *MPL* gene mutations were ET patients. The two MPL mutations were W515K and W515L mutations (Figure 4).

DNA sequencing for *c-kit* exon 17 mutation was

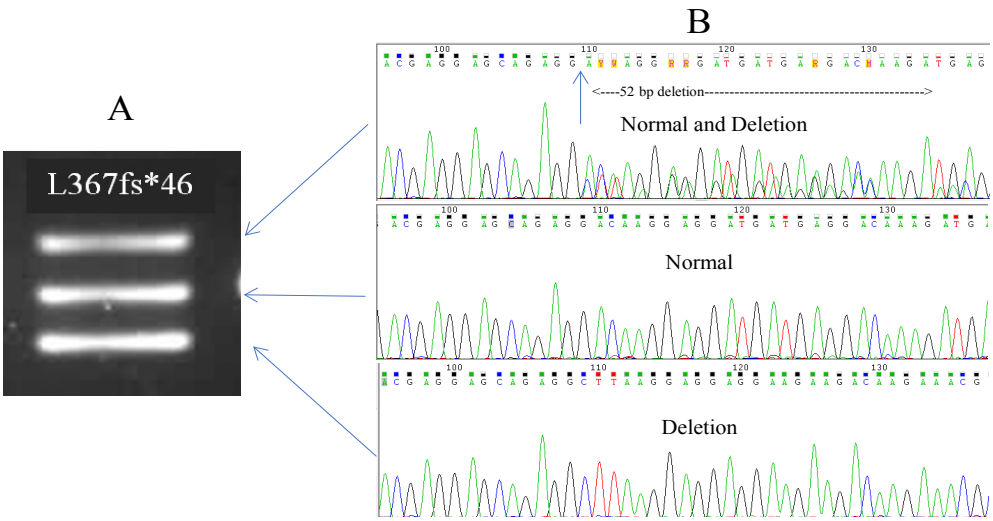


Figure 2. Sequencing Results Demonstrate Mixture of 52 bp Deletion Allele and Wild-Type Allele in Extra-Band of Agarose Gel Analysis. (A) Gel electrophoresis result demonstrate the upper extra-band of mixture wild-type and mutant allele followed by band of normal and mutant allele. (B) Sequencing result demonstrate the heterozygous of 52 bp deletion mutant allele mix with wild type allele of CALR gene. The comparison sequencing result of homozygous normal and mutant allele showed in the lower panel, respectively.

Table 2. Clinical Parameters of ET and AMM/IMF Patients with Wild-Type or Mutated CALR Gene

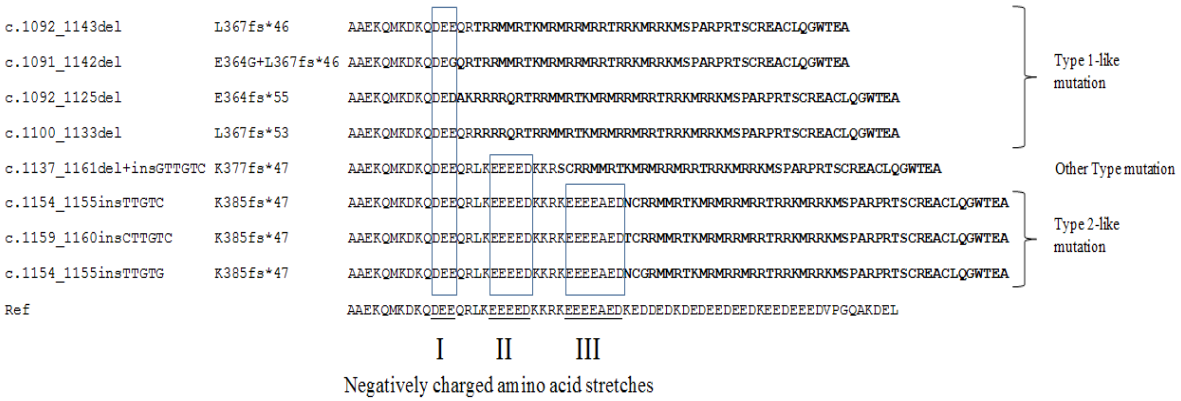
Parameter	ET			PMF		
	CALR WT	CALR Mutant	P-value	CALR WT	CALR Mutant	P-value
Patient no.	26	23	-	34	9	-
Age at onset, years, median (range)	56 (22–81)	66 (26–83)	0.1204[1]	65.5 (33–87)	63 (39–80)	0.3628[2]
WBC count, x10^9/L, median (range)	10.95 (4.3–81.19)	11.25 (5.78–22.1)	0.9920[1]	9.245 (1.45–146)	12.2 (4–24.71)	0.4923[1]
PLT count, x10^9/L, median (range)	808.5 (266–3221)	1395 (462–7500)	0.0002[1]	72.5 (8–883)	273 (71–506)	0.1284[2]
Hemoglobin, g/dL, median (range)	9.45 (6.2–19.5)	10.9 (6.9–14.3)	0.1380[1]	8.1 (3–13.5)	8 (6.3–13.4)	0.6536[2]
Hematocrit, L, median (range)	28.95 (20–56)	33 (22.8–43.5)	0.0904[1]	26.05 (10–407)	26.55 (20.4–41)	0.6602[1]
c-kit mutation SNP position c.74978						
+ *c-kit* SNP G/A	1/22 (4.5%)	3/18 (16.7%)		1/30 (3.3%)	2/8 (25.0%)	
+ *c-kit* SNP G/G	21/22 (95.5%)	15/18 (83.3%)		29/30 (96.7%)	6/8 (75.0%)	
SNP position c.75255						
+ *c-kit* SNs T/C	8/22 (36.4%)	10/18 (55.6%)		13/30 (43.4%)	6/8 (75.0%)	
+ *c-kit* SNP T/T	0/22 (0%)	2/18 (11.1%)		1/30 (3.3%)	0/8 (0%)	
+ *c-kit* SNP C/C	14/22 (63.6%)	6/18 (33.3%)		16/30 (53.3%)	2/8 (25.0%)	

