GENE ABERRATIONS AND METHYLATION ANALYSIS OF JAK/STAT AND TOLL-LIKE RECEPTOR DOWNSTREAM SIGNALLING IN *BCR-ABL*-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS AND MYELODYSPLASTIC SYNDROME/MYELOPROLIFERATIVE NEOPLASMS OVERLAP SYNDROMES

CHIA YUH CAI

UNIVERSITI SAINS MALAYSIA

2021

GENE ABERRATIONS AND METHYLATION ANALYSIS OF JAK/STAT AND TOLL-LIKE RECEPTOR DOWNSTREAM SIGNALLING IN *BCR-ABL*-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS (MPN) AND MYELODYSPLASTIC SYNDROME/MYELOPROLIFERATIVE NEOPLASMS OVERLAP SYNDROMES

by

CHIA YUH CAI

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

OCTOBER 2021

ACKNOWLEDGEMENT

First of all, I would like to use this opportunity to express my gratitude and thanks to my honourable supervisor, Dr Marini Ramli for her supervision, continuous guidance, advice and support throughout my study. I am also sincerely grateful to my co-supervisors, Associate Professor Dr Muhammad Farid Johan, Professor Dr Rosline Hassan and Dr Md Asiful Islam for assisting in solving problems and helping me in gaining new knowledge. This study project would not have been completed without all my supervisors.

Most grateful to the School of Medical Sciences, Universiti Sains Malaysia (USM) for all the support and facilities provided to perform this study. Many thanks to all staff at the Laboratory of Molecular Biology, Central Research Laboratory (CRL), especially Puan Abdah Karimah Che Md Nor, Encik Zulkefli Sanip and Puan Afzan Hawani Alias for their patience, teaching and helping me in polishing my skill in lab techniques. I am also grateful to the staff at Haematology Laboratory, Hospital USM and Institute for Research in Molecular Medicine (INFORMM). Many sincere thanks also go to all academic lecturers, staff and postgraduate students in Haematology Department, USM who offered me considerable help and advice and thank you for all the unforgettable experiences and memories.

I would like to take this opportunity to record my deepest appreciation and thanks to my parents and siblings for their endless love and support. I am also grateful to the Division of Research and Innovation, USM and Ministry of Higher Education, Malaysia for financial assistance to continue my higher study. Last but not least, I am thankful to USM for awarding the RUI grant (1001/PPSP/812187) for a financial grant which enabled me to conduct and complete this study successfully.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

5-caC	5-carboxylcytosine
5-fC	5-formylcytosine
5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
ABL1	ABL proto-oncogene 1
aCML	Atypical chronic myeloid leukaemia
Akt	Protein kinase B
AFLP	Amplification fragment length polymorphism
AML	Acute myelogenous leukaemia
AS-PCR	Allele-specific PCR
ASXL1	Additional sex combs like-1
BCR-ABL	Philadelphia chromosome
BM	Bone marrow
CALCA	Calcitonin related polypeptide alpha
CALR	Calreticulin
CBL	Casitas B-lineage lymphoma
CCM	Chemical cleavage of mismatch
CD	Cys-rich domain
CEBPA	CCAAT/enhancer-binding protein alpha
CFU-GM	Granulocyte-macrophage progenitors
CH_3	Methyl group
CI	Confidence interval
CML	Chronic myelogenous leukaemia
CMML	Chronic myelomonocytic leukaemia
CNL	Chronic neutrophilic leukaemia
CpG	Cytosine-phosphate guanine
CSF3R	Colony-stimulating factor 3 receptor
CSGE	Conformation sensitive gel electrophoresis
CXCR4	C-X-C motif chemokine receptor 4
DAMP	Damage-associated molecules patterns
dC	Deoxycytidine

DD	Death domain
ddNTPs	Dideoxynucleotide triphosphates
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3 alpha
dNTPs	Deoxynucleotide triphosphates
DSBH	Double-stranded helix
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ET	Essential thrombocythaemia
ETNK1	Ethanolamine kinase 1
EZH2	Enhancer of zeste homolog 2
FERM	N-terminal Band 4.1, ezrin, radixin, moesin domain
FGFR1	Fibroblast growth factor receptor 1
FLT3	Fms-like tyrosine kinase 3
G	Guanine
GM-CSF	Granulocyte macrophage-colony stimulating factor
Hb	Haemoglobin
HPLC-UV	High performance liquid chromatography-ultraviolet
HRM	High-resolution melt
HSC	Haematopoietic stem cell
HxD	His-x-Asp
IDH1	Isocitrate dehydrogenase 1
IDH2	Isocitrate dehydrogenase 2
IFN	Interferon
IgG	Immunoglobulin G
IKZF1	IKAROS
INFORMM	Institute for Research in Molecular Medicine
INPP5D	Inositol polyphosphate 5-phosphatase D
IL	Interleukin
IL-1R	Interleukin-1 receptor
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
JAK2	Janus kinase 2

JEPEM	The Human Research Ethics Committee of USM
JH	JAK homology
JMML	Juvenile myelomonocytic leukaemia
KIR	Kinase inhibitory region
KIT	KIT Proto-oncogene, receptor tyrosine kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
lncRNA	Long non-coding RNA
LUMA	Luminometric methylation assay
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
MDS/MPN	Myelodysplastic syndrome/myeloproliferative neoplasms syndromes
MDS/MPN-RS-T	MDS/MPN with ring sideroblasts and thrombocytosis
MDS/MPN-U	MDS/MPN-unclassifiable
MF	Myelofibrosis
MPL	Thrombopoietin receptor
MPN	Myeloproliferative neoplasms
MLL	Lysine Methyltransferase 2A
MLPA	Multiplex ligation-dependent probe amplification
mRNA	Messenger RNA
MS-PCR	Methylation-specific polymerase chain reaction
MyD88	Myeloid differentiation primary response 88
ncRNA	Non-coding RNA
NGS	Next-generation sequencing
NF-κB	Nuclear factor-kappa B
NF1	Neurofibromatosis type 1
NPM1	Nucleophosmin 1
NRAS	Neuroblastoma RAS viral oncogene homolog
OLA	Oligonucleotide ligation assay
PAMP	Pathogen-associated molecular patterns
PCM1	Pericentriolar material 1
PCR	Polymerase chain reaction
PDGFRA	Platelet-derived growth factor receptor alpha
PDGFRB	Platelet-derived growth factor receptor beta

