DETECTION OF FMS-LIKE TYROSINE KINASE 3 (*FLT3*) AND NUCLEOPHOSMIN 1 (*NPM1*) MUTATIONS FROM FORMALIN FIXED PARAFFIN EMBEDDED MARROW TISSUES IN PATIENTS WITH MYELOID NEOPLASMS

BY

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ABSTRACT

Acute myeloid leukemia (AML) and myeloproliferative neoplasms (MPN) are the most common entities of myeloid neoplasms. In AML, among the most frequent genetic alterations that carries both diagnostic and prognostic values are mutations in Nucleophosmin 1 (*NPM1*) and FMS-like tyrosine kinase 3 (*FLT3*) genes. Nevertheless, their frequencies among AML patients in Kuantan, Pahang have not been studied. Additionally, published literatures on both of these mutations in MPN are scarce although they have been shown to confer MPN in animal model. This cross-sectional study therefore aimed to determine the proportion of *FLT3-ITD*, *FLT3-D835* and *NPM1* mutations among patients diagnosed with AML and MPN in Hospital Tengku Ampuan Afzan of Kuantan, Pahang from the year 2016 to 2019. A total of 56 cases were studied, of which 43 cases were AML and 13 cases MPN. Molecular methods employed were polymerase chain reaction-based assays for mutation detection, from the retrieved trephine biopsy tissue blocks. The mutation positivity was subsequently validated by Sanger DNA sequencing. Six of the 43 cases (14.0%) of AML were positive for *FLT3-ITD* and a similar proportion (6/43, 14.0%) were also positive for *NPM1* mutations. *FLT3-D835* mutation was identified in three of the AML cases (7.0%) while concurrent mutations of *NPM1* and *FLT3-ITD* were seen in two of the mutation positive cases (4.7%). One of the 13 (7.7%) MPN cases was positive for *FLT3-ITD*. None of the MPNs cases were positive for either *FLT-D835* or *NPM1* mutations. When the mean haematological values were compared with the mutation status in AML cases, only the total white cell count was significantly higher with *FLT3* mutations (p=0.001). In conclusion, the frequency of *FLT3* mutations in the AML cases concurs with others, while the frequency of *NPM1* mutations in our study was relatively lower as compared to other reports. The significance of the *FLT3-ITD* mutation positivity found in our series of MPN remains to be elucidated.

خلاصة البحث

سرطان الدم النقوي الحاد (AML) والأورام التكاثرية النقوية (MPN) هي أكثر الكيانات شيوعًا للأورام النخاعية. في AML، من بين التغييرات الجينية الأكثر شيوعًا التي تحمل القيم التشخيصية والإنذارية هي الطفرات في جينات Nucleophosmin 1 (*NPM1*) و Tyrosine kinase (*FLT3*) 3 الشبيهة FMS. ومع ذلك ، لم يتم دراسة انتشارهم بين مرضى سرطان الدم النقوي الحاد في كوانتان ، باهانج. بالإضافة إلى ذلك ، فإن الابحاث المنشورة على كل من هذه الطفرات في MPN نادرة على الرغم من أنها أثبتت أنها تمنح MPN في النموذج الحيواني. وبالتالي تهدف هذه الدراسة المستعرضة إلى تحديد نسبة طفرات *FLT3-ITD* و *FLT3-D835* و *NPM1* بين المرضى الذين تم تشخيصهم بـ AML و MPN في مستشفى Tengku Ampuan Afzan (HTAA) في Kuantan، Pahang من عام 2016 إلى 2019. تمت دراسة ما مجموعه 56 حالة ، 43 حالة منها AML و 13 حالة MPN. الطرق الجزيئية القائمة على تفاعل سلسلة البوليميراز للكشف عن الطفرة ، من كتل أنسجة خزعة التريفين المستردة. تم التحقق من إيجابية الطفرة لاحقًا من خلال تسلسل الحمض النووي Sanger. كانت ست من الحالات الـ 43 (14.0٪) من مرضى AML إيجابية بالنسبة *FLT3-ITD* وكانت نسبة مماثلة (43/6 ، 14.0٪) إيجابية أيضًا لطفرات *NPM1*. تم تحديد طفرة *FLT3-D835* في ثلاث من حالات مرضى سرطان AML (7.0٪) بينما شوهدت طفرات متزامنة من *NPM1* و *FLT3-ITD* في حالتين (4.7٪). كانت إحدى حالات MPN (7.7٪) إيجابية *FLT3-ITD*. لم تكن أي من حالات MPN إيجابية لطفرات *FLT-D835* أو *NPM1*. عندما يعني متوسط القيم الدموية مقارنة بحالة الطفرة في حالات AML ، كان إجمالي عدد الخلايا البيضاء فقط أعلى بكثير مع طفرات (p = 0.001) *FLT3*. في الختام ، كان تواتر طفرات *NPM1* في حالات مرضى سرطان الدم النقوي الحاد في دراستنا أقل نسبيًا مقارنة بالتقارير الأخرى. لا يزال يتعين توضيح أهمية إيجابية الطفرة *FLT3-ITD* الموجودة في سلسلتنا من MPN.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Medical Science.

