FLT3, NPM1, DNMT3α AND NRAS GENES MUTATION IN ACUTE MYELOID LEUKAEMIA IN HUSM

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by

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ABBREVIATIONS

ADD	ATRX, DNMT3 and DNMT3L-type finger
AML	acute myeloid leukaemia
APML	acute promyelocytic leukaemia
ATRA	*all-trans*-retinoic acid
BAP	1,4-bis acryloylpiperazine
BM	bone marrow
bp	base pairs
°C	degree Celcius
CA	cytogenetically abnormal
CBF	core binding factor
CEBPA	CCAAT enhancer binding protein alpha
CI	confidence intervals
CN	cytogenetically normal
CRM1	chromosomal region maintenance 1
CSGE	conformation sensitive gel electrophoresis
del	deletion
DNA	deoxyribonucleic acid
DNMT3a	DNA (cytosine-5-)-methyltransferase 3 alpha
EDTA	ethylenediamine tetraacetic acid
FAB	French-American-British
FLT3	*fms*-like tyrosine kinase-3
FLT3-ITD^{+}	FLT3-ITD mutant

FLT3-ITD^{-}	FLT3-ITD non-mutant
g	gram
GDP	guanosine diphosphate
GRB2	receptor-bound protein 2
GTP	guanosine triphosphate
h	hour
Hb	haemoglobin
IDH	isocitrate dehydrogenase
Ig	immunoglobulin
ins	insertion
ITD	internal tandem duplication
JM	juxtamembrane
KI	kinase insert
M	Molar
MDS	myelodysplasia syndrome
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mm	millimeter
MPD	myloproliferative disease
MRC	Medical Research Council
Mtase	methyltransferase
NES	nuclear export signal

NLS	nuclear localization signal
NoLS	nucleoli localization signal
ng	nanogram
NRAS	neuroblastoma RAS
NPM1	nucleophosmin
NPM1$^+$	NPM1 mutant
NPM1$^-$	NPM1 non mutant
nt	nucleotide
μg	microgram
μl	microlitre
μM	micromolar
OD	optical density
PCR	polymerase chain reaction
PI3K	phosphoinositide-3 kinase
Plt	platelet
PWWP	proline-tryptophan-tryptophan-proline
RBC	red blood cell
RTK	receptor tyrosine kinase
rpm	revolutions per minute
SAM	s-adenosyl methionine
sec	second
SNP	single nucleotide polymorphism
SOS	son of sevenless
SSCP	single strand conformational polymorphism

TBE	tris borate-EDTA buffer
TEMED	tetramethylethylenediamine
TE	tris-EDTA buffer
TET2	tet methylcytosine dioxygenase 2
TKD	tyrosine kinase domain
TKI	tyrosine kinase inhibitor
Tm	melting temperature
TM	transmembrane
U	unit
WBC	white blood cell
WHO	World Health Organization
WT	wild-type

LIST OF PUBLICATIONS

Journal

Noraini Mat Yunus, Muhammad Farid Johan, Hamid Ali Nagi Al-Jamal, Azlan Husin, Abdul Rahim Hussein, Rosline Hassan (2015). Characterisation and clinical significance of *FLT3*-ITD and non-ITD of Acute Myeloid Leukemia (AML) patients in Kelantan, Northeast of Peninsular Malaysia. *Asian Pac J Cancer Prev,* **16(2)**, 4869-4872.

Abstract in journal

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Abstract in proceeding

Mutational analysis of *FLT3*, *NPM1* and *DNMT3A* in patients with Acute Myeloid Leukaemia. 10th Malaysia Genetics Congress, 3rd-5th December 2013, Palm Garden Hotel IOI Resort, Putrajaya.

Abstracts in conferences

Conformational Sensitive Gel Electrophoresis (CSGE) as a method for *NPM1* mutational screening in patients Acute Myeloid Leukemia. 1st International Conference on Molecular Diagnostic and Biomarker Discovery, 23th-25th October 2013, Equatorial Hotel, Penang, Malaysia.

Mutation analysis of *FLT3-ITD, NPM1* and *DNMT3A* in AML patients at Hospital Universiti Sains Malaysia. 37th Annual Conference of the Malaysian Society for Biochemistry & Molecular Biology 18th -19th July 2012, Sime Darby Convention Centre, Kuala Lumpur.

MUTASI PADA GEN *FLT3, NPM1, DNMT3a* DAN *NRAS* DALAM LEUKEMIA MIELOID AKUT DI HUSM

ABSTRAK

Leukemia mieloid akut (AML) adalah sejenis penyakit yang jarang berlaku namun ia di antara penyakit yang paling kerap terjadi dalam kumpulan penyakit malignan darah dan selalu dikaitkan dengan kematian dalam kalangan orang dewasa. Mutasi pada gen FMS-tirosina kinase 3 duplikasi dalaman secara rawak (*FLT3*-ITD) dan nukleofosmin 1 (*NPM1*) merupakan mutasi genetik yang kerap berlaku dalam kalangan orang dewasa yang menghidapi AML dan masing-masing dikaitkan sebagai prognosis buruk dan prognosis baik. Data insiden mutasi bagi gen-gen mutasi yang lazimnya terlibat dalam kalangan pesakit AML dewasa masih belum diterbitkan. Dengan itu, kajian ini dijalankan adalah untuk menentukan frekuensi bagi gen *FLT3-ITD, FLT3-TKD, NPM1, DNMT3a* dan *NRAS*, jenis mutasi pada gen-gen tersebut, median kelangsungan hidup pesakit bagi kumpulan *FLT3*-ITD$^+$/*FLT3*-ITD$^-$ dan *NPM1*$^+$/*NPM1*$^-$ dalam masa 2 tahun, dan perhubungan antara parameter klinikal dengan mutasi gen *FLT3*-ITD dan *NPM1* dalam kalangan pesakit AML dewasa di Malaysia. Dalam kajian ini, DNA daripada 54 pesakit dewasa AML yang baru didiagnosis diambil daripada Hospital Universiti Sains Malaysia di Kelantan. Kaedah PCR-CSGE telah digunakan untuk penyaringan mutasi pada gen *FLT3*-ITD dan *NPM1* dalam kalangan pesakit AML. Frekuensi mutasi bagi gen *FLT3*-ITD dan *NPM1* masing-masing adalah sebanyak 11% dan 13% yang mana frekuensinya adalah kurang berbanding data-data yang telah diterbitkan. Analisis jujukan menunjukkan kesemua mutasi pada gen *FLT3*-ITD dan *NPM1* adalah unik

