PREVALENCE OF *JAK2V617F* AND ITS CLINICAL CORRELATION IN THAIS WITH MYELOPROLIFERATIVE NEOPLASM

Mr. Namo Suksomyos

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ความชุกและความสัมพันธ์ทางคลินิกของ *JAK2V617F* ในผู้ป่วยกลุ่มโรคไขกระดูกสร้างเม็ดเลือดมากผิดปกติชาวไทย

นายนะโม สุขสมยศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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By	Mr. Namo Suksomyos
Field of Study	Clinical Hematology Sciences
Thesis Advisor	Supantitra Chanprasert, Ph.D.

Accepted by the Faculty of Allied Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Faculty of Allied Health Sciences

(Assistant Professor Vanida Nopponpunth, Ph.D.)

THESIS COMMITTEE

…….. Chairman

(Assistant Professor Tewin Tencomnao, Ph.D.)

…….. Thesis Advisor

(Supantitra Chanprasert, Ph.D.)

…….. Examiner

(Sirikalaya Brimson, Ph.D.)

…….. External Examiner

(Moltira Promkan, Ph.D.)

นะโม สุขสมยศ: ความชุกและความสัมพันธ์ทางคลินิกของ *JAK2V617F* ในผู้ป่วยกลุ่มโรคไขกระดูกสร้างเม็ดเลือดมากผิดปกติชาวไทย (PREVALENCE OF *JAK2V617F* AND ITS CLINICAL CORRELATION IN THAIS WITH MYELOPROLIFERATIVE NEOPLASM) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ดร.สุพันธิตรา ชาญประเสริฐ, 101 หน้า.

กลุ่มโรคไขกระดูกผลิตเม็ดเลือดมากผิดปกติ (MPN) ประกอบด้วยโรคมะเร็งแบบเรื้อรังชนิดต่างๆ ของเซลล์มัยอีลอยด์ ข้อกำหนดองค์การอนามัยโลกปี 2008 ระบุให้การกลายพันธุ์ชนิด *JAK2V617F* เป็นหนึ่งในเกณฑ์สำหรับวินิจฉัยโรคในกลุ่มดังกล่าว โดยเฉพาะกลุ่มโรคที่ไม่พบฟิลาเดลเฟียโครโมโซม (Ph- MPN) รายงานความชุกของการกลายพันธุ์ชนิดนี้มีความแตกต่างกันไปตามประชากรที่ศึกษา และไม่พบว่าเคยมีการศึกษาในประชากรไทย การศึกษานี้มีวัตถุประสงค์เพื่อสำรวจความชุกของการกลายพันธุ์ชนิด *JAK2V617F* ในผู้ป่วย MPN ชาวไทย และตรวจหาความสัมพันธ์ของการกลายพันธุ์ชนิดนี้ต่ออาการทางคลินิก ผู้วิจัยรวบรวมข้อมูลจากผู้ป่วย 103 ราย จำแนกเป็นผู้ป่วย Ph- MPN 96 ราย และผู้ป่วยโรคมะเร็งเม็ดเลือดขาวเรื้อรัง (CML) 7 ราย ผู้วิจัยได้เก็บตัวอย่างเลือดและนำไปสกัดแยกสารพันธุกรรมเพื่อตรวจหาการกลายพันธุ์ด้วยวิธี AS-PCR และ PCR-RFLP จากนั้นนำผลมาประกอบกับผลตรวจทางคลินิกในขั้นตอนการวิเคราะห์ จากการศึกษาพบว่าความชุกของ *JAK2V617F* ในผู้ป่วย Ph- MPN เท่ากับร้อยละ 68.8 (66/96) จำแนกเป็นร้อยละ 59.2 (29/49) ในผู้ป่วยโรคเกล็ดเลือดสูงโดยไม่ทราบสาเหตุ (ET) ร้อยละ 80.6 (25/31) ในผู้ป่วยโรคเม็ดเลือดแดงสูงโดยไม่ทราบสาเหตุ (PV) ร้อยละ 70.0 (7/10) ในผู้ป่วยโรคไขกระดูกฝ่อแบบปฐมภูมิ (PMF) และร้อยละ 83.3 (5/6) ในผู้ป่วยโรคไขกระดูกผลิตเม็ดเลือดมากผิดปกติที่ไม่สามารถระบุชนิดได้ (unclassifiable MPN) ในผู้ป่วย CML ไม่พบว่ามีการกลายพันธุ์ดังกล่าว (0/7) ผู้ป่วยที่มีการกลายพันธุ์มีประวัติ bleeding และ MCV น้อยกว่ากลุ่มที่ไม่กลายพันธุ์ (P = 0.039 และ = 0.006) ผู้วิจัยไม่พบความแตกต่างทางคลินิกระหว่างผู้ป่วย ET ทั้งสองกลุ่ม ในผู้ป่วย PV พบว่ากลุ่มที่มีการกลายพันธุ์มีระดับฮีโมโกลบินต่ำกว่า (P = 0.038) และมีจำนวนเกล็ดเลือดสูงกว่ากลุ่มไม่กลายพันธุ์ (P= 0.014) โดยสรุป การกลายพันธุ์ชนิด *JAK2V617F* สามารถพบได้ในผู้ป่วย MPN ชาวไทยและมีความชุกใกล้เคียงกับประชากรเชื้อชาติอื่นๆ ผู้ป่วยที่มีการกลายพันธุ์มีความแตกต่างทางคลินิกจากผู้ป่วยที่ไม่กลายพันธุ์บ่งชี้ถึงความสัมพันธ์ต่ออาการทางคลินิกของการกลายพันธุ์ดังกล่าว อย่างไรก็ดีปัจจัยอื่นๆเช่น สภาวะของหลอดเลือดและโมเลกุลต่างๆในวิถีการส่งสัญญาณภายในเซลล์อาจร่วมส่งผลต่อพยาธิสรีรวิทยาของโรคด้วยเช่นกัน

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สาขาวิชา วิทยาศาสตร์โลหิตวิทยาคลินิก ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS : *JAK2V617F*/ ESSENTAIL THROMBOCYTHAEMIA/ POLYCYTHAEMIA VERA/ BLEEDING & THROMBOSIS

NAMO SUKSOMYOS: PREVALEVCE OF *JAK2V617F* AND ITS CLINICAL CORRELATION IN THAIS WITH MYELOPROLIFERATIVE NEOPLASM. ADVISOR: SUPANTITRA CHANPRASERT, Ph.D., 101pp.

Myeloproliferative neoplasm (MPN) is a group of various chronic myeloid cancers. According to the 2008 WHO recommendation, *JAK2V617F* mutation is a major diagnostic criterion for these diseases, particularly the Philadelphia chromosome-negative MPN (Ph- MPN). Prevalence of such mutation, however, has been reported differently in numbers based on a study population; never before in Thais. This study aims to explore the prevalence of *JAK2V617F* mutation in Thai MPN patients and examine its correlation to clinical features. 103 individuals diagnosed MPN after the 2008 WHO recommendation were recruited: 96 Philadelphia-negative MPN and 7 CML. Blood samples were taken for DNA isolation. *JAK2V617F* detection was examined using AS-PCR and PCR-RFLP. Clinical data at the diagnosed date were matched to the molecular outcome. In the Ph- MPN population, prevalence of *JAK2V617F* was 68.8 % (66/96): 59.2% (29/49) in ET, 80.6% (25/31) in PV, 70.0% (7/10) in PMF, and 83.3% (5/6) in unclassifiable MPN. None of CML patients exhibited the mutation (0/7). The mutant group had less bleeding history (P = 0.039) and MCV than the wild type (P = 0.006). No difference of all parameters between both groups showed in ET, except slightly higher white cells and platelets level of the mutant than the wild type without significance. In PV, the haemoglobin level of the mutant was lower than the wild type (P = 0.038) while the platelet level was higher (P= 0.014). In conclusion, *JAK2V617F* does exist in Thai MPN patients. Its prevalence is close to other populations with ethnical difference. The mutant displayed some difference in parameters to the wild type, referring to clinical contribution of the mutation. Nevertheless, other factors can not be overlooked. Vascular circumstances and other intracellular signal transduction molecules could altogether participate in pathophysiology.

Department Clinical Microscopy Student's Signature .....

Field of Study Clinical Hematology Sciences Advisor's Signature .....

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LIST OF ABBREVIATIONS

°C	=	Degree Celsius
µL	=	Microlitre
µM	=	Micromolar
AML	=	Acute myeloid leukaemia
AMM	=	Agnogenic myeloid metaplasia
AS-PCR	=	Allele specific-polymerase chain reaction
bp	=	Base pair
cm	=	Centimetre
CML	=	Chronic myelogenous leukaemia
dL	=	Decilitre
dNTP	=	Deoxynucleiotide triphosphate
DIC	=	Disseminated intravascular coagulation
DNA	=	Deoxyribonucleic acid
EBV	=	Ebstein-Barr virus
ECP	=	European Clinical & Pathological
EDTA	=	Ethyldyldiamine tetraacitate
EPO	=	Erythropoietin
EPO-R	=	Erythropoietin receptor
ET	=	Essential thrombocythaemia
FERM	=	Band 4.1-erazin-radixin-moesin
GM-CSF-R	=	Granulocyte-macrophage colony stimulating factor receptor
g	=	Gramme
Hb	=	Haemoglobin
Hct	=	Haematocrit
HHV	=	Human herpesvirus

HSCs	=	Haematopoietic stem cells
IL	=	Interleukin
IMF	=	Idiophathic myelofibrosis
INF	=	Interferon
JAB	=	JAK binding protein
JAK	=	Janus kinase
JH	=	Janus kinase homology
L	=	Litre
LAP	=	Leukocyte alkaline phosphatase
min	=	Minute(s)
mm	=	Millimetre
mL	=	Millilitre
mM	=	Millimolar
MALT	=	Mucoid associated lymphoid tissue
MCV	=	Mean corpuscular volume
MDS	=	Myelodysplatic syndrome
MPD	=	Myeloproliferative disorder
MPN	=	Myeloproliferative neoplasm
MPN un	=	Unclassifiable MPN
ng	=	Nanogramme
NK	=	Natural killer
PCR	=	Polymerase chain reaction
Ph+	=	Philadelphia chromosome positive
Ph-	=	Philadelphia chromosome negative
PLT	=	Platelet(s)
PMF	=	Primary myelofibrosis
PRV	=	Polycythaemia rubra vera
PV	=	Polycythaemia vera

PVSG	=	Polycythaemia vera study group
rpm	=	Round per minute
RBC	=	Red blood cell
RFLP	=	Restriction fragment length polymorphism
RNA	=	Ribonucleic acid
sAML	=	Secondary acute myeloid leukaemia
sec	=	second(s)
SH2	=	Src-homology 2
SHP	=	SH2-containing phosphatase
S.E.	=	Standard error of mean
SOCS	=	Suppressor of cytokine signalling
SSI	=	STAT-induced STAT inhibitor
STAT	=	Signal transducer and activator of transcription
TNF-α	=	Tumour necrotic factor-α
TYK	=	Tyrosine kinase
vWD	=	von Willebrand disease
vWF	=	von Willebrand factor
V	=	Volt
w/v	=	Weight/Volume
WBC	=	White blood cell
WHO	=	World Health Organization

CHAPTER I

INTRODUCTION

Background

Haematopoiesis is the maturating process of blood corpuscles from a very naïve to a fully grown form ready to function. It situates in varied organs depending on ages: originating at yolk sack in embryos, switching to long and flat bones in newborns after that, and settling permanently in bone marrow. Unfortunately, some problem affecting cell production, such as blood cancers, could occur. Categorising after their severity, they are the acute with blast cells in massive number and the chronic with more mature cells in slightly lower amount. Those cells are classified after their origin into either myeloid or lymphoid.

According to the World Health Organization 2008 classification, the chronic cancers of the myeloid could be categorised into 4 clusters; one of them called myeloprolierative neoplasm (MPN). Formerly known as myeloproliferative disorders (MPD), MPN pictures excessive cells production in one lineage or over. The overproduced disturbs affects normal haemostasis causing vascular complications, such as bleeding and thrombosis. These could be life-threatening if occur in certain vital organs. Moreover, the disease could transform itself into either secondary acute leukaemia or myelofibrosis unfavourable for treatment after a long-term chronic phase.

Studies in molecular perspective have revealed several mutations of the intracellular signalling molecule, eminently *JAK2V617F*. Due to the molecule is a part of the intracellular signal transduction, substitution of amino acid in certain codon shifts away its regular function. There is suggestion of clinical contribution of the mutation: to levels of haematological parameters, bleeding and thrombosis, for instances. Currently, the mutation has been considered to be a major criterion in MPN diagnosing recommended by the WHO since 2008. Its prevalence has been reported

continuously, but varied according to a study population. However, the information was collected in Americans and Europeans as majority. Few studies in Asians were conducted, never before in Thais.

This study aimed to extend this knowledge into Thai MPN population by exploring *JAK2V617F* prevalence and investigating its clinical correlation. This would be an evidence to ensure clinical implication of the mutation in Thailand.

Objectives

1. To report the *JAK2V617F* prevalence in Thais with myeloproliferative neoplasm
2. To reveal clinical correlation of *JAK2V617F* in Thais with myeloproliferative neoplasm

Limitations

1. The mutation detection was conducted by allele specific-polymerase chain reaction and polymerase chain reaction-restriction length polymorphism
2. The clinical correlation of the mutation was analysed by statistical comparison between the mutant and the wild type

Terminology

A. Myeloproliferative neoplasm (MPN)

A group of bone marrow neoplasms

B. Chronic myelogenous leukaemia (CML)

A myeloproliferative neoplasm with appearance of Philadelphia chromosome as typical

C. Philadelphia chromosome negative MPN (Ph- MPN)

Myeloproliferative neoplasms with no evidence of Philadelphia chromosome

D. Essential thrombocythaemia (ET)

A myeloproliferative neoplasm compatible to the 2008 WHO ET diagnostic criteria

E. Polycythaemia vera (PV)

A myeloproliferative neoplasm compatible to the 2008 WHO PV diagnostic criteria

F. Primary myelofibrosis (PMF)

A myeloproliferative neoplasm compatible to the 2008 WHO PMF diagnostic criteria

G. Unclassifiable myeloproliferative neoplasm (MPN un)

A myeloproliferative neoplasm incompatible to the 2008 WHO diagnostic criteria for PV, ET nor PMF

H. Mutant, the

Those without evidence of *JAK2V617F* examined by allele specific-polymerase chain reaction and polymerase chain reaction-restriction fragment length polymorphism

I. Wild type, the

Those with evidence of *JAK2V617F* examined by allele specific-polymerase chain reaction and polymerase chain reaction-restriction fragment length polymorphism

Benefits

1. As a clinical reference for myeloproliferative neoplasm care and treatment in Thailand
2. As a reference for development of *JAK2V617F* examination and for future studies in related field

CHAPTER II

LITERATURE REVIEW

In this chapter, three core issues in this study are reviewed. The first part provides knowledge on myeloproliferative neoplasm in general perspective and also in detail for each specific disease. The second part on Janus kinase describes its biochemistry and *JAK2V617F* mutation. The final belongs to bleeding & thrombosis. Haemostasis and the complication related theory are explained.

Myeloproliferative Neoplasm

Myeloproliferative neoplasm (MPN) is a group of chronic bone marrow dysfunction resulting overproduction of cells in myeloid linage. It is renamed from the former Myeloproliferative disorder (MPD) after the 2008 World Health Organization classification of haematopoietic and lymphoid neoplasm as shown in **Table 1**. The disease has clinical similarity to another myeloid neoplasm, Myelodysplastic/ Myeloproliferative disease (MDS/MPD), in increase of marrow cellularity and fibrosis, but differently in absence of marrow dysplastic sign. MPN can be subcategorised roughly into Philadelphia chromosome positive (Ph+), particularly typical chronic myelogenous leukaemia (CML), and Philadelphia chromosome negative (Ph-) which is the focus of this study. Each Ph- MPN could be distinguished on the basis of cells with problem.

Generally, the overproduced cells disturb normal haemostasis causing vascular conditions, such as bleeding and thrombosis, as consequences. These evidences present in various life threatening complications, i.e., alimentary bleeding, deep vain thrombosis, Budd-Chiari syndrome, cerebral vein thrombosis, cerebral infraction, myocardial infraction, etc. (Thiele, 2009). Moreover, long termed chronic phase could enter either myelofibrosis or secondary acute myeloid leukaemia. Patients in these

phases have poor survival rate and depend on symptomatic treatment. They also risk opportunistic infection and adverse effect from treatment.

Table 1 The 2008 WHO classification of haematopoietic and lymphoid neoplasm (Arber and Couser, 2009)

Myeloproliferative neoplasm
Chronic myelogenous leukaemia, *BCR-ABL1*
Chronic neutrophilic leukaemia
Polycythaemia vera
Primary myelofibrosis
Essential thrombocythaemia
Chronic eosinophilic leukaemia, not otherwise categorised
Mast cell disease
Myeloproliferative neoplasm, unclassifiable
Myeloid neoplasm associated with abnormalities of *PDGFRA, PDGFRB,* or *FGFR1*
Myelodysplastic/Myeloproliferative disease
Chronic myelomonocytic leukaemia
Atypical chronic myelogenous leukaemia
Juvenile myelomonocytic leukaemia
Myelodysplastic/myeloproliferative disease ,unclassifiable
Myelodysplastic syndrome
Refractory anaemia with uniliniage dysplasia
Refractory anaemia with ringed sideroblasts
Refractory cytopaenia with multilineage dysplasia
Refractory anaemia with excess blasts
Myelodysplastic syndrome associated with isolated del(5q)
Myelodysplastic syndrome, unclassifiable
Acute myeloid leukaemia

Mature B-cell neoplasm (continued)
Extranodal marginal zone B-cell lymphoma (MALT lymphoma)
Nodal marginal zone B-cell lymphoma
Follicular lymphoma
Primary cutaneous follicle centre lymphoma
Mentle cell lymphoma
Diffuse large B-cell lymphoma (DLBCL)
Primary mediastinal (thymic) large B-cell lymphoma
T-cell/histiocyte-rich large B-cell lymphoma
Intravascular large B-cell lymphoma
Primary DLBCL of the central nervous system
Primary cutaneous DLBL, leg type
ALK+ DLBCL
Plasmablastic lymphoma, oral cavity type
DLBCL associated with chronic inflammation
Primary effusion lymphoma
Lymphoma associated with HHV8-associated multicentric Castleman disease
Burkitt lymphoma (BL)
B-cell lymphoma with features intermediate between DLBCL and BL
B-cell lymphoma with features intermediate between DLBCL and classic Hodgkin lymphoma
Lymphomatoid granulomatosis
B-cell posttransplant lymphoproliferative disorder
Other/non-transplant-associated iatrogenic immunodeficiency-associated B-cell lymphoproliferative disorder
Age-related EBV-positive lymphoproliferative

AML with recurrent genetic abnormalities

AML with t(8;21)(q22;q22)(*RUNX1-RUNX1T1*)

AML with inv(16)(p13q22) or t(16,16)(p13;22)(*CBFB-MYH11*)

Acute myeloid leukaemia (continued)

Acute promyelocytic leukaemia with t(15;17)(q22;q11-2)(*PML-RARA*)

AML with t(9;11)(p22;q23)(*MLLT3-MLL*)

AML with t(6;9)(p23;q34)(*DEK-NUP214*)

AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2)(*RPN1-EVI1*)

AML (megakaryoblastic) with t(1;22)(p13;q13)(*RBM15-MKL1*)

Provisional entity: AML with mutated *NPM1*

Provisional entity: AML with mutated *CEBPA*

AML with myelodyaplasia-related changes

Therapy-related AML, myelodysplastic syndrome, and myelodysplastic/myeloproliferative neoplasm

AML not otherwise specified

AML minimally differentiated

AML without maturation

AML with maturation

Acute myelomonocytic leukaemia

Acute monoblastic and monocytic leukaemia

Acute erythroid/myeloid leukaemia and pure erythroid leukaemia

Acute megakaryocytic leukaemia

Acute basophilic leukaemia

Acute panmyelosis with myelofibrosis

Myeloid sarcoma

Acute leukaemia of ambiguous lineage

Precursor B-cell neoplasm

Precursor B-lymphoblastic leukaemia/lymphoma disorder

Mature (peripheral) T-cell neoplasm

T-cell prolymphocytic leukaemia

Mature (peripheral) T-cell neoplasm (Continued)

T-cell large granular lymphocytic leukaemia

Indolent large granular NK-cell lymphoproliferative disorder

Aggressive NK-cell leukaemia

Fulminant EBV-positive T-cell lymphoproliferative disorder of childhood

Adult T-cell lymphoma/leukaemia

Extranodal NK/T-cell lymphoma

Enteropathy-type T-cell lymphoma

Hepatosplenic T-cell lymphoma

Subcutaneous panniculitis- like T-cell lymphoma

Mycosis fungoides/Sezary syndrome

Primary cutaneous aggressive epidermortropic CD8+ cytotoxic T-cell lymphoma

Cutaneous $\gamma\delta$ T-cell lymphoma

Provision entity: Primary cutaneous small/medium CD4+ T-cell lymphoma

Peripheral T-cell lymphoma, unspecified

Angioimmunoblastic T-cell lymphoma

Anaplastic large cell lymphoma, *ALK*+

Anaplastic large cell lymphoma, *ALK*-

T-cell lymphoproliferative disorder of variable malignant potential

Cutaneous CD30+ lymphoproliferative disorder

T-cell posttransplant lymphoproliferative disorder

Hodgkin lymphoma

Nodular lymphocyte-predominant Hodgkin lymphoma

Precursor T-lymphoblastic leukaemia/lymphoma	Classic Hodgkin lymphoma
	Nodular sclerosis Hodgkin lymphoma
	Mixed cellularity Hodgkin lymphoma
Mature B –cell neoplasm	**Hodgkin lymphoma (continued)**
Chronic lymphocytic leukaemia/small lymphocytic leukaemia	Lymphocyte-rich classic Hodgkin lymphoma
	Lymphocyte-depleted Hodgkin lymphoma
B-cell prolymphocytic leukaemia	
Lymphoplasmacytic lymphoma	**Histiocytic and dendritic cell neoplasm**
Splenic marginal zone lymphoma	Histiocytic sarcoma
Hairy cell leukaemia	Langerhans cell histiocytosis/sarcoma
Plasma cell myeloma	Interdigitating dendritic cell sarcoma/tumour
Plasmacytoma	Follicular dendritic cell sarcoma/tumour
Heavy- and light- chain deposition disease	Dendritic cell sarcoma, not otherwise specified
Heavy-chain disease	

AML, acute myeloid leukaemia; EBV, Ebstein-Barr virus; HHV8, human herpesvirus-8; MALT, mucoid associated lymphoid tissue; NK, natural killer.