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DECLARATION

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TO MY PARENTS

TO MY TEACHERS

TO MY BELOVED WIFE AND KIDS

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LIST OF ABBREVIATIONS

˚C	Celsius degree
ADP	Adenosine triphosphate
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APL	Acute promyelocytic leukaemia
Ara-C	Cytarabine
Arf	ADP ribosylation factor
ARMS	Amplification-refractory mutation system
AS-PCR	Allele-specific polymerize chain reaction
ASXL1	Additional sex-comb like 1
BM	Bone marrow
B-MYH11	Beta-myosin heavy chain 11
bp	Base pair
C/EBPα	CCAAT/enhancer binding protein alpha
CALR	Calreticulin
CBF	Core-binding factor
CBFβ	Core-binding factor beta
CD	Common differentiation
CDKs	Cyclin dependent kinases
CFU	Colony forming unit
CLL	Chronic lymphocytic leukaemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukaemia
CMML	Chronic myelomonocytic leukaemia
CMP	Common myeloid progenitor
CN-AML	Cytogenetically normal acute myeloid leukaemia
CR	Complete remission
CRM1	Chromosomal region maintenance 1
del	Deletion
DNA	Deoxyribonucleic acid
DNMT3a	DNA methyltransferase 3 alpha
EDTA	Ethylenediamine tetra acetic acid
Epo	Erythropoietin
ERK	Extracellular signal regulated kinase
ET	Essential thrombocythemia
EZH2	Enhancer of Zest 2 polycomb repressive complex
FAB	French-American-British
FFPE	Formalin fixed paraffin embedded
FISH	Florescent in situ hybridization
FLT3	FMS-related tyrosine kinase 3
g	gram
GDP	guanosine diphosphate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte and macrophage progenitor

GRB2	Geceptor-bound protein 2
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
h	Hour
Hb	Haemoglobin
HCS	Haematopoietic stem cell
HLA	Human leukocyte antigen
HPSCs	Haematopoietic stem/progenitor cells
H-RAS	Harvey sarcoma virus
HTAA	Hospital Tingku Ampuan Afzan
IDH	Isocitrate dehydrogenase
Ig	Immunoglobulin
IIUM	International Islamic University of Malaysia
IKZF1	IKOROS family zinc finger 1
IL	Interleukin
Indels	Insertions/deletions
IREC	IIUM research ethical committee
ITD	Internal tandem duplication
ITD	Internal tandem duplication
JAK2	Janus kinase 2
JM	Juxta membrane
K-RAS	Kirsten sarcoma virus
KRC	Kulliyyah of medicine research committee
LT	Long term
M	Molar
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte and erythroid progenitor
mg	Milligram
min	Minute
mL	Milliliter
MLL	Myeloid/lymphoid leukaemia
μM	Micro molar
MPD	Myeloproliferative disease
MPL	Myeloproliferative leukaemia virus
MPN	Myeloproliferative neoplasia
MPP	Multipotent progenitor cell
MRD	Minimal residual disease
NADPH	Nicotinamide adenine dinucleotide phosphate
NES	Nuclear export signal
NF1	Neurofibromatosis 1
NK	Neutral killer
NLS	Nuclear localization signal
NoLS	Nucleoli localization signal
NOS	Not otherwise specific
NPM1	Nucleophosmin 1
NRAS	Neuroblastoma RAS
NTC	No template control
PCR	Polymerase chain reaction

