DECIPHERING S-PHASE PROTEIN KINASE 2 (SKP2) MEDIATED REGULATION OF TELOMERASE REVERSE TRANSCRIPTASE (TERT) IN KASUMI-1, t (8;21) ACUTE MYELOID LEUKEMIA (AML) CELL LINE

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2023

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by

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

June 2023

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful.

All praises to Allah and His blessing for the completion of this thesis. I thank God for all the opportunities, trials and strength that have been showered on me to finish writing the thesis.

First and foremost, special appreciation goes to my supervisor Dr. Emmanuel Jairaj Moses, for his supervision, understanding, and constant support and most importantly, he has provided positive encouragement and a warm spirit to finish this thesis. His invaluable help of constructive comments and suggestions throughout the experimental and thesis works have contributed to the success of this research. It has been a great pleasure and honour to have him as my supervisor and not forgotten, my appreciation to my co-supervisor, Prof Dr. Narazah Mohd. Yusoff for her support.

I'm incredibly grateful to Adam Azlan and Raja Yaashini, who have always given me a hand in dealing with the practicalities of working in a research center. Let me use this opportunity to express my sincerest gratitude to you for taking time out of your busy schedule to listen, offer advice, and support me through this process. These brilliant friends and colleagues inspired me over years.

My deepest gratitude goes to all of my family members. It would not be possible to write this thesis without the support from them. I am thankful for their unconditional love, support, blessing, wishes and encouragement for the successful of this project.

Getting through my dissertation required more than academic support, and I have a friend to thank for listening to and, at times, having to tolerate me over the past two years. Thank you to Fatin Fazrina who have been unwavering in her personal and

professional support during my time at AMDI. To those who indirectly contributed to this research, your kindness means a lot to me. Thank you very much.

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LIST OF SYMBOLS

°C	Degree celcius
%	Percentage
CO_2	Carbon dioxide
ml	Milliliters
µl	Microliters
(v/v)	Volume/volume
w/v	weight per volume
nM	nanometer
mm	Millimetre
V	Volume
msec	millisecond
mg/ml	Milligrams per milliliter
g	Gram
µg	microgram
α	Alpha
β	Beta

LIST OF ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myelocytic leukemia
BM	bone marrow
CDK	cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
CDKN1B	cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$)
CEBPA	CCAAT enhancer-binding protein alpha
ChIp	Chromatin Immunoprecipitation Assay
CKI	cyclin-dependent kinases inhibitors
CLL	chronic lymphocytic leukemia
CML	chronic myelocytic leukemia
DBD	DNA binding domain
FAB	French-American-British
FOXO	forkhead box O
FOXO3	Transcription factor forkhead box O-3
HSC	hematopoietic stem cells
LRR	leucine-rich repeats
NES	nuclear export sequence
NLS	nuclear localization sequence
PKB	protein kinase B
PKC	protein kinase C
POT1	protection of telomeres
qPCR	Quantitative reverse transcription polymerase chain reactions
Rap1	TRF2- interacting protein 1

Rb	Retiblastoma
RQ-TRAP	real-time quantitative telomeric repeat amplification protocol
SKP2	S-phase kinase associated protein 2
TADL	Transcription activation domain
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TF	Transcriptions factors
TIN2	TRF1-interacting nuclear factor 2
TPP1	TIN2-interacting protein 1
TRAP	Telomerase Repeated Amplification Protocol
TRF1	Telomeric repeat-binding factors 1
TRF2	Telomeric repeat-binding factors 2
UPS	ubiquitin–proteasome system
WHO	World Health Organization

LIST OF APPENDICES

MERUNGKAI PENGAWALATURAN S-PHASE PROTEIN KINASE 2 (SKP2) OLEH TELOMERASE TRANSKRIPTASE BERBALIK (TERT) DALAM TURUNAN SEL LEUKEMIA MYELOID AKUT (AML) t (8;21), KASUMI-1

ABSTRAK

Leukemia mieloid akut (AML) adalah leukemia akut yang sering berlaku dalam kalangan golongan dewasa disebabkan oleh proliferasi mieloid yang tidak matang dan kegagalan sumsum tulang. S-Phase Protein Kinase 2 (SKP2), pengawal atur kitaran sel telah terbukti menyebabkan ekspresi yang berlebihan di dalam pesakit AML. SKP2 berpotensi dalam percambahan dan pembahagian sel. Kajian menunjukkan perencatan gen *SKP2* yang berpanjangan menyekat ekspresi enzim telomerase transkriptase berbalik (TERT) dalam sel AML t(8;21). Walau bagaimanapun, mekanisme molekul SKP2 mengawal ekspresi TERT masih tidak jelas. Oleh itu, tujuan kajian ini adalah untuk menyiasat mekanisme pengawalaturan TERT oleh SKP2 dalam AML. Perencatan gen *SKP2* menggunakan kaedah siRNA dilakukan di dalam sel Kasumi-1 dan THP-1. Dalam kajian ini, ekspresi TERT berkurang pada peringkat gen dan protein selepas perencatan gen SKP2 dalam sel AML bukan t(8;21). Berikutan ini, aktiviti telomerase juga berkurang dalam sel AML bukan t(8;21). Hasil kajian mendapati *c-Myc* dan *FOXO3* tidak memainkan peranan secara lansung dalam pengawalaturan *TERT* disebabkan oleh *SKP2* di dalam sel AML t(8;21) dan bukan t(8;21) pada peringkat gen. Namun, c-Myc menunjukkan corak yang berbeza pada peringkat protein di dalam sel AML bukan t (8; 21).Hasil kajian mendapati ekspresi c-Myc meningkat pada peringkat gen dan protein selepas