A. Polycythaemia vera

Also called 'Polycythaemia rubra vera', Polycythaemia vera (PV) was described first in 1892 by Vaquez as persistent polythaemia distinctive from relative and transient form. In 1900s, Osler and Turk clarified its clinical picture as increase of immature red blood cells and leukocytes depicted bone marrow hyperplasia (Means Jr, 2009). Dameshek grouped the disease into MPD after that. Its original diagnostic criteria were proposed by Polycythaemia Vera Study Group (PVSG) in 1967 and had been revised after discover of JAK2 mutation into the current 2008 WHO's.

According to the 2008 WHO diagnostic criteria, patients must meet at least one from two major criteria and two supportive minor criteria. They should present red cells volume reflected on the haemoglobin level which is over 18.5 g/dL in men and 16.5 g/dL in women. Reactive erythrocytosis must be ruled out. Serum erythropoietin (EPO) level should be in a normal range to affirm no involvement of the growth factor to the increased cells. Presence of JAK2 mutation either *JAK2V617F* or *JAK2 exon 12* mutations is preferred as a lesion in the molecule dealing with cell proliferation directly. Bone marrow histopathology is a confirmative but unnecessary document. It should exhibit hypercellularity in trilineage with remarkable erythorid cells. Increased myeloid cells and megakaryocytes show occasionally. Myelofibrosis could be observed in relevancy to disease progression. For *in vitro* culture of the marrow cells, the erythriod series must be capable in forming colony endogenously with no requirement of cytokines or growth factors. Another diagnostic recommendation available is the European Clinical & Pathological (ECP) criteria. It embraces pathological finding to clinical symptoms becoming an effective tool in defining stage of the disease. The additional criteria over the WHO's includes palpable splenomegaly, thrombocytosis, thrombosis, pruritus, etc.

Table 2 The 2008 WHO diagnostic criteria for PV (Landolfi, Nicolazzi et al. 2010)

Diagnosis requires meeting of both major criteria and one minor criterion or the first major criterion and two minor criteria
Major criteria
Haemoglobin > 18.5 g/dL in men, 16.5 g/dL in women or other evidence of increased red cell volume [a]
Presence of *JAK2V617F* or other functionally similar mutation such as *JAK2 exon 12* mutations
Minor criteria
Bone marrow biopsy showing hypercellularity for age with thilineage growth (panmyelosis) with prominent erythriod, granulocytic, and megakaryocytic proliferation
Serum erythropoietin level below the reference range for normal
Endogenous erythroid colony formation *in vitro*

[a] Haemoglobin or haematocrit > 99th percentile of method-specific reference range for age, sex, and altitude of residence; or haemoglobin > 17 g/dL in men, 15 g/dL in women if associated with a documented and sustained increase of at less 2 g/dL from an individual's baseline value that cannot be attributed to correlation of iron deficiency; or elevated red cell mass > 25% above mean normal predicted value

Table 3 The European Clinical & Pathological diagnostic criteria for PV (Means Jr, 2009)

Pathologic criteria

- P1. Marrow morphology
- P2. Bone marrow endogenous erythroid colony formation
- P3. *JAK2V617F* mutation

Clinical criteria

- C1. Increased red cell masses
- C2. Haematocrit 0.45-0.51 in male, 0.43-0.46 in female
- C3. Low serum erythropoietin
- C4. Persistent thrombocytosis
  - Grade 1: 400 – 1,500 x 10^3 platelets /μL
  - Grade II: over 1,500 x 10^3 platelets/μL
- C5. Palpable splenomegaly or radiologic splenomegaly over 12 cm.
- C6. Granulocytosis over 10 x 10^3 cells/μL; or leukocytosis over 12 x 10^3 cells/μL; or increased LAP score; or increased *PRV-1* expression, in absence of fever or infection
- C7. Platelet-mediated microvascular disease or thrombosis
- C8. Typical PV signs/symptoms of hypervolaemia
- C9. Pruritus, fatigue, upper abdominal discomfort
- C10. Absence of secondary erythrocytosis

Diagnosis

- P1 + P2 + P3 = PV
- P1 + P2 + P3 + C1= Overt PV
- P1 + P2 + P3 + C2 + any(C3-C10) = Early PV

LAP, leukocyte alkaline phosphatase, PRV, polycythaemia rubra vera

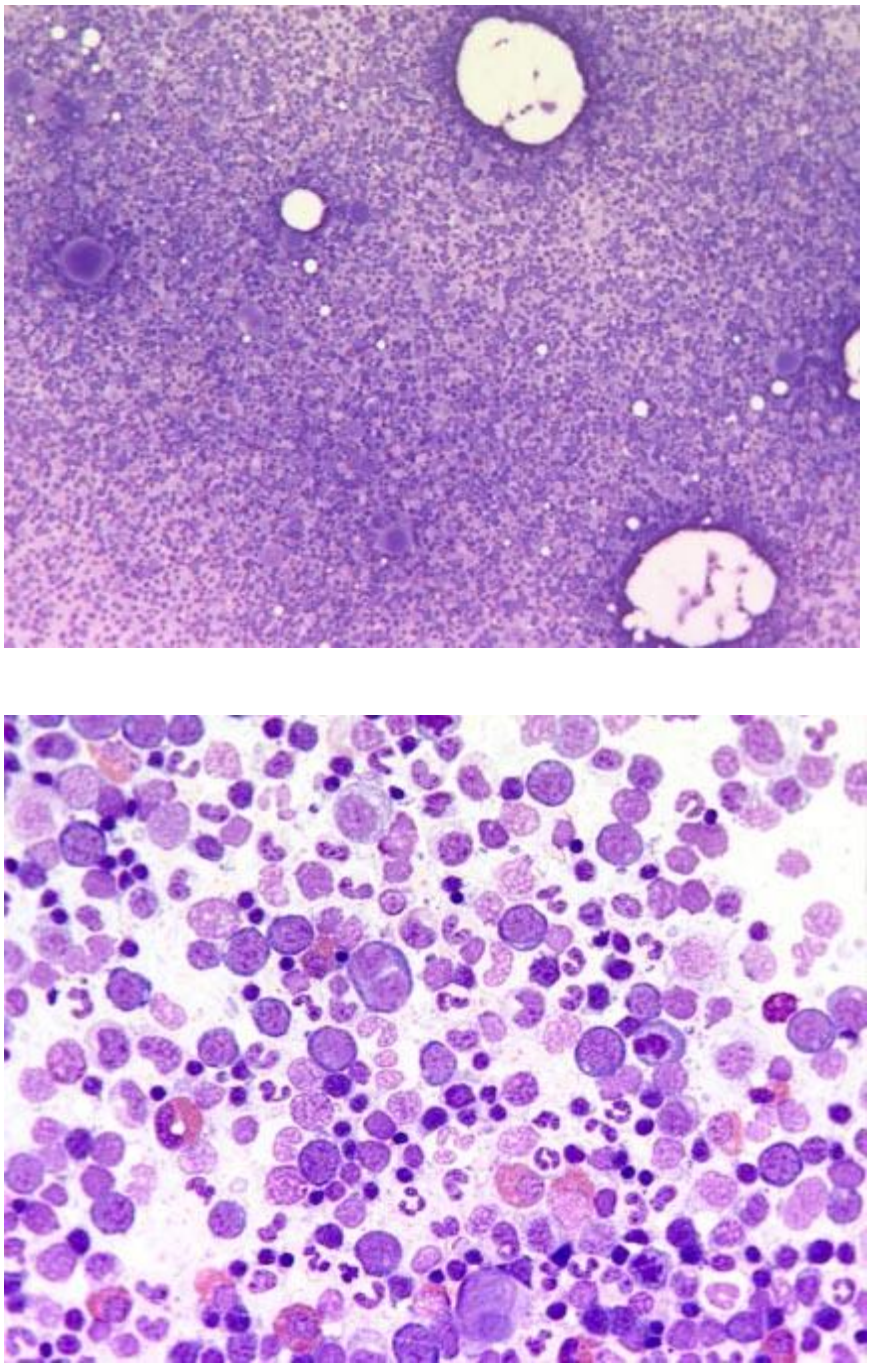


Figure 1 Bone marrow of PV shows hypercellularity, eminently erythroid series (Besa, 2012)

PV patients usually have few clinical symptoms in less severity. They often complain of non-specific discomforts: headache, sweating and pruritus after bathing. The mostly concerned complications could be life-threatening are bleeding and thrombosis. On the skin and mucous membrane, they would appear in echymoses and purpura. Several studies also reported bleeding tendency in gastrointestinal system of the PV than the healthy. The evidence, moreover, affects nervous system resulting neurological problems caused by occlusion of cerebral vessels. The clot also disrupts normal function of other systems, such as cardiovascular and respiratory, and could be fatal as well. Progression of PV into either acute leukaemia or myelofibrosis could happen after years of treatment.

B. Essential Thrombocythaemia

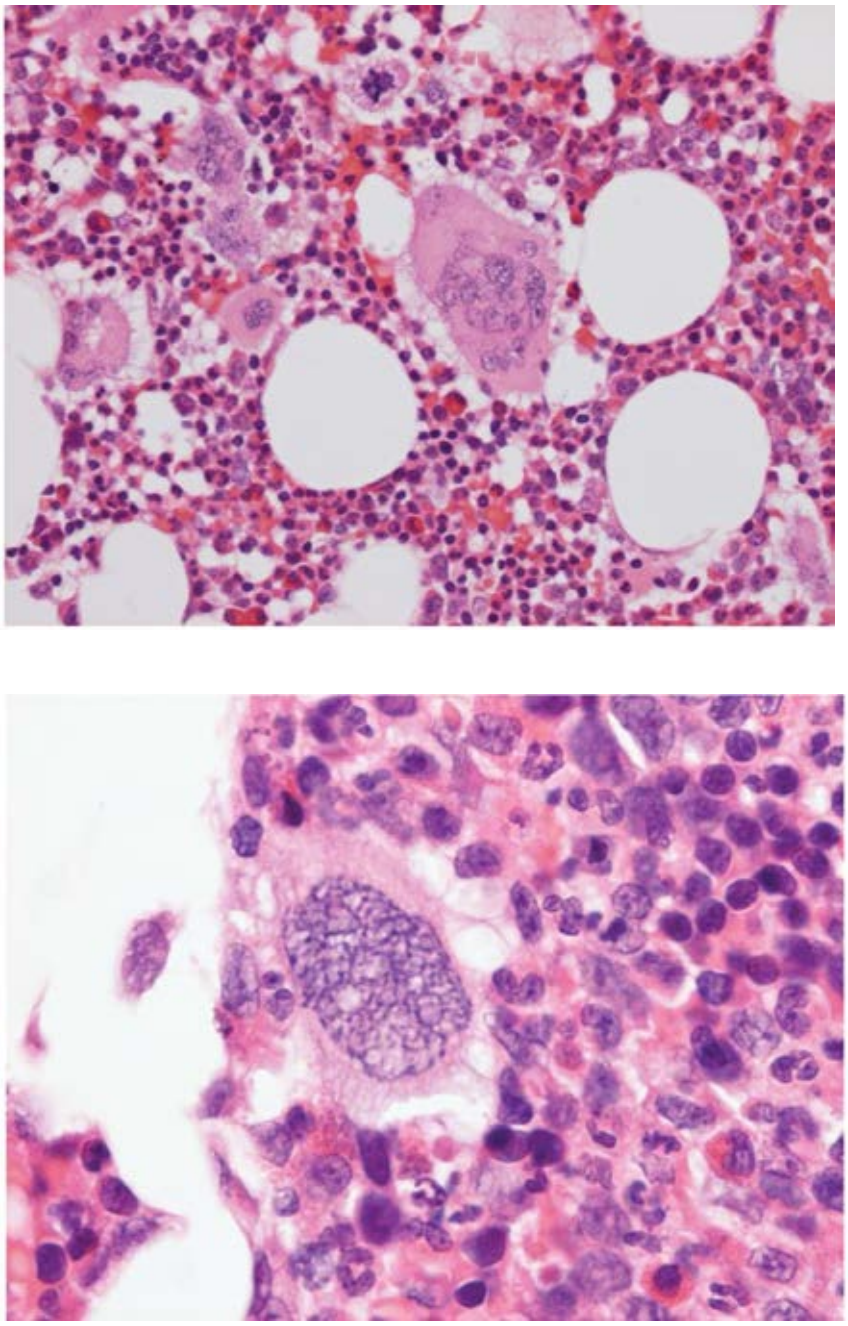
Firstly described by Epstein E. and Goedel A. in 1930s, it was named "haemorrhagic thrombocythemia". The disease is also mentioned in various alternative titles, such as primary/idiopathic/essential thrombocytosis. In 1951, it was classified as MPD along with CML, PV, PMF and erythroleukaemia by Dameshek. In 1970s, the first diagnostic criteria were established by the PVSG. In 2005, the gain-of function mutation *JAK2V617F* has been discovered. With high relevancy to molecular pathogenesis, it was included into the 2008 WHO diagnostic criteria.

ET patients usually exhibit thrombocytosis over 450 x 10^9 platelets/L that must be proved not to be reactive response; other conditions capable in inducing thrombocytosis such as congenital disorder, chronic disorder, infection, malignancy, haemolytic anaemia, postsplenectomy, blood loss, etc. Bone marrow histopathology is required in diagnosing. It should display hypercellularity eminently megakaryocytes. Morphology of the cells is bizarre with enlarged cell and cloud-liked or staghorn-liked nucleus. Increase of myeloid and erythroid cells can be seen sometimes but, in less level than megakaryocytes. Fibrous tissue could appear gradually during disease

progression. In cytogenetic aspect, Philadelphia chromosome or *BCR*/*ABL* is not expected unlike typical CML. Presence of the mutation involving cell proliferation, particularly *JAK2V617F* mutation, is an additional but unnecessary requirement. Clonal markers of other gene in relevancy, such as *MPL*, *LNK*, *IDH*, *TET2*, *IKZF* and *ASXL*, are also preferred (Vainchenker, Delhommeau et al., 2011).

Table 4 The 2008 WHO criteria for ET (Tefferi, 2009)

Major criteria
Sustained platelets count at 450 x 10^9cells/L
Bone marrow biopsy specimen showing proliferation mainly of the megakaryocytic lineage with increased number of enlarged, mature megakaryocytes. No significant increase or left-shift of neutrophils granulopoiesis or erythropoiesis
Not meeting WHO criteria for PV or PMF, *BCR-ABL* positive CML, or MDS or other myeloid neoplasm
Demonstration of *JAK2V617F* or other clonal marker or,
In the absence of *JAK2V617F*, no evidence of reactive thrombocytosis



A

B

Figure 2 Bone marrow of ET displays hypercellularity and bizarre giant megakaryocytes (A) and cloud-liked nucleus (B) (Wilkins, Erber et al., 2008)

Thrombocytosis in ET is often found accidentally with no other severe symptom. The patients could present some 'microvascular symptoms', i.e., headaches, visual symptoms, atypical chest pain and erythromelagia (Tefferi, 2009). Certain life-threatening complications could occur, mostly bleeding and/or thrombosis associated. Degree of severity varies according to a troubled organ. Risk category should be assigned to each patient for the most appropriate treatment as shown in **Table 5**. It includes ages, platelet count and thrombosis history. Younger age than 60 years old, less platelet than 1,000 x 10^3 /μL and no prior history of bleeding are the criteria of low risk. The treatment for this group focuses only on thrombosis prevention. Those with older ages and previous thrombosis require cytoreductive treatment along with clot prevention. If the disease proceeds into acute leukaemia or myelofibrosis, the treatment will be different and symptomatic dependent.

Table 5 Risk-based treatment algorithm in ET (Tefferi, 2009)

Risk factor	**Variables**	**Treatment**
Low risk	Age over 60 years, and no history of thrombosis, and platelet count less than 1,000 x $10^3/\mu L$	Aspirin
Intermediate risk	Neither low risk or high risk	Individualised
High risk	Age 60 years or older, or a positive history of thrombosis	Hydroxyurea and aspirin

C. Primary myelofibrosis

Named as 'chronic idiopathic myelofibrosis' by WHO, Primary myelofibrosis (PMF) has various alternative titles: idiopathic myelofibrosis (IMF) and agnogenic myeloid metaplasia (AMM). It was described firstly in 1879 and classified into the classic MPD together with CML, PV and ET by Dameshek in 1951. Like ET, *JAK2V617F* mutation was discovered in PMF (Baxter, Scott et al., 2005). Mutation of thrombopoietin receptor, *MPLW515K/L*, was disclosed later in few PMF population (Pardanani, Levine et al., 2006; Pikman, Lee et al., 2006; Chaligne, James et al., 2007; Beer, Campbell et al., 2008).

In diagnosing PMF, PV and ET must be ruled out since the early stage of PMF often mimics ET in clinical presentation. It is also similar, but not identical to the fibrotic stage of PV and ET. According to the 2008 WHO criteria, three major criteria must be met with two additional minor criteria as show in **Table 6**. Bone marrow histopathology is required, fibrous tissue is expected as well as increased megakaryocytes and myeloid cells. Megakaryocytes morphology is more atypical than ET, varied in size from gigantic to miniature with aberration in nuclear/cytosol ratio. The nucleus appears in bulbous and hyperchromatic cloud-shaped; some naked with no cytosol presents. For erythroid cells, they often decrease in number with additional maturation arrest (Thiele, 2009). In distinguishing PMF from the fibrotic stage of other MPN, previous medical documentation is required. The patients must meet no WHO criteria for other MPN, MDS or other myeloid disorders. They should present anaemia and leukoerythroblastosis blood picture in parallel with the marrow. Raised serum lactate dehydrogenase level and splenomegaly could be observed reflecting destruction of cells. Molecular lesion associated cell proliferation is preferable including *JAK2V617F*, *MPLW515K/L* or others.