PDGFR α/β	Platelet derived growth factor alfa and beta
PI3K	Phosphoinositide-3 kinase
PI3K	Phosphatidylinositol 3-kinase
Plt	Platelet
PMF	Primary myelofibrosis
PRC	Polycomb repressive complex
PV	Polycythaemia vera
RARA	Retinoic acid receptor alfa
RBC	Red blood cell
RFLP	Restriction fragment length
RTK	Receptor tyrosine kinase
RUNX1	RUNT-related transcription factor 1
SCF	Stem cell factor
SD	Standard deviation
SNPs	Single nucleotide polymorphisms
SNVs	Single nucleotide variants
SSC	Side scatter
ST	Short term
STAT	Signal transducer and activating transcription
TBE	Tris/Borate/EDTA
TET2	Ten-eleven translocation 2
TGF-β	Transforming growth factor beta
TKD	Tyrosine kinase domain
TNF-α	Tumour necrosis factor alfa
TP53	Tumor protein 53
TPO	Thrombopoietin
UK	United Kingdom
USA	United states of America
WBC	Wight blood cell
WT	Wild type
WT1	Wilms tumour 1
µg	Microgram
µL	Microliter
µM	Micromolar

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Myeloid neoplasms are a group of related disorders characterized by defective haematopoiesis originating from abnormal clones of haematopoietic stem/progenitor cells that show myeloid differentiation (Docking & Karsan, 2019). Among the categories included as myeloid neoplasms based on the revised 2016 World Health Organization (WHO) Classification, acute myeloid leukaemia (AML) and myeloproliferative neoplasms (MPN) are among the common types. MPN have preleukaemic features and do evolve into AML (A. Arber & Orazi, 2016).

AML is a malignant disorder characterized by abnormal growth and differentiation of haematopoietic stem cells, accompanied by complex network of cytogenetic aberrations and molecular mutations. In this disorder , there is accumulation of immature myeloid precursors namely the blast cells in the bone marrow as well as the peripheral blood (Estey, 2018).

The overall incidence of leukaemia in the Asian population (7.5 per 100 000 population) is lower than that of the White population (13.5 per 100 000 population) (Azzwali & Azab, 2019). Leukemia is the sixth most common cancer in Malaysia, with an incidence of 2.9 per 100,000 population (Manan et al., 2019; Meng et al., 2013). AML being the most common form of acute leukemia among adults accounts for the largest number of annual deaths from leukemias globally (Tallman et al., 2019). Its incidence increases with age and the majority of patients are older than 60 years at the

time of diagnosis. In Malaysia the age at presentation ranged from four months to 81 years with the median age being 39 years old (Meng et al., 2013).

The aetiology of AML remains poorly understood although the occurrence of this disease has been linked to a combination of genetics and environmental factors (Azzwali & Azab, 2019; Zahrani et al., 2015). The clinical features are predominantly related to the replacement of bone marrow by the malignant blast cells which will lead to suppression of normal haemopoiesis and infiltration of tissues by the malignant cells (Kulsoom et al., 2017). The clinical manifestations therefore include sign and symptoms of infection and anaemia, bleeding tendencies and organ infiltration (Kulsoom et al., 2017; Mei et al., 2019). In the presence of hyperleucocytosis the patients are at higher risks of signs and symptoms related to tumour lysis syndrome and leucostasis (Shahab & Raziq, 2014).

Although traditionally, AML classification and risk stratification relied on the cytogenetic findings, in the last decade, studies have shown that the presence or absence of specific gene mutations and/or changes in gene expression can further classify AML cases and have an effect on the patients' prognosis (Alrajeh et al., 2017; Pankaj K., 2016). As a matter of fact, genetic mutations are identified in more than 97% of cases, often in the absence of any large chromosomal abnormality (De Kouchkovsky & Abdul-Hay, 2016). Among the most frequent mutations found in AML including cytogenetically normal-AML are mutations in the *FLT3* and *NPM1* genes. Mutations in the *MLL, CEBPA*, *NRAS* and *WT1* genes are also commonly documented (Döhner et al., 2017).