perencatan gen *AML1/ETO* dalam sel AML t(8;21). Disebabkan oleh peningkatan ekspresi c-Myc, imunopresipitasi kromatin (ChIP) telah dijalankan di mana c-Myc didapati mengikat kepada promoter TERT selepas perencatan gen *AML1/ETO*. Namun demikian, c-Myc gagal untuk mengaktifkan transkripsi *TERT* di dalam AML t(8;21) sel.Protein lain yang berkaitan dengan mekanisme TERT turut diuji termasuklah Rb, E2F1 dan CDKN1B. Hipofosforilasi Rb didapati meningkat dalam sel AML bukan t(8;21) selepas perencatan gen SKP2 namun tiada perbezaan yang ketara direkodkan pada ekspresi protein E2F1. Peningkatan CDKN1B adalah sangat berkaitan dengan penindasan *SKP2* dalam kajian ini.

DECIPHERING S-PHASE PROTEIN KINASE 2 (SKP2) MEDIATED REGULATION OF TELOMERASE REVERSE TRANSCRIPTASE (TERT) IN KASUMI 1, t (8;21) ACUTE MYELOID LEUKEMIA (AML) CELL LINE

ABSTRACT

Acute myeloid leukemia (AML) is the most common acute leukemia in adults and is characterized by immature myeloid cell proliferation. S-Phase Protein Kinase 2 (SKP2) is a cell cycle regulator and shown to be overexpressed in AML patients. SKP2 potentiates to cause cell proliferation and division. Studies shown prolonged *SKP2* knockdown suppress telomerase reverse transcriptase (TERT) expression in t(8;21) AML cells in vitro. Nevertheless, the molecular mechanism of TERT regulation by SKP2 remain unclear. Therefore, the aim of this study was to investigate the TERT mechanism by SKP2 in AML.SKP2 was suppressed in Kasumi-1 and THP-1 via siRNA mediated gene knockdown. In this study, TERT expression reduced at gene and protein level after *SKP2* suppression in non t(8;21) AML cells. Accordingly, telomerase activity was also reduced in non t(8;21) AML cells. Result obtained show that *c-Myc* and *FOXO3* did not play a direct role in *SKP2* mediated TERT regulation in t(8;21) and non-t(8;21) AML cells at gene level. However, different pattern in c-Myc protein expression was observed in non t(8;21) AML cells. Another observation were made in t(8;21) AML cells after AML1/ETO knockdown where c-Myc was up-regulated at gene and protein level. Due to increase in c-Myc expression levels, chromatin immunoprecipitation (ChIP) was carried out and increased observed binding of c-Myc to the TERT promoter was observed after *AML1/ETO* down-regulation. However, c-Myc binding to the TERT promoter failed to induce *TERT* transcription in t(8;21) AML cells.Other proteins related to TERT mechanism

observed were Rb, E2F1 and CDKN1B. Hypophosphorylation of Rb was observed up-regulated in non t(8;21) AML cells after *SKP2* knockdown yet no significant difference in E2F1 protein expression was observed. Accumulation of CDKN1B was markedly related with suppression of SKP2 in this studies.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Leukemia is a cancerous blood disease occurring in the bone marrow and is caused by the dysfunctional proliferation of abnormal white blood cells. Over time, the malignancy causes uncontrolled proliferation of hematopoietic stem cells in the bone marrow and suppresses the development of normal cells (Davis, Viera, and Mead, 2014). Based on GLOBOCAN 2020 estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer, a total of 474,519 cases and 311,594 deaths of leukemia cases were registered in 2020 globally (Sung *et al.*, 2021). In GLOBOCAN 2020, leukemia in Malaysia is ranked as the ninth most common cancer among the Malaysian population with 1,905 cases and 1,377 deaths in 2020 (Sung *et al.*, 2021)

There are four major subtypes of leukemia with distinct genetic and molecular abnormalities. Four major subclasses of leukemias include acute lymphoblastic leukemia (ALL), acute myelocytic leukemia (AML), chronic myelocytic leukemia (CML), and chronic lymphocytic leukemia (CLL) (Ashwini and Aswini, 2017; Koohi1 *et al.*, 2015).