dalam setiap kes. Berdasarkan analisis tersebut, dua jenis mutasi yang dikenalpasti bagi gen *FLT3* ekson 14 adalah ITD (sebanyak enam kes) dan bukan ITD (sebanyak satu kes). Kedua-dua jenis mutasi ini akan menggangu fungsi 'juxtamembrane' pada FLT3, dengan itu mengakibatkan pengaktifan isyarat pertumbuhan secara terus-menerus. Menariknya, satu penemuan baru dikenalpasti dalam mutasi gen *NPM1*. Walaupun mutasi tersebut dijumpai di kawasan domain terminal C, mutasi tersebut tidak mengganggu motif NoLs pada protein NPM. Mutasi gen *FLT3*-ITD berkaitan secara signifikan dengan kehadiran peratusan blast yang tinggi (nilai $p = 0.008$) dan kiraan sel darah putih yang lebih tinggi (nilai $p = 0.023$) berbanding gen *FLT3* yang normal. Manakala mutasi pada gen *NPM1* pula dikaitkan secara signifikan lebih tinggi dalam kalangan wanita berbanding lelaki (nilai $p = 0.038$). Tiada sebarang mutasi dikesan pada gen *FLT3*-TKD, *DNMT3a, dan NRAS*. Analisis kelangsungan hidup selama 2 tahun pesakit menunjukkan tiada perbezaan secara signifikan bagi keseluruhan kelangsungan hidup (OS) dalam kedua-dua kumpulan *FLT3*-ITD$^+$/*FLT3*-ITD$^-$ (nilai $p = 0.660$) dan *NPM1*$^+$/*NPM1*$^-$ (nilai $p = 0.714$). Walaupun OS dalam kumpulan *FLT3*-ITD$^+$/*FLT3*-ITD$^-$ tidak menunjukkan perbezaan secara signifikan, median OS bagi pesakit AML yang mempunyai *FLT3*-ITD adalah lebih pendek berbanding pesakit yang mempunyai *FLT3* yang normal (masing-masing 9 dan 52 minggu). Frekuensi bagi mutasi pada gen *FLT3*-ITD dan *NPM1* adalah lebih tinggi dalam kalangan pesakit AML di Malaysia berbanding mutasi pada gen lain iaitu *FLT3*-TKD, *DNMT3a*, dan *NRAS*.

FLT3, NPM1, DNMT3a AND *NRAS* GENES MUTATION IN ACUTE MYELOID LEUKAEMIA IN HUSM

ABSTRACT

Acute myeloid leukaemia (AML) is a rare but the most common occurrence among hematologic malignancy and associated with the greatest mortality in adults. FMS-like tyrosine kinase 3-internal tandem duplication (*FLT3*-ITD) and nucleophosmin 1 (*NPM1*) mutations are the most common genes mutation found in adult AML and have been associated with poor and good prognosis, respectively. Published data on the prevalence of *FLT3*-ITD and *NPM1* mutations of AML adult patients in Malaysia are not available online. Thus, this study was conducted to determine the frequency of *FLT3*-ITD and *NPM1* mutation as well as *FLT3*-TKD, *DNMT3a,* and *NRAS*, the types of mutations, two-years overall survival/median survival time of *FLT3*-ITD$^+$/*FLT3*-ITD$^-$ and *NPM1*$^+$/*NPM1*$^-$ group, and the related clinical data for mutant *FLT3*-ITD and *NPM1* within the adult Malaysian AML patients. In this study, the genomics DNA from 54 newly diagnosed adult AML patients were retrieved from Hospital Universiti Sains Malaysia, Kelantan. Polymerase chain reaction followed by conformation sensitive gel electrophoresis (PCR-CSGE) technique was used to detect gene mutations in AML. The frequency of *FLT3*-ITD and *NPM1* mutations were found to be 11% and 13%, respectively which were less compared to published data. With respect to sequence analysis, all of the mutant *FLT3*-ITD and *NPM1* resulted in variety of amino acid sequences in every case. Based on the analysis, two types of mutation have been identified in *FLT3* exon 14; ITD (six cases) and non-ITD (a single nucleotide deletion)

(one case). Both kind of mutations would disturb the juxtamembrane domain function in FLT3 receptor, thus activate the continuous growth signal. Interestingly, a different finding was found in *NPM1* mutation. Although the mutation was identified in the coding region of C-terminal domain, the mutation does not disturb the NPM nucleolar localization signal (NoLs) motif. *FLT3*-ITD mutation was associated with a significantly higher blast percentage (*p*-value = 0.008) and white blood cell count (*p*-value = 0.023) than the *FLT3* wild type. On the other hand, *NPM1* mutation were found not to associate with blast percentage and white blood cell count but occurred significantly higher in female patients (*p*-value = 0.038). Mutation was not detected in *FLT3*-TKD, *NRAS*, and *DNMT3a* genes. Two years overall survival (OS) analysis indicated no significant difference in both group, *FLT3*-ITD+/*FLT3*-ITD- (*p*-value = 0.660) and *NPM1*+/*NPM1*- (*p*-value = 0.714). Although the OS in *FLT3*-ITD+/*FLT3*-ITD- group showed not significantly difference, the median OS of AML patients with the *FLT3*-ITD was shorter than those with the wild type (9 versus 52 weeks, respectively). *FLT3*-ITD and *NPM1* mutations were more prevalent in adult Malaysian AML patients compared to the other genes; *FLT3*-TKD, *DNMT3a,* and *NRAS*.

CHAPTER 1

INTRODUCTION

1.1   Acute Myeloid Leukaemia

1.1.1   Definition

Acute myeloid leukaemia (AML) is heterogenous with respect to clinical manifestation, therapy, cytogenetic rearrangement and genetic alteration. AML is defined as a clonal proliferation of immature haematopoietic progenitors (myeloblasts) with varying degree of myeloid differentiation in the bone marrow, peripheral blood, or extramedullary tissues (Vardiman *et al.*, 2009).

1.1.2   Clinical manifestations and diagnosis

Clinical manifestations of AML result from proliferation (overproduction) of myeloid blast cells and bone marrow failure that leads to decrease in the production of normal blood cells; red blood cells (RBCs), other types of white blood cells (WBCs), and platelets. As a result of bone marrow failure, AML patients would have anaemia, neutropenia and/or thrombocytopenia manifested as fatigue, lethargy, dyspnea, fever, weight loss, infection and haemorrhage. AML progresses quickly and typically fatal within weeks or months if not treated (Hoffman *et al.*, 2005).