The FMS-like tyrosine kinase 3 gene (*FLT3*) encodes a class III receptor tyrosine kinase that is normally expressed in early bone marrow precursors. It plays a significant role in the regulation of hematopoietic cell proliferation, differentiation and survival

through recruitment and activation of several signaling molecules (Mahmood et al., 2019). Although the frequency may varied, published studies have revealed that *FLT3* gene mutations are found in approximately 20-30% of all AML patients (Bhattacharyya et al., 2018; Mahmood et al., 2019; Naseem et al., 2021; Patnaik, 2018; Rezaei et al., 2017; Yunus et al., 2015; Yusoff et al., 2019). The two types of mutations that have been identified in the *FLT3* gene include internal tandem duplication (*FLT3-ITD*) of the region between exon 14 and 15 in the juxtamembrane domain and a point mutation at codon 835 of exon 20 in the tyrosine kinase domain (*FLT3-D835* or *FLT3-TKD)* (Patnaik, 2018). Both mutations contribute to constitutive activation of the FLT3 receptor (Yusoff et al., 2019). It has been shown that the *FLT3-ITD* mutation is a poor prognostic factor that has an inverse correlation with survival of AML patients (Grimwade et al., 2016). The identification of *FLT3-ITD* as a negative prognostic marker, serves to highlight the importance of *FLT3-ITD* testing at diagnosis. Earlier identification of *FLT3* mutations will help provide a better understanding of the patient's disease and enable targeted treatment (FLT3 inhibitors) that may help patients achieve longer and more durable remissions (Daver et al., 2019; Kim & Williams, 2018). The prognostic value of *FLT3-D835* mutation , which has a lower incidence in AML is however uncertain (Daver et al., 2019; Naseem et al., 2021; Yunus et al., 2015).

The nucleophosmin 1 (*NPM1*) gene encodes the NPM1 protein, which functions as a chaperone that shuttles between the nucleus and cytoplasm (Yingyu Chen & Hu, 2020; López et al., 2020; Yusoff et al., 2019). *NPM1* regulates different intracellular processes such as transport of preribosomal particles, responses to stress stimuli, DNA repair, centromere duplications, and the activity and stability of tumour suppressor genes like *p53* (Yohe, 2015). Mutation within the *NPM1* gene, occurring in about 25-35% of adult AML patients as documented by some published studies, results in

abnormal expression and localization of the protein within the cytoplasm (Bhattacharyya et al., 2018; Mahmood et al., 2019; Saultz & Garzon, 2016) The most common *NPM1* mutation is the insertion of the TCTG tetranucleotide at position 860-863 in exon 12, while other less common mutations in exon 12 have also been described (Jung et al., 2019). There are various reports describing co-occurring of *NPM1* and *FLT3-ITD* mutations (Boddu et al., 2017; Juliusson et al., 2020a). In the absence of *FLT3-ITD* mutation *NPM1* mutation carries a favourable prognosis in that it predicts better overall survival (Jung et al., 2019; Prada-Arismendy et al., 2017).

The other major category of myeloid neoplasms is MPN. They are a group of diseases that are characterized by clonal expansion of haematopoietic precursor cells followed by increased production of differentiated cells of the myeloid lineage (erythroid, megakaryocytic, or granulocytic) (Skoda et al., 2015; M. Wang et al., 2014). The non-leukaemic MPN include polycythemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF). PV is characterized by increased red blood cell production independent of the mechanisms that normally regulate erythropoiesis while ET primarily involves the megakaryocytic series and is characterized by sustained increased in the platelet count (Mangaonkar et al., 2018). As for PMF, there are predominantly proliferation of abnormal megakaryocytes and granulocytes in the bone marrow with reactive fibrosis and extramedullary haemopoiesis (Swerdlow et al., 2017).

The documented incidence of MPN most likely varies due to the high heterogeneity of MPN (Titmarsh et al., 2014). MPN are generally diagnosed over the age of 60. The median life expectancies for such patients are estimated at 20 years for ET, 14 years for PV, and 6 years for PMF (Tefferi & Pardanani, 2015).

The clinical picture of PV is a direct consequence of elevated number of red cell mass often accompanied by the increased production of granulocytes and megakaryocytes and is mostly characterized by hyperviscosity of the blood (Rumi & Cazzola, 2017) . Although ET is typified by persistent thrombocytosis, most patients are asymptomatic. Those who are symptomatic generally present with thrombosis or haemorrhage (Barbui et al., 2018). Similarly at diagnosis, most PMF patients are asymptomatic, although in 80% of patients there is splenomegaly (Tefferi, 2016). The major causes of death and complications in MPN include thrombosis, bleeding, and transformation into overt myelofibrosis or AML (Iurlo et al., 2019; Yap et al., 2018).

The underlying causes of MPN are largely unknown. The majority of MPN result from somatic mutations in the three driver genes, *JAK2*, *CALR*, and *MPL* which represent major diagnostic criteria in combination with haematologic and morphological abnormalities (Rumi & Cazzola, 2017). In a minor fraction of MPN cases, cytogenetic abnormalities are found at diagnosis (Zoi & Cross, 2017). With disease progression, an abnormal karyotype is more frequently detected, even in the absence of disease-specific genetic defects (Azzato & Bagg, 2015).