Among these four types, acute myeloid leukemia (AML) is one of the most prevalent types of leukemia. AML is a heterogeneous disorder of the bone marrow and is characterized by the uncontrolled proliferation of immature myeloid blast cells (Connerty *et al.*, 2021; Gambacorta *et al.*, 2019; Pippa, Raffaella & Odero, 2020; Zhao *et al.*, 2018).

AML is commonly diagnosed in adults who are more than 60 years of age with a median age at diagnosis of 68 to 70 years (Huang *et al.*, 2022; Mendes and Fahrenkrog, 2019; Short *et al.*, 2018). AML remains life-threatening to most adult patients, contributing to about 80% of all AML cases and approximately 15%-20% of pediatric cases (De Kouchkovsky and Abdul-Hay, 2016; Garba and Usman, 2018; Lagunas-Rangel *et al.*, 2017; Loke *et al.*, 2017).

AML is largely characterized by chromosomal abnormalities. Several distinct chromosomal translocations, inversions, and gene mutations have been found in AML patients. Two general types of clonal chromosome abnormality including balanced abnormalities and unbalanced abnormalities. Certain balance cytogenetic abnormalities are an association of acute promyelocytic leukemia (APL) with t(15;17) and association of acute myelomonocytic leukemia with abnormal eosinophils with t(16;16) or inv(16) were reported. On the other hand, several unbalanced abnormalities, including deletion 7q , deletion 5q, trisomy 8 and monosomy 7 were also reported (De Kouchkovsky and Abdul-Hay, 2016; Pourrajab *et al.*, 2020; Yang *et al.*, 2017). Furthermore, gene mutation that can be found in AML including Fms-related tyrosine kinase 3 (FLT3), nucleophosmin (NPM1), proto oncogene c-KIT and DNA methyltransferase 3A (DNMT3A) (Di Nardo and Cortes, 2016; Wallwitz *et al.*, 2021; Zarka *et al.*, 2020). One of the most frequent AML with recurrent genetic abnormalities is t (8;21)(q22;q22), which results in the fusion of RUNX1 (AML1) and RUNX1T1 (ETO) (Al-Harbi *et al.*, 2020; Anetta Ptasinska *et al.*, 2019).

Several studies of human AML have suggested that various genes and proteins affect AML. These include S-phase kinase-associated protein 2 (SKP2) (Kulinski *et al.*, 2018), forkhead box O (FOXO) family (Huang and Tindall, 2007), c-Myc (Leon *et al.*,

2015), Telomerase reverse transcriptase (TERT) (Yik *et al.*, 2021), Retinoblastoma (Rb) (Mandigo *et al.*, 2021) and E2F transcription factor (Janostiak *et al.*, 2022).

S-phase kinase-associated protein 2 (SKP2) belongs to the F-box protein family playing a vital function in cell development and its overexpression leads to the oncogenesis of various solid tumors including haematological malignancies (Bretones *et al.*, 2011; Chan *et al.*, 2010; Hao and Huang, 2015). Studies show that SKP2 specifically recognizes cyclin-dependent kinase (CDK) inhibitor p27 ^{Kip1} in a phosphorylation-dependent manner to be polyubiquitinated for degradation (Bretones *et al.*, 2011; Chen *et al.*, 2008; Min *et al.*, 2004; Šimečková *et al.*, 2019; Wenwen Zhang *et al.*, 2016).On the other hand, studies found that p27 ^{Kip1} inhibits telomerase activity by down-regulating TERT expression translationally (Kanzawa *et al.*, 2003; Lee *et al.*, 2005). TERT activities have been reported overexpressed in cancer cells (Yuan *et al.*, 2019). TERT dysfunctional was found to be associated with uncontrolled cell growth leading to cancer initiation and disease progression including leukemogenesis (Guzman *et al.*, 2018; Yik *et al.*, 2021).

Many oncogenes and tumor suppressor genes have been implicated in regulating TERT expression including c-Myc, Rb, and E2F1. The oncogene c-Myc stimulates TERT expression by forming a complex with the oncogene Max (c-Myc/Max) followed by binding to the E-box region of the TERT loci (Beaulieu *et al.*, 2020; Hong *et al.*, 2022; Yik *et al.*, 2021). Recent studies have proved that c-Myc is active and overexpressed in AML which has been shown to induce leukemogenesis (Brondfield *et al.*, 2015; Mudgapalli *et al.*, 2019; Ohanian *et al.*, 2019). Overexpression of c-Myc has also been associated with the Rb/E2F1 axis of gene-mediated control (Mandigo *et al.*, 2021).In addition, the induction of c-Myc repressed FOXO3-mediated activation in the control of cell cycle progression and apoptosis (Chandramohan *et al.*, 2008; Ferber *et al.*, 2012; Yik *et al.*,

2020). FOXO3 and c-Myc are becoming more widely recognized as important regulatory genes in the initiation and progression of leukemia (Yik *et al.*, 2020).