PI3K/Akt	Phosphatidylinositol 3-kinase/protein kinase B
PI3K	Phosphatidylinositol 3-kinase
piRNA	Piwi-interacting RNA
PMF	Primary myelofibrosis
PPT	Protein truncation test
PRMT5	Protein arginine methyltransferase 5
PRR	Pattern-recognition receptors
PRV1	Polycythemia rubra vera 1
PTPN6	Protein tyrosine phosphatase non-receptor type 6
PTPN11	Protein tyrosine phosphatase non-receptor type 11
PV	Polycythaemia vera
PVSG	Polycythemia Vera Study Group
RARβ2	Retinoic acid receptor beta 2
RARA	Retinoic acid receptor alpha
RARS-T	Refractory anaemia with ring sideroblasts associated with marked thrombocytosis
RAS	Ras-GTPase
RBC	Red blood cell
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
RUI	Research University Grants
SETBP1	SET binding protein 1
SF3B1	Splicing factor 3b subunit 1
SFRP2	Secreted frizzled-related protein 2
SH2	Src homology 2
SHIP1	Inositol polyphosphate 5-phosphatase D
siRNA	Short interfering RNA
SMF	Secondary myelofibrosis
SNP	Single nucleotide polymorphisms
SOCS	Suppressor of cytokine signalling
SOCS1	Suppressor of cytokine signalling 1
SOCS3	Suppressor of cytokine signalling 3
SSCP	Single strand conformational polymorphism
SRSF2	Serine and arginine-rich splicing factor 2

STAT	Signal transducers and activators of transcription
T	Thymine
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TET2	Ten-eleven translocation 2
TIR	Toll/interleukin-1 receptor
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptors
TP53	Tumour protein p53
TPO	Thrombopoietin
TRAF	Tumour necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-beta
TSS	Transcription start site
USM	Universiti Sains Malaysia
WBC	White blood cell
WES	Whole exome sequencing
WGBS	Whole genome bisulfite sequencing
WGS	Whole-genome sequencing
WHO	World Health Organization
WIF1	WNT inhibitory factor 1
WM	Waldenström Macroglobulinaemia

x g	Acceleration due to gravity
bp	Base pair
kb	DNA size
kDa	Protein size
ng	Nanogram
RLU	Relative light units
rpm	Revolutions per minute
µg	Microgram
µl	Microlitre
µM	Micromolar

LIST OF APPENDICES

ANALISIS ABERASI GEN DAN PEMETILAN PADA ISYARAT HILIRAN JAK/STAT DAN RESEPTOR TOLL-LIKE DALAM *BCR-ABL*-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS DAN MYELODYSPLASTIC SYNDROME/MYELOPROLIFERATIVE NEOPLASMS OVERLAP SYNDROMES

ABSTRAK

BCR-ABL-negative myeloproliferative neoplasms (MPN) dan myelodysplastic syndrome/myeloproliferative neoplasms overlap syndromes (MDS/MPN) adalah kemalignanan myeloid hasil daripada kejadian mutasi dalam genetik, epigenetik dan kromosom, terutamanya dalam isyarat hiliran janus kinase/signal transducers dan activators of transcription (JAK/STAT) dan reseptor toll-like (TLR). Kajian ini adalah untuk mengenal pasti prevalens, status mutasi gen *JAK2*, *TET2* dan *MyD88* dan profil pemetilan gen *SOCS3* dan *INPP5D* dalam kedua-dua penyakit tersebut. Mutasi dalam gen *TET2* dalam *BCR-ABL*-negative MPN telah dipilih untuk menjalankan analisis meta. Arkib sampel DNA pesakit telah digunakan untuk kajian mutasi dan kajian pemetilan. Status mutasi untuk gen *JAK2*, *TET2* dan *MyD88* dalam *BCR-ABL*-negative MPN dan MDS/MPN telah dikaji dengan penjujukan langsung. Profil pemetilan untuk kawasan promoter gen *SOCS3* dan *INPP5D* telah dikaji dengan menggunakan pyrosequencing dan exon 26 gen *INPP5D* telah dianalisis dengan menggunakan PCR pemetilan spesifik. Kawalan normal telah digunakan. Dianggarkan prevalens mutasi gen *TET2* dalam *BCR-ABL*-negative MPN adalah 15.5%. Mutasi *JAK2* V617F, lima varian missense (M532I, M535R, V536G, R541K dan D544N), satu polimorfisme nukleotida tunggal (SNP) intronik yang baru (c.1194+12G>A) dan dua deletan (c.1157delT dan c.1160delT) dalam exon 12 *JAK2* serta satu SNP intronik (rs4988457)

dalam *MyD88* berjaya dikesan. Mutasi *JAK2* V617F kerap dikesan dalam *BCR-ABL*-negative MPN (85.4%) dan MDS/MPN (50.0%). Varian missense dalam exon 12 *JAK2* (27.1%) dan *MyD88* (7.3%) hanya dikesan dalam *BCR-ABL*-negative MPN. Status pemetilan kawasan promoter bagi *SOCS3*, *INPP5D* dan exon 26 *INPP5D* tidak menunjukkan perbezaan yang ketara berbanding dengan kawalan normal. Mutasi dalam gen *TET2* didapati menyumbang kepada permulaan dan perkembangan *BCR-ABL*-negative MPN. Mutasi dalam gen tersebut dipercayai berkaitan dengan trombosis, transformasi leukemia dan mutasi gen lain yang dikenal pasti dalam penyakit yang disebutkan. Namun begitu, lebih banyak kajian diperlukan. *JAK2* V617F sangat berkaitan dengan *BCR-ABL*-negative MPN dan MDS/MPN. Mutasi dalam exon 12 *JAK2* pula lebih spesifik kepada *BCR-ABL*-negative MPN dan dicadangkan untuk dibuat dalam granulosit kerana mutasi nampaknya berkumpul dalam granulosit. Kajian ekspresi untuk gen *MyD88* yang menunjukkan rs4988457 dalam kemalignanan darah juga disyorkan. Analisis status pemetilan dalam kawasan promoter gen *SOCS3* yang berhampiran dengan tempat permulaan transkripsi dicadangkan dalam *BCR-ABL*-negative MPN.