JAK2 gene, which plays a major role in cytokine signal transduction and carries an activating point mutation (*JAK2*V617F), which strongly correlates with MPN (Azzato & Bagg, 2015). This mutation has also been found other types of myeloid neoplasms (Webersinke & Rumpold, 2009). *JAK2*V617F is a somatic G to T point mutation at position 1849 in exon 14 of *JAK2* and is present in approximately 95% of PV patients and half of ET and PMF cases (Azzato & Bagg, 2015; Rumi & Cazzola, 2017). On the contrary, *JAK2* exon 12 mutations, which seem to be specific to the *JAK2*V617F mutation-negative PV, are absent from other types of MPN (Zoi & Cross, 2017). *MPL* mutation, which is an activating mutation in the thrombopoietin receptor,

is seen in up to 4% of ET cases and 5% of PMF cases (Langabeer et al., 2015) while *CALR* mutation occurs in approximately 15-25% of ET and 20-25% of PMF cases. Approximately 70% of ET and PMF patients without a *JAK2* or *MPL* mutation harbour *CALR* mutation (Langabeer et al., 2015; Zoi & Cross, 2017). These mutations affect the hematopoietic stem cells and result in cytokine-independent activation of the JAK-STAT signal transduction pathway (Azzato & Bagg, 2015). This pathway also appears to be hyperactive in patients with none of these mutations, which indicates that these mutation-free cases may have yet unidentified mutation(s) that is functionally similar to a *JAK2* mutation (Schischlik & Kralovics, 2017; Skoda et al., 2015).

Few studies have shown that knock-in of an ITD mutation into murine *FLT3* conferred myeloproliferative disease in a mouse model, which indicated the potential involvement of *FLT3-ITD* in MPN (Li et al., 2008; M. Wang et al., 2014; M. L. Wang & Bailey, 2015). One conventional" knock-in model of *NPM1* mutation also demonstrated that *NPM1* mutation can result in myeloproliferative disease but is insufficient for leukemogenesis (Chou et al., 2012; M. Wang et al., 2014). To date, the data on *FLT3* and *NPM1* mutations in human MPN remain poorly defined as their possible roles are rarely investigated.

1.2 PROBLEM STATEMENT

From a clinical perspective, there are two important points to be considered in relation to the study of genetic alterations in AML. Firstly, according to the current WHO classification it is necessary to search for genetic defects in AML patients, because each defect could define different clinical entities and pathological processes. Secondly these genetic alterations including both the cytogenetic and molecular genetic abnormalities are also known to have prognostic implication, and hence play crucial roles in the risk

stratification of patients for customization of therapy. Both the numerical and structural cytogenetic alterations have been recognized as the strongest predictive factor for AML patients' response to therapy and survival, for a long period of time (Wiernik et al., 2018). However, the lack of apparent chromosomal aberrations in what is known as CN-AML has proven to be challenging for the management of this clinically heterogeneous disease. Nevertheless, with progress made in the recent years, several molecular markers in AML were discovered within a growing list of genes and have allowed for more precise prognostic predictions and therapeutic decisions.

FLT3 and *NPM1* genes have been chosen in this study because research have indicated that mutations involving these genes have prognostic values and implication on patient risk stratification and therapeutic options namely the use of FLT 3-inhibitors (Alrajeh et al., 2017; Juliusson et al., 2020a). Nevertheless, in Malaysia, published research works related to *FLT3* and *NPM1* mutation in AML are scarce and one of which is by Yusoff et al., 2019. To the best of our knowledge, there is no published data on the status of *FLT3* and *NPM1* mutations among myeloid neoplasms cases in Kuantan, Pahang, Malaysia, a center for treating haematological malignancies for the state of Pahang. The prevalence of *FLT3*, and *NPM1* mutations are unknown.

As for MPN, there are limited publications that look into these mutations (*FLT3* and *NPM1*) and their possible roles in the pathogenesis. Also, there are very few studies that investigate the association of *JAK2*V617F with *FLT3* and/ or *NPM1* in MPN.

1.3 SIGNIFICANCE OF RESEARCH

1. Since there are limited publications on *FLT3 and NPM1* mutation status in myeloid neoplasms in Malaysia, this research will indeed contribute to the body of knowledge.