Table 6 WHO 2008 criteria for PMF (Tefferi, 2009)

Diagnosis requires meeting all three majors criteria and two minor criteria
Major criteria
Presence of megakaryocyte proliferation and atypia, accompanied by either reticulin or collagen fibrosis, or
In the absence of significant reticulin fibrosis, the megakaryocyte changes must be accompanied by an increased marrow cellularity characterised by granulocytic proliferation and often decreased erythropoiesis (i.e. prefibrotic cellular-phase disease)
Not meeting WHO PV , CML, MDS, or other myeloid disorders
Demonstration of *JAK2V617F* or other clonal marker (e.g. *MPLW515K/L*) or,
In the absence of the above clonal markers no evidence of secondary bone marrow fibrosis
Minor criteria
Leukoerythroblastosis
Increased serum lactate dehydrogenase level
Anaemia
Splenomegaly

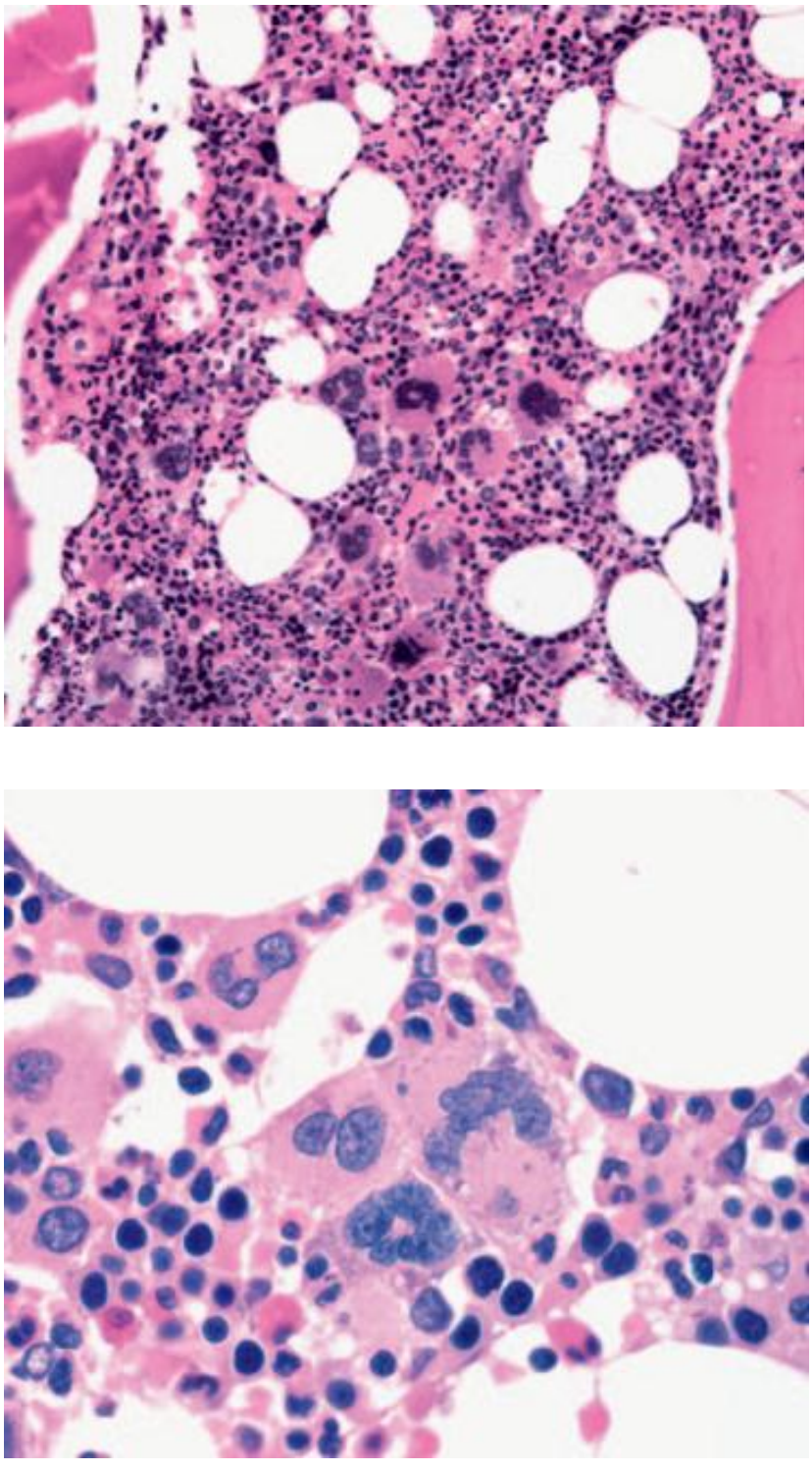


Figure 3 Bone marrow of early stage PMF displays hypercellularity and megakaryocytes with abnormal morphology, differently from ET (Thiele, 2009)

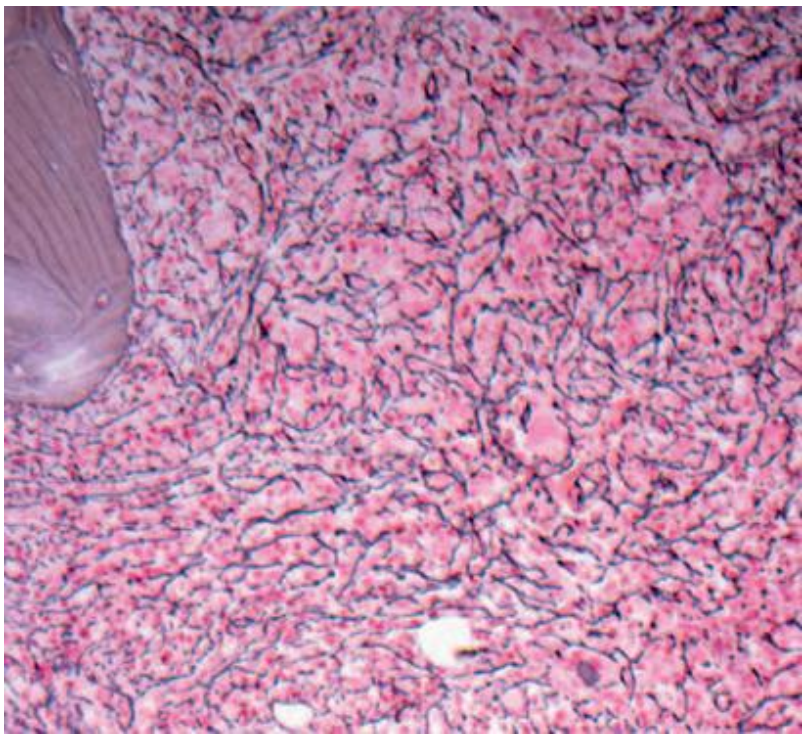


Figure 4 Bone marrow of late stage PMF displays reticulin and fibrous (Thiele, 2009)

Table 7 International working group for MPN research and treatment (IWG-MRT) recommended criteria for post-PV and post-ET myelofibrosis (Tefferi, 2011)

Criteria for post-PV myelofibrosis

Required Criteria:

- Documentation of a previous diagnosis of PV as defined by the WHO criteria
- Bone marrow fibrosis grade 2-3 (on 0-3 scale) or grade 3-4 (on 0-4 scale)

Additional criteria (two are required):

- Anaemia or sustained loss of requirement of phlebotomy in the absence of cytoreductive treatment
- A leukoerythroblastic peripheral blood picture
- Increased splenomegaly defined as either an increase in palpable splenomegaly of > 5 cm (distance of the tip of the spleen from the left costal margin) or the appearance of a newly palpable splenomegaly
- Development of > 1 of three constitutional symptoms: > 10% weight loss in 6 months, night sweats, unexplained fever (>37.5°C)

Criteria for post-ET myelofibrosis

Required criteria:

- Documentation of a previous diagnosis of ET as defined by the WHO criteria
- Bone marrow fibrosis grade 2-3 (on 0-3 scale) or grade 3-4 (on 0-4 scale)

Additional criteria:

- Anaemia and a > 2 g/dL decrease from baseline haemoglobin level
- A leukoerythroblastic peripheral blood picture
- Increasing splenomegaly defined as either an increase in palpable splenomegaly of > 5 cm (distance of the tip of the spleen from the left costal margin) or the appearance of a newly palpable splenomegaly
- Increased lactate dehydrogenase
- Development of > 1 of three constitutional symptoms: > 10% weight loss in 6 months, night sweats, unexplained fever (>37.5°C)

Grade 2-3 according to the European classification: diffused, often coarse fibre network with no evidence of collagenisation (negative trichrom stain) or diffuse, coarse fibre network with the areas of collagenisation (positive trichrom stain). Grade 3-4 according to the standard classification: diffuse and dense increase in reticulin with extensive intersections, occasionally with only focal bundles of collagen and/or focal osteosclerosis or diffuse and dense increase in reticulin with extensive intersections with coarse bundles of collagen, often associated with significant osteosclerosis

PMF patients usually present remarkable anaemia and splenomegaly. Others non-specific symptoms can be observed such as, night sweat, fatigue, abnormal bowel movement, etc (Tefferi, 2009). Few patients develop progressive blast-phase.

Janus Kinase

Proliferation and differentiation is the key process in haematopoeisis, growth and development of haematological cells. Even if there are several kinds of cells served each specific function, they grow from the same origin called haematopoietic stem cells (HSCs). Differently located in any organ based on periods of life, HSCs receive signals from outsides essential for their growth. These signals stimulate intracellular process to trigger them to develope further. The process is called 'signal transduction' consists of to many complicated pathways and several molecules in function. One of those dominates initiation of DNA transcription directly is Janus kinase-Signal transducer and activator of transcription (JAK-STAT) pathway.

Named after the two-faced Roman god who is the gate guardian, Janus kinase (JAK) is an intracellular molecule locating at a cytoplasmic region of transmembrane receptors. The protein includes several members into its family, only in mammalians they are JAK1, JAK2 JAK3 and TYK2. JAK1 and JAK2 were discovered from large numbers isolation with other kinases. They were named 'Just another kinase' firstly and changed later to the current one after scientists revealed the cooperating structure of its kinase and pseudokinase domian (Vainchenker, Dusa et al., 2008). TYK2 was discovered from T-lymphocytes as an intracellular molecule in the interferon α/β signalling pathway (Vainchenker et al., 2008). JAK1, JAK2 and TYK2 function transferring signal from cytokine receptors, both type I and type II (Kaushansky, 2006). Meanwhile, JAK3 fits specifically to the γ_c subunit of IL-2R subfamily members, i.e., IL-4R, IL-7R, IL-9R, IL-15R and IL-21R (Kaushansky, 2006). These following receptors also employ JAKs in their signalling pathway: homodimeric

myeloid receptors (MPL, G-CSF-R), prolactin and growth hormone receptors, heterodimeric receptors sharing the common β chain of IL-3R and glycoprotein 130 (GM-CSF-R, IL-3R, and IL-5R, of receptors) and IFN-γR 2 (Vainchenker et al., 2008).

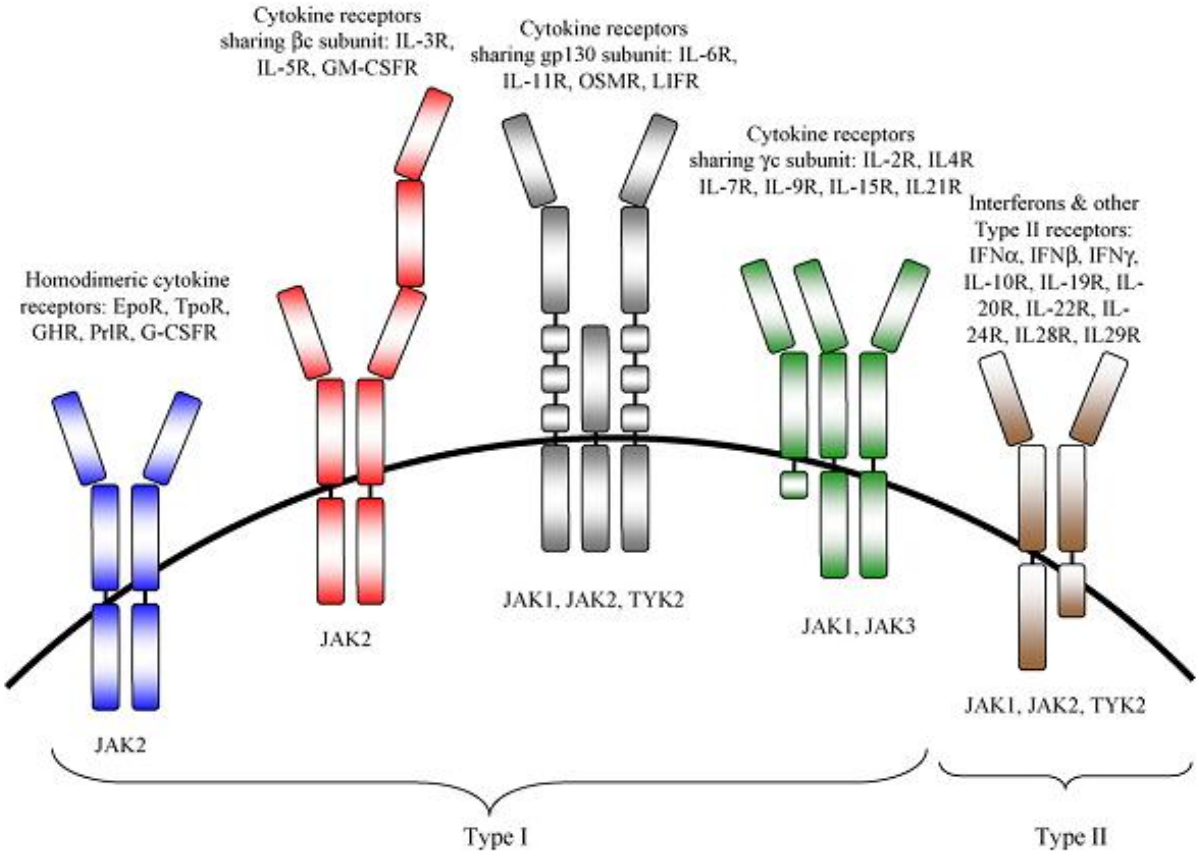


Figure 5 JAKs and cytokine receptors (Vainchenker et al., 2008)

A. Biochemistry

Janus kinase composes of 7 domains: JAK homology1-JAK homology7 (JH1-JH7) as shown in **Figure 6**. On the N-terminus in the vicinity of cytokine receptors, JH6 and JH7 are the crucial parts linking the molecule to receptors. The region is known as 'FERM domain' (band 4.1, Erazin, Radixin and Moesin). Additionally, the domain is responsible for cell surface localisation and stabilisation of certain receptors (Vainchenker et al., 2008). Not only tethering the molecule to the receptor, but FERM domain also corresponds closely to enzymatic activity of the kinase domain. Aberration of the domain causes poor activity of the kinase, and vice versa (Haan, Margue et al., 2008). These phenomena were described in erythroid cells as well (Funakoshi-Tago, Pelletier et al., 2008). Beside the FERM domain, there are JH3 and JH4. They are called 'SH2-liked domain' as their structural similarity to Src-homology 2 domain. Nevertheless, JAKs' SH2 domain is different in function from the classical SH2 domain which is a location where the negative regulatory molecule, phosphatases, to bind to. The study on the domain with alteration in core residues exhibited no effect to JAK's function (Haan, Kreis et al., 2006). Thus, the biological function of this domain is still questioned. Next to the SH2-like domain, it is JH2. This region has a complete kinase domain structure, but lacking certain amino acid residues essential for catalytic activity. It is, therefore, called 'pseudokinase' or 'kinase-liked' domain. Albeit it is functionally inactive, the domain plays the crucial regulatory roles to the kinase domain in both positive and negative way. The study on JAK molecule with modified amino residues in the JH2 revealed inactive kinase activity (Yeh, Dondi et al., 2000). On the contrary, deletion of the entire JH2 resulted in enhancement of ability of the kinase domain causing automatic activation of STAT by phosphorylation with no cytokine bound to the receptor as consequence (Saharinen and Silvennoinen, 2002). The region is also responsible for promoting affinity of cytokines to the receptors (Yeh et al., 2000). The terminal region on the C-terminus is JH1 or kinase

domain. It contains a tyrosine residue and a conserved aspartic residue for phosphate transferring in the activation loop with the canonical GXGXXG motif in the nucleotide binding loop (Vainchenker et al., 2008). It functions in phosphorylation to itself, cytokine receptors and down stream signalling molecules similarly to other kinases.

With cytokine stimulation, JAKs cause activation of cytokine receptors by phosphorylation recruiting several substrates of tyrosine kinases to initiate various responses. Additionally, JAKs themselves also enter the nucleus to trigger changes in cell cycle and epigenetic control directly (Zouein, Duhe et al., 2011). The molecules are negative regulated by Suppressor of cytokine signalling (SOCS) and SH2-containing phosphatase (SHP). These proteins contain SH2 domain to react with phosphorylated tyrosine on the activated receptor complexes, cytokine receptors, JAKs and other substrates of tyrosine kinases, in order to terminate the signal by dephosphorylation (Ihle, 2006). SOCS is known as JAK binding protein (JAB) and STAT-induced STAT inhibitor (SSI).

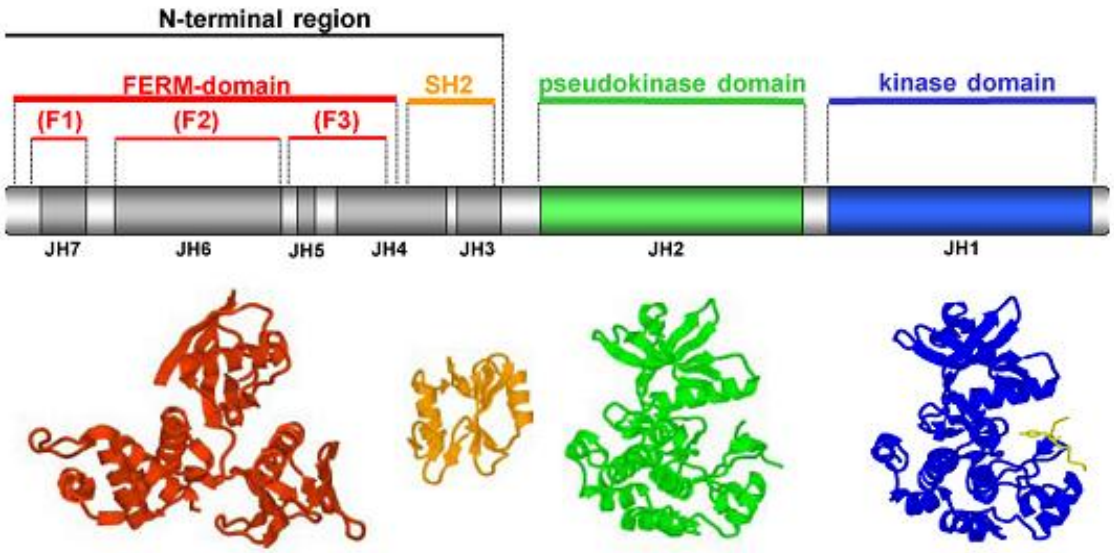


Figure 6 Illustration of JAK2 structure and crystallography (Haan et al., 2006)

Table 8 Size, chromosomal regions and sites of expression of JAKs members (Ihle, 2006)

Kinase	**Size (kDa)**	**Chromosome**	**Expression**
JAK1	135	1p31.1	Ubiquitous
JAK2	130	10q23-24	Ubiquitous
JAK3	120	19p13	Haematopoietic
TYK2	140	19p13.2	Ubiquitous

Table 9 Tyr-kinase substrates (Ihle, 2006)
Phospholipase C γ1 and γ2
P85α and p85β; regulatory subunits of PI-3 kinase
SH-2containing tyrosine phosphatase (SHP-2)
Vav
SHC adapter protein
Stam
SHIP
Cbl
Insulin response substrates (IRS)
Signal transducers and activator if transcription (STATs)
Crk, Crkl
P130Cas, p105Cas1
Hepatocyte growth factor regulated kinase substrate (Hrs)
Linker for activation of T cells (LAK)
Lnk
SH2 domain-containing leukocyte protein of 76 kDa (SLP-76)
IL-4 receptor interacting protein (FRIP)
$P62^{dok}$

C. *JAK2V617F*

Insight into JAK biochemistry has brought comprehension of pathogenesis of MPN. Studies in past has disclosed JAK2 as a JAK member with great relevancy to the disease, especially the single nucleotide switch on the molecule from guanine to tyrosine. It results mistranslation of the amino acid on codon 617 from the original valine to phenylalanine instead (*V617F*). This point mutation appears on the JH2 domain known as pseudokinase that functions in negative regulatory of the kinase domain. The alteration affects chemical structure of the molecule, thereby JAK works independently from cytokine and/or growth factor causing phosphorylation to receptors and also STAT to trigger cell proliferation further. Nonetheless, the mutation does not appear in every patient with Ph- MPN. Its prevalence is nearly 90% in PV and nearly 40-60% in ET as well as PMF (Jones, Kreil et al., 2005; Levine, Wadleigh et al., 2005).