GENE ABERRATIONS AND METHYLATION ANALYSIS OF JAK/STAT AND TOLL-LIKE RECEPTOR DOWNSTREAM SIGNALLING IN *BCR-ABL*-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS AND MYELODYSPLASTIC SYNDROME/MYELOPROLIFERATIVE NEOPLASMS OVERLAP SYNDROMES

ABSTRACT

BCR-ABL-negative myeloproliferative neoplasms (MPN) and myelodysplastic syndrome/myeloproliferative neoplasms (MDS/MPN) overlap syndromes are myeloid malignancies result from genetics, epigenetics and chromosomal mutational events, particularly in janus kinase/signal transducers and activators of transcription (JAK/STAT) and toll-like receptor (TLR) signalling pathway. This study was to estimate the prevalence, identify the mutational status of *JAK2*, *TET2* and *MyD88* genes, and methylation status of *SOCS3* and *INPP5D* genes in these diseases. *TET2* gene mutations in *BCR-ABL*-negative MPN was selected for a meta-analysis. The same archived DNA samples were used for mutational and methylation analysis. The mutational status of *JAK2*, *TET2* and *MyD88* genes in *BCR-ABL*-negative MPN and MDS/MPN were studied through direct sequencing. The methylation status of the promoter region for *SOCS3* and *INPP5D* genes were studied using pyrosequencing. For exon 26 of the *INPP5D* gene was analysed using methylation-specific PCR. Normal controls were included. It was estimated that the overall pooled prevalence of *TET2* gene mutations in *BCR-ABL*-negative MPN was 15.5%. *JAK2* V617F, five missense variants (M532I, M535R, V536G, R541K and D544N), one novel intronic single nucleotide polymorphisms (SNP) (c.1194+12G>A) and two novel deletions (c.1157delT and c.1160delT) in *JAK2* exon 12 and an intronic SNP in *MyD88*

(rs4988457) were detected. *JAK2* V617F was frequently found in *BCR-ABL*-negative MPN (85.4%) and MDS/MPN (50.0%). The missense variants in *JAK2* exon 12 (27.1%) and *MyD88* (7.3%) were detected in *BCR-ABL*-negative MPN only. The methylation level of *SOCS3* promoter, *INPP5D* promoter and *INPP5D* exon 26 showed no significant difference with normal controls. *TET2* gene mutations could contribute to the initiation and development of *BCR-ABL*-negative MPN. The mutations were also believed to be related to thrombosis, leukaemic transformation and had a close relationship with other gene mutations found in the disease. However, more studies were needed. *JAK2* V617F was highly associated with *BCR-ABL*-negative MPN and MDS/MPN. Mutations in *JAK2* exon 12 seemed to be specific to *BCR-ABL*-negative MPN and were suggested to be studied in granulocytes since the mutations were found in granulocytes. A study on the expression of the *MyD88* gene with rs4988457 in blood malignancies is recommended in the future. The methylation status of the *SOCS3* promoter near the transcription start site can also be analysed in *BCR-ABL*-negative MPN.

CHAPTER 1

INTRODUCTION

1.1 Introduction to the study

World Health Organization (WHO) and Polycythemia Vera Study Group (PVSG) classify myeloproliferative neoplasms (MPN) as Philadelphia chromosome (*BCR-ABL*)-negative polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) distinguish from *BCR-ABL*-positive chronic myelogenous leukaemia (CML) (Barbui *et al.*, 2018; Michiels *et al.*, 2015). *BCR-ABL*-negative MPN is a group of rare blood cancers characterized by the overproduction of erythroid, granulocytic or megakaryocytic cells. This group of blood disorders are often accompanied by thromboembolic events and transformation to acute myelogenous leukaemia (AML) or overt myelofibrosis (MF), which in turn be the major causes of death of the diseases (Skoda *et al.*, 2015).

The first discovery of a somatic mutation in the janus kinase 2 domain (*JAK2* V617F) of erythropoietin (EPO) receptor over a decade ago provides an insight into the pathogenesis, pathophysiology and molecular biology of *BCR-ABL*-negative MPN. *JAK2* V617F mutation is found in >95% in PV, 50% to 75% in ET, and 40% to 75% in PMF (Cristina *et al.*, 2018; Skoda *et al.*, 2015). *JAK2* exon 12 mutations are found to be exclusively present in PV without *JAK2* V617F mutation (Scott *et al.*, 2007). The recent identification of thrombopoietin receptor (*MPL*) and calreticulin (*CALR*) mutations bring a sense of completeness to the biological basis for *BCR-ABL*-negative MPN (Nangalia and Green, 2017). Nevertheless, despite the discovery of the three main driver mutations (*JAK2*, *MPL* and *CALR*), some patients do not demonstrate these mentioned mutations, so-called “triple-negative” *BCR-ABL*-negative MPN,

indicating that *BCR-ABL*-negative MPN is still not thoroughly understood (Spivak, 2017).

In adults, the acquisition of somatic *JAK2*, *CALR* and *MPL* mutations are normally sporadic, and only about 7% of the cases are familial *BCR-ABL*-negative MPN (Landgren *et al.*, 2008). An acquisition of any of the *BCR-ABL*-negative MPN driver mutations does not necessarily indicate the expansion of the mutated clonal to the unaffected stem cells (Hinds *et al.*, 2016). Generally, the mutations are age- and sex-dependent (Spivak *et al.*, 2014; Stein *et al.*, 2010). For instance, *JAK2* V617F mutation can occur at any age, but *BCR-ABL*-negative MPN with *JAK2* V617F is more common in those who are over 50 years old. The incidence of *BCR-ABL*-negative MPN also increases exponentially with age along with the higher frequency of *JAK2* V617F, ten-eleven translocation 2 (*TET2*), additional sex combs like-1 (*ASXL1*), DNA methyltransferase 3 alpha (*DNMT3A*) and tumour protein 53 (*TP53*) mutations (Spivak, 2017; Xie *et al.*, 2014). As for sex-dependent, there is a higher prevalence of females with *JAK2* V617F than males in PV and ET, and more males with *JAK2* V617F than females in PMF (Moliterno *et al.*, 2006). Besides, another gene myeloid differentiation primary response 88 (*MyD88*) is overexpressed in myeloid malignancies (Dimicoli *et al.*, 2013) and mutation is also detected in the gene in one ET patient with concomitant Waldenström Macroglobulinaemia (WM) (Lu *et al.*, 2020).

Aside from the three main driver mutations, another feature of *BCR-ABL*-negative MPN is aberrant DNA methylation that could contribute to the diseases (Pérez *et al.*, 2013). Abnormal DNA methylation can be caused by occurrences of gene mutations in genes that are involved in methylation. Both *TET2* and *DNMT3A* genes

encode proteins that regulate the methylation and demethylation of DNA (Mahfoudhi *et al.*, 2016; Ren *et al.*, 2018) and these genes are somehow associated with the phenotypes of *BCR-ABL*-negative MPN (Saeidi, 2016). Other than gene mutations, abnormal DNA methylation is related to ageing as well (De and Michor, 2011). Telomere shortening is one of the causes of ageing and this phenomenon is also found to be related to *BCR-ABL*-negative MPN, but its underlying mechanisms are undefined (Ruella *et al.*, 2013).