1.2 Problem Statement

Overexpression of TERT expression and telomerase activity can be seen in hematological malignancies including leukemogenesis (Gessner *et al.*, 2010; Moses, 2015). The underlying mechanisms of TERT regulation, their role associated with AML, and the other potential regulation factors in TERT control are not fully elucidated.

Previous studies have shown that prolonged suppression of *SKP2* and *AML1/ETO* led to TERT down-regulation in AML t(8;21). Additionally, AML1/ETO was also found to regulate TERT via SKP2/ CDKN1B axis with Rb and E2F1 involvement in AML t(8;21) (Moses, 2015). Furthermore, studies showed that FOXO3 induced MYC-mediated gene suppression (Eijkelenboom *et al.*, 2013; Peck *et al.*, 2013). Nevertheless, the mechanism by which SKP2 regulates TERT is currently unknown. Therefore, investigating these mechanisms will be the focus of this study.

1.3 Research Objectives

1.1.1 General Objective

To elucidate the mechanism of TERT regulation by SKP2 in AML.

1.1.2 Specific Objective

1 To study the expression level of *SKP2*, TERT, *CDKN1B*, *c-Myc*, and *FOXO3* gene after down-regulation of *SKP2* in AML cells via qPCR

2 To study the expression level of SKP2, FOXO3, c-Myc, CDKN1B, TERT, Rb, and E2F1 protein after SKP2 silencing in AML cells using immunoblotting.

3 To investigate the expression level of c-Myc and FOXO3 gene and protein level after suppression of *AML1/ETO* in AML t(8;21) cells.

4 To elucidate the c-Myc binding at TERT promoter upon suppression of SKP2 and AML1/ETO in AML cells using chromatin immunoprecipitation.

5 To analyze telomerase activity after suppression with *SKP2* in AML cells using RQ-TRAP analysis.

1.4 Hypothesis

i. SKP2 regulates TERT expression and telomerase activity in AML.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancers in Malaysia

Cancer is a significant public health problem worldwide leading cause of death globally. This includes leukemia, a group of blood cancers that are the most common type in adults. GLOBOCAN 2020, part of IARC's Global Cancer Observatory providing global cancer estimates and statistics, shows a rise in total number of cancer cases (19.3 million) and cancer deaths in 2020 (10 million). Furthermore, GLOBOCAN database estimates that 1 in 5 people will develop cancer during their lifetime, and one in eight men as well as one in 11 women will eventually die from the disease.

In Malaysia, a total of 48639 new cancer cases and 29530 death cases were diagnosed and registered in 2020 to GLOBOCAN by National Cancer Patient Registry (NCPR). The NCPR, a hospital-based patient database, is in charge of gathering data on Malaysian patients who have been officially diagnosed with cancer and who have visited one of the participating facilities. Approximately 23, 052 were males and 25, 587 were females. Among the cancers reported in Table 2.1 and 2.2, leukemia is the ninth most common cancer occurring in the Malaysian population with 1, 905 new cases and 1, 481 deaths cases recorded. A total number of cases were registered indicating that 838 cases were among males and 829 were among females. The Age-standardised incidence rates (ASR) were used to standardize cancer data in the Malaysian population. Overall data

collected by GLOBOCAN 2020 reported that AML commonly occurred among the elderly compared to other types of leukemia.

Table 2.1 Ten most common new cancer cases recorded in Malaysia, 2020 (Adapted from GLOBOCAN 2020)

Cancer	Number	Rank	(%)	Cum. risk
Breast	8418	1	17.3	5.29
Lung	5139	2	10.6	1.87
Colon	3816	3	7.8	1.33
Rectum	2690	4	5.5	0.94
Nasopharynx	2222	5	4.6	0.69
Liver	2149	6	4.4	0.77
Prostate	2146	7	4.4	1.57
Non-Hodgkin lymphoma	1940	8	4.0	0.62
Leukemia	1905	9	3.9	0.51
Ovary	1836	10	3.8	1.16

Table 2.2 Ten most common death of cancer cases recorded in Malaysia, 2020 (Adapted from GLOBOCAN 2020)

Cancer	Number	Rank	(%)	Cum. risk
Lung	4509	1	15.3	1.64
Breast	3503	2	11.9	2.24
Liver	2050	3	6.9	0.73
Colon	2035	4	6.9	0.61
Leukemia	1377	5	5.0	0.73
Nasopharynx	1450	6	4.9	0.50
Rectum	1385	7	4.7	0.43
Ovary	1175	8	4.0	0.67
Stomach	1174	9	4.0	0.36
Non-Hodgkin lymphoma	1104	10	3.7	0.41

2.2 Acute Myeloid Leukemia

Leukemia blood cancer resulting in a neoplastic proliferation of hematopoietic or lymphoid cells, which causes excessive production of immature white blood cells. It is a heterogeneous cancer group that differs from other subtypes with different pathogenesis, prognosis, and responsiveness to treatment. Therefore, an accurate diagnosis is necessary for the identification of the disease. Mainly, leukemia is classified into two subgroups, acute and chronic leukemia. This is further sub-classified as acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL), and other less common types (Mudgapalli *et al.*, 2019).