Although *JAK2V617F* has contribution to the disease pathogenesis, it also dominates clinical features. The studies in MPN population revealed remarkable high level of haematological parameters as well as thrombosis and haemorrhagic history in the mutant than the wild type (Antonioli, Guglielmelli et al., 2005; Vannucchi, Antonioli et al., 2008; Lussana, Caberlon et al., 2009; Patriarca, Pompetti et al., 2010; Finazzi, Carobbio et al., 2011). Moreover, homo/heterozygosity of the mutation also influences clinical characteristics (De Stefano, Za et al., 2010). There is suggestion that PV patients are homozygous *JAKV617F* mostly while ET and PMF are heterozygous. Few ET patients with the homozygosity can be found occasionally with presentation of clinical symptoms similarly to PV such as erythrocytosis, raised haemoglobin and haematocrit level unlike typical ET. *JAK2V617F* has meaning to therapeutic method as well. The pharmacological treatment is currently changing from suppression of cells levels to mutation targeting. Hydroxyurea or interferon has been applied to patients conventionally coordinating with Aspirin or Wafarin to prevent intravascular clot. This

treatment strategy seems to cause unfavourable outcome and remission of the disease in some case (Antonioli, Carobbio et al., 2010; Hasselbalch, Kiladjian et al., 2011). To date, the JAK2 specific inhibitor, Anaglilide, is prescribed and the novel gene therapy targeting JAK2 is under development.

Seeing that *JAK2V617* mutation does involve MPN in every step from diagnosis to treatment, its detection is greatly concerned. Late after discovery of the mutation, Campbell developed an examination method combined two polymerase chain reaction (PCR) based techniques: Allele specific-PCR (AS-PCR) and PCR-restriction fragmentation length polymorphism (PCR-RFLP) (Campbell, Scott et al., 2006). Heller proposed different concept of a suitable specimen for the examination which was platelets-derived RNA (Heller, Lev et al., 2006). Toyama, after that, compared those methods and reported identical outcome in most cases. However, some cases with the negative result by DNA method were found positive by using RNA, still it was not much enough to show significance (Toyama, Karasawa et al., 2007).

Albeit the approximate prevalence and clinical correlation of *JAK2V617F* in MPN have been studied, they were conducted mostly in Westerners. Little information is available in Asians, never before in Thais. There is a space to fulfil this valuable knowledge by the study in Thai MPN population. This could be useful for clinical practice and further research.

Bleeding & Thrombosis

In the condition when blood flows fluently in vessels, it is called ‘Haemostasis’ which clotting and clot degrading meets each other. The clot should not present in absence of wound and it must form rapidly in urgent need to prevent massive blood loss. The harmony of these two mechanisms is crucial in maintaining fluidity of blood in order to deliver nutrients and oxygen to every organ properly. Those complicated

mechanisms could be summarised into three basic compartments: primary, secondary and tertiary haemostasis. The primary haemostasis bases on endothelial cells, various plasma proteins and platelets; it initiates the secondary involving cascades of coagulation factors. In activated form, these factors work corporately to generate fibrin for covering an injured region. The tertiary responds by starting clot dissolving process after that, which is called fibrinolysis.

Out of this balance, clotting could occur in an extreme way. This causes medical conditions known as ‘thrombosis’ and ‘bleeding/ haemorrhage’. Bleeding, by itself, results only chronic anaemia with less severity in clinical appearance, but it could be fatal when happening in a sensitive organ such as brain. A thrombus appears after that and obstructs the vascular flow. Certain complications could follow as consequence. The ischemic condition results in death of cells causing damaged organ and disability. Its severity would be grater if happens in cardiovascular, nervous, respiratory, or gastrointestinal system. The theory on pathogenesis of thrombosis was proposed in 1850s, known as Virchow triad. Theses three factors explain the condition principally until present: (a) stasis of blood flow, (b) vascular endothelial injury, and (c) hypercoagulability (Deitcher and Rodgers, 2009).

Table 10 Classification of disorders of haemostasis (Seligsohn and Kaushansky, 2006)

Major type	**Disorder**	**Examples**
Acquired	Thrombocytopaenia	Autoimmunue and alloimmune, drug-induced hypersplenism, hypoplastic (primary, suppressive. myelophthisic), DIC, thrombotic thrombocytopaenic purpura
	Liver diseases	Cirrhosis, acute hepatic failure, liver transplantation, thrombopoietin deficiency
	Renal failure, vitamin K deficiency	Malabsorption syndrome, haemorrhagic disease of the new born, prolonged antibiotic therapy, malnutrition, prolonged billiary obstruction
	Haematologic disorders	Acute leukaemia (particularly promyelocytic), myelodysplasia, monoclonal gammopathies, essential thrombocythaemia
	Acquired antibodies against coagulation factors	Neutralising antibodies against factors V, VIII and XIII, accelerated clearance of antibody-factor complexes e.g., vWD, hypoprothrombinaemia associated with antiphospholipid antibodies
	DIC	Acute (sepsis, malignancies, trauma, obstetric complications) and chronic (malignancies, giant haemagiomas, missed abortion)
	Drugs	Antiplatelet agents, anticoagulants, antithrombin and thrombolytic,

		myelosuppresive, hepatotoxic and nephrontoxic agents
	Vascular	Nonpalpaple purpura, use of corticosteroids, vitamin C deficiency, child abuse, thromboembolic, purpura fulminans; palpable purpura, amyliodosis
Inherited	Deficiencies of coagulation factors	Hamophilia A, haemophilia B, deficiencies of fibrinogen factors II, V, VII, X, XI and XIII, vWD
	Platelet disorders	Glanzmann thrombasthaenia, Bernard-Soulier syndrome, platelet granule disorders
	Fibrinolytic disorders	α_2-antiplasmin deficiency, plasminogen activator inhibitor-1 deficiency
	Vascular	Haemorrhagic telangiectasia
	Connective tissue disorders	Ehlers-Danlos syndrome

DIC, Disseminated intravascular coagulation, vWD, von Willebrand disease.

A. Stasis of blood flow

Rapidly flow in vessels, blood stream creates high shear stress which is necessary for primary haemostasis in expansion of von Willebrand factor (vWF). The condition prepares the factor to be prompt to attachment of circulating platelets in case of requirement. However, this force generates difficulty for the platelet/endothelial complex to form tightly and prevents accumulation of platelets, coagulation promoting proteins and coagulation factors in plasma also (Deitcher et al., 2009). This helps maintaining harmonious haemostasis. Disturbance to the flow, such as obstruction of the vessel, decelerates the stream velocity affecting the balance results in clotting. Even if in a healthy condition, certain anatomical structure then to allow a clot to occur easily. Venous system differently from the arterial in presence of valves along its distance, it could become a storage place of various colt promoting factors if that individual experiences immobility too long.

B. Vascular endothelial injury

Endothelium cells are not only the core structure of blood vessel, but also functions in haemostasis. Underneath these cells, there is a structural protein know as collagen. It is capable in binding the stream flowing platelets ‘platelet adhesion’- the initial step of platelet activation in the primary haemostasis. This phenomenon impacts platelets in reshaping and secreting intra-platelet granules results in recruitment of extra platelets, platelet aggregation and initiation of coagulation cascades (Deitcher et al., 2009). Endothelium, moreover, stores the tissue factor playing the major role in the secondary haemostasis regulating. With injury, the endothelial tissue factor leaks and reacts with activated coagulation factor VII, IX and phosphatidylserine onto a plasma membrane to form ‘Extrinsic Tenase’ activating coagulation further (Deitcher et al., 2009).

C. Hypercoagulability

Hypercoagulability means abnormalities of blood with emphasis on platelets, coagulation factors and negative regulatory to coagulation. The phenomenon is also referred to as 'thrombophilia' and could be caused inherited or acquired. Certain genetic mutations and polymorphisms are considered as a cause of thrombosis; von Willebrand disease (vWD), Glanzmann thrombasthaenia, Bernard-Soulier syndrome and platelet granules disorders, for instances, abolish regular platelets functions deteriorating the primary haemostasis (Deitcher et al., 2009). Prothrombin *G20210A* and factor V Leiden affect the secondary haemostasis similarly to fibrinolytic disorders do to the tertiary (Deitcher et al., 2009). Malignancy, infection, autoimmunity and other acquired causes could result generation of auto-antibodies affecting coagulation and further lead to thrombosis as consequence.

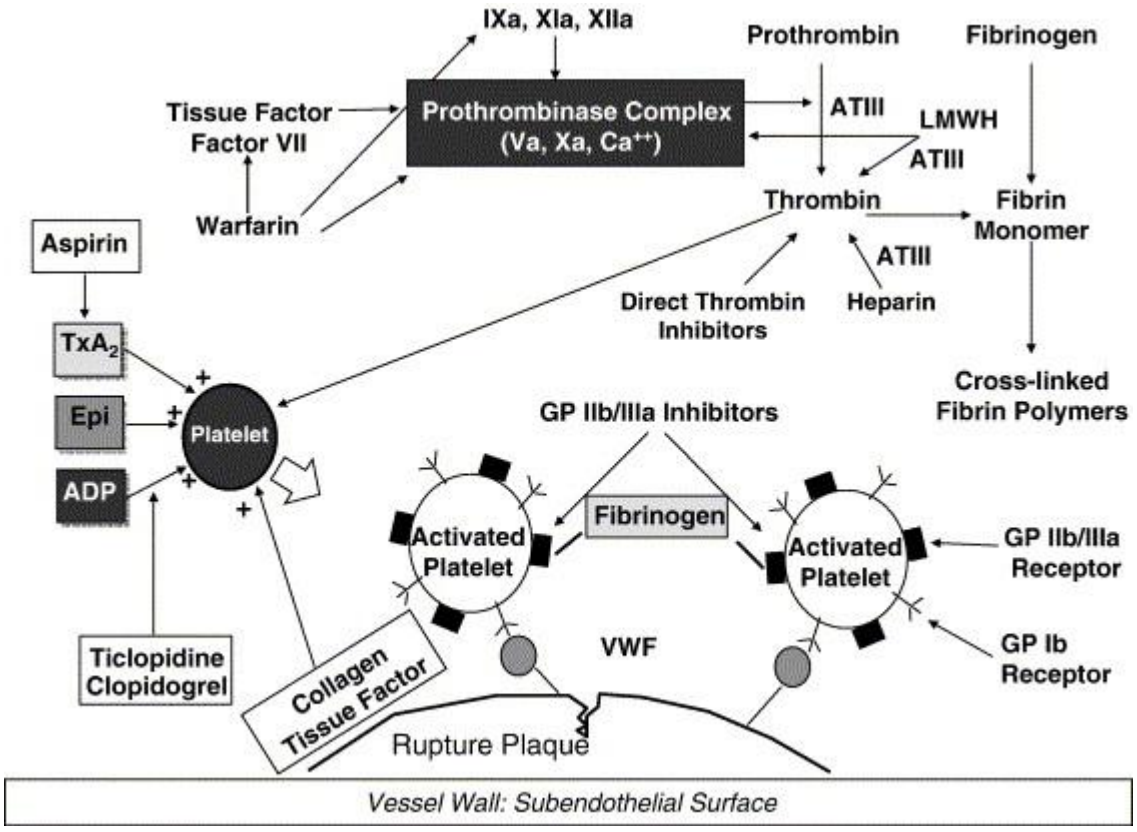


Figure 7 Integration of 3 factors in Virchow triad promotes thrombosis (Brener, 2006)

CHAPTER III

METHODOLOGY

This chapter describes the entire methodology of this study. It begins with the population of study in calculation of sample size and the source of the sample. After that, it mentions all scientific equipments and chemicals in use. Technical and statistical methods are explained in the final.

Study Population

According to the WHO manual for a study in health study, estimation of sample size for prevalence study could be computed by using the following equation (Lwanga and Lemeshow, 1991):

$$n = \frac{Pq}{(E/1.96)^2}$$

When **n** = the minimum required sample size

p = the maximum expected prevalence rate (%)

q = 100-p

E = the margin of sampling error tolerated (%)

As the average prevalence of *JAK2V617* in MPN from previous reports is approximately 50% and the acceptable margin of error in this study is 10%, the equation should be replaced as follows;

$$n = \frac{50 \times 50}{(10/1.96)^2}$$

$$= 97$$

Subsequently, we extracted information from the medical records of MPN patients who had visited King Chulalongkorn memorial hospital. There were 103 in total, 7 CML and 96 Ph- MPN (31 PV, 49 ET, 10 PMF and 6 unclassifiable MPN). All patients were diagnosed according to the WHO 2008 criteria during 2009-2011.

Ethical consideration

This study has been approved by The Institutional Review Board, Faculty of Medicine, Chulalongkorn University, according to the document COA No. 637/2011 IRB No. 269/54 date September 27, 2011.

Research equipments

A. Instruments

-20°C freezer	Sanyo, Osaka, Japan
4°C refrigerator	Mitsubishi, Tokyo, Japan
Analytical balance	Mettler Toledo, Greifensee, Switzerland
Autoclave	Tomy, Tokyo, Japan
Autopipitte 0.2-2 μL	CAPP, Odense S, Denmark
Autopipitte 2-20 μL	Labnet, New Jersey, USA
Autopipitte 10-100 μL	Biohit, Helsinki, Finland
Autopipitte 100-1000 μL	Nichiryo, Tokyo, Japan
Class II biosafety cabinet	Faster, Milan, Italy
DC power supplier	Hoeler, California, USA
Electrophoresis chamber	Kodak, New York, USA
Gel Doc	Syngene, Cambridge, UK
Microwave oven	Turbora, Pathumthani, Thailand
Microcentrifuge	Eppendorf, Hamburg, Germany

Nano Drop	Thermo, Massachusetts, USA
Nanofuge	Tomy, Tokyo, Japan
Plate Sharker	Desaga, Wiesloch, Germany
Thermo cycler	Eppendorf, Hamburg, Germany
Vortex mixer	Labnet, New Jersey, USA
Water bath	Mgw Lauda, Lauda-Königshofen, Germany

B. Glass & Plastic wares

Cylinder	Kartell, Milan, Italy
EDTA tube (3mL)	Vacuette, Kremsmünster, Austria
Flask (100,125 mL)	Schott Duran, Mainz, Germany
Glass bottle (500 mL)	Schott Duran, Mainz, Germany
Microtube (1.5 mL)	Hycon, New Hampshire, USA
PCR tube	SSI, California, USA
Tips (10, 100, 1000 μL)	Sorenson, Utah, USA

Chemicals

10 mM dNTP	Promega, Wisconsin, USA
100 bp DNA ladder	Promega, Wisconsin, USA
Absolute ethanol	Merck, Darmstadt, Germany
Agarose	ISC Bioexpress, Utah, USA
AmpliTaq GOLD	Applied Biosystems, California, USA
BsaX1	NEB, Massachusetts, USA
DNA blood mini kit	Qiagen, Hilden, Germany
Ethidium Bromide	Invitrogen, California, USA

Glacial acetic acid	Merck, Darmstadt, Germany
Nuclease free water	Promega, Wisconsin, USA
Tris-base	Sigma-Aldich, Missouri, USA

Research methods

A. Clinical data collection

The clinical features at the diagnosed date were extracted to match to the mutation result. The information includes red blood cells count, haemoglobin level, haematocrit, white blood cells count, platelets count, and bleeding and thrombosis history.

B. Specimen processing

Blood was collected in an ethylenediamine tetraacitate anti-coagulated tube. Whole blood DNA was isolated by QIAamp DNA blood mini kit after that following manufacturer's instruction. The DNA samples were confirmed their validity by optical density measurement and stored in a freezer under -20°C awaiting *JAK2V617* examination.

C. DNA isolation

The isolation was conducted by using QIAmp DNA bloods mini kits. Cell membrane is disrupted with the activity of proteinase K. The enzyme promotes denaturation of plasma membrane by degrading protein and inactivating nuclease enzyme simultaneously to preserve nucleic acids. The genetic material would be adsorbed onto the silica membrane during a brief centrifugation. Contaminants are removed by washing twice to increase purity of DNA. The elution buffer is applied in the last step to elute the attached DNA. To isolate DNA from whole blood sample, the following procedure was conducted

1. Pipette 20 μL proteinse K into the bottom of a 1.5 mL microcentrifuge tube
2. Add 200 μL whole blood sample to the microcentrigue tube
3. Add 200 μL buffer AL to the sample, mix by purse vortexing for 15 sec
4. Incubate at 56°C for 10 min
5. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid
6. Add 200 μL ethanol (96-100%) to the sample, and mix again by pulse-vortexing for 15 sec. After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid
7. Carefully apply the mixture from the step 6 to the spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6000g (8000 rpm) for 1 min. Place the spin column in a clean 2 mL collection tube, and discard the collection tube containing the filtrate
8. Carefully open the spin column and add 500 μL buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000g (8000 rpm) for 1 min. Place the spin column in a clean 2 mL collection tube, and discard the collection tube containing the filtrate
9. Carefully open the spin column and add 500 μL buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000g; 14,000 rpm) for 3 min
10. Place the spin column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge al full speed for 1 min
11. Place the spin column in a clean 1.5 mL microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the

spin column and add 200 μL buffer AE. Incubate at room temperature (15-25°C) for 1 min, and then centrifuge at 6000g (8000 rpm) for 1 min

D. DNA validity affirmation

The affirmation is conducted by NanoDrop. The equipment quantifies concentration of nucleic acid in specimens principally depending on modification of the Beer-Lambert equation to use a factor as ng-cm/μL as follows:

$$c = (A*\varepsilon)/b$$

c = nucleic concentration in ng/μL

A = absorbance in AU

ε = the wavelength-dependent extinction coefficient in ng-cm/μL

b= the pathlength in cm

The acceptable wavelength-dependent extinction coefficient for double-stranded DNA in general is 50 ng-cm/μL and the pathlength of NanoDrop is between 1.0 mm to 0.05 mm. However, the software would normalise to 1 cm in calculation. To measure the concentration the following procedure is conducted as follows:

1. Open the NanoDrop software on the monitor
2. Select nucleic acid and click OK
3. Apply distilled water for 2 μL onto the equipment bottom pedestal, close and select OK on the screen to initialise the measurement
4. Select double-stranded DNA, and click blank
5. Clean the pedestal, and then apply sample onto. Close the pedestal and select measure

6. After measurement, clean the upper and the bottom pedestal with a dry laboratory wipe

The screen-displayed result includes concentration in the ng/μL unit, and absorbance ratio at 260/280 and 260/230. The 260/280 ratio indicates purity of DNA that should be 1.8 approximately while the 260/230 ratio is the secondary indicator that should be in1.8-2.2.

E. *JAK2V617F* examination

JAK2V617F was examined using two polymerase chain reaction-based methods modified from Campbell's procedure: allele specific PCR and PCR-restriction fragmentation length polymorphism (Campbell et al., 2006).

The polymerase chain reaction (PCR) is a molecular biological technique to amplify a targeted DNA template by fabricating natural DNA duplication. Its degree of success depends on the optimised temperature for annealing of primers pair compatible to particular target. The reaction requires Taq DNA polymerase enzyme for nucleotide extension, buffer to the enzyme, building blocks deoxy-nucleotidetriphospate, and $MgCl_2$ as a co-factor. During the reaction, temperature would be varied into 3 signified points: for template denaturation, for annealing, and for extension. Based on this same principle, the reaction can be adapted into various patterns for particular reason. Allele specific-PCR (AS-PCR), one of them, requires 2 forward primers in the reaction. An inner one is added for specific detection of a single base mutation while an outer amplifies other non-mutated templates in full length. In examining *JAK2V617F*, 2 bands with difference in size would be observed under UV light: 264 bp and 203 bp.

While AS-PCR could be accomplished in a single reaction, restriction fragment length polymorphism (RFLP) must be awaited a general PCR to be completely done prior using the product as a template for the reaction after that. RFLP

causes a cleft of nucleotide template in a specific residue by a restriction enzyme. Even though it is time consuming over AS-PCR, it is more advantageous since its capability in revealing homo/heterozygosity of the mutation. In this study, we followed Campbell's procedure by selecting BsaX1 as a restriction enzyme (Campbell et al., 2006). Because of the restriction site of the enzyme is 5'…GGAG$(N)_5$GT…3', only a wild type JAK2 with this site would be cleft displaying 3 bands differently in size under UV light, 189 bp, 145 bp, and small undetectable 30 bp. *JAK2V617F* would leave the whole 364 bp-band to be observed.

In both reactions, DNA samples are amplified in a total volume of 25 μL containing nuclease free water, 10x AmpliTaq Gold buffer, 25 mM $MgCl_2$, 10 mM dNTPs mix, primer F1, primer F2, primer R, and AmpliTaq Gold DNA polymerase. For AS-PCR, dNTPs mix and primers are prepared in 2.5 mM and 12.5 μM concentration respectively while 10 mM dNTPs mix and 50 μM primers are favourable in PCR-RFLP. The reactions are conducted at 58°C as the annealing temperature for 35 cycles. The products after that are applied onto a 1.5% agarose gel for electrophoresis in a 100V electric field for 50 min. The gel is stained with ethidium bromide solution for 15 min, and then de-stained twice in tap water for 15 min. Band presence is observed under UV light in Gel Doc. For PCR-RFLP, its products are incubated with BsaX1 restriction enzyme solution for 30 min before electrophoresis.