WHO announced Myelodysplastic syndrome/myeloproliferative neoplasms (MDS/MPN) overlap syndromes as a new category of myeloid neoplasms and acute leukaemia to include chronic clonal myeloid malignancies that display both proliferative and dysplastic features but are not grouped as myelodysplastic syndrome (MDS) or MPN (Orazi and Germing, 2008). Different from *BCR-ABL*-negative MPN, the reasons giving rise to MDS/MPN can be divided into cytogenetic abnormalities and somatic mutations (Pati and Veetil, 2019). About 70% of MDS/MPN patients are detected with an abnormal karyotype (Tiu *et al.*, 2011). The common chromosomal abnormalities are aneuploidies (monosomy 7, trisomy 8 and trisomy 9), chromosomal deletions (del7q, del13q and del20q) (Delhommeau *et al.*, 2006; Foucar, 2009) and reciprocal translocation involving tyrosine kinases (fibroblast growth factor receptor 1 (*FGFR1*), platelet-derived growth factor receptor alpha (*PDGFRA*) and platelet-derived growth factor receptor beta (*PDFRB*)) (Chase *et al.*, 2013a; Chase *et al.*, 2013b; Cools *et al.*, 2003; James *et al.*, 2005; Lierman *et al.*, 2012). The somatic mutations in MDS/MPN occur in genes that play roles in several important cellular activities, such as signal transduction, RNA splicing, DNA transcription and translation and DNA damage response (Pati and Veetil, 2019).

Besides gene mutations, the initiation and development of *BCR-ABL*-negative MPN and MDS/MPN are somehow associated with the abnormal epigenetic modifications that remodel chromatin and eventually alter the gene expression (Chim *et al.*, 2010; Zhang *et al.*, 2013). The mechanisms for epigenetic modifications include DNA methylation that covalently adds a methyl group ($-CH_3$) to cytosine-rich cytosine-phosphate guanine (CpG) site (Métivier *et al.*, 2008; Pérez *et al.*, 2013), post-translational modifications of histones by methylation, acetylation, ubiquitination, phosphorylation and ADP-ribosylation of glycosylation (Huang *et al.*, 2013; Zee *et al.*, 2010), and transcriptional or post-transcriptional regulation of gene expression by non-coding RNA (ncRNA). The involved ncRNA are short interfering RNA (siRNA), microRNA, long non-coding RNA (lncRNA) and piwi-interacting RNA (piRNA) (Berdasco and Esteller, 2010). Non-coding RNA can repress the translation of protein from messenger RNA (mRNA) and degrade mRNA to halt the activities of cells (Ambros, 2004).

There are two different categories of epigenetic dysregulations in *BCR-ABL*-negative MPN. The first category is the presence of somatic mutations in genes that are involved in regulating the chromatin structure. The genes involved are *JAK2* (Dawson *et al.*, 2009; Nischal *et al.*, 2010), *ASXL1*, *TET2* (Hussein *et al.*, 2010; Schaub *et al.*, 2010; Tefferi *et al.*, 2009c), *DNMT3A* (Ren *et al.*, 2018), enhancer of zeste homolog 2 (*EZH2*) (Ernst *et al.*, 2010), isocitrate dehydrogenase 1 (*IDH1*), isocitrate dehydrogenase 2 (*IDH2*) (Green and Beer, 2010; Pardanani *et al.*, 2010b; Tefferi *et al.*, 2010), IKAROS (*IKZF1*) (Jäger *et al.*, 2010) and protein arginine methyltransferase 5 (*PRMT5*) (Liu *et al.*, 2011). The second category involves the different methylation levels at the promoter sites of genes that regulate cellular activities like cell proliferation, cell differentiation and apoptosis. The genes involved are ABL proto-

oncogene 1 (*ABL1*) (Aviram *et al.*, 2003), calcitonin related polypeptide alpha (*CALCA*), C-X-C motif chemokine receptor 4 (*CXCR4*) (Bogani *et al.*, 2008), polycythaemia rubra vera 1 (*PRV1*) (Jelinek *et al.*, 2007), retinoic acid receptor beta 2 (*RARβ2*) (Jones *et al.*, 2004), secreted frizzled-related protein 2 (*SFRP2*) (Bennemann *et al.*, 2010; Mascarenhas *et al.*, 2011), suppressor of cytokine signalling 1 (*SOCS1*) (Capello *et al.*, 2008; Fernández-Mercado *et al.*, 2008), suppressor of cytokine signalling 3 (*SOCS3*) (Capello *et al.*, 2008; Fernández-Mercado *et al.*, 2008; Fourouclas *et al.*, 2008) and WNT inhibitory factor 1 (*WIF1*) (Suzuki *et al.*, 2007).

In *BCR-ABL*-negative MPN, abnormal DNA methylation may lead to defective functioning of negative regulators. SOCS3, a negative regulator from the suppressor of cytokine signalling proteins (SOCS) family, possess suppressive ability against normal and mutated JAK2 proteins. The suppression helps to control the proliferation of cells and inhibits tumourigenesis (Funakoshi-Tago *et al.*, 2019). SOCS3 induces ubiquitination on JAK2 and stops the activity of the mutated JAK2 (Kershaw *et al.*, 2014). The promoter region of the *SOCS3* gene is hypermethylated in *BCR-ABL*-negative MPN (Fourouclas *et al.*, 2008; Torun *et al.*, 2013), implying the contribution of the epigenetic down-regulation of this crucial tumour suppressor in the pathogenesis of *BCR-ABL*-negative MPN (Quentmeier *et al.*, 2008). Another negative regulator encoded by the (inositol polyphosphate-5-phosphatase D) *INPP5D* gene from the TLR signalling pathway also plays role in the pathogenesis of *BCR-ABL*-negative MPN. Deficiency in the negative regulators causes dysregulated BM haematopoiesis in *BCR-ABL*-negative MPN (Helgason *et al.*, 1998).

Thus, the causes of *BCR-ABL*-negative MPN and MDS/MPN are closely associated with the abnormality in genetics and epigenetics. Five genes (*JAK2*, *TET2*, *MyD88*, *SOCS3* and *INPP5D*) are selected for this study. The first aim of the study is

to identify the prevalence of *TET2* gene mutations in *BCR-ABL*-negative MPN by meta-analysis. Prevalence studies on another gene (*JAK2*, *MyD88*, *SOCS3* and *INPP5D*) are not done because the number of existing studies for the *JAK2* gene is too large and impossible to be done in a two-year study duration with works to be completed. As for the *MyD88*, *SOCS3* and *INPP5D* genes, only very few studies are available and are not enough for a meta-analysis study. The other two aims are to determine the mutational status of *JAK2*, *TET2* and *MyD88* gene in *BCR-ABL*-negative MPN and MDS/MPN patients and to investigate the methylation status of important negative regulators (*SOCS3* and *INPP5D*) in the janus kinase/signal transducers and activators of transcription (JAK/STAT) and toll-like receptors (TLR) signalling pathway among patients with *BCR-ABL*-negative MPN and patients with MDS/MPN.