[1], Wilcoxon rank-sum (Mann–Whitney); [2], T-test

performed for 40 ET, 38 PMF and 10 PV patients. We did not detect c-kit exon 17 mutation in any patient. However, we found single nucleotide polymorphisms (SNPs) at intron positions between exon 16 and exon 17 as well as between exon 17 and exon 18: c.74,978 and c.75,255 (Figure 5). For the SNP at c.74,978, the heterozygous G/A allele was found in 8 patients and the homozygous G/G allele was found in 80 patients. For the SNP at c.75,255, the heterozygous T/C allele was found in 40 patients while the homozygous T/T allele was found in 3 patients and the homozygous C/C allele was found in 45 patients. There were no significant differences in Hb, Hct, Plt and WBC between patients of each SNP type (Table 2).

Discussion

There are limit data of JAK2 V617F mutation in Thai patients. Our previous study showed that JAK2 V617F mutation of 186 Thai MPN patients were 49% for PV, 54% for ET and 43% for PMF patients (Bunyoo et al., 2018). The other study showed that JAK2 V617F was detected in 41 from 58 patients (71%) of ET patients (Limsuwanachot et al., 2017). In this study, 28.3% of the overall MPN patient group with no JAK2 V617F mutation carried *CALR* gene mutation. Approximately 46.9% of the ET patient group showed CALR mutation, while 20.9% of PMF patients showed CALR mutation. None of the



DNA change	c.1092_1143del	c.1091_1142del	c.1092_1125del	c.1100_1133del	c.1137_1161del +insGTTGTC	c.1154_1155 insTTGTC	c.1159_1160 insCTTGTC	c.1154_1155 insTTGTG
Amino Acid change	L367fs*46	E364G+L367fs*46	E364fs*55	L367fs*53	K377fs*47	K385fs*47	K385fs*47	K385fs*47
No. of case	17	1	1	1	1	7	2	2

Figure 3. Amino Acid Sequence Alignment of the C-terminal Domain of Wild-Type and CALR exon 9 Mutations. The three groups included type 1-like (I), type 2-like (II) and other type (III). The bold sequences indicate frame shift mutations. The boxes indicated negatively charge amino acid sequences. The table shown number of cases that were found each mutation.

Table 3. The Prevalence of CALR and MPL Mutations Reported in Different Countries

	CALR mutation (%)				MPL mutation (%)				Reference
	MPN	PV	ET	PMF	MPN	PV	ET	PMF	
USA	N/A	N/A	N/A	25	N/A	N/A	N/A	8.3	Tefferi et al.
Italy	N/A	0	24	N/A	N/A	0	4	N/A	Rumi E et al.
Korea	12.6	0	17.7	14.8	3.5	0	2.5	9.3	Kim SY et al.
Vietnam	N/A	N/A	27.6	N/A	N/A	N/A	1	N/A	Vu HA et al.
Japan	N/A	0	19.6	30.4	N/A	0	8	4.4	Shirane S et al.
Thai	28.3	0	46.9	20.9	1.8	0	4.3	0	This study