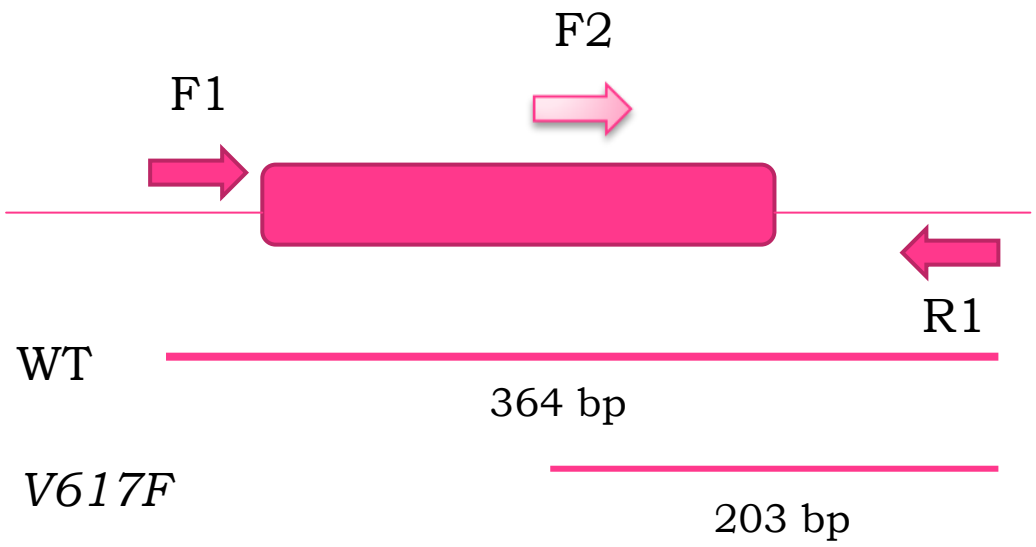


Figure 8 Wild type JAK2 is amplified by the outer primers while *JAK2V617F* is amplified by the inner, adapted from Campbell et al., 2006

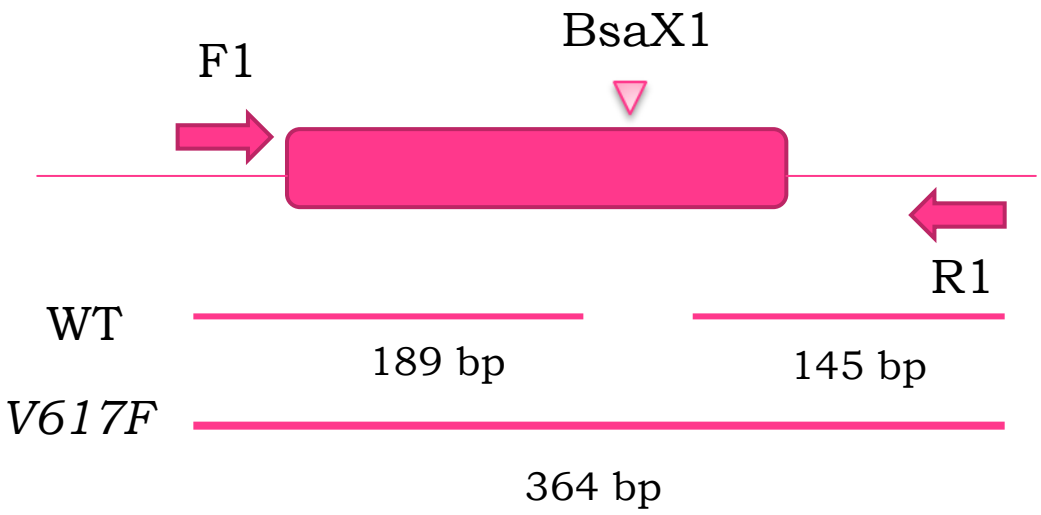


Figure 9 Wild type JAK2 is amplified in PCR and cleft by BsaX1 in RFLP, but *JAK2V617F* remains, adapted from Campbell et al., 2006

Table 11 The oligonucleotide primers in the study and their product sizes

Primer	Sequence	Product length	Ref
JAK2_F1	ATCTATAGTCATGCTGAAAGTAGGAGAAAG	364 bp	(Campbell,
JAK2_F2	AGCATTTGGTTTTAAATTATGGAGTAT	203 bp	et al., 2006)
JAK2_R	CTGAATAGTCCTACAGTGTTTTCAGTTTCA		
GAPDH_F	TGGACCTGACCTGCCGTCTA	243 bp	-
GAPDH_R	CCCTGTTGCTGTAGCCAAATTC		

Table 12 The PCR recipe for *GAPDH* detection

Reagents	Volume/Reaction	Concentration/Reaction
PCR water	12.75	-
10X Taq buffer	2.5	1X
2.5 mM dNTP (each)	2	0.2
25 mM $MgCl_2$	1.5	1.5
12.5 μM primer F1	2.5	1.25
12.5 μM primer R	2.5	1.25
Taq polymerase	0.25	-
DNA template	1	
Total	**25**	

Table 13 The AS-PCR recipe for *JAK2V617F* detection

Reagents	Volume/Reaction	Concentration/Reaction
PCR water	10.75	-
10X Taq buffer	2.5	1X
2.5 mM dNTP (each)	2	0.2
25 mM $MgCl_2$	1.5	1.5
12.5 μM primer F1	1.25	0.625
12.5 μM primer F2	1.25	0.625
12.5 μM primer R	2.5	1.25
Taq polymerase	0.25	-
DNA template	3	
Total	**25**	

Table 14 The PCR recipe for JAK2 amplification prior RFLP

Reagents	Volume/Reaction	Concentration/Reaction
PCR water	13.25	-
10X Taq buffer	2.5	1X
10 mM dNTP (each)	1.5	0.6
25 mM $MgCl_2$	1.5	1.5
50 μM primer F1	0.5	1.0
50 μM primer R	0.5	1.0
Taq polymerase	0.25	-
DNA template	5	
Total	**25**	

Table 15 The RFLP recipe for *JAK2V617F* detection

Reagents	Volume/Reaction	Concentration/Reaction
PCR water	12	-
10X BsaXI buffer	2	1X
BsaX1	1	-
DNA template	10	
Total	**25**	

F. Agarose gel electrophoresis

Gel electrophoresis is a molecular technique for nucleic acids separation according to their sizes in an electric field. As chemical characteristic of DNA provides the negative charge, DNA mobilises to the positive polar in the field. In practice, DNA samples are applied into a porous gel variously in pore size according to its concentration fitting for DNA separation. Nucleic acids with smaller size could pass through those pores easily while the larger move slowly. There are 2 kinds of gel: agarose and acrylamide. Acrylamide generates more delicate pore, thus it is preferable in separation of DNA products nearly indistinguishable. Nevertheless, the chemical is recognised a carcinogen. Here, agarose is chosen.

Another chemical ingredient required in the separation is TAE buffer. It is a mixture of Tris base, Acetate, and EDTA using as an electrophoresis buffer and a solvent for dissolving the gel. Here, 50X concentration is prepared as a stock, and then diluted to 10X and 1X concentration finally as the working solution. The concentration of the agarose gel solution depends on efficiency of nucleic acids separation required. In this study, we prepare 1.5% w/v concentration and heat it roughly in a microwave oven for 2 min until dissolved completely. The solutions is, then, poured onto the tray and left at room temperature for 15 min awaited until well solidified. The gel is placed into the electrophoresis chamber where the working TAE buffer would be poured onto until its level higher than the gel surface. DNA samples are applied onto the gel, and the chamber would be connected to a DC power supplier after that to create an electric field at 100 V for 50 min.

Table 16 The recipe for TAE buffer preparation

Reagents	50X	10X	1X
Glacial acetate	5.71 mL	-	-
Tris base	24.2 g	-	-
EDTA	3.72 g	-	-
Distilled water	100 mL	400 mL	450 mL
50X TAE buffer	-	100 mL	-
10X TAE buffer	-	-	50 mL
Total volume	**100**	**500**	**500**

Table 17 The recipe for agarose gel solution preparation

Reagents	2.0%	1.5%	1.0%	0.5%
Agarose	0.7 g	0.53 g	0.35 g	0.18 g
1X TAE buffer	35 mL	35 mL	35 mL	35 mL
Total volume	**35**	**35**	**35**	**35**

G. Products detection

Fundamentally, DNA can be attached by ethidium bromide into its grooves. Under UV light, the chemical would be luminous and detectable. The agarose gel is stained in the ethidium bromide solution for 15 min after electropheresis, and then de-stained twice in tap water for 10 min. The gel is placed into the Gel Doc and adjusted its position under normal light. The light source is switched to the UV light to detect the product bands after that.

H. Statistical analysis

The SPSS version 15.0 software is utilised for conducting data analysing. The statistics includes:

1. One-way ANOVA for comparison of parameters among MPN
2. Scheffe for comparison of parameters between each specific MPN
3. Welch test for comparison of parameters with unequal variance among MPN
4. Kruskal Wallis H test for comparison of parameters among MPN asymmetrically distributed
5. Student-*t* test for comparison of parameters between the mutant and the wild-typed
6. Pearson Chi-square for comparison of parameters between the mutant and the wild type asymmetrically distributed
7. Mann-Whitney *u* test for comparison of nonparametric data between the mutant and the wild type

CHAPTER IV

RESULTS

In this chapter, the contents are divided into 3 sections. Clinical features of the study population are described in the first section. That includes comparison of clinical data among specific MPN types. Prevalence of *JAK2V617F* mutation in the overall population and in each MPN type is mentioned in the following section. In the final, it is clinical correlation of the mutation.

Clinical features

Clinical features including ages at the diagnosed date, bleeding and thrombosis history, and general haematological parameters are summarised in the **table 18**. Age at the diagnosed date was 56.8 ± 2.0 years old closely to each specific MPN type. Bleeding tendency was recorded 13.7% of the population; no significant difference among each MPN type. 32.4% from the population was recorded thrombosis. Numbers of cases with that complication were significantly different among MPN types (P = 0.026, **Table 18**). It was at 34.4% in ET, much more than only PMF (P = 0.029, **Table 19**). The evidence in PV was 43.5%, much more than PMF as well (P = 0.028, **Table 19**). Red blood cells count was at 5.0 ± 0.2 $x10^6$ cells/μL, significantly different among each MPN type (P < 0.001, **Table 18**). PV patients showed the highest averaged value at 6.9 ± 0.3 x 10^6 cells/μL, significantly higher than ET, PMF, and CML (P < 0 .001, < 0.001, and < 0.001, **Table 20**). Haemoglobin level was at 13.7 ± 0.4 g/dL totally, varied similarly to red blood cells seeing that there was significant difference among each MPN (P < 0.001, **Table 18**). PV held the highest level at 16.7 ± 0.5 g/dL, significantly higher than ET, PMF, and CML (P < 0.001, < 0.001, and < 0.001, **Table 20**). Likewise, haematocrit varied following other red cells indicators; its

averaged value was at 42.5 ± 1.2. Significant difference among each MPN showed (P < 0.001, **Table 18**). PV belonged the highest haematocrit at 52.0 ± 1.5, significantly higher than ET, PMF, and CML (P < 0.001, < 0.001, and < 0.001, **Table 20**).

Averaged white blood cells were at 19.5 ± 2.2 x 10^3 cells/μL, significantly different among each specific MPN (P < 0.001, **Table 18**). CML as the disease with the problem in this lineage carried the cell level at 72.5 ± 12.5 x 10^3 cells/μL, significantly higher than ET, PV, PMF, and MPN un (P < 0.001, < 0.001, = 0.001, = 0.003, **Table 21**). For platelets levels, the overall average was 769.7 ± 77.3 x 10^3 platelets/μL. The level was various in numbers among each specific MPN type; significant difference was observed (P < 0.001, **Table 18**). ET held the first place in level at 1073.2 ± 133.8 x 10^3 platelets/μL, significantly higher than PV and PMF (P < 0.001, = 0.001, **Table 21**).

Table 18 Clinical data of the study population

			Ph- MPN				
	Overall	**CML**	**ET**	**PV**	**PMF**	**MPN un**	***P*-value**
Age	56.8 ± 2.0	60.6 ± 7.7	52.9 ± 2.8	60.6 ± 3.1	62.4 ± 5.8	59.3 ± 9.2	-
Bleeding	14/102 (13.7%)	2/7 (28.6%)	6/49 (12.2%)	4/30 (13.3%)	1/10 (10.0%)	1/6 (16.7%)	.816a
Thrombosis	33/102 (32.4%)	0/7 (0.0%)	17/49 (34.7%)	14/30 (46.7%)	0/10 (0.0%)	2/6 (33.3%)	*.026***a
RBC10^6	5.0 ± 0.2	3.9 ± 0.4	4.6 ± 0.1	6.5 ± 0.3	3.6 ± 0.5	5.9 ± 0.5	*.000***b
Hb	13.7 ± 0.4	10.4 ± 0.8	12.7 ± 0.3	16.7 ± 0.5	10.4 ± 0.9	15.4 ± 0.8	*.000***c
Hct	42.5 ± 1.2	28.8 ± 4.2	38.9 ± 0.9	52.0 ± 1.5	32.8 ± 3.0	49.6 ± 2.2	*.000***c
MCV	84.0 ± 1.2	85.2 ± 2.9	86.6 ± 1.7	80.2 ± 2.1	35.3 ± 2.5	79.8 ± 3.4	.115d
WBC10^3	19.5 ± 2.2	72.5 ± 12.5	14.4 ± 1.8	13.2 ± 1.6	19.9 ± 4.6	13.7 ± 2.7	*.000***d
PLT10^3	769.7 ± 77.3	771.0 ± 254.7	1073.2 ±133.8	397.5 ± 47.6	455.2 ± 168.4	623.4 ± 144.3	*.000***d
JAK2V617F	66/103 (64. 1%)	0/7 (0.0%)	29/49 (59.2%)	25/31 (80.6%)	7/10 (70.0%)	5/6 (83.3%)	-

Haematological parameters in mean ± S.E., Bleeding, thrombosis and *JAK2V617F* in frequency/total case, a. Pearson chi-square, b. Welch test, c. one-way ANOVA and d. Kruskal Wallis H test, RBC (Red blood cell count), Hb (Haemoglobin), Hct (Haematocrit), MCV (Mean corpuscular volume), WBC (White blood cell count), PLT (Platelet count), MPN (Myeloproliferative neoplasm), Ph-MPN (Philadelphia chromosome negative MPN), ET (Essential thrombocythaemia), PV (Polycythaemia vera), PMF (Primary myelofibrosis), MPN un (unclassifiable myeloproliferative neoplasm), CML (Chronic myelogeneous leukaemia), **statistical difference.

Table 19 Thrombosis incidence in each MPN type

		Thrombosis			
	ET	**CML**	**PV**	**PMF**	**MPN un**
Cases	17/49 (34.7%)	0/7 (0.0%)	14/30 (46.7%)	0/10 (0.0%)	2/6 (33.3%)
***P*-value**		.147a	.293a	*.029***a	.969a

		Thrombosis			
	PV	**CML**	**ET**	**PMF**	**MPN un**
Cases	14/30 (46.7%)	0/7 (0.0%)	17/49 (34.7%)	0/10 (0.0%)	2/6 (33.3%)
***P*-value**		.059a	.293a	*.028***a	.634a

Thrombosis in frequency/total case, a. Mann-Whitney *u test*, ET (Essential thrombocythaemia), PV (Polycythaemia vera), PMF (Primary myelofibrosis), MPN un (unclassifiable myeloproliferative neoplasm), CML (Chronic myelogeneous leukaemia), **statistical difference.

Table 20 Haematological parameters of each MPN type (RBC, Hb and Hct)

		RBCx10^6			
	PV	**CML**	**ET**	**PMF**	**MPN un**
mean ± S.E.	6.5 ± 0.3	3.9 ± 0.4	4.6 ± 0.1	3.6 ± 0.5	5.9 ± 0.5
***P*-value**		*.000***a	*.000***a	*.000***a	.493a

		Hb			
	PV	**CML**	**ET**	**PMF**	**MPN un**
mean ± S.E.	16.7 ± 0.5	10.4 ± 0.8	12.7 ± 0.3	10.4 ± 0.9	15.4 ± 0.8
***P*-value**		*.000***b	*.000***b	*.000***b	.935b

		Hct			
	PV	**CML**	**ET**	**PMF**	**MPN un**
mean ± S.E.	52.0 ± 1.5	28.8 ± 4.2	38.9 ± 0.9	32.8 ± 3.0	49.6 ± 2.2
***P*-value**		*.000***b	*.000***b	*.000***b	.980b

Haematological parameters in mean ± S.E., a. student *t*-test, b. Schffee, RBC (Red blood cell count), Hb (Haemoglobin), Hct (Haematocrit), ET (Essential thrombocythaemia), PV (Polycythaemia vera), PMF (Primary myelofibrosis), MPN un (unclassifiable myeloproliferative neoplasm), CML (Chronic myelogeneous leukaemia), **statistical difference.

Table 21 Haematological parameters of each MPN type (WBC and PLT)

		WBCx10^3			
	CML	**ET**	**PV**	**PMF**	**MPN un**
mean ± S.E.	72.5 ± 12.5	14.4 ± 1.8	13.2 ± 1.6	19.9 ± 4.6	13.7 ± 2.7
***P*-value**		*.000***a	*.000***a	*.001***a	*.003***a

		PLTx10^3			
	ET	**CML**	**PV**	**PMF**	**MPN un**
mean ± S.E.	1073.2±133.8	771.0 ± 254.7	397.5 ± 47.6	455.2 ± 168.4	623.4 ± 144.3
***P*-value**		.274a	*.000*** a	*.001***a	.123a

Haematological parameters in mean ± S.E., a. Mann-Whitney *u* test, WBC (White blood cell count), PLT (Platelets count), ET (Essential thrombocythaemia), PV (Polycythaemia vera), PMF (Primary myelofibrosis), MPN un (unclassifiable myeloproliferative neoplasm), CML (Chronic myelogeneous leukaemia), **statistical difference

Prevalence of *JAK2V617F*

Availability of DNA in the specimens was determined by the detection of a house keeping gene. *GAPDH* as a general gene coding an enzyme in the Krebs cycle had been selected as a representative. The determination was conducted using PCR technique; the product presented a 347 bp band under the UV light as shown in **Figure 10**.

In *JAK2V617F* examination, dual PCR based techniques were conducted. Principally, AS-PCR bases on concurrent capture of two distinct primers onto the DNA template; the outer for the wild type molecule and the inner for the mutation as described in the previous chapter. Two nucleotide products with difference in size were thence observed. They were 364 bp for the wild type JAK2 and 203 bp for *JAK2V617F* (**Figure 10**). Another examining technique, PCR-RFLP, whereby amplifies the products only by capture of the outer primers prior cleaving with BsaX1 restriction enzyme. The mutation causes alteration of the enzyme restriction site located into the genome originally. Therefore, the mutant product is impossible to be cleft showing a 364 bp band under the UV light. The wild type, on the contrary, carrying the restriction site could be cleft into 189/145 bp and the small unobservable 30 bp band (**Figure 10**).

Here, we reported 64.1 % as prevalence of *JAK2V617F* mutation in Thais with MPN; it was 65.6% for the Ph- MPN population. The prevalence for each specific MPN type was as follows: 59.2% for ET, 80.6% for PV, 70% for PMF, and 83.3% for unclassifiable MPN. In CML, there was no evidence of the mutation (**Table 18**). No difference in outcomes between both techniques showed. For a homo/heteozygosity perspective of the mutation detectable by PCR-RFLP, we found that all PV with the mutation were homozygous while almost ET patients with the same condition were heterozygous (**Figure 10**). The only case of ET with homozygous *JAK2V617F* we had observed in this population presented erythrocytosis similarly to PV.