1.2 Statement of the problem

It is suggested that the main cause of *BCR-ABL*-negative MPN is somatic mutations. Around 90% of *BCR-ABL*-negative MPN patients are found to carry at least one somatic mutation, which includes *JAK2* V617F (69%), *CALR* (15%), *TET2* (12%), *ASXL1* (5%), and *DNMT3A* (5%) (Lundberg *et al.*, 2014). Recently, defective toll-like receptors (TLR) signalling pathway that lead to prolonged TLR signalling is demonstrated as a potential predisposition to acquire *BCR-ABL*-negative MPN and could contribute to the chronic inflammatory state of *BCR-ABL*-negative MPN (Lai *et al.*, 2019; Marín Oyarzún *et al.*, 2020). Mutations in the same genes are associated with MDS/MPN as well (Pati and Veetil, 2019). The association of *JAK2*, *CALR* and *MPL* with *BCR-ABL*-negative MPN are confirmed and included in the diagnosis of *BCR-ABL*-negative by WHO (Barbui *et al.*, 2018), but not the other genes. Therefore, a meta-analysis on the prevalence of *TET2* gene mutation is believed can help to reveal

the connection between *TET2* gene mutations and *BCR-ABL*-negative MPN. All currently available data are mainly from the Caucasian population, with only a few from Asian countries. So, a better understanding of the genetic changes among patients with *BCR-ABL*-negative MPN and MDS/MPN from Asian countries such as Malaysia may help to gain a more thorough insight into the prevalence, diagnosis and surveillance of *BCR-ABL*-negative MPN as well as MDS/MPN.

Epigenetic mechanisms have roles in the pathogenesis of *BCR-ABL*-negative MPN (McPherson *et al.*, 2017) and MDS/MPN (Deininger *et al.*, 2017). Aberrant DNA methylation is frequently found in MDS/MPN, such as modified epigenetic landscape in chronic myelomonocytic leukaemia (CMML) (Perez *et al.*, 2012; Yamazaki *et al.*, 2012) and hypermethylation of several genes in juvenile myelomonocytic leukaemia (JMML) (Fluhr *et al.*, 2016; Olk-Batz *et al.*, 2011; Wilhelm *et al.*, 2016). In *BCR-ABL*-negative MPN, important negative regulators are studied for their methylation status. *SOCS* family, protein tyrosine phosphatase non-receptor type 6 (*PTPN6*) and *TET2* gene are found to be methylated in the diseases (Chim *et al.*, 2010; Zhang *et al.*, 2013). There are methylation studies done on *BCR-ABL*-negative MPN and MDS/MPN, however, the number was small, with only a few studies on JAK/STAT signalling pathway and no studies on the TLR signalling pathway were available. It is believed that a better understanding of the epigenetic landscape in *BCR-ABL*-negative MPN and MDS/MPN can provide a clear picture of the role of epigenetics in the pathogenesis of diseases since the epigenetic landscape shapes the biological and clinical expression which contribute to the development of the diseases.

1.3 Research question

1. What are the DNA mutational status and methylation patterns in patients with *BCR-ABL*-negative MPN and patients with MDS/MPN?
2. What is the prevalence of JAK/STAT associated gene (*TET2*) gene mutations in patients with *BCR-ABL*-negative MPN?
3. What are the mutational status of JAK/STAT associated genes (*JAK2* V617F, *JAK2* exon 12 and *TET2*) and TLR adaptor gene (*MyD88*) in patients with *BCR-ABL*-negative MPN and patients with MDS/MPN?
4. What are the methylation status of negative regulators to JAK/STAT (*SOCS3*) and TLR downstream signalling (*INPP5D*) in patients with *BCR-ABL*-negative MPN and patients with MDS/MPN?

1.4 Justification of the study

Besides the main driver mutations, *TET2* gene mutation appears to be related to *BCR-ABL*-negative MPN based on the findings of previous studies, however, there is no precise answer on how common is *TET2* gene mutation in *BCR-ABL*-negative MPN. Thus, this study is expected to gather all the data of *TET2* gene mutations in *BCR-ABL*-negative MPN and analyse the data to learn about the commonness of *TET2* gene mutations in *BCR-ABL*-negative MPN.

The genomics era has brought with it plenty of dramatic advances in our understanding of the molecular basis of diseases. Many studies have been carried out to study different kinds of diseases, but only a very small number of studies are related to *BCR-ABL*-negative MPN and MDS/MPN as compared to other myeloid malignancies. Thus, this study is expected to collect more genetic (*JAK2*, *TET2* and *MyD88*) and epigenetic information (*SOCS3* and *INPP5D*) on patients with *BCR-ABL*-

negative MPN and patients with MDS/MPN. The data collected can be used for future reference such as in assisting to find more strategic approaches to treat *BCR-ABL*-negative MPN and MDS/MPN, identifying any novel molecular prognostic marker or searching for some good candidates for gene therapy.

1.5 Hypothesis

1. There are presence of gene mutations and abnormal methylation patterns in patients with *BCR-ABL*-negative MPN and patients with MDS/MPN patients.
2. *TET2* gene mutation is prevalent in patients with *BCR-ABL*-negative MPN.
3. There are the presence of gene mutations and/or gene polymorphisms in *JAK2*, *TET2* and *MyD88* genes in patients with *BCR-ABL*-negative MPN and patients with MDS/MPN patients.
4. There are presence of abnormal methylation patterns in *SOCS3* and *INPP5D* genes in patients with *BCR-ABL*-negative MPN and patients with MDS/MPN patients.

1.6 Objective of the study

1.6.1 General objective

To study the DNA mutation and methylation of JAK/STAT and TLR downstream signalling genes in patients with *BCR-ABL*-negative MPN and patients with MDS/MPN.

1.6.2 Specific objectives

1. To estimate the prevalence of JAK/STAT associated gene (*TET2*) in patients with *BCR-ABL*-negative through meta-analysis.

2. To determine the mutational status of JAK/STAT associated genes (*JAK*2 V617F, *JAK2* exon 12 and *TET2*) and TLR adaptor gene (*MyD88*) in patients with *BCR-ABL*-negative MPN and patients with MDS/MPN patients using direct DNA sequencing.
3. To determine the methylation status of negative regulators of JAK/STAT (*SOCS3*) and TLR downstream signalling (*INPP5D*) in patients with *BCR-ABL*-negative MPN and patients with MDS/MPN using pyrosequencing and/or methylation-specific polymerase chain reaction (MS-PCR).

CHAPTER 2

LITERATURE REVIEW

2.1 Myeloproliferative neoplasms (MPN)

MPN are clonal bone marrow stem cells disorders characterised by the overproduction of erythroid, myeloid and megakaryocytic lineages (Campbell and Green, 2006). MPN consists of two categories, the *BCR-ABL*-positive CML and the *BCR-ABL*-negative MPN (Kiladjian, 2012).