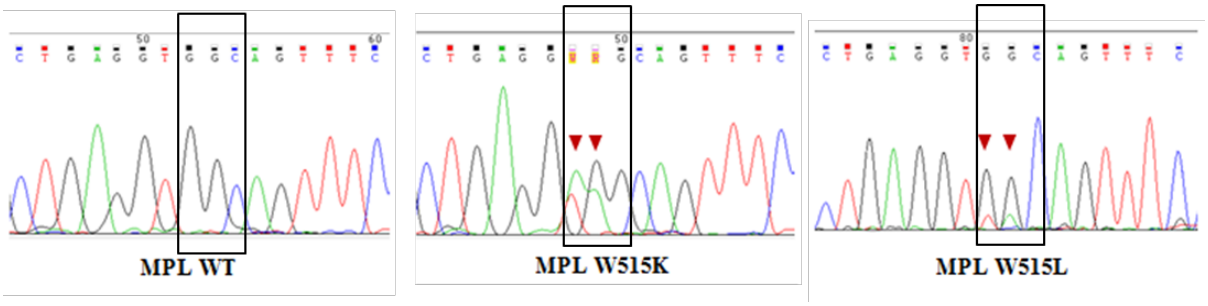


Figure 4. Sequencing Results for MPL Mutations. The red triangles indicate the position of nucleotide substitution leading to a point mutation at codon 515.

PV patients in this study showed CALR mutation. The incidence of CALR mutation in our overall MPN patient group was similar to those reported in other Caucasian and Asian populations (Rumi et al., 2014; Kim et al., 2015; Shirane et al., 2015; Vu et al., 2019) (Table 3).

Pietra et al, (2016). classified the CALR mutations into three types based on the three negatively charged amino acid stretches at the C-terminal domain. Type 1–like CALR mutations group result in the loss of two negatively charged amino acid stretches, while the type 2–like mutations group maintain all three negatively charged amino acid stretches. And the other type group mutations resulted in the loss of one negatively charged amino acid stretch. Type 2–like CALR mutations group are mainly associated with an ET phenotype, a low risk of thrombosis and indolent clinical course, while type 1–like mutations group are mainly associated with a myelofibrosis phenotype and a high risk of progression from ET to myelofibrosis. The PML patients (without JAK2 V617F mutation) with type 1-like CALR mutation

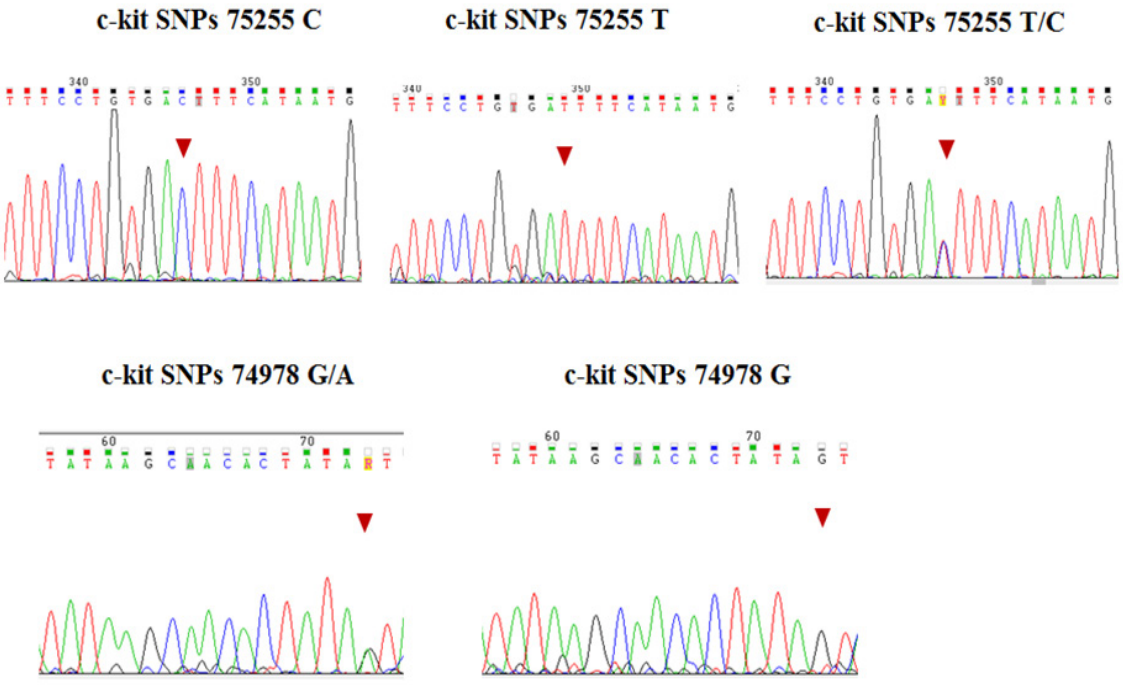


Figure 5. Diagram Show SNPs in *c-kit*. The red triangles indicate positions of nucleotide changes.