The recent definitive diagnosis for AML requires bone marrow aspiration for morphologic examination to identify the presence of 20% or more blasts in the marrow. In rare cases, for blast count below than 20%, but if cytogenetic aberrations are present with a t(8;21)(q22;q22), inv(16)(p13.1q22), t(16;16)(p13.1;q22), or t(15;17)(q22;q12), it has to be considered as AML as well (Vardiman *et al.*, 2009). Aside from that, cytochemistry, immunology and immunohistochemistry techniques are also used for confirmation of AML.

1.1.3 AML classification

Although AML patients share many common clinical manifestations, their leukaemic cell morphology, cytogenetics and molecular genetics are obviously heterogenous. Two systems have been commonly used to classify AML, the French-American-British (FAB) classification and the World Health Organization (WHO) classification.

The older FAB classification divided AML into eight subtypes, M0 through M7, based on the type of cell from which the leukaemia developed and its degree of maturity (Table 1.1). This classification was based primarily on leukemic cell morphology and cytochemical stains (Lowenberg *et al.*, 1999).

The WHO classification of AML is a newer system that incorporates and relates morphology, cytogenetic, molecular genetic and immunologic marker (Table 1.2). Using WHO criteria, the diagnosis of AML is defined as at least 20% of blasts in the bone marrow (BM) required. However, it is not applicable if one of the chromosome

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abnormalities such as t(8;21)(q22;q22), inv(16)(p13.1q22), or (15;17)(q22;q12) in acute pro-myelocytic leukemia (APML) is detected. The presence of these genetic abnormalities is sufficient for the diagnosis of AML without blast percentage (Vardiman *et al.*, 2009). The WHO classification of AML is more universally applicable and prognostically valid.

Although WHO classification is more detailed and perhaps more useful today, the FAB classification system is still used in Malaysia. FAB classification can be achieved faster and easier as it is only based on morphological examination and cytochemical stains.

Table 1.1: FAB classification system of AML (Lowenberg et al., 1999).

FAB subtype	Common name
M0	Acute myeloblastic leukaemia with minimal maturation
M1	Acute myeloblastic leukaemia without maturation
M2	Acute myeloblastic leukaemia with maturation
M3	Acute promyelocytic leukaemia (APML)
M4	Acute myelomonocytic leukaemia
M4Eo	Acute myelomonocytic leukaemia with abnormal eosinophils
M5	Acute monocytic leukaemia
M6	Acute erythroid leukaemia
M7	Acute megakaryoblastic leukaemia

Table 1.2: WHO classification system of AML (Vardiman et al., 2009).

1. AML with recurrent genetic abnormalities

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

      AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*

      APL with t(15;17)(q22;q12); *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) or t(3;3)(q21;q26.2); *RPN1-EVI1*

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

      AML with mutated *NPM1*

      AML with mutated *CEBPA*

2. AML with myelodysplasia-related changes

3. Therapy – related myeloid neoplasms (MRC)

4. AML, not otherwise specified (NOS)

      AML with minimal differentiation

      AML without maturation

      AML with maturation

      Acute myelomonocytic leukaemia

      Acute monoblastic / monocytic leukaemia

      Acute erythroid leukaemia

         Pure erythroid leukaemia

         Erythroleukemia, eryhthroid / myeloid

      Acute megakaryoblastic leukaemia

      Acute basophilic leukaemia

      Acute panmyelosis with myelofibrosis

5. Myeloid sarcoma

6. Myeloid proliferations related to Down syndrome

7. Transient abnormal myelopoiesis

8. Blastic plasmacytoid dendritic cell neoplasm

1.1.4   Epidemiology of AML in Malaysia

The incidence of all 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) (Howlader *et al.*, 2014). In Malaysia leukaemia was the seventh most common cancer in females and fourth in males, with only 2.8 per 100,000 populations (Lim and Halimah, 2004). AML is a rare but it is the most common type of leukaemia in adults, and its incidence increases with age. The age of Malaysian AML at presentation ranged from four month to 81 years with median age of 39 years (Meng *et al.*, 2013). Chromosome aberrations were detected in 30% Malaysian AML and the most common chromosome abnormalities are t(8;21), trisomy 8, and t(15;17) (Meng *et al.*, 2013).

1.2   Genetic pathogenesis in AML

The heterogeneity of AML results from a complex network of chromosome aberrations and genetic mutations. Chromosome aberrations, revealed by GTG-banding (G bands by Trypsin using Giemsa) are found in 30-50% of AML patients. Genetic mutations occur both in cytogenetically normal AML (CN-AML) and in cytogenetically abnormal AML (CA-AML) patients (Grimwade and Hills, 2009). Lately, the genetic mutations are increasingly being recognized as important diagnostic and prognostic markers in AML. In parallel to this important insight, researchers nowadays are trying to develop targeted treatments based on interference with molecular genetics as well as epigenetic mechanisms.

1.2.1 Model of leukaemogenesis

Many of the mutations identified in AML when existing alone do not lead to leukaemogenesis. This is because at least two different types of mutations are required for the development of leukaemia. For many years back, the "two-hit" model of hypothesis by Gilliland (2001) was accepted as model of leukaemogenesis which suggested that development of AML required two different types of genetic mutations, class I (proliferative mutation) and class II (blocking mutation) .

However recently, scientists discovered the presence of a new class of mutation (class III) involving epigenetic modifications to the AML genome would also contribute towards leukaemogenesis of AML and they cannot be categorized in class I or class II mutations (Figure 1.2). Epigenetics is defined as the study of heritable changes in gene expression that are not due to modifications in the DNA sequence. Class III mutations that confer epigenetic regulation includes genes which encode transcription like *DNMT3a, IDH1, IDH2* and *TET2* (Galm *et al.*, 2006; Figueroa *et al.*, 2009; Jiang *et al.*, 2009; Barrero *et al.*, 2012).

Class I mutations confer proliferation and survival advantage. They involve gene coding for receptor and non-receptor protein tyrosine kinase like *FLT3* and *KIT* as well as for proteins of GTPase activity such as *NRAS*, which affect proliferative signaling pathways, leading abnormal growth of leukemic cells (Kelly and Gilliland, 2002).

Class II mutations lead to arrest differentiation of myeloid progenitor cells by affecting genes of transcription factors. Genes coding for transcription factors become disrupted by their fusion, as a result of chromosome changes (AML1-ETO and PML-RARα), or by point mutation (*NPM1* and *CEBPA*). This class of mutation occurs during early leukaemogenesis and is stable throughout the disease course. These mutations have been considered as initiating mutations (Kelly and Gilliland, 2002).