2.1.1 *BCR-ABL*-negative MPN

BCR-ABL-negative MPN is a condition in which the Philadelphia chromosome involving a translocation from chromosome 22 to chromosome 9 is absent and no *BCR-ABL* fusion gene is formed (Chopra *et al.*, 1999). The three classical *BCR-ABL*-negative MPN include PV, ET and PMF (Elf, 2020). PV is defined by an increased red blood cell (RBC) mass and occasion raise in white blood cell (WBC) counts. ET is characterised by a rise in platelet counts but with normal RBC mass. PMF manifests a fibrosis condition in bone marrow (BM) (Yow *et al.*, 2020). These *BCR-ABL*-negative MPN share common features, including an increased risk of thrombosis, hypercellularity in BM, haemorrhages, and transformation to AML (Marchetti and Falanga, 2007). Patients with PV and ET have chances to develop secondary myelofibrosis (SMF) by transforming to post-PV myelofibrosis or post-ET myelofibrosis. Besides, patients with ET can subsequently develop erythrocytosis and have overlap PV and ET at the same time (Meyer and Levine, 2014).

BCR-ABL-negative MPN is closely associated with gene mutations. There are three somatic driver gene mutations (*JAK2*, *CALR*, *MPL*) in *BCR-ABL*-negative MPN. All three main driver mutations are involved in JAK/STAT signalling pathway. *JAK2* mutations are mostly found in PV, whereas *CALR* and *MPL* gene mutations are almost

exclusively present in ET and PMF. Majority of the patients with *BCR-ABL*-negative MPN carry at least one of the three driver mutations, and sometimes they can carry two of the mutations at the same time (Nangalia and Green, 2017; Tefferi and Pardanani, 2015; Ye *et al.*, 2020).

2.1.1(a) Polycythaemia vera (PV)

PV is the most common type of the three classical *BCR-ABL*-negative MPN and is characterised by the overproduction of red blood cells in the BM. The cause of PV is unclear but *JAK2* mutations are found to be associated with the disease. *JAK2* mutations lead to an excess number of RBC in patients with PV. Increased RBC causes symptoms like headache, migraine, dizziness, visual disturbances, burning pains in the extremities and weakness in limbs or face. An elevated platelet count may result in nose bleeds and easy bruising. A high WBC number tends to induce gout in patients. Occasionally, patients may suffer from the inability to eat a full meal, abdominal pain and abdominal fullness due to splenomegaly (Spivak, 2013).

For testing of PV, patients show erythrocytosis, leukocytosis, thrombocytosis and are often accompanied by splenomegaly. PV patients may experience expanded RBC numbers alone or with combinations of increased RBC, WBC or platelet numbers. Since there is a high prevalence of *JAK2* mutations in PV (95%), *JAK2* mutational test is included as a diagnostic criterion for PV. However, *JAK2* mutations are present in both ET and PMF (50%), PV can be distinguished from them by referring to the elevated RBC count in patients (Spivak, 2013).

According to the 2016 WHO classification and diagnostic criteria, patients who fulfil either all three major criteria or the first two major criteria and the minor criterion are diagnosed as PV (Table 2.1) (Passamonti and Maffioli, 2016).

Table 2.1 2016 WHO diagnostic criteria for PV (Passamonti and Maffioli, 2016).

Major criteria	
Criterion 1 (clinical)	
Hb, or	>16.5 g/dL in men, >16.0 g/dL in women
Haematocrit, or	>49% in men, >48% in women
Red cell mass	Increased 25% above mean normal predicted value
Criterion 2 (morphologic)	
BM morphology*	Hypercellularity for age with trilineage growth (panmyelosis), including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size)
Criterion 3 (genetic)	
JAK2 V617F, or	Presence
JAK2 exon 12 mutation	Presence
Minor criterion	
Serum EPO level	Subnormal

*Criterion number 2 (BM biopsy) may not be required in cases with sustained absolute erythrocytosis: Hb levels >18.5 g/dL in men (haematocrit 55.5%) or >16.5 g/dL in women (haematocrit 49.5%) if major criterion 3 and the minor criterion are present.

2.1.1(b) Essential thrombocythaemia (ET)

ET is a type of rare blood cancer in which the body produces too many platelets. The prevalence of ET is around two per 100,000 people and is more common in the aged (>60 years old). Patients with ET have chances to transform into post-ET myelofibrosis when the BM is replaced by scarred tissues and develop into a form of leukaemia. One of the factors leading to ET is the presence of genetic changes in genes that are responsible for blood cells production. About 90% of the patients with ET are found to carry gene mutations in *JAK2*, *CALR* or *MPL* (Double and Harrison, 2015).

Around half of the patients with ET are asymptomatic. For those patients who show symptoms, the symptoms can be varied in a wide range and often include headache and fatigue. Inappropriate formation of blood clots due to an elevated number of platelets is the main problem faced by the patients. The blood clots can block arteries and veins in the body and causing thrombosis, heart attacks, stroke and pulmonary embolism. Also, a great number of platelets can introduce a 'thick blood' condition and affect the smoothness of blood flow. This causes headache, tiredness, night sweat, splenomegaly, visual disturbances, bone pain and burning or itching feeling in the four limbs. Bleeding problems may also occur since the clotting factors accumulate to the platelet and do not function properly. ET is more common in the aged (>60 years old), and those who have a previous history of arterial or venous thrombosis, high platelet counts and presence of cardiovascular risk factors (Double and Harrison, 2015).

According to the 2016 WHO classification and diagnostic criteria, patients who fulfil either all four major criteria or the first three major criteria and the minor criterion are diagnosed as ET (Table 2.2) (Passamonti and Maffioli, 2016).

Table 2.2 2016 WHO diagnostic criteria for ET (Passamonti and Maffioli, 2016).

Major criteria	
Criterion 1 (clinical)	
Platelet count	>450 x 10^9/L
Criterion 2 (morphologic)	
BM morphology	Proliferation mainly of the megakaryocytes lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis, and very rarely minor (grade 1) increase in reticulin fibres
Criterion 3 (clinical)	
WHO criteria for *BCR-ABL*-positive CML, PV, PMF, MDS, or other myeloid neoplasms	Not meeting
Criterion 4 (genetic)	
JAK2, *CALR* or *MPL* mutation	Presence
Minor criterion	
Clonal marker, or	Presence
Reactive thrombocytosis	Absence

2.1.1(c) Myelofibrosis

Myelofibrosis is very rare and occurs in one out of 100,000 people and is more common in the aged (>60 years old). Myelofibrosis is a type of rare blood cancer in which the spongy tissue in BM is replaced by fibrous scar tissues through a process called fibrosis. Fibrosis disrupts the normal function of BM and affects the ability of the body to produce normal blood cells. Since the BM is affected by myelofibrosis, the blood cells are made in other organs, for example, the spleen and liver. This causes

spleen enlargement by 10 to 20 folds. There are two types of myelofibrosis, primary myelofibrosis (PMF) and secondary myelofibrosis (SMF). PMF occurs in patients without any previous history of BM problem, whereas SMF is another type of myelofibrosis that occur after a prior diagnosis of other blood disorders. For the myelofibrosis that develops in patients diagnosed with PV and ET, they are called post-PV myelofibrosis and post-ET myelofibrosis respectively. About 50% of myelofibrosis patients have a mutation in the *JAK2* gene. Although the rest 50% of patients show no mutation in the *JAK2* gene, their activity of normal *JAK2* gene also shows increment as in *JAK2* gene mutated patients. Apart from that, other gene mutations are detected in patients with myelofibrosis as well (Harrison and McLornan, 2014).