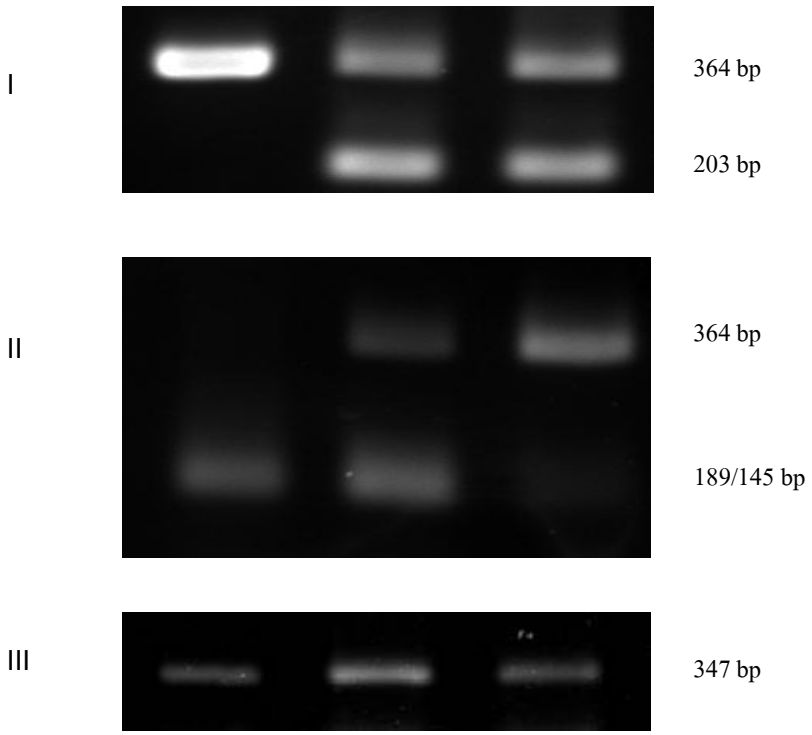


Figure 10 Product bands on 1.5% agarose gel after electrophoresis under UV light (I) AS-PCR, PCR-RFLP, and (III) *GAPDH*; Lane 1 DNA from healthy volunteer, lane 2 DNA from ET patient, lane 3 DNA from PV patient.

Clinical correlation of *JAK2V617F*

In order to investigate clinical correlation of *JAK2V617F*, we had moved forward to comparison of clinical features and haematological parameters between the wild type and the mutant. The overall study population displayed similarity in statistical documents thoroughly, except bleeding history and MCV. 4.9% of overall patients presented thrombosis and the mutation, meanwhile 8.7% of those with thrombosis carried no mutation. This meant the mutant had significantly lower thrombosis history than the wild type (P = 0.039, **Table 22**). The mutant, moreover, held significantly lower MCV than the wild type at 81.7 ± 0.7 to 87.3 ± 0.7 (P = 0.006, **Table 22**). In the focus group without Philadelphia chromosome, only significant difference at MCV was observed similarly to the overall, 81.5± 1.6 for the mutant and 88.7 ± 1.5 for the wild type (P = 0.005, **Table 23**)

In investigation of *JAK2V617F*'s clinical correlation on each specific MPN, comparison between the wild type and the mutant group were conducted in patients with certain MPN. As both ET and PV are the mostly found MPN than others, we had selected them as representatives. In patients with ET, there was no significant difference on any clinical features between the wild type and the mutant. Nevertheless, some haematological parameters of the mutant were slightly higher without significance. As seen in the **Table 24**, the mutant held white blood cells at 14.8 ± 1.8 x 10^3 cells/μL and its platelet level at 1170 ± 207.5 x 10^3 platelets/μL. Meanwhile, averaged values of the wild type were 13.8 ± 3.8 x 10^3 cells/μL and 903.6 ± 56.5 x 10^3 platelets/μL. These represented a trend of slightly higher white blood cells and platelets of the mutant than the wild type (P= 0.098**,** and 0.850, **Table 24**). In PV population, the mutation appeared to have much effect on clinical symptoms. The patients with *JAK2V617F* carried platelets at 449.7 ± 60.9 x 10^3 platelets/μL significantly higher than the wild type with 211.3 ± 15.2 platelets/μL (P = 0.014,

Table 25).To our surprise, the averaged haemoglobin level of the mutant at 15.9 ± 0.6 g/dL was significantly lower than the wild type (P = 0.038, **Table 25**). Haematocrit and MCV of the mutant were at 49.5 ± 1.8 to 54.8 ± 4.1 and 77.7 ± 2.6 to 88.9 ± 3.4 respectively; they were slightly lower as well though they were not significant (P = 0.099 and 0.067, **Table 25**).

Table 22 Comparison of clinical data between the wild type and the *JAK2V617F* in the overall study population

	Overall MPN		
	Wild type	***JAK2V617F***	***P*-value**
Bleeding	9/103 (8.7%)	5/103 (4.9%)	*0.039***a
Thrombosis	13/103 (12.6%)	20/103 (19.4%)	0.980a
RBC10^6	4.6 ± 0.2	5.4 ± 0.2	0.051b
Hb	13.2 ± 0.7	13.8 ± 0.5	0.453c
Hct	39.2 ± 2.2	42.7 ±1.5	0.157c
MCV	87.3 ± 1.7	81.7 ± 1.7	*0.006***b
WBC10^3	28.3 ± 6.5	14.4 ± 1.2	0.471b
PLT10^3	777.5 ± 92.8	647.4 ± 75.0	0.852b
Total	37/103 (35.9%)	66/103 (64.1%)	-

Haematological parameters in mean ± S.E., Bleeding, thrombosis and *JAK2V617F* in frequency/total case, a. Pearson chi-square, b. Mann-Whitney *u* test, c. student *t*-test, RBC (Red blood cell count), Hb (Haemoglobin), Hct (Haematocrit), MCV (Mean corpuscular volume), WBC (White blood cell count), PLT (Platelet count), **statistical difference.

Table 23 Comparison of clinical data between the wild type and the *JAK2V617F* in the Ph- MPN population

	Ph- MPN		
	Wild type	***JAK2V617F***	***P*-value**
Bleeding	9/103 (8.7%)	5/103 (4.9%)	0.066a
Thrombosis	13/103 (12.6%)	20/103 (19.4%)	0.487a
RBC10^6	4.8 ± 0.2	5.4 ± 0.2	0.084b
Hb	14.0 ± 0.7	13.9 ± 0.4	0.836b
Hct	43.1 ± 1.9	43.6 ±1.3	0.832b
MCV	88.7 ± 1.5	81.5 ± 1.6	*0.005***c
WBC10^3	14.6 ± 2.7	14.5 ± 1.2	0.331c
PLT10^3	752.5 ± 76.0	727.9 ± 105.5	0.138c
Total	33/96 (34.4%)	63/96 (65.6%)	-

Haematological parameters in mean ± S.E., Bleeding, thrombosis and *JAK2V617F* in frequency/total case, a. Pearson chi-square, b. Mann-Whitney *u* test, c. student *t*-test, RBC (Red blood cell count), Hb (Haemoglobin), Hct (Haematocrit), MCV (Mean corpuscular volume), WBC (White blood cell count), PLT (Platelet count), **statistical difference.

Table 24 Comparison of clinical data between the wild type and the *JAK2V617F* in the ET population

	ET		
	Wild type	***JAK2V617F***	***P*-value**
Bleeding	4/49(8.2%)	2/49 (4.1%)	.169a
Thrombosis	7/49 (14.3%)	10/49 (20.4%)	.970a
RBC10^6	4.4 ± 0.1	4.7 ± 0.2	.183b
Hb	12.8 ± 0.4	12.7 ± 0.5	.903b
Hct	38.8 ± 1.1	39.0 ± 1.4	.943b
MCV	89.9 ± 2.0	83.9 ± 11.7	.083b
WBC10^3	13.8 ± 3.8	14.8 ± 1.8	.098c
PLT10^3	903.5 ± 56.5	1171.0 ± 207.5	.850c
Total	20/49(40.8%)	29/49 (59.2%)	-

Haematological parameters in mean ± S.E., Bleeding, thrombosis and *JAK2V617F* in frequency/total case, a. Pearson chi-square, b. student *t* test, c. Mann-Whitney *u* test, RBC (Red blood cell count), Hb (Haemoglobin), Hct (Haematocrit), MCV (Mean corpuscular volume), WBC (White blood cell count), PLT (Platelet count), ET (Essential thrombocythaemia), **statistical difference.

Table 25 Comparison of clinical data between the wild type and the *JAK2V617F* in the PV population

	PV		
	Wild type	***JAK2V617F***	***P*-value**
Bleeding	1/31 (3.2%)	3/31 (9.7%)	0.788a
Thrombosis	4/31 (13.0%)	10/31 (32.3%)	0.272a
RBC10^6	6.2 ± 0.5	6.8 ± 0.3	0.786b
Hb	18.5 ± 1.3	15.9 ± 0.6	*0.038***b
Hct	54.8 ± 4.1	49.5 ± 1.8	0.099b
MCV	88.9 ± 3.4	77.7 ± 2.6	0.067b
WBC10^3	10.4 ± 2.3	10.3 ± 1.7	0.484b
PLT10^3	211.3 ± 15.2	449.7 ± 60.9	*0.014***b
Total	6/31 (19.4%)	25/31 (80.6%)	-

Haematological parameters in mean ± S.E., Bleeding, thrombosis and *JAK2V617F* in frequency/total case, a. Pearson chi-square, b. Mann-Whitney *u* test, RBC (Red blood cell count), Hb (Haemoglobin), Hct (Haematocrit), MCV (Mean corpuscular volume), WBC (White blood cell count), PLT (Platelet count), ET (Essential thrombocythaemia), PV (Polycythaemia vera), **statistical difference.

CHAPTER V

DICUSSION & CONCLUSION

Discovery of *JAK2V617F* in myeloid disorders since 2005 has brought new insight into haemato-oncology, especially its contribution to pathogenesis of the classical MPD. Thenceforth, it has become a remarkable marker in diagnosing the Ph- MPN and was added into the newly revised diagnostic criteria of the WHO in 2008. The mutation also plays important roles in prediction of clinical symptoms, such as levels of haematological parameters and bleeding-thrombosis risk. Furthermore, it is now focused as a target of treatments either by pharmaceutical or other alternative technology. Its information, however, has been gathered mostly on Westerners in Europe and North America; few documents in Asians and other population available. This study has refined the view on *JAK2V617F* in Thai MPN population emphasising its prevalence and clinical correlation.

Prevalence of *JAK2V617F* is reported differently in number as shown in **Table 26**: approximately 80-90% in PV, 50-60% in ET, 40-50% in PMF, and 20% in unclassifiable MPN (Jones et al., 2005; Levine et al., 2005; Lippert, Boissinot et al., 2006). The numbers in other populations are not much different from Europeans and Americans. The study in Africans also showed prevalence at 89.5% in PV, 62.5% in ET and 66.6% in PMF, whereas it is 48% in Hispania with ET (Heller et al., 2006; Benmoussa, Dehbi et al., 2011). Focusing Asians, the numbers are quite similar in either East or South Asians. It is 80-90% in PV, 50-60% in ET, and 30-50% in PMF (Hattori, Fukuchi et al., 2008; Lieu, Wu et al., 2008; Xu, Li et al., 2009; Kim, Cho et al., 2010; Sazawal, Bajaj et al., 2010; Wong, Kam et al., 2010). The prevalence we reported here in Thais, especially in ET and PV patients, are close to both regional and

international. It could be assumed that the prevalence of *JAK2V617F* has no difference among ethnicity.

Table 26 *JAK2V617F* mutation prevalence in other populations

Researchers	Study population	*JAK2V617F* prevalence
Jones et al., 2005	British	PV 81%, ET 41%, PMF 43%, MPN un 20%
Levine et al., 2005	American	PV 73.8%, ET 32.2%, PMF 34.8%
Lippert et al., 2006	French	PV 92%, ET 75%
Heller et al., 2006	Argentine	ET 48%
Kittur, Knudson et al. 2007	American	ET 55%
Antonioli et al., 2008	Italian	ET 63.4%
Hattori et al., 2008	Japanese	PV 100%, ET 63.9%, PMF 50%
Lieu et al., 2008	Chinese (Taiwan)	PV 85%, ET 59%, PMF 33%
Xu et al., 2009	Chinese (Mainland)	PV 34%, ET 80%, PMF 78%, MPN un 75%
Kim et al., 2010	Korean	PV 91.6%, ET 52.8%, PMF 46.2%, MPN un 66.7%
Sazawal et al., 2010	Indian	PV 82%, ET 70%, PMF 52%
Wong et al., 2010	Singaporean (Chinese, Malay, Indian and Western)	ET 34%
Benmoussa et al., 2011	Moroccan	PV 89.5%, ET 62.5%, PMF 33.3%

PV, polycythaemia vera, ET, essential thrombocythaemia, PMF, primary myelofibrosis, MPN un, unclassifiable myeloproliferative neoplasm

Still, differences of our mutation incidence in PMF and unclassifiable MPN to others could be seen. This can be explained with the effect of sample size; study in larger scale is required for more precision. In addition, it must be noted that difference in mutation detecting method could interfere to the prevalence. The study of Lucia et al in Italian MPN population with two different *JAK2V617F* examining techniques exhibited shift of the incidence. With Allele-specific PCR, the prevalence was 78.5% while the semi-quantitative 5'fluorescence TaqMan assay revealed the number at 92.9% (Lucia, Martino et al., 2008).

Clinical correlation of *JAK2V617F* is another topic to be discussed. The previous study in 106 ET individuals reported association of the mutation to thrombosis risk, but not to haemorrhage. Correlations of the mutation to higher haemoglobin, to higher white blood cells count and to lower platelets were also disclosed in the mutant (Patriarca et al., 2010). Here in ET population, we found no significant difference of any haematological parameters, bleeding and thrombosis; but only trends of slightly higher platelets, leukocytes and thrombosis history of the mutant than the wild type. We considered these could be clues of clinical contribution of the mutation in ET. In PV which bleeding is more common, still there is no prior document to proof direct correlation of the mutation to the complication. Our study in PV population also showed no significant data to clarify this question, but the mutant displayed slightly higher bleeding and thrombosis record than the wild type. For haematological parameters, we reported significant higher haemoglobin of the wild type than the mutant. It is the only red cells indicators we observed significant data. We suggest that the others could be interfered by red cells volume, seeing that the mutant had significant less MCV and bleeding history than the wild type in the overall population. These might reflect unrecorded chronic bleeding prior visiting a hospital. In addition, it must be noted that the individuals with bleeding complication were originally low in this population. This means early enough diagnosis prior the complication and/or

unrecorded data as discussed. Although the other parameters had no significant data, it provided trends in clinical difference between the mutant and the wild type. Our finding has confirmed contribution of *JAK2V617F* to clinical features of MPN in accordance with other previous reports.

Though *JAK2V617F* mutation does influence clinical features, it is not the only factor. According to the Virchow's triad, vascular circumstance could be interfered by pathologic conditions, such as infection and chronic disorder. Those situations induce generation of cytokines affecting the vascular flow directly. It is commonly known that bacterial and viral infection could result cytokine storming. Chronic disorders, especially malignancies, could also disrupt physiologic cytokine regulation. Kurzrock in 1997 proposed deregulation of an inflammatory cytokine, interleukin-6 (IL-6), in haematological malignancies, particularly lymphomas (Kurzrock, 1997). This cytokine connects to pathophysiology of multiple myeloma as well (Kallen, 2002). In MPN, Panteli et al in 2005 reported significantly raised IL-2 and soluble IL-2 receptor (sIL-2R) level in PV, ET, PMF and CML patients than the healthy, plus IL-6 in transformation of PV and ET to myelofibrosis and of PMF to AML (Panteli, Hatzimichael et al., 2005). Additionally, Goette et al had discovered increased plasma sIL-6R level and monocyte IL-2Rα in ET, suggesting their clinical contribution to the increased platelets level and thrombosis (Marta, Goette et al., 2004; Goette, Lev et al., 2010; Goette, Lev et al., 2010). Investigation by protein arrays for 79 cytokines in 20 MPN individuals disclosed significant higher levels of tissue inhibitor of metalloproteinase-1 TIMP-1, macrophage inflammatory protein-1β (MIP-1β) and insulin-like growth factor binding protein-2 (IGHBP-2) (Ho, Lasho et al., 2007). Another inflammatory related cytokine, tumour necrotic factor-α (TNF-α), was recently disclosed its capability in promoting expansion of JAK2V617F clones (Fleischman, Aichberger et al., 2011). These documents ensure cytokines' role as a contributor in pathophysiology and vascular complications of MPN. Other chronic

disorders are capable in causing these troubles as well. In cardiovascular disease, atherosclerotic plaque and thrombus are not the ultimate cause of the obstruction of the circulation. The impaired vascular evidence promotes platelets-leukocytes formation via P-selectin/PSGL1 while the conventional platelets-endothelial cells formation occurs (Bournazos, Rennie et al., 2008). These phenomena triggers platelets activation, aggregation, and release of granules and also activate coagulation factors further. The evidence has been proofed recently in ET on cells surfaces directly and onto cell-derived microparticles (Maugeri, Malato et al., 2011).

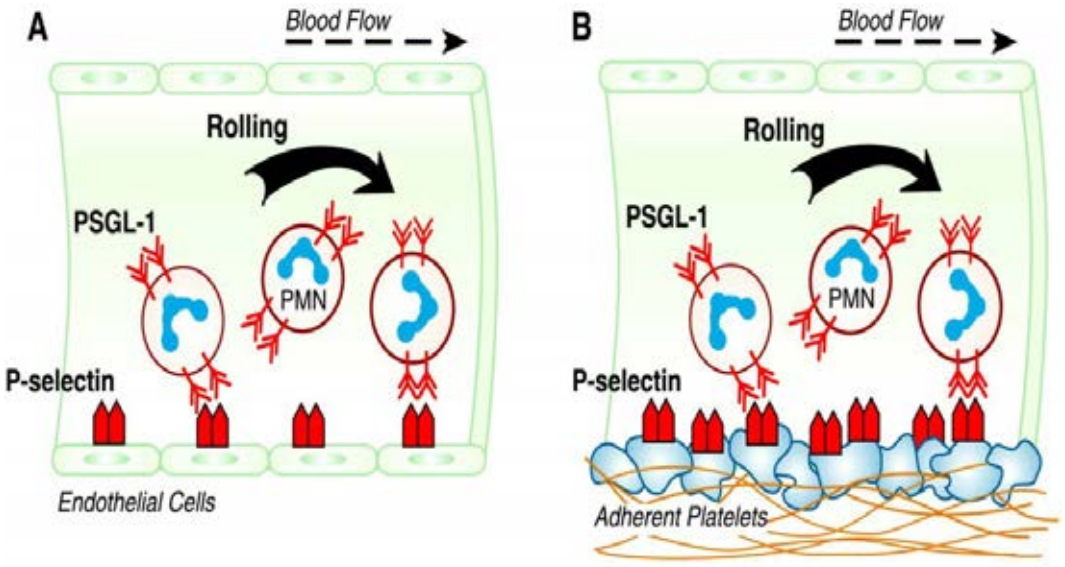


Figure 11 Formations of (A) leukocytes/endothelial cells and (B) leukocytes/platelets via P-selectin/PSGL-1 promote thrombosis (Zimmerman, 2001).

In molecular aspect, JAK2 is not the only molecule involving cell proliferation and differentiation; several others also function simultaneously for fully effective process. Other reports have highlighted association of those to the disease directly, indirectly via negative feedback, correspondingly to leukaemic progression and epigenetically (Vainchenker et al., 2011). Other mutations on JAK2, *JAK2 exon 12* mutations have been described in PV with the absence of *JAK2V617F* while the mutation of thrombopoietin receptor, *MPLW515K/L*, has been discovered in both ET and PMF (Pardanani et al., 2006; Scott, Tong et al., 2007). Alteration of negative regulators of signalling, such as *LNK*, suppressor of cytokine signalling (*SOCS*) and Casitas B-cell lymphoma (*CBL*), have been proofed their relevancy to pathogenesis (Fourouclas, Li et al., 2008; Sanada, Suzuki et al., 2009; Sanada, Suzuki et al., 2009; Baran-Marszak, Magdoud et al., 2010). Isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*), Ikaros transcription factor (*IKZR*), *NRAS/KRAS*, *TP53* and *AML1/RUNX1* with their molecular lesion involve AML and also leukaemic phase of MPN (Bacher, Schnittger et al., 2009; Ding, Harada et al., 2009; Beer, Ortmann et al., 2010; Jager, Gisslinger et al., 2010; Tefferi, Lasho et al., 2010). Currently, researchers are working on epigenetic causes. The genes in association to MPN are *TET2*, *ASXL1* and *EZH*, for instances (Carbuccia, Murati et al., 2009; Delhommeau, Dupont et al., 2009; Herrera-Merchan, Arranz et al., 2012). Further study on those molecules is required to understand their exact contribution to the disease. Not just for clarifying ambiguous pathogenesis and pathophysiology, but the insight into those would also provide more customised treatment to prevent any fatal complication and promote curable chance in very near future.