If mutations of class I (excessive myeloproliferation) or class II (differentiation arrest) of hematopoietic progenitors occur on its own, it results in the development of myeloproliferative syndrome (MPD) or myelodysplastic syndrome (MDS), respectively. When these two kinds of mutations occuring sequentially in a single cell, these two-hit lead to a fully penetrating AML (Figure 1.1) (Kelly and Gilliland, 2002).

AML with multilineage dysplasia was introduced in the WHO classification 2001 to enclose cases of AML characterized by myelodysplastic syndrome–like features. The WHO classification 2008 revised this group into a new category, AML with myelodysplasia-related changes (AML-MRC). The category now includes patients with at least 20% blasts in peripheral blood or bone marrow and any of the following: (1) AML arising from a previous MDS or mixed MDS/myeloproliferative neoplasm, (2) AML with a specific MDS-associated cytogenetic abnormality and/or (3) AML with multilineage dysplasia (Vardiman *et al.*, 2009; Vardiman and Reichard, 2015).
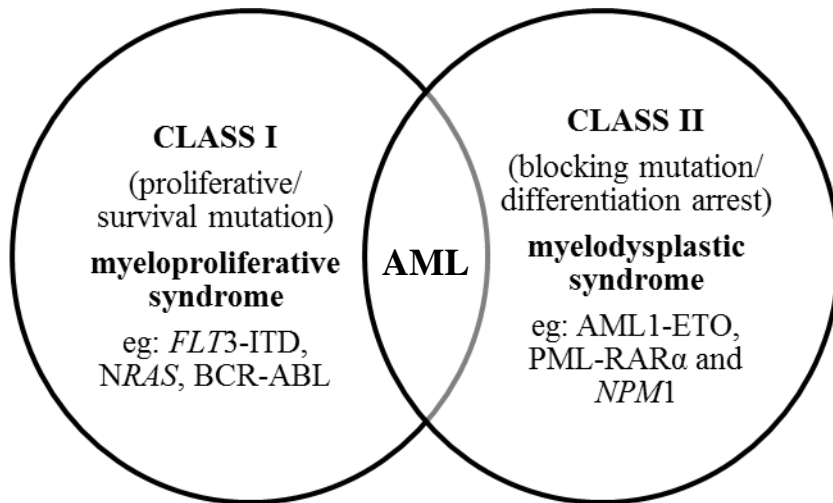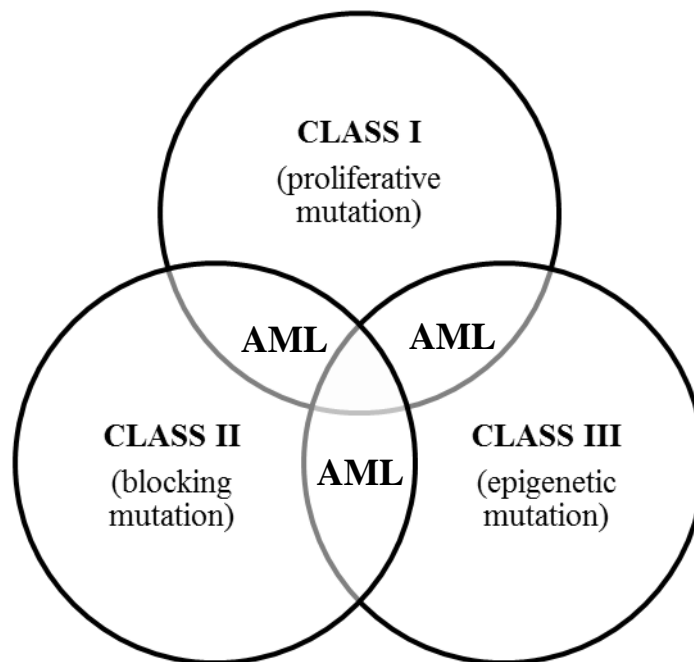
Figure 1.1: "Two-hit" model of leukemogenesis.

Figure 1.2: Venn diagram including a new class model of leukemogenesis.

1.3   Molecularly Targeted Therapies

For over past 40 years, the induction treatment for AML has not changed. The standard induction chemotherapy for AML patients remains cytarabine and anthracycline combination, also known as 7+3 treatment. This treatment has been troubled by low survival rates (10% for 5 years) and deaths due to toxicity. For these reasons, the ability to identify specific patients who may get benefit from molecularly targeted therapies would give a great promise for AML to improve remission and cure rate. By screening for the presence of genetic mutations that are targeted by specific drugs, specific therapy would be given based on genetic abnormalities characteristic of the individual patient's.

The first targeted therapy in AML was the use of all–trans-retinoic acid (ATRA) for acute promyelocytic leukemia (APML/AML-M3) patients. This treatment specifically blocks PML-RARA fusion protein function and it appears curable in more than 70% of APML patients (Quignon *et al.*, 1997).

A number of new targeted molecular therapies are still under study and clinical trials. FLT3 gene mutations is a potentially attractive marker towards molecularly targeted therapy in AML as they constitute 1/3 mutations in AML. Nowadays, researchers have explored in clinical trials of tyrosine kinase inhibitor (TKI) drugs, including lestaurtinib, sorafenib, sunitinib and midostaurin, which were shown to have in-vitro activity against FLT3 mutants. During therapy with FLT3 TKIs, the induction of acquired resistance has emerged as a clinical problem. Thus, the current focus in clinical research is to find out

the optimization of the targeted therapy and the potential treatment options to overcome resistance in this situation.

On the other hand, *NPM1* mutations which account in 50-60% of CN-AML show a great promise for minimal residual disease, as it is stable and persists at relapse (Mrozek *et al.*, 2007). It appears that *NPM1* mutations will become important markers for disease monitoring in patients with CN-AML.

The advent of these molecular agents provides us with a new approach toward AML treatment and monitoring.

1.4   Rationale of the study

Cytogenetic alterations have been recognized as the strongest predictive factor for AML patients' response to therapy and survival for a long time. The lack of chromosome aberration in cytogenetically normal AML has proved challenging for the management of this clinically heterogeneous disease. However in recent years, several molecular markers in AML were discovered within a growing list of genes and allowed more precise prognostic predictions and therapeutic decisions. *FLT3* (exon 14-15 and 20) and *NPM1* (exon 12) genes have been chosen in this study because they are the two most extensively studied genes for their significant role in prognostic predictions of AML and they are the most commonly identified in AML throughout the world. Besides that, we also interested to look at the frequency and types of mutation in *DNMT3a* (exon 23) and *NRAS* (exon 1 and 2) genes. Unfortunately, we have limited information regarding the alteration of these genes in Malaysian AML patients, and also the correlation between these genes alteration status and the important clinical parameters associated with AML prognosis and treatment. Concerned with that, this study aimed to explore these genetic mutations in Malaysian AML cases particularly in the North Eastern State of the Peninsular Malaysia and it is truly hoped that the results obtained from this study would give us better understanding towards the molecular pathogenesis of AML in Malaysia; also would lead to improve diagnostic strategy as well as treatment outcome among Malaysian AML population.