were good prognosis than patients with type 2-like CALR mutation. Whereas the prognosis in PML patients (without JAK2 V617F mutation) with type 2-like CALR mutation were not different with PML patients contained only JAK2 V617F mutation (Guglielmelli et al., 2015). MPN patients with CALR mutation treated with Ruxolitinib, a JAK2 inhibitor, showed decreased spleen size and increased overall survival compared with patients treated with the best available therapy (Guglielmelli et al., 2016). Furthermore, Fedratinib, a JAK inhibitor, reduced both spleen size and Plt count in myelofibrosis patients with CALR mutation (Passamonti et al., 2014). In our study, the Plt count of ET patients with CALR mutation was higher than ET patients with wild-type CALR, and these findings were similar to previous reports in other populations (Klampfl et al., 2013; Nangalia et al., 2013; Shirane et al., 2015; Fu et al., 2014) (Table 3).

The *c-kit* gene proto-oncogene encodes a tyrosine kinase that is expressed in mast cells and hematopoietic stem cells (Yarden et al., 1987). The c-kit mutations are found in CML and CMML patients at a prevalence rate of approximately 1%–4%. Almost c-kit mutation in CML and CMML associated with systemic mastocytosis case (Valent et al., 2010). Over 90% of systemic mastocytosis patients carry the c-kit D816V mutation (Nagata et al., 1995; Valent et al., 2017). In this study, we did not find this c-kit mutation at exon 17 in the overall MPN patients. However, we detected SNPs at introns between exon 16 and 17 as well exon 17 and 18. We did not observe any relationship between the intronic variations and hematological parameters including Hb, Hct, Plt and WBC. Because of the limited data of mastocytosis in our study, the result of no c-kit mutation in sample may from MPN patients have no mastocytosis. These data are similar to the recent report showing that c-kit mutations were only found in systemic indolent mastocytosis cases, while no c-kit mutations were observed in aggressive mastocytosis and myelodysplastic syndrome patients (Fritsche-Polanz et al., 2001).

MPN patients are identified MPL mutations approximately 1% to 8% of patients. Almost MPL mutation found in ET patients whereas this mutation rarely found in PMF patients. The majority of MPL mutations are W515L/K (Kim et al., 2015; Shirane et al., 2015; Shams et al., 2018). These findings are consistent with our results showing *MPL* gene mutations in two ET patients (W515K and W515L). Because of the small numbers of MPL mutant cases, we were unable to compare hematological parameters between wild-type and mutant. In addition, there are previous studies reported CALR mutation patients showed higher Plt count than patients who carry MPL mutation (Kim et al., 2015; Shirane et al., 2015; Shams et al., 2018; Tefferi et al., 2014). In conclusion, 70% of the overall MPN patients, which were negative for *JAK2* gene mutation, did not have *CALR* and *MPL* gene mutations; this patient group can be classified as triple-negative (TN) mutation MPN cases. Next-generation sequencing has been used to identify other gene mutations in triple-negative MPN patients. Whole-exome sequencing in TN MPN cases identified other mutations, including MPL T119I, S204F, E230G, P453R, R537W, S505N, R537W, and Y591D and germ-line mutations of MPL and JAK2 including MPL S204P, V285E and R321W and JAK2 F556V and V625F (Milosevic et al., 2016). Interestingly, the JAK2 V617F mutation was detected in a TN ET patient by next-generation sequencing (Alimam et al., 2021), suggesting that highly sensitive methods may be necessary for the diagnosis of low-level mutations in MPN patients. A JAK1/2-inhibitor, Ruxolitinib (RUX), was used for MPN patients with JAK2 V617F or CALR mutation. But the overexpress of MPL leads to RUX-resistance in CALR mutation cell line (Yasuda et al., 2022). The vaccine called "CALRLong36" contained CALR exon 9 mutant peptides, was tested in CALR mutation MPN patients. The result showed that the vaccine could activate the immune respond in PV patients but it could not activate in PMF patients (Handlos et al., 2021). According to previous data shown by various studies that CALR and MPL mutations were related to the response of treatment and prognosis of the patients. Although, there are low prevalence of CALR exon 9, MPL exon 10 mutations, the analysis of these mutation is still necessary for diagnosis and treatment in Thai patients.

Author Contribution Statement

The authors confirm contribution to the paper as follows: Miss Supanee Nimsanor, Mr. Nithiphut Tantirukdham, Mr. Jin Tongsom; help with MPN sample processing: Mr. Chakrit Bunyoo; MPL gene mutation analysis: Miss Kamonwan Soonklang; statistical analysis: The authors thank Prof. Dr Chirayu Auewarakul, Dr. Narongrit Sritana; advised in reviewing the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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