AML is the most common acute type of leukemia in adults which can occur in any age group but is predominantly detected in older adults, with a median age of diagnosis at 68 years (Short *et al.*, 2018; Wallwitz *et al.*, 2021). AML is characterized by the abnormal proliferation of hematopoietic stem cells (HSC), resulting in the accumulation of immature malignant cells, fewer differentiated red blood cells, low platelet count, and over redundant white blood cells (Khwaja *et al.*, 2016). AML is a heterogeneous hematological cancer and has numerous genetic and epigenetic abnormalities. The abnormalities are characterized by clonal growth of immature myeloid stem cells with abnormal proliferation and differentiation, culminating in poor hematopoiesis and bone marrow failure (Wallwitz *et al.*, 2021).

Historically, AML has been classified according to morphology and immunophenotype. Advancements in sequencing technologies and other molecular biology methods led to proposals for updated genomic classifications (Hwang, 2020; Yu and Zheng, 2017).

2.3 Classification of Acute Myeloid Leukemia

Currently, two classification systems use laboratory hematology to categorize AML into subtypes. These are the French-American-British (FAB) classification and the newer World Health Organization (WHO) classification currently used globally. The traditional classification of AML used criteria proposed by the French-American-British Cooperative Group (FAB) solely evaluates the blast cells based on morphological and cytochemical criteria and categorizes AML into eight subtypes (M0, M1, M2, M3, M4, M5, M6, and M7) (Garba and Usman, 2018). AML classification system by FAB is presented in table 2.3.

Over recent decades, improvements in the therapeutic strategy throughout the induction and post-remission stages, as well as therapy stratification, have all contributed to develop improved methods in the treatment of AML. Specifically, morphology, cytochemistry, immunophenotype, genetics, and clinical aspects are all included in determining that all characteristics describe clinically important disease entities (Vardiman *et al.*, 2009; Yang *et al.*, 2017). In 2016, the World Health Organization (WHO) in conjunction with the Society for Hematopathology and the European Association of Hematopathology revised the classification. It was categorized into several major groups including AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related myeloid neoplasms, NOS, myeloid sarcoma, myeloid proliferations related to Down syndrome (Arber, 2016; Garba and Usman, 2018; Naeim *et al.*, 2018). The classification of AML and related neoplasms are described in table 2.4 below.

Table 2.3 AML classification according to French-American-British system. The table is taken from Garba and Usman,2018.

FAB subtype	Description	Description
M0	Undifferentiated	Myeloperoxidase negative; myeloid marker positive
M1	Myeloblastic without maturation	Some evidence of granulocytic differentiation
M2	Myeloblastic with maturation	Maturation at or beyond the promyelocytic stage of differentiation; can be divided into those with t (8,21) AML1-ETO fusion and those without
M3	Promyelocytic	APL; most cases have t (5,17) PML-RARα or another translocation involving RARα
M4	Myelomonocytic	
M4EO	Myelomonocytic with bone-marrow eosinophilia	Characterized by inversion of chromosome 16 involving CBFβ, which normally forms a heterodimer with AML1
M5	Monocytic	
M6	Erythroleukemia	
M7	Megakaryoblastic	GATA1 mutations in those associated with Down's syndrome

Table 2.4 AML classification according to WHO system.
The table is based on Arber *et al,*2019.

Acute Myeloid Leukemia Classification
Acute myeloid leukemia (AML) and related neoplasms
AML with recurrent genetic abnormalities
• AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1
• AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
• APL with PML-RARA
• AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A
• AML with t(6;9)(p23;q34.1);DEK-NUP214
• AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
• AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
• Provisional entity: AML with BCR-ABL1
• AML with mutated NPM1
• AML with biallelic mutations of CEBPA
• Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, NOS
• AML with minimal differentiation
• AML without maturation
• AML with maturation
• Acute myelomonocytic leukemia
• Acute monoblastic/monocytic leukemia
• Pure erythroid leukemia
• Acute megakaryoblastic leukemia
• Acute basophilic leukemia

2.4 Common cytogenetic abnormalities and genetic mutations in Acute Myeloid Leukemia

Numerous karyotype abnormalities have been discovered in AML. The prevalence of aberrant karyotypes in AML is between 55 to 60 percent in adults and 70 percent in pediatrics (Kumar, 2011; Nunes *et al.*, 2019; Shi *et al.*, 2017). To date, cytogenetic analysis has become an essential diagnostic tool in diagnosing, classifying, prognosis, managing AML, and recognizing specific subtypes (Gadhia *et al.*, 2016).