1.5   Target Genes

1.5.1 *FLT3* gene

The *FLT3* (FMS-like tyrosine kinase receptor III) gene is mapped to chromosome 13q12 in humans and contains 24 exons. It encodes for the FLT3 receptor protein that belongs to class III receptor tyrosine kinase (RTKIII) and composed of 993 amino acids (Zuo *et al.*, 2009). The FLT3 receptor is a membrane-bound RTK, which plays a crucial role in proliferation, differentiation and apoptosis of normal haematopoietic cells. It comprises five immunoglobulin-like domains (Ig-like) in the extracellular ligand binding region, a transmembrane domain (TM), a juxtamembrane domain (JM), two intracellular kinase domains (TKD1 and TKD2) divided by a kinase insert domain (KI), and a C-terminal domain (Figure 1.3) (Abu-Duhier *et al.*, 2001a).

In normal haematopoietic cells, activation of FLT3 receptor is ligand-dependent. Figure 1.4 represents FLT3 receptor signaling in normal haematopoietic cell. In the absence of ligand, FLT3 receptor is an inactive conformation. Ligand binding to FLT3 receptor leads to dimerization on the cell membrane and conformational change of the receptor as well as rapid phosphorylation of tyrosine residues on the FLT3 receptor. This in-turn activates RAS and PI3K pathways. The activated FLT3 receptor interacts with growth factor-bound protein 2 (GRB2) through SHC and activates GRB2 which binds Son of Sevenless homolog 1 (SOS). The GRB2-SOS complex is recruited to the membrane associated RAS. This activates the MAPK pathway which includes RAF, MEK and ERK proteins. Ligand-stimulation of FLT3 activates the GRB2 associated protein 1/2

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(GAB1/2), and also activates the PI3K-AKT pathway. Phosphorylated ERK and AKT result in activation of transcription factors involved in cell proliferation and anti-apoptosis, respectively. Mutation in the structure FLT3 receptor will lead to a continuous growth signal and promote leukaemogenesis (Kiyoi *et al.*, 2002; Stirewalt and Radich, 2003).
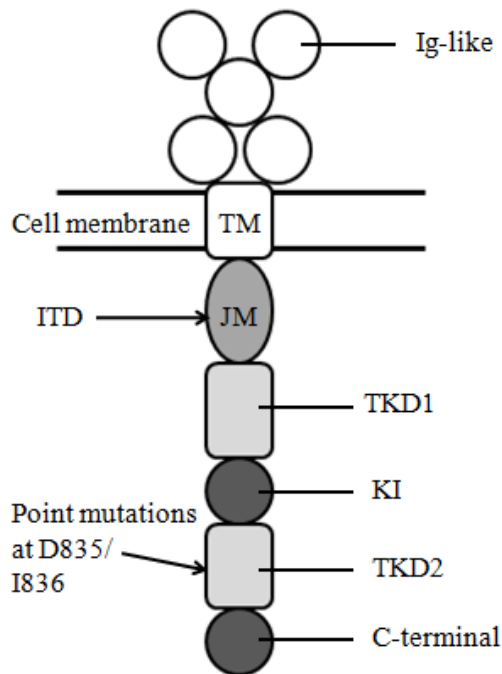

Figure 1.3: Structure of FLT3 and commonly occurring mutation sites in FLT3.
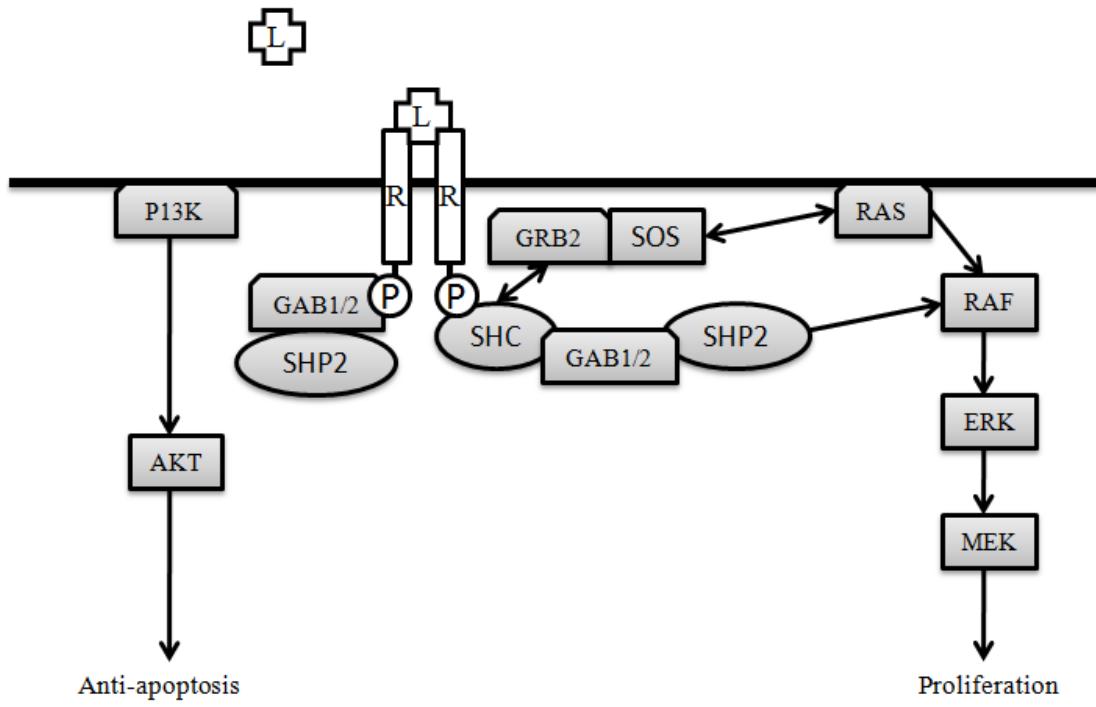
Figure 1.4: FLT3 receptor signaling in normal hematopoietic cell.