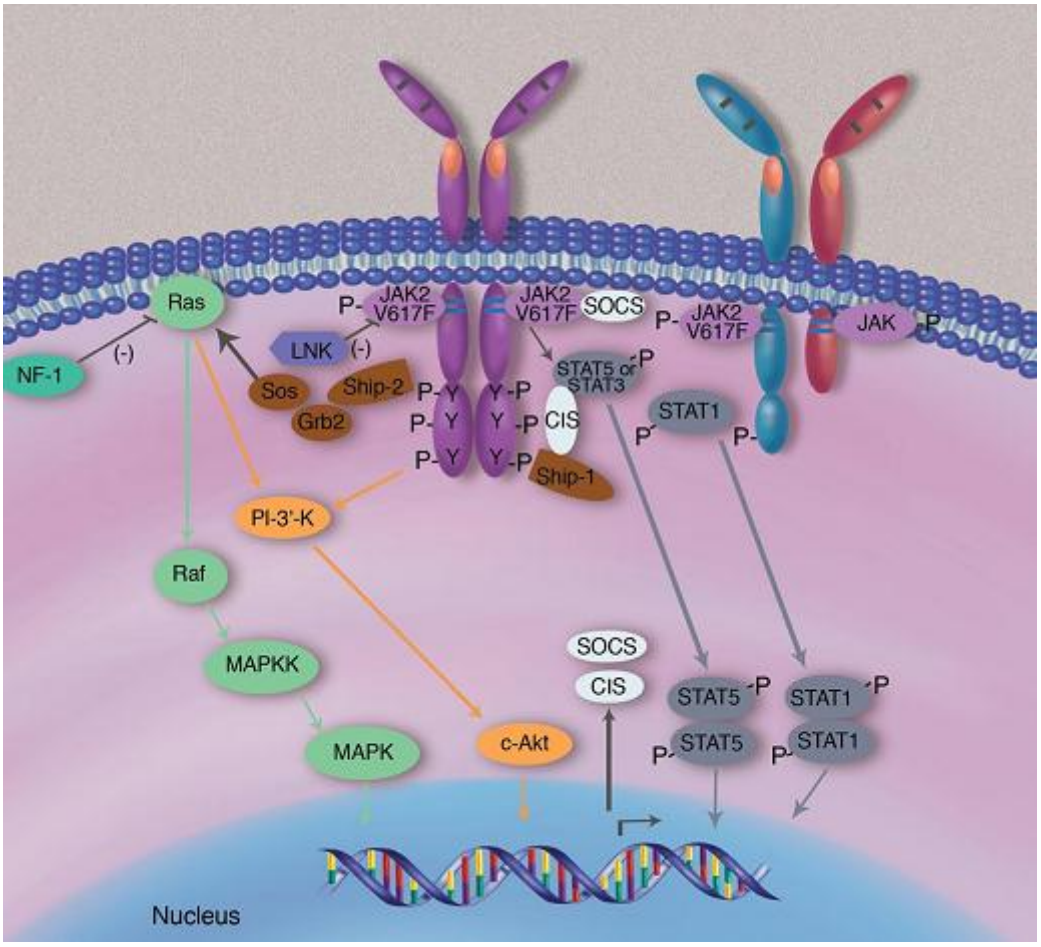


Figure 12 Intracellular signalling pathways associating MPN (Vainchenker et al., 2011)

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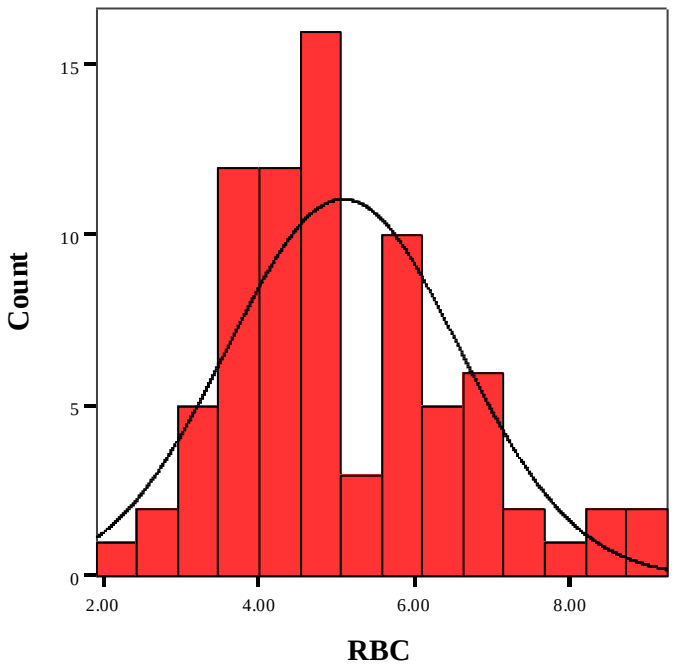
APPENDICES

Appendix A

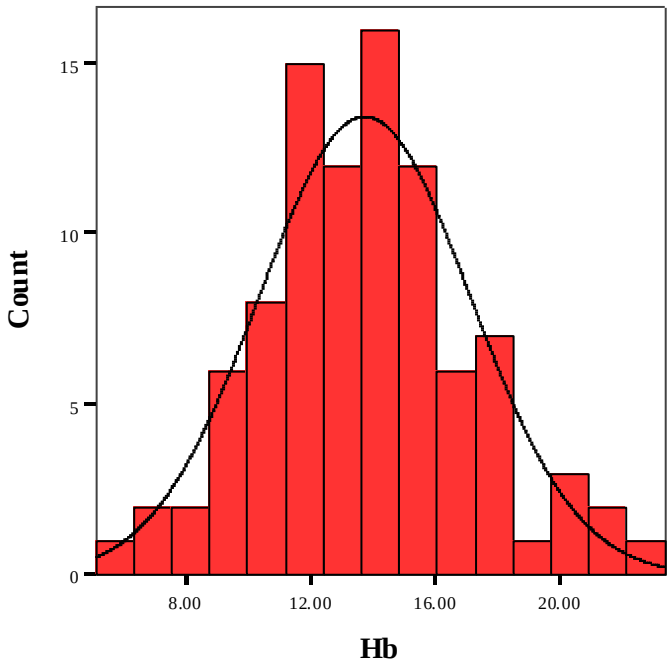
Tests of Normality: Overall MPN population

	Kolmogorov-Smirnov(a)		
	Statistic	df	Sig.
RBC	.101	74	.058
Hb	.067	74	.200
Hct	.067	74	.200
MCV	.121	74	.009
WBC	.232	74	.000
PLT	.088	74	.200

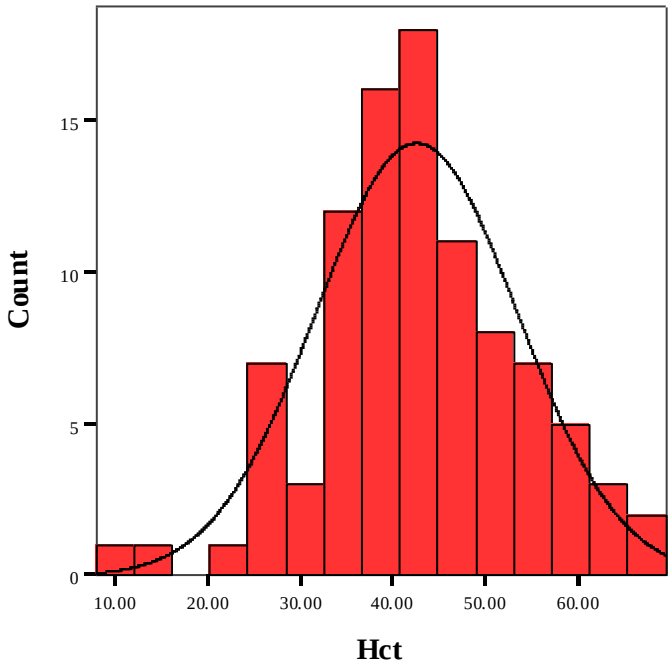
Distribution of RBC



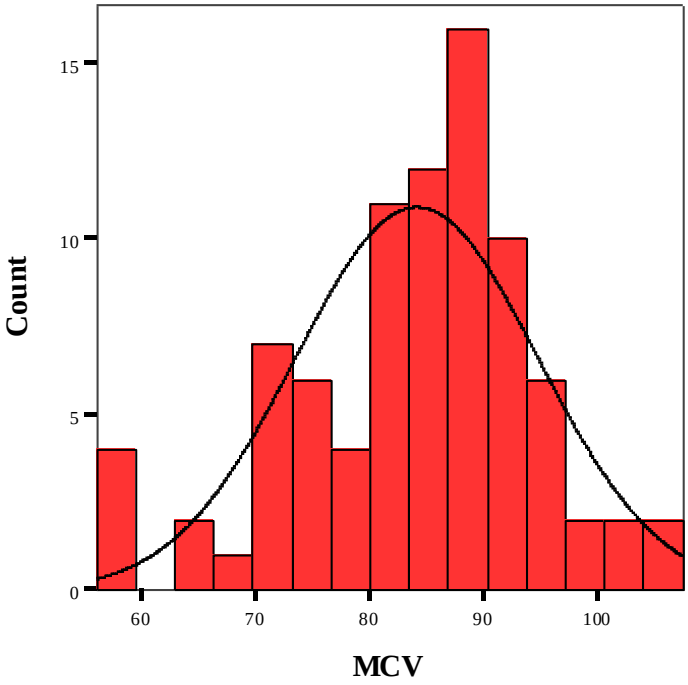
Distribution of Hb



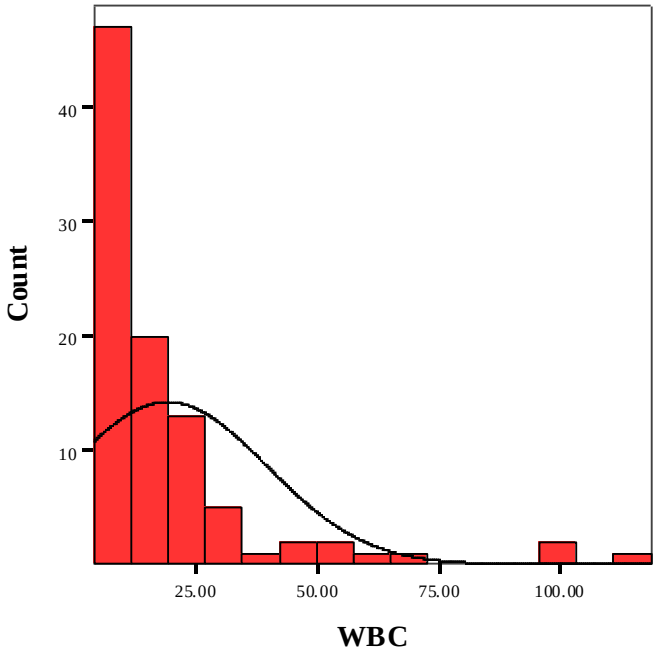
Distribution of Hct

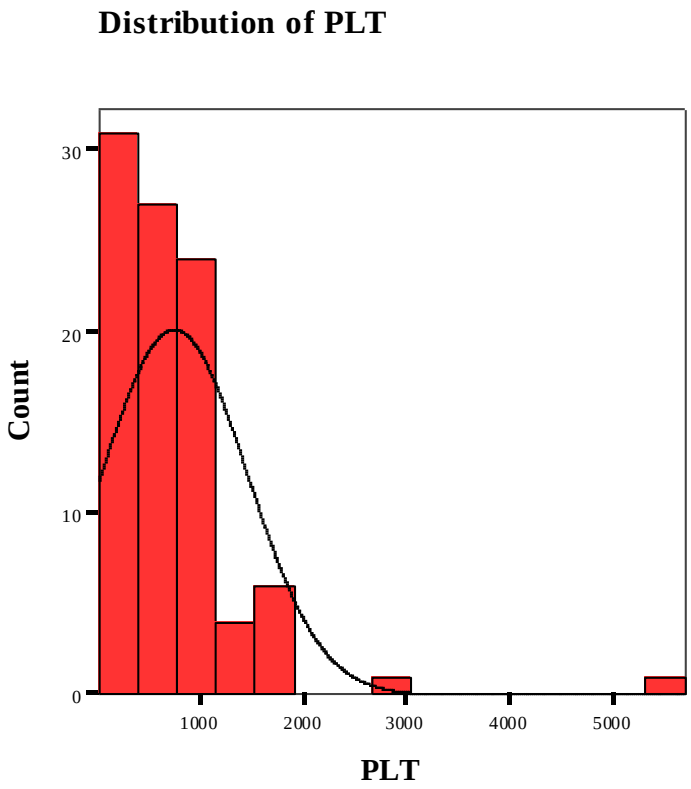


Distribution of MCV



Distribution of WBC



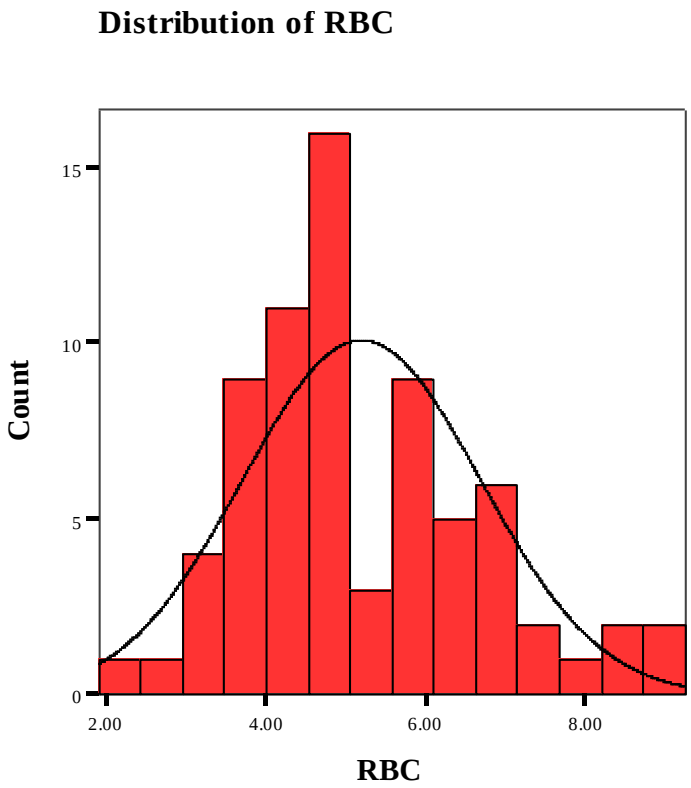


Test of Homogeneity of Variances: Overall MPN population

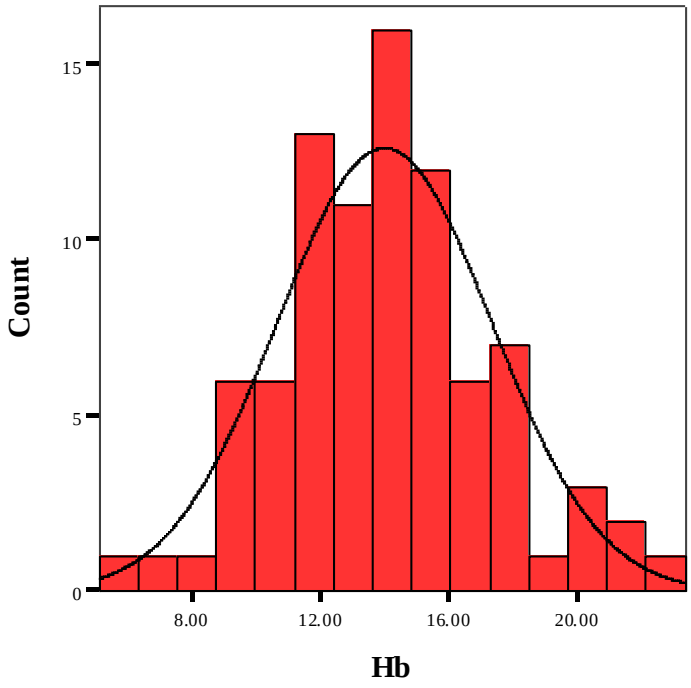
	Levene Statistic	df1	df2	Sig.
RBC	2.701	4	74	.037
Hb	1.444	4	89	.226
Hct	2.228	4	90	.072
MCV	.568	4	80	.687
WBC	11.175	4	90	.000
PLT	1.053	4	89	.385

Tests of Normality: Ph- MPN population

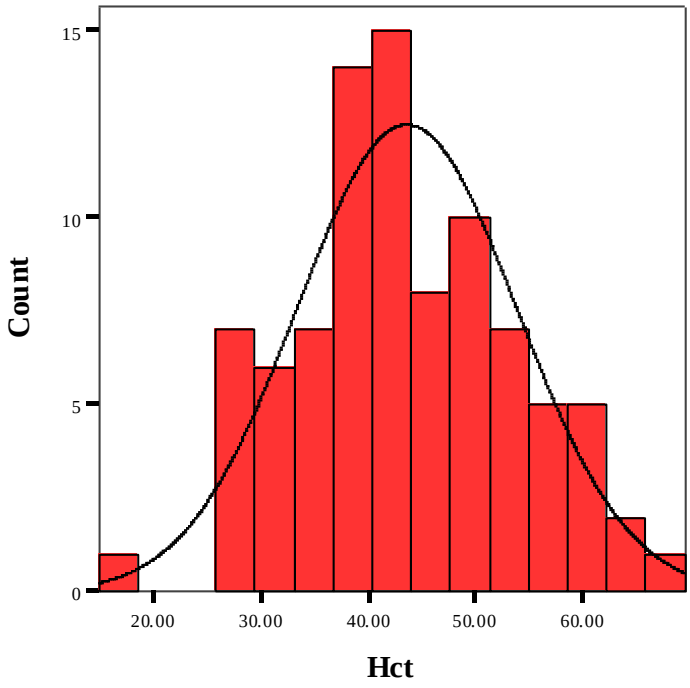
	Kolmogorov-Smirnov(a)		
	Statistic	df	Sig.
RBC	.103	67	.077
Hb	.073	67	.200
Hct	.076	67	.200
MCV	.135	67	.004
WBC	.158	67	.000
PLT	.086	67	.200