For myelofibrosis patients, they may be asymptomatic during diagnosis. However, symptoms and signs can develop over time. As the disease progresses, patients with myelofibrosis may experience fever, fatigue, bleeding complications, loss of weight, sweating at night, poor appetite and bone pain. If splenomegaly develops, the patients may suffer discomfort in the abdomen or pain under the ribs at the left, particularly after meals. Sometimes, anaemia, painful joints and gout may occur (Harrison and McLornan, 2014).

According to the 2016 WHO classification and diagnostic criteria, patients who fulfil either all three major criteria and at least one minor criterion are diagnosed as PMF (Table 2.3) (Passamonti and Maffioli, 2016).

Table 2.3 2016 WHO diagnostic criteria for PMF (Passamonti and Maffioli, 2016).

Major criteria	
Criterion 1 (morphologic)	
BM morphology	Presence of megakaryocytic proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis grades 2 or 3
Criterion 2 (morphologic)	
WHO criteria for PV, ET, *BCR-ABL*-positive CML, MDS, or other myeloid neoplasms	Not meeting
Criterion 3 (genetic)	
JAK2, *CALR* or *MPL* mutation, or	Presence
Clonal marker*, or	Presence
Reactive BM reticulin fibrosis**	Absence
Minor criterion	
Anaemia not attributed to a comorbid condition	Presence
WBC count	$\geq 11 \times 10^9$/L
Spleen size	Palpable
Serum lactate dehydrogenase	Increased to the above upper normal limit of the institutional reference range
Leuko-erythroblastosis	Presence

*In the absence of any of the 3 major clonal mutations, the search for the most frequent accompanying mutations (eg, *ASXL1*, *EZH2*, *TET2*, *IDH1*/*IDH2*, *SRSF2*, and *SF3B1*) are of help in determining the clonal nature of the disease.

**Minor (grade 1) reticulin fibrosis secondary to an infection, autoimmune disorder or other chronic inflammatory conditions, hairy cell leukaemia or other lymphoid neoplasms, metastatic malignancy, or toxic (chronic) myelopathies.

2.2 Myelodysplastic syndrome/myeloproliferative neoplasms (MDS/MPN) overlap syndromes

A new MDS/MPN category was introduced in the 3rd edition of the WHO classification of myeloid neoplasms and acute leukaemia to include myeloid disorders that overlap between MDS and MPN (Orazi and Germing, 2008). In the latest 2016 version, MDS/MPN category consists of atypical chronic myeloid leukaemia (aCML), CMML, myelodysplastic/myeloproliferative neoplasms with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T), JMML and MDS/MPN-unclassifiable (MDS/MPN-U) (Arber *et al.*, 2016). MDS/MPN are different from pure MDS or MPN and have their features. For example, a high percentage of monocytes in CMML, severe granulocytic dysplasia in aCML and high platelet count in MDS/MPN-RS-T. Nowadays, advanced genomic and molecular studies have improved our understanding of the pathogenesis of MDS/MPN, but there is still a lack of specific treatments for MDS/MPN. More studies and efforts are needed (Thota and Gerds, 2018).

2.2.1 Chronic myelomonocytic leukaemia (CMML)

The most common type of MDS/MPN is CMML (Srour *et al.*, 2016). CMML is commonly seen in older individuals, shows higher prevalence in males, presents with monocytosis, hepatosplenomegaly, dysplastic changes in BM, cytopenia and risk of transformation into AML (Arber *et al.*, 2016; Saarni and Linman, 1971). Mutations in five categories are associated with CMML: epigenetic regulators, chromatin modification, DNA repair, signalling transduction and spliceosome (Gur *et al.*, 2018; Thota and Gerds, 2018). CMML can be classified into three different groups, CMML-0 (peripheral blood: <2% blasts, BM: <5% blasts), CMML-1 (peripheral blood: 2% to 4% blasts, BM: 5% to 9%) and CMML-2 (peripheral blood: 5% to 19% blasts, BM: 10% to 19% blasts) (Thota and Gerds, 2018).

The diagnosis criteria for CMML based on the 2016 WHO classification and diagnostic criteria of myeloid neoplasms are as shown in Table 2.4 (Arber *et al.*, 2016).

Table 2.4 2016 WHO diagnostic criteria for CMML (Arber *et al.*, 2016).

CMML diagnostic criteria
• Persistent peripheral monocytosis ≥1 x 10^9/L, with monocytes accounting for ≥10% of the WBC count • Not meeting WHO criteria for *BCR-ABL*-positive CML, PMF, PV, or ET* • No evidence of *PDGFRA*, *PDGFRB*, or *FGFR1* rearrangement or *PCM1-JAK2* (should be specifically excluded in cases with eosinophilia) • <20% blasts in the blood and BM** • Dysplasia in 1 or more myeloid lineages. If myelodysplasia is absent or minimal, the diagnosis of CMML may still be made if the other requirements are met and • An acquired clonal cytogenetic or molecular genetic abnormality is present in hemopoietic cells*** Or • The monocytosis (as previously defined) has persisted for at least 3 months and • All other causes of monocytosis have been excluded

*Cases of MPN can be associated with monocytosis or they can develop it during the disease. These cases may simulate CMML. In these rare instances, a previously documented history of MPN excludes CMML, whereas the presence of MPN features in the BM and/or of MPN-associated mutations (*JAK2*, *CALR*, or *MPL*) tend to support MPN with monocytosis rather than CMML.

**Blasts and blast equivalents include myeloblasts, monoblasts, and promonocytes. Promonocytes are monocytic precursors with abundant light grey or slightly basophilic cytoplasm with a few scattered, fine lilac-coloured granules, finely distributed, stippled nuclear chromatin, variably prominent nucleoli, and delicate nuclear folding or creasing. Abnormal monocytes, which can be present both in the PB and BM, are excluded from the blast count.