Mutation in the *FLT3* accounts for 25% of all AML cases. They fall into two main categories of mutations; internal tandem duplications (*FLT3*-ITD) in JM and point mutations in TKD (*FLT3*-TKD) (Kiyoi *et al.*, 2002; Meshinchi *et al.*, 2008). *FLT3*-ITD is also known as *FLT3* length mutation (*FLT3*-LM). The length of the ITD varies from 3-400bp nucleotides (Stirewalt and Radich, 2003). *FLT3*-ITD accounted in 25% for adult (Frohling *et al.*, 2002; Schnittger *et al.*, 2002; Thiede *et al.*, 2002) and 15 % for pediatric (Meshinchi *et al.*, 2008). Another most common type of *FLT3* mutation is a point mutation at codon D835 (aspartate) or I836 (isoleucine) in exon 20 within the activation loop of TKD. *FLT3*-TKD usually is less frequently seen in AML and they account for 5% of adult and 3% of peadiatric AML (Abu-Duhier *et al.*, 2001b; Yamamoto *et al.*, 2001). Both *FLT3*-ITD and *FLT3*-TKD result in ligand independent activation and leads to continuous activation of FLT3 receptor (Betz and Hess, 2010; Gulley *et al.*, 2010).

FLT3-ITD in AML patients confer poor prognosis which relates to high percentage of blast cells, increased risk of relapse from complete remission, and reduced survival (Kottaridis *et al.*, 2001; Frohling *et al.*, 2002; Small, 2006; Feng *et al.*, 2012). However, the prognosis of *FLT3*-TKD in AML is still unclear (Whitman *et al.*, 2010; Smith *et al.*, 2011). The high incidence of *FLT3* mutations as well as the poor prognosis status in AML eventually stimulated the development of specific treatment for *FLT3* aberration.

1.5.2 *NPM1* gene

The nucleophosmin 1 (*NPM1*) gene is located on chromosome 5q35 in humans and contains 12 exons. It encodes for the nucleophosmin (NPM) proteins which contains 294 amino acids. The NPM is a multifuntional nucleocytoplasmic shuttling protein localized mainly in the granular regions of the nucleolus (Cordell *et al.*, 1999). It has several functions which are transporting ribosome components to the cytoplasm for ribosome biogenesis (Olson *et al.*, 1986), controlling centrosome duplication during cell division (Okuda *et al.*, 2000), and interacting with tumor-suppressors gene (p53 and p19Arf) for controlling cell proliferation and apoptosis (Colombo *et al.*, 2002).

Mutations in *NPM1* gene are documented in 26-30% of whole adult AML cases (Falini *et al.*, 2005; Roti *et al.*, 2006; Boonthimat *et al.*, 2008) and 40-60% of adult CN-AML (Mrozek *et al.*, 2007). Their incidences are lower among peadiatric AML (7.5-8%) (Bacher *et al.*, 2010; Braoudaki *et al.*, 2010). These mutations are restricted to exon 12 which encodes for the C-terminus of NPM protein and generally heterozygous. Various *NPM1* gene mutations were reported in AML at nucleotide positions 956 through 971 (Chen *et al.*, 2006; Boonthimat *et al.*, 2008). All these mutations are either insertions or insertions/deletions producing the frameshift mutation in the C-terminus region of the *NPM1* gene and these mutations arrest the protein production in the cytoplasm (Mardis *et al.*, 2009).

More than 40 different mutations have been identified in exon 12 of the *NPM1* gene. The most common type of mutations in *NPM1* is a duplication of a TCTG between

position 956 and 959 and its accounts for 75% of the cases. Figure 1.5 represents the *NPM1* mutations type A in C-terminal domain (Grisendi and Pandolfi, 2005).
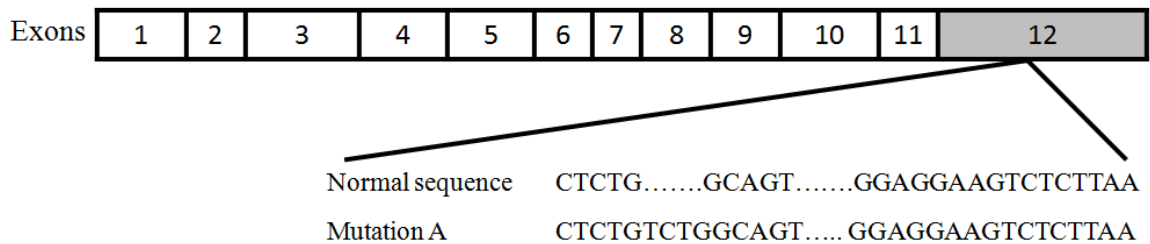

Figure 1.5: Diagram of the *NPM1* mutation type A in exon 12.

NPM1 mutations are related with an increased in WBCs count (Suzuki *et al.*, 2005a; Yan *et al.*, 2007; Boonthimat, 2008), female (Dohner *et al.*, 2005; Schnittger *et al.*, 2005; Thiede *et al.*, 2006; Gale *et al.*, 2008) and M4/M5 FAB subtypes (Kassem *et al.*, 2011). *NPM1* mutations generally coexist with *FLT3*-ITD (Dohner *et al.*, 2005; Falini *et al.*, 2005; Schnittger *et al.*, 2005; Suzuki *et al.*, 2005a; Verhaak *et al.*, 2005). Only *NPM1*$^+$/*FLT3*-ITD$^-$ compilation is recognized as favorable prognostic factor, especially in patients aged more than 70 years. AML with *NPM1*$^-$/*FLT3*-ITD$^+$ is related with poor prognosis, while AML with *NPM1*$^+$/*FLT3*-ITD$^+$ and *NPM1*$^-$/*FLT3*-ITD$^-$ is associated with intermediate prognosis (Thiede *et al.*, 2006; Bacher *et al.*, 2010; Becker *et al.*, 2010).

NPM1 functions are based on its nucleocytoplasmic shuttling process which is controlled by three important domains such as nuclear export signal (NES), nuclear

localization signal (NLS) and nucleoli localization signal (NoLS) (Figure 1.6). The NES is crucial for transportation of NPM protein from nucleoplasm into cytoplasm. The NLS and NoLS are crucial for localization of the NPM protein in nucleoplasm and nucleolus (Chen *et al.*, 2006; Falini *et al.*, 2007).