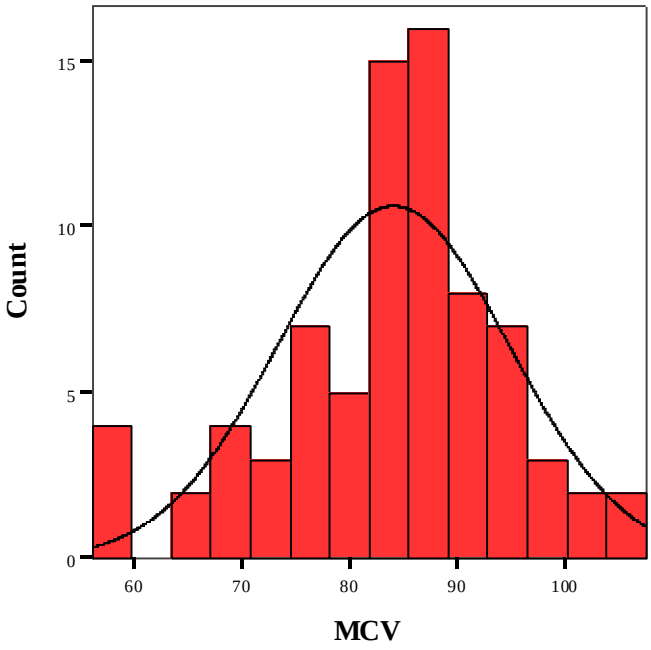
Distribution of Hb



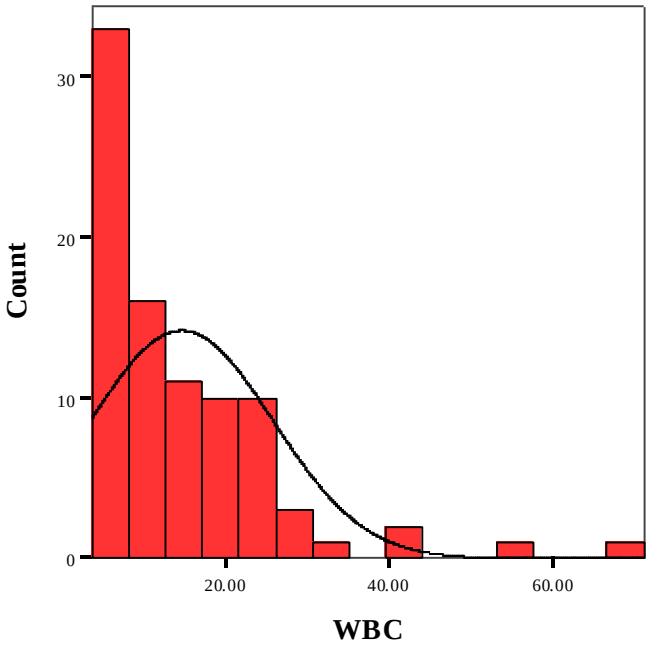
Distribution of Hct



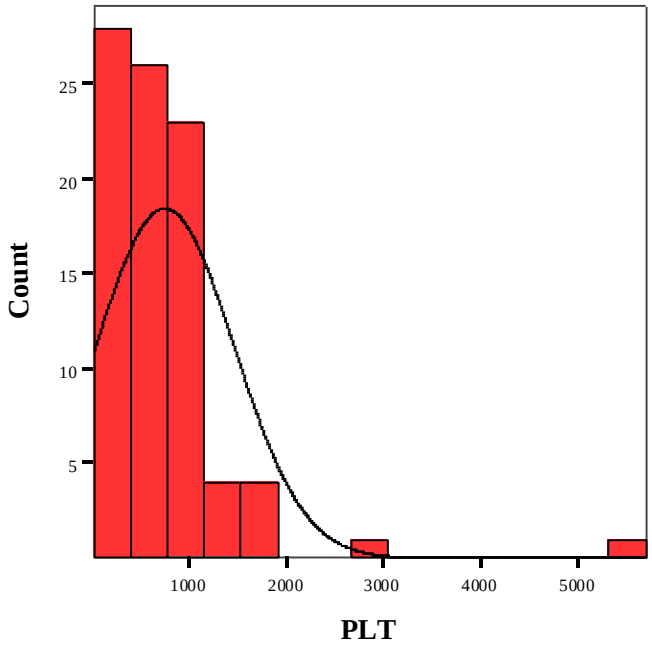
Distribution of MCV



Distribution of WBC



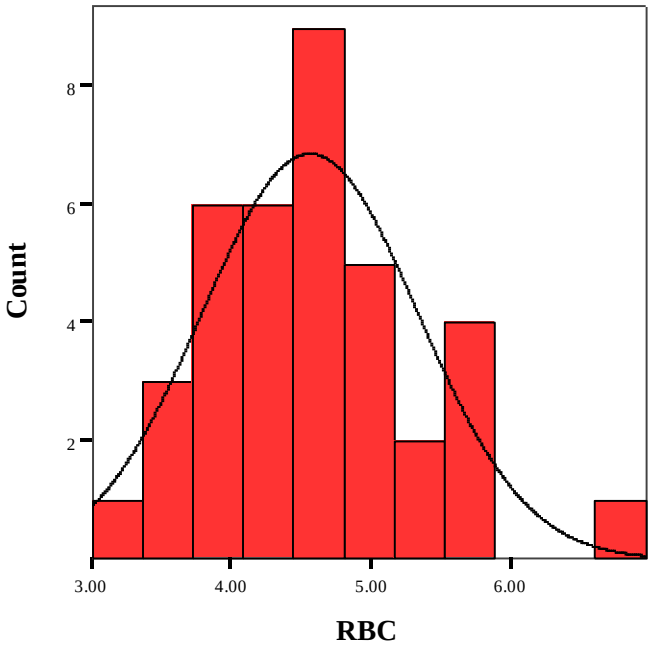
Distribution of PLT



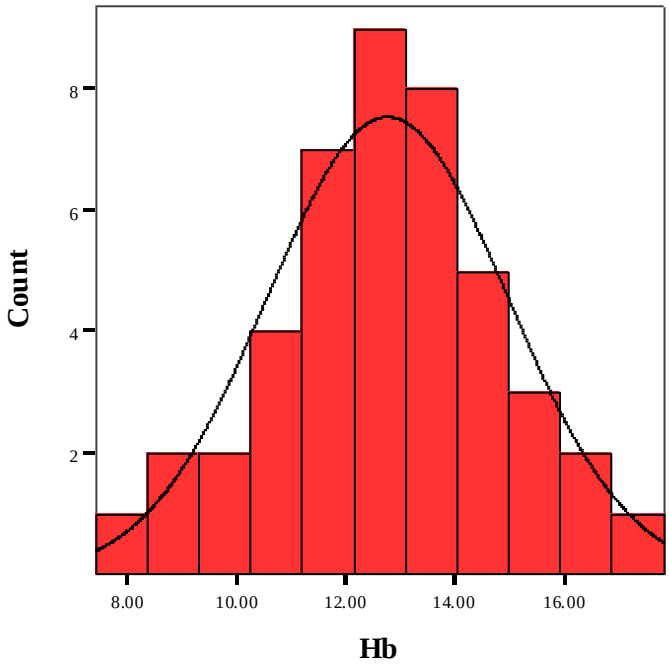
Tests of Normality: ET population

	Kolmogorov-Smirnov(a)		
	Statistic	df	Sig.
RBC	.113	32	.200
Hb	.096	32	.200
Hct	.121	32	.200
MCV	.130	32	.182
WBC	.172	32	.017
PLT	.186	32	.007

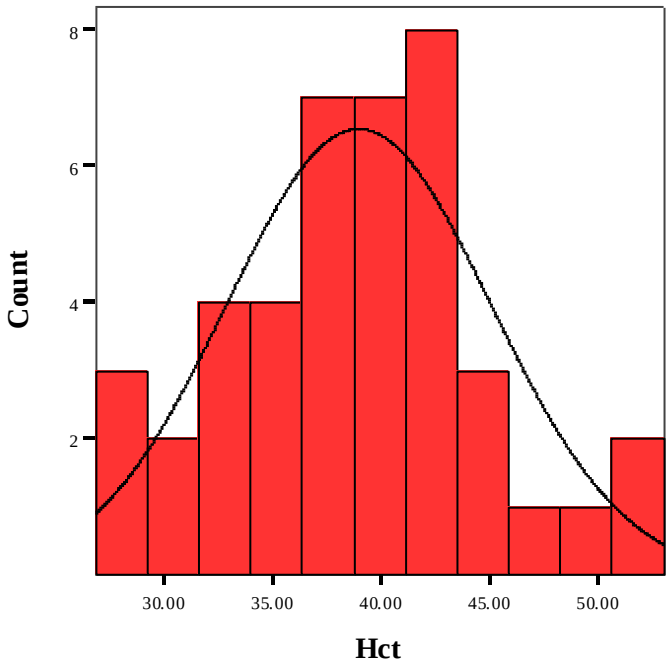
Distribution of RBC



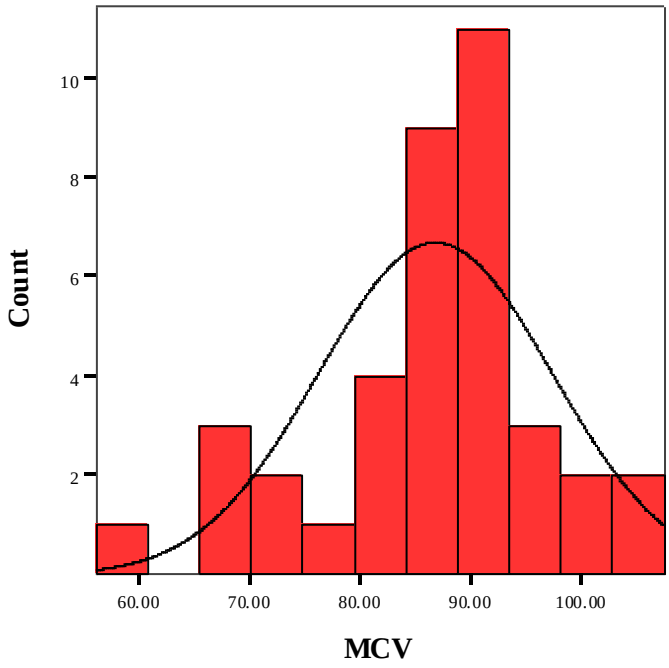
Distribution of Hb



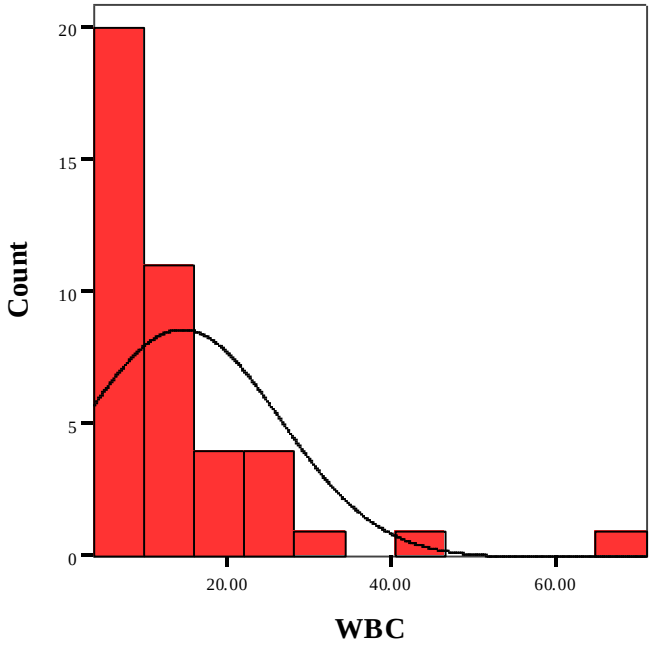
Distribution of Hct



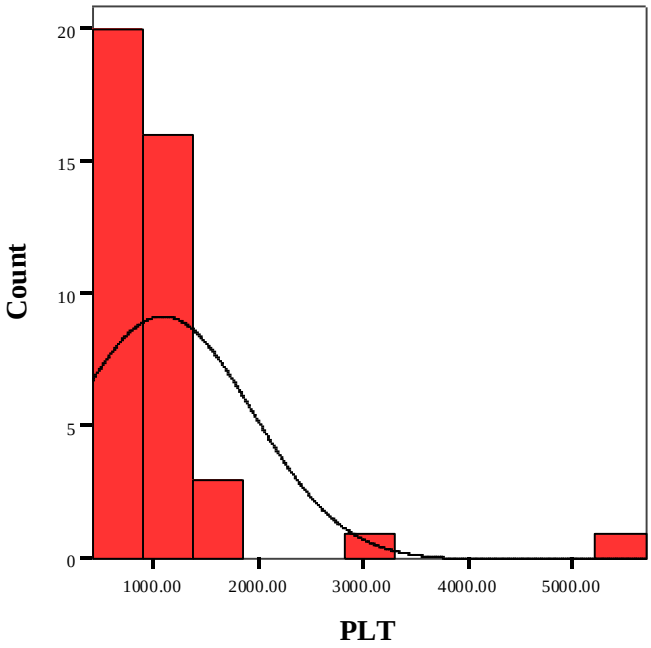
Distribution of MCV



Distribution of WBC



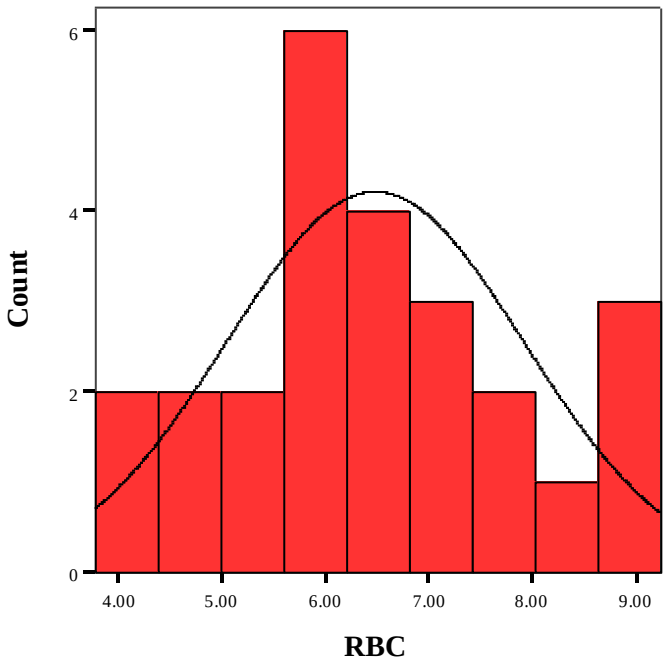
Distribution of PLT



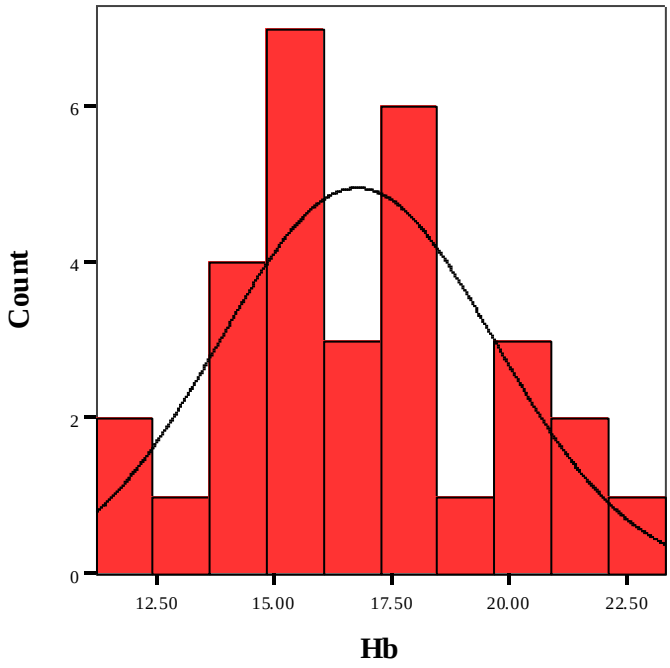
Tests of Normality: PV population

	Shapiro-Wilk		
	Statistic	df	Sig.
RBC	.977	25	.828
Hb	.958	25	.383
Hct	.968	25	.584
MCV	.911	25	.032
WBC	.889	25	.010
PLT	.884	25	.008

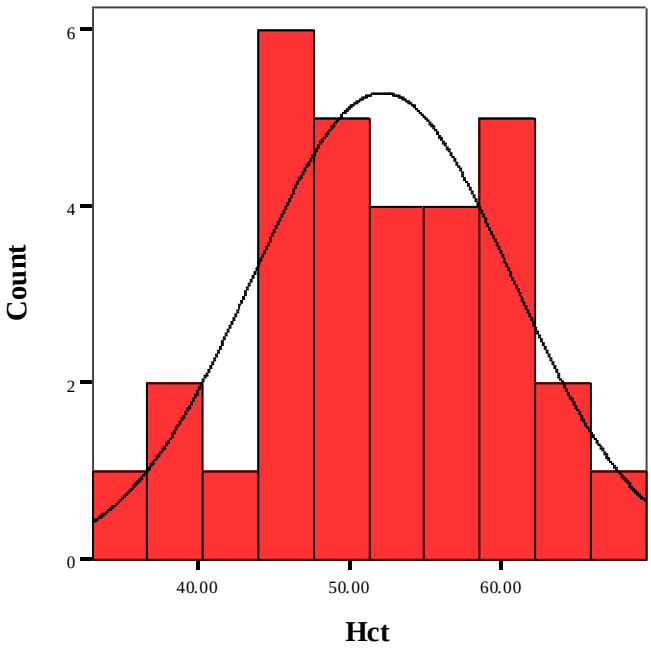
Distribution of RBC



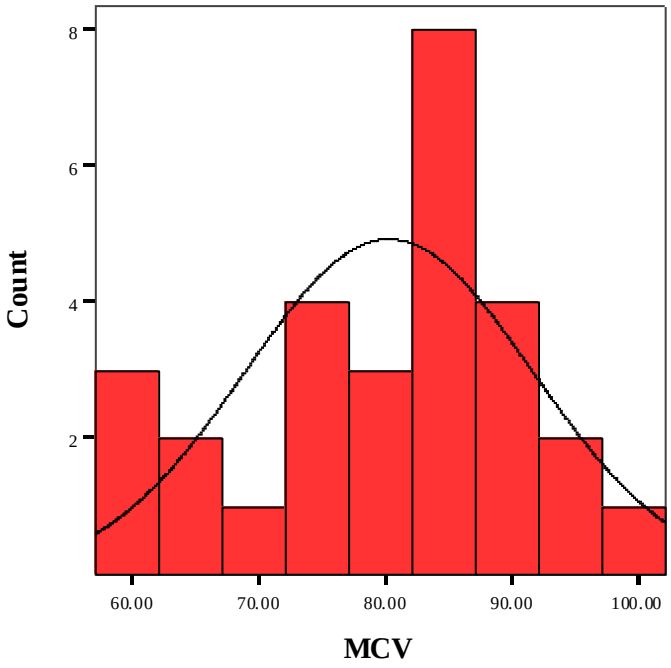
Distribution of Hb



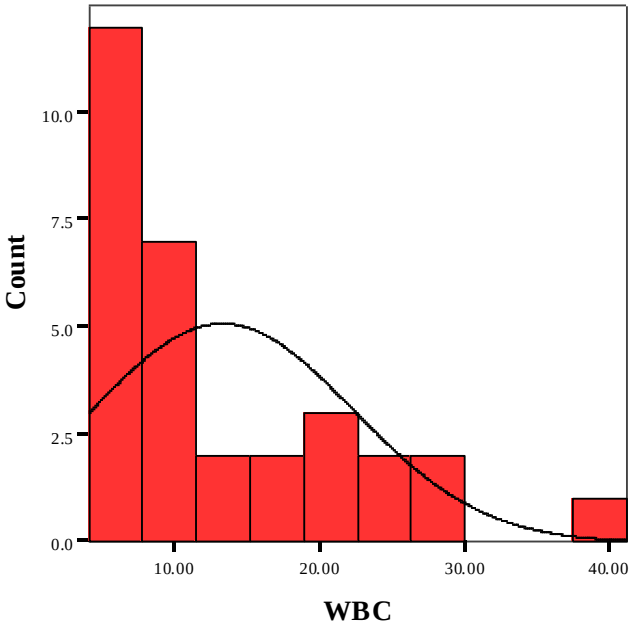
Distribution of Hct

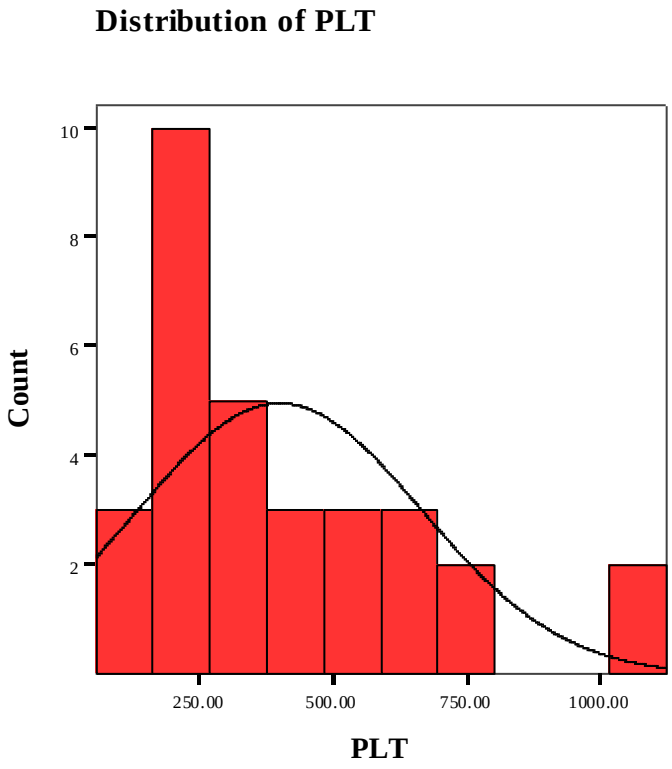


Distribution of MCV



Distribution of WBC




Distribution of PLT
Count
10
8
6
4
2
250.00
500.00
750.00
1000.00
PLT

Appendix B

This study has been published in

Suksomyos, N., S. Chanprasert, et al. (2012). Prevalence of JAK2V617F mutation and its clinical correlation in Thais with myeloproliferative neoplasm. Int J Biol Med Res 3(2): 1801-1805

BIOGRAPHY

Namo Suksomyos, born August 13th 1987, is half Chinese-Thai. He is a great grandson of the world's great personality of the 20th century awarded by United Nations Educational, Scientific and Cultural Organization (UNESCO), the senior statesperson and the former Prime Minister of Thailand, late H.E. Professor Dr. Pridi Banomyong. His father was in the military service as a finance and accountancy lecturer in the Royal Thai Army Academy. His Chinese Thai mother was a businesswoman running her family's businesses in goldsmith and automobile. N. Suksomyos was raised up with his younger sister in Bangkok with close care from his paternal grandmother who strictly emphasised importance of education and social manners. He earned the primary education in a catholic school and went to the Demonstration School of Suan Sunandha Rajabhat University for the lower and upper secondary education. There, he found his interest in Biology, particularly medical-related. Thus, he chose studying Medical Technology with the minor programme in Molecular Biology at the Faculty of Allied Health Sciences, Chulalongkorn University, and was awarded the Bachelor of Sciences with the second class honour in 2008. He started his career as a research assistant at the Division of Immunology, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, and left later for postgraduate study in Haematology. During the course, he was awarded Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej, the 90thAnniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot endowment fund) and the University Prestigious Student. His specialties are Haemato-Oncology, Thrombosis, and Platelet biology.