***The presence of mutations in genes often associated with CMML (eg, *TET2*, *SRSF2*, *ASXL1*, *SETBP1*) in the proper clinical contest can be used to support a diagnosis. It should be noted, however, that many of these mutations can be age-related or be present in subclones. Therefore, caution would have to be used in the interpretation of these genetic results.

2.2.2 Atypical chronic myeloid leukaemia (aCML)

aCML is a rare subtype of MDS/MPN (Arber *et al.*, 2016) and is not related to CML but it shares some characteristics with CML. Patients with aCML are older, present with splenomegaly, granulocytosis and circulating myeloid precursors (Hernandez *et al.*, 2000; Kurzrock *et al.*, 2001; Kurzrock *et al.*, 1990; Wang *et al.*, 2014). In separating aCML from CML, a lack of *BCR-ABL* fusion gene, presence of dysgranulopoiesis and BM dysplasia can be referred (Arber *et al.*, 2016; Hernandez *et al.*, 2000; Kurzrock *et al.*, 2001; Kurzrock *et al.*, 1990; Wang *et al.*, 2014). The clinical features of aCML include organomegaly, BM failure and increased leukaemic burden (Hernandez *et al.*, 2000).

Patients with aCML tend to present with a trisomy chromosomal condition, such as trisomy 8, trisomy 13 and trisomy 21. Other than that, deletion in chromosome 20 (del(20q)) and isochromosome 17q (i(17q)) are also found in patients with aCML. Somatic mutations in SET binding protein 1 (*SETBP1*), ethanolamine kinase 1 (*ETNK1*), colony-stimulating factor 3 receptor (*CSF3R*) and ras-GTPase (*RAS*) are seen in aCML (Thota and Gerds, 2018). The three driver mutations for MPN (*JAK2*, *CALR* and *MPL*) are not found in aCML (Arber *et al.*, 2016).

The diagnosis criteria for aCML based on the 2016 WHO classification and diagnostic criteria of myeloid neoplasms are as shown in Table 2.5 (Arber *et al.*, 2016).

Table 2.5 2016 WHO diagnostic criteria for aCML (Arber *et al.*, 2016).

aCML diagnostic criteria
• Peripheral leukocytosis due to increased numbers of neutrophils and their precursors (promyelocytes, myelocytes, metamyelocytes) comprising ≥10% of leukocytes • Dysgranulopoiesis, which may include abnormal chromatin clumping • No or minimal absolute basophilia; basophils usually <2% of leukocytes • No or minimal absolute monocytosis; monocytes <10% of leukocytes • Hypercellular BM with granulocytic proliferation and granulocytic dysplasia, with or without dysplasia in the erythroid and megakaryocytic lineages • <20% blasts in the blood and BM • No evidence of *PDGFRA*, *PDGFRB*, or *FGFR1* rearrangement, or *PCM1-JAK2* • Not meeting WHO criteria for *BCR-ABL*-positive CML, PMF, PV, or ET*

*Cases of MPN, particularly those in accelerated phase and/or in post-PV or post-ET myelofibrosis, if neutrophilic, may simulate aCML. A previous history of MPN, the presence of MPN features in the BM and/or MPN-associated mutations (in *JAK2*, *CALR*, or *MPL*) tend to exclude a diagnosis of aCML. Conversely, a diagnosis of aCML is supported by the presence of *SETBP1* and/or *ETNK1* mutations. The presence of a *CSF3R* mutation is uncommon in aCML and if detected should prompt a careful morphologic review to exclude an alternative diagnosis of CNL or other myeloid neoplasms.

2.2.3 Myelodysplastic/myeloproliferative neoplasms with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T)

MDS/MPN-RS-T was formerly known as refractory anaemia with ring sideroblasts associated with marked thrombocytosis (RARS-T). Ring sideroblasts are erythroblasts in which perinuclear mitochondrion iron abnormally accumulates on the nuclear circumference of the cells (Cazzola and Invernizzi, 2011). In the 2016 revised 4th edition WHO classification, MDS/MPN-RS-T is officially listed as a new entity in MDS/MPN (Thompson *et al.*, 2021). Patients with MDS/MPN-RS-T have features of MDS with ring sideroblasts and also show persistent thrombocytosis associated with abnormal megakaryocytes resembling MPN (Patnaik and Tefferi, 2017). MDS/MPN-RS-T is found in older people, equally distributed among males and females, and present with higher haemoglobin (Hb) and high platelet counts (Broseus *et al.*, 2012; Jeromin *et al.*, 2015).

At the genetic and molecular level, a high frequency of splicing factor 3b (*SF3B1*) mutations is found in MDS/MPN-RS-T and induces abnormal erythropoiesis and the formation of ring sideroblasts. Similar to *BCR-ABL*-negative MPN, *JAK2* V617F is detected in MDS/MPN-RS-T and causes the proliferation of different lineages of blood cells in patients (Norris and Stone, 2008; Wardrop and Steensma, 2009). Both *JAK2* and *SF3B1* mutations usually co-exist at the same time (Arber *et al.*, 2016). Besides, *TET2*, *ASXL1*, *DNMT3A* and *MPL* genes are also found to be mutated in MDS/MPN-RS-T (Broseus *et al.*, 2012; Jeromin *et al.*, 2015).

The diagnosis criteria for MDS-RS-T based on the 2016 WHO classification and diagnostic criteria of myeloid neoplasms are as shown in Table 2.6 (Arber *et al.*, 2016).

Table 2.6 2016 WHO diagnostic criteria for MDS/MPN-RS-T (Arber *et al.*, 2016).

MDS/MPN-RS-T diagnostic criteria
• Anaemia associated with erythroid lineage dysplasia with or without multilineage dysplasia, ≥15% ring sideroblasts,* <1% blasts in peripheral and <5% blasts in the BM
• Persistent thrombocytosis with platelet count ≥450 x 10^9/L
• Presence of a *SF3B1* mutation or, in the absence of *SF3B1* mutation, no history of recent cytotoxic or growth factor therapy that could explain the myelodysplastic/myeloproliferative features**
• No *BCR-ABL* fusion gene, no rearrangement of *PDGFRA*, *PDGFRB*, or *FGFR1*; or *PCM1-JAK2*; no (3;3)(q21;q26), inv(3)(q21q26) or del(5q)***
• No preceding history of MPN, MDS (except MDS-RS), or other types of MDS/MPN

*At least 15% ring sideroblasts required even if *SF3B1* mutation is detected.

**A diagnosis of MDS/MPN-RS-T is strongly supported by the presence of *SF3B1* mutation together with a mutation in *JAK2* V617F, *CALR*, or *MPL* genes.

***In a case that otherwise fulfils the diagnostic criteria for MDS with isolated del(5q)-no or minimal absolute basophilia; basophils usually <2% of leukocytes.