All *NPM1* mutations have in a distinct sequence in the NPM protein at the C-terminal domain. All of them show loss of at least one of the tryptophan residues (W) at amino acid positions 288 and 290 at the C-terminal domain, which lead the frameshift mutations and create the common last 5 amino acid residues of VSLRK. The C-terminal tryptophans 288 and 290 are essentials for NoLS which is responsible for NPM nucleolar localization. The aberration disrupts the NoLS and also brings about an additional leucine-rich NES motif at the C-terminal. Both of them play crucial role in disrupting the normal nucleocytoplasmic transportation of the NPM protein and creating the abnormal cytoplasmic accumulation of the mutant NPM proteins (Figure 1.7) (Chen *et al.*, 2006; Falini *et al.*, 2007).

Falini *et al.* (2007) reported that cytoplasmic accumulation of the mutant NPM protein is NES dependent and normal NPM protein contains only one NES domain at amino acid position 92-104 (Figure 1.6) (Falini *et al.*, 2006). It binds to the chromosomal region maintenance 1 (CRM1) export receptor and facilitates the downstream nuclear export of the normal NPM protein for normal functions. The mutant NPM protein however contains an extra creation of NES motif at C-terminal domain in which might reinforces the accessibility of the NPM mutants towards CRM1 receptor. It would leads to more

efficient nuclear export activity than the nuclear import activity and result in the uncontrolled cytoplasmic accumulation of NPM protein (Falini *et al.*, 2007).
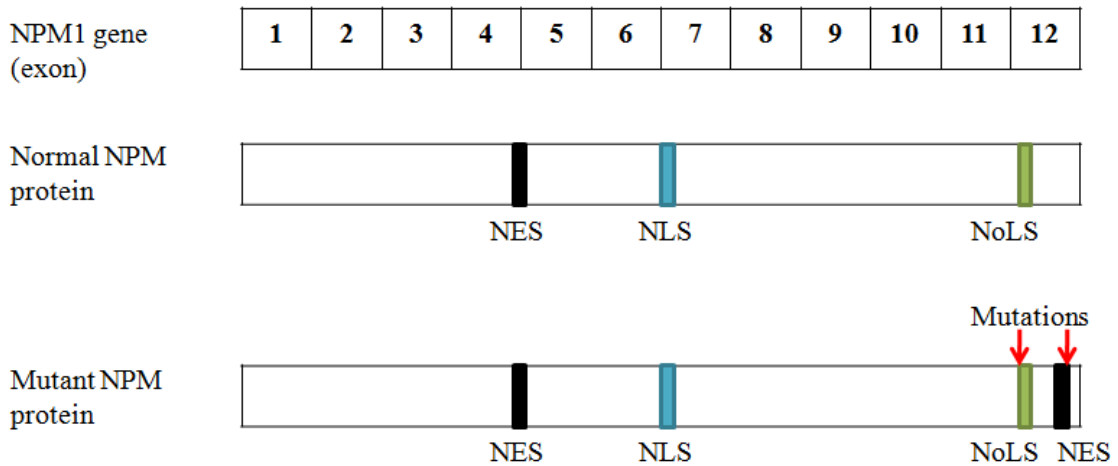

Figure 1.6: Diagram of *NPM1* gene, mutant NPM protein and normal NPM protein (Leung, 2010).
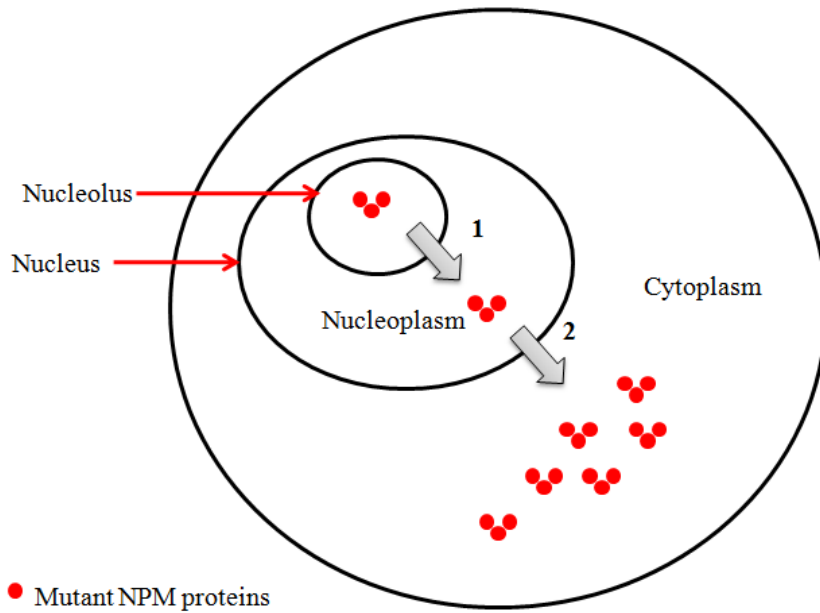
Figure 1.7: Accumulation of mutant NPM proteins in cytoplasm (Leung, 2010).

1: Dislocation of the mutant NPM proteins from the nucleolus to the nucleoplasm due to disruption of the NoLS. 2: Dislocation of the mutant NPM proteins from the nucleoplasm to the cytoplasm due to the addition of NES in the C-terminal mutant NPM proteins.

1.5.3 *DNMT3a* gene

DNMT3a (DNA methyl transferase 3α) gene has 23 exons and maps to chromosome 2p23 in humans. *DNMT3a* gene encodes a DNA methyltransferase enzyme that is actively involved in epigenetic regulation. The DNMT3a enzyme contains three main domains stucture, a proline-tryptophan-tryptophan-proline (PWWP) domain, an ATRX, DNMT3 and DNMT3L-type finger (ADD) domain, and the methyltransferase (Mtase) domain (Figure 1.8)(Weisenberger *et al.*, 2004). This enzyme localizes in the nucleus and uses S-adenosyl methionine (SAM) as the methyl group donor being transferred to the carbon 5 (C5) position of the cytosine ring in CpG dinucleotide in DNA (Figure 1.9). It is responsible for de novo methylation during mammalian development (Brenner and Fuks, 2006).

DNA methylation occurs mainly in the cytosine residues at the C5 positions of CpG di nucleotides and is important in regulating gene expression (Chen and Li, 2006). Aberrant DNA methylation plays a significant role in the pathogenesis of AML (Rosenbauer and Tenen, 2007).