Chromosomal translocation in AML can be classified as balanced structural translocation, unbalanced karyotype, and complex karyotype (Arber *et al.*, 2016; Khan *et al.*, 2020; Pourrajab *et al.*, 2020). Balanced cytogenetic abnormalities, including reciprocal translocations and inversions generally result in a leukemia-specific fusion gene. Recurrent chromosomal rearrangements in AML with t(8;21)(q22;q22) /AML1/ETO, inv(16)(p13q22)/CBFβ-MYH11 and t(15;17)(q22;q12)/PML-RARα are frequently identified as a core binding factor AML and shown to have a more favorable prognosis (Foucar and Anastasi, 2015; Khamidullina *et al.*, 2021; Wilde *et al.*, 2019). Other balanced chromosomal abnormalities are AML with t(9;11)(p22;q23), AML (megakaryoblastic) with t(1;22)(p13;q13), AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A and AML with t(6;9)(p23;q34.1);DEK-NUP214 (Nunes *et al.*, 2019; Quessada *et al.*, 2021).

In contrast, unbalanced aberrations include trisomies, monosomies, deletions, and isochromosomes leading to the gain and/or loss of chromosomal material (Arber *et al.*, 2016; Pourrajab *et al.*, 2020; Shi *et al.*, 2017). The most common unbalanced

abnormalities include deletion 5q, trisomy 21, monosomy 7, trisomy 8, deletion 9q, deletion 7q, and trisomy 11 (Hemsing *et al.*, 2019; O'Hagan Henderson *et al.*, 2022; Pitel *et al.*, 2021; Shimada, 2021; Sitges *et al.*, 2020). Other unbalanced abnormalities include deletion 20q, deletion 12p, deletion 11q (Zahid *et al.*, 2017). Complex karyotypes found in AML patients with more than three unrelated cytogenetic abnormalities happen in a single karyotype (Daneshbod *et al.*, 2019).

On the other hand, mutations in the gene encoding nucleophosmin 1 (NPM1), Fms-like tyrosine kinase-3 (FLT3) and DNA methyltransferase 3A (DNMT3A) genes are the most common genetic mutated occur in AML (Dinardo and Cortes, 2016; Lagunas-Rangel *et al.*, 2017; Yu *et al.*, 2020). Mutation of CCAAT/enhancer-binding protein alpha (CEBPA) is also associated with AML (Shumilov *et al.*, 2018; Yang *et al.*, 2017).

All AML patients are classified into cytogenetic risk groups based on their pre-treatment cytogenetic, including the favorable, intermediate or unfavorable-risk group (Estey, 2018; Pelcovits and Niroula, 2020; Short *et al.*, 2018).

2.5 S-phase kinase-associated protein 2 (SKP2)

S-phase kinase-associated protein 2 (SKP2) is a proto-oncogene and cell cycle regulator. SKP2 protein is a critical component of the SKP1-cullin 1-F-box (SCF) E3 ligase complex, one of the primary groups of E3 ligases in which the F-box protein is in charge of substrate recognition. This complex is comprised of three core subunits ROC1/RBX1 (108 residues), CUL1 (776 residues) and SKP1 (163 residues). This E3 ligase family is part of the ubiquitin-proteasome system (UPS), which regulates numerous biological processes by managing the timely turnover of proteins (Wang *et al.*, 2012).

SKP2 is responsible in mediating ubiquitination and subsequent proteasomal degradation of cyclin-dependent kinases inhibitors (CKI) by the 26S proteasome. Examples of CKI are $p21^{Cip1}$,$p27^{Kip1,}$ $p57^{Kip2}$, p130, and FOXO1. By doing so, SKP2 elevates S phase entry to promote cell proliferation, migration, and metastasis.

2.5.1 SKP2 structure

SKP2 is an F-box protein with approximately 40 amino acid motifs. Structurally, SKP2 consists of a destruction box (D-box) domain, putative nuclear localization sequence (NLS), F-box motif, and leucine-rich repeats (LRR). The NLS component consists of 66–70 amino acids located near the N-terminal, followed by the F-box motif (100–105 amino acids) at the N-terminal of the protein. Another component is LRR, a protein-protein interaction module (200–300 amino acids) near the C- terminal responsible for its interaction with various substrates (Figure 2.1) (Kulinski *et al.*, 2018; Ting Wu *et al.*, 2021).

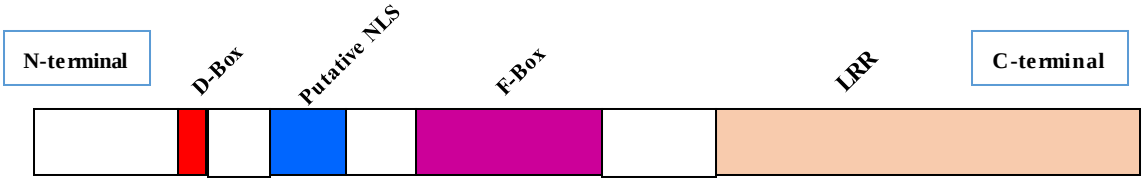

Figure 2.1 Functional structure and domains of the human SKP2 protein.

D-Box plays a significant role in SKP2 stability and the ten LRR on the F-box motif is responsible for its interaction with various substrates (Wang *et al.*, 2004). Each LRR form a concave surface made up of a β strand and an α helix. The α helix is arranged around as sickle-shaped lined with β strands (Hao *et al.*, 2005).

2.5.2 SKP2 dysregulation in cancer initiation and progression

S-phase kinase-associated protein 2 (SKP2) is an oncogenic protein frequently overexpressed in human cancers including hematological (Kulinski *et al.*, 2018; Ting Wu *et al.*, 2021), lymphoma (Kulinski *et al.*, 2018), breast cancer (Li *et al.*, 2019), prostate cancer (Šimečková *et al.*, 2019), pancreatic cancer (Kulinski *et al.*, 2018) and multiple myeloma (Yang *et al.*, 2019). SKP2 is involved in cell proliferation, apoptosis, migration, invasion, angiogenesis, and metastasis. SKP2 positively regulates G1-to-S transition during proliferation by induction of cell cycle regulators.

SKP2 recognizes and targets cyclin-dependent kinase inhibitor $p27^{Kip1}$ (CDKN1B) for ubiquitination in many human malignancies including AML (Hydbring *et al.*, 2017; Uras *et al.*, 2017). SKP2 overexpression is frequently associated with oncogenic and

correlated with suppression of CDKN1B protein expression in cancer including hematological malignancies (Bretones *et al.*, 2011; Chan *et al.*, 2010; Kulinski *et al.*, 2018). Indeed, SKP2 specifically targets CDKN1B as a critical regulator of T-cell proliferation and its accumulation is crucial for restraining the cell cycle.

In AML, the PIM family members, serine/threonine kinases, control the cytoplasmic abundance of CDKN1B. The CDKN1B is phosphorylated at Thr-187 and targeted by SKP2 for degradation through the 26S proteasome (Carrano and Pagano, 2001; Chen *et al.*, 2008; Hao and Huang, 2015). The CDKN1B provokes an erythroid differentiation response and suppresses myeloid differentiation (Uras *et al.*, 2017). Several studies in AML suggested that complete SKP2 knockdown prevents proteasomal degradation of CDKN1B (Khamidullina *et al.*, 2021; Min *et al.*, 2004; Zou and Lin, 2021).

SKP2 and CDKN1B influence the progression and prognosis of leukemia by controlling the proliferation, apoptosis, and differentiation of leukemic cells (Xiao *et al.*, 2009). As a result, levels of CDKN1B expression in cancer cells provide new insights into leukemia prognosis in tumor progression and therapeutics.

2.6 Cyclin-dependent kinase inhibitor $p27^{Kip1}$ (CDKN1B)

Cyclin-dependent kinase inhibitor $p27^{Kip1}$ (CDKN1B), a CDK inhibitor, is a cell cycle inhibitor and inducer of anti-apoptotic responses. CDKN1B is located on chromosome 12p13 and acts as a negative regulator of the cell cycle (Farsani *et al.*, 2018; Zou and Lin, 2021). The fact that CDKN1B has a wide range of functions, it could act either as an oncogene or tumor suppressor gene (Cusan *et al.*, 2018; Roy and Banerjee, 2015; Uras *et al.*, 2017).

CDKN1B can interact with a variety of proteins and is a target for a variety of signal transduction pathways, allowing it to perform various signaling pathways. CDK inhibitors inactivate cell cycle progression while the binding of cyclins activates CDKs.

Cyclin-dependent kinases (CDKs) represent a family of 20 serine/threonine kinases that coordinate the cell cycle progression. The G1-S phase transition of cell cycle depends upon the activity of specific CDK complexes. In a complex with cyclin, CDKs act as catalytic components to change cell division in response to external and intracellular inputs. CDKs are found overexpressed in hematological neoplasms, leading to enhanced cell proliferation and cancer progression (Amani *et al.*, 2021; Richter *et al.*, 2021). CDK and CDK inhibitors are key regulators in controlling critical cell cycle checkpoints. Their mutations have the potential to cause tumorigenesis.

CDK inhibitors are divided into two groups based on their structures and CDK targets. It is known as CDK interacting protein/kinase inhibitory protein (CIP/KIP) proteins which including $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, have an enormous influence on the functioning of cyclin A, cyclin D, and cyclin E dependent kinase complexes. The other group is classified as inhibitors of CDK4 (INK4) proteins which include $P16^{INK4a}$,

$p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, specifically target CDK4 and CDK6 (Amani *et al.*, 2021; Hume *et al.*, 2020; Quereda *et al.*, 2016; Richter *et al.*, 2021). The CIP/KIP and INK4 protein are involved in cell cycle, transcription, apoptosis, and cell migration (Amani *et al.*, 2021). CDKN1B, like other members of the CIP/KIP family, is an unstructured or disordered protein (Roy and Banerjee, 2015).

Notably, CDKN1B directly inhibits cell cycle progression by binding to cyclin E/CDK 2 and cyclin A/CDK2 kinases complexes, which drive the cells from the G1 to the S phase (Amani *et al.*, 2021; Min *et al.*, 2004; Roy and Banerjee, 2015; Zou and Lin, 2021). It has been established that Rb is involved in preventing S phase entry and cell proliferation. Remarkably, CDK4 and CDK6 kinases are widely overexpressed in leukemia and are involved in the G1 to S phase cell cycle transition via Rb-dependent activation of the E2F1 transcription factor (Spirin *et al.*, 2021). The cyclin D-CDK4/CDK6 and the cyclin E-CDK2 complexes can phosphorylate and partially suppress Rb while proportionally increasing E2F activity (Amani *et al.*, 2021; Haronikova *et al.*, 2021; Hume *et al.*, 2020). E2Fs, in turn, repress their transcriptional activity. In addition, CDKN1B able to reduce telomerase activity and TERT expression. CDKN1B suppresses telomerase activity by interfering with Myc binding sites in the TERT core promoter in cancer including malignant glioma cells.

2.7 c-Myc

c-Myc proto-oncogene is a critical member of the Myc family, which serves as a transcription factor of the basic helix-loop-helix–leucine (bHLHZ) zipper family of proteins that significantly maintains cell cycle progression, cell proliferation, differentiation, DNA repair, protein translation, immune response, and apoptosis. In addition, c-Myc is also involved in cellular transformation leading to tumor pathogenesis in various animal and human tumors. These are including ovarian cancer, liver cancer, squamous lung cancer, leukemia, esophageal cancer, and breast cancer (Beaulieu *et al.*, 2020; Duffy *et al.*, 2021; Melnik *et al.*, 2019; Mudgapalli *et al.*, 2019).

As both a transcriptional activator and a repressor, c-Myc is required in cell proliferation and to prevent differentiation (Ahmadi *et al.*, 2021; Sammak *et al.*, 2019). c-Myc generally recruits co-activator partners through its transcription activation domain (TAD) active expression. It can also repress the transcription of some target genes (Beaulieu *et al.*, 2020).

Furthermore, c-Myc signalling occurs in non-cancerous and cancerous cells. Growth factors stimulate c-Myc expression in non-cancerous cells by activating growth factors including p53, ARF, BIM, and PTEN that can cause cell growth arrest or death. In cancerous cells, on the other hand, the occurrence of abnormal enhancer activation, chromosomal translocation and deregulated signaling events leads to growth factor–independent MYC metabolic activities and proliferation (Stine *et al.*, 2015).

2.7.1 c-Myc structure

c-Myc gene is found on chromosome 8 (8q24), comprised of three exons and 439 amino acids (Gao *et al.*, 2021; Madden *et al.*, 2021; Sammak *et al.*, 2019). The structure of c-Myc consists of a central region, N-terminal transactivation domain (NTD) and a C-terminal domain (CTD) (Sammak *et al.*, 2019). The N-terminal region domain contains the transcription activation domain (TAD) and three highly conserved sequence elements, known as "MYC boxes" (MB0, MBI and MBII), involved in transcription regulation and protein stability (Beaulieu *et al.*, 2020; Madden *et al.*, 2021; Sammak *et al.*, 2019). The various MB domains bind to different proteins and consequently play varied roles in MYC function (Duffy *et al.*, 2021).

The proto-oncogene c-Myc is ubiquitous and is highly abundant in proliferating cells, whereby transcription is initiated by complex formation. The C-terminal domain with 360-439 amino acids comprises the bHLHZip motif domain responsible for DNA binding on promoters of specific target genes and heterodimerization with MAX. The crystal MYC-MAX heterodimer binds to DNA containing an E-box (5′-CACGTG-3′) motif in the promoter region of target genes (Madden *et al.*, 2021; Melnik *et al.*, 2019; Sammak *et al.*, 2019). Binding allows them to recruit multiple interacting proteins leading to activation of transcription and chromatin remodeling (Beaulieu *et al.*, 2020).