DNMT3a gene mutations in AML accounted for 7-30% and were related to CN-AML, older age, M4/M5 FAB subtypes and poor prognosis (Ley *et al.*, 2010; Thol *et al.*, 2011; Hou *et al.*, 2012). Ho *et al.* (2011) demonstrated that frequency of *DNMT3a* mutations was rare in peadiatric AML. The most frequently mutated site in *DNMT3a* is Aginine 882 (R882) residue located in the catalytic domain which led to reduced DNA methylation (Ley *et al.*, 2010; Thol *et al.*, 2011).
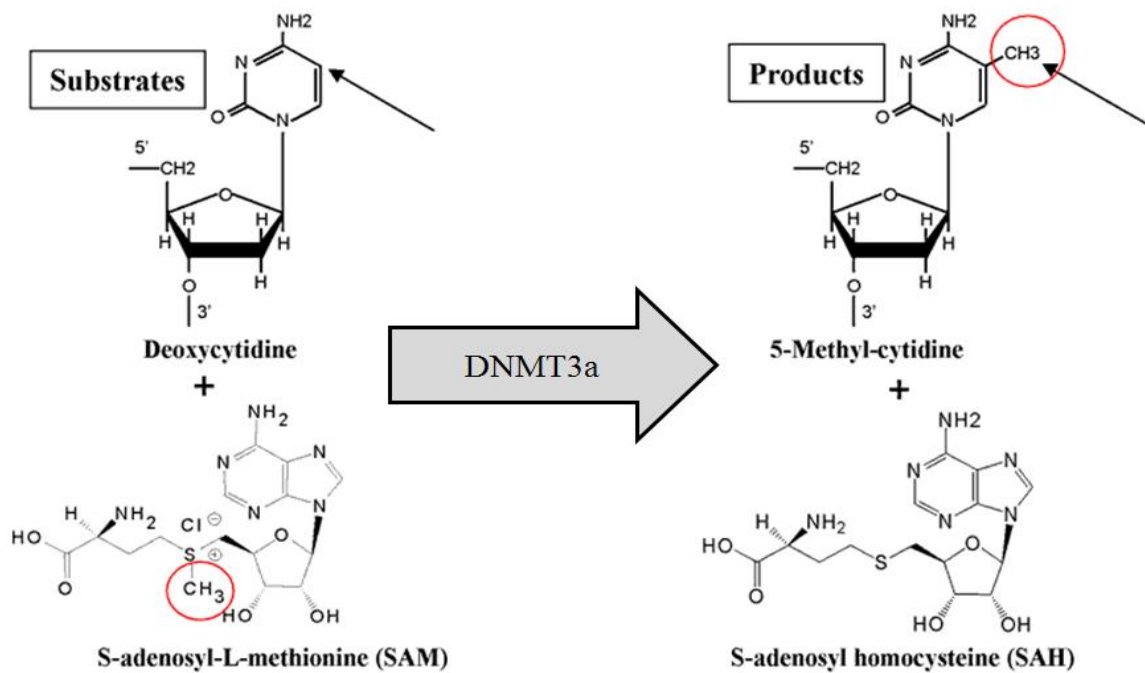
Figure 1.8: Structure of DNMT3a.

Figure 1.9: Methylation of cytosine catalyzed by DNMT3a (Brenner and Fuks, 2006).

1.5.4 *RAS* gene

The RAS proteins are a family of guanine nucleotide-binding proteins which has GTP/GDP binding and GTPase activity. They play important roles in regulatory processes including proliferation, differentiation as well as apoptosis (Stirewalt *et al.*, 2001).
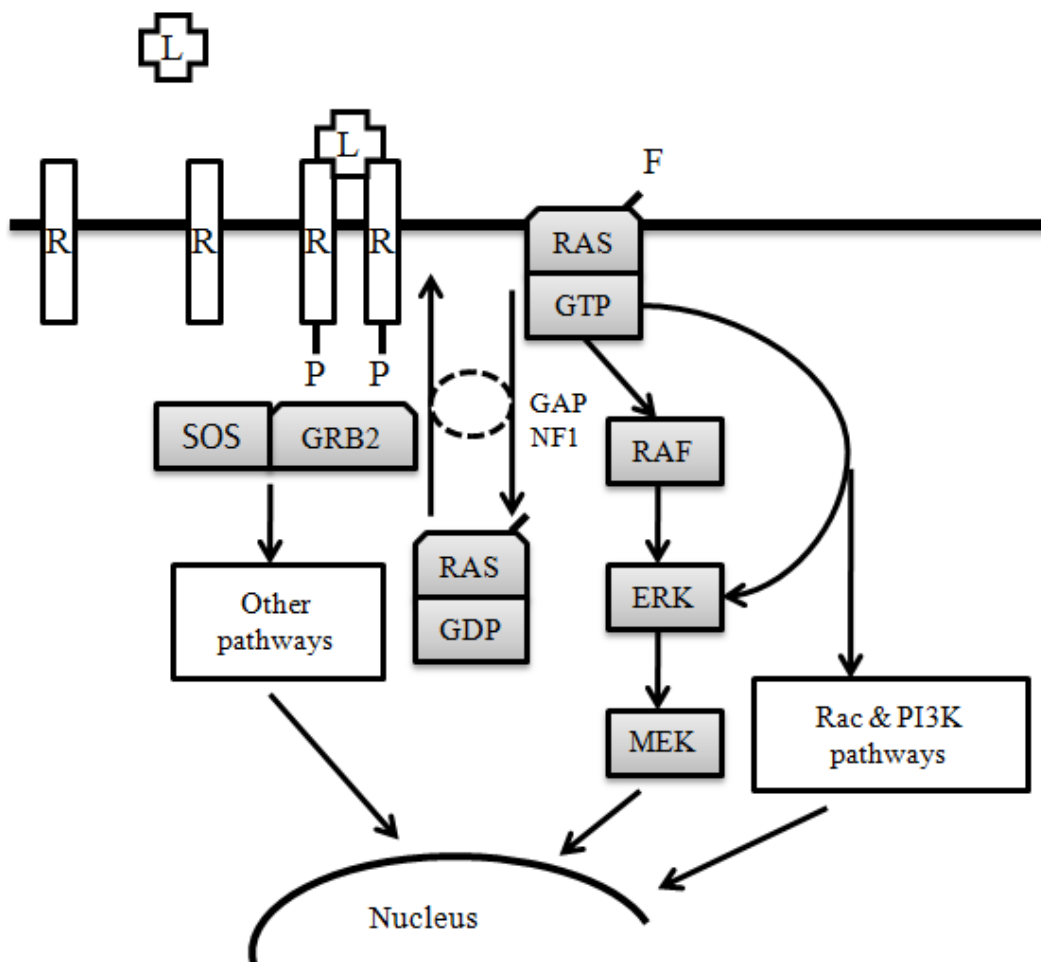

Figure 1.10: RAS signaling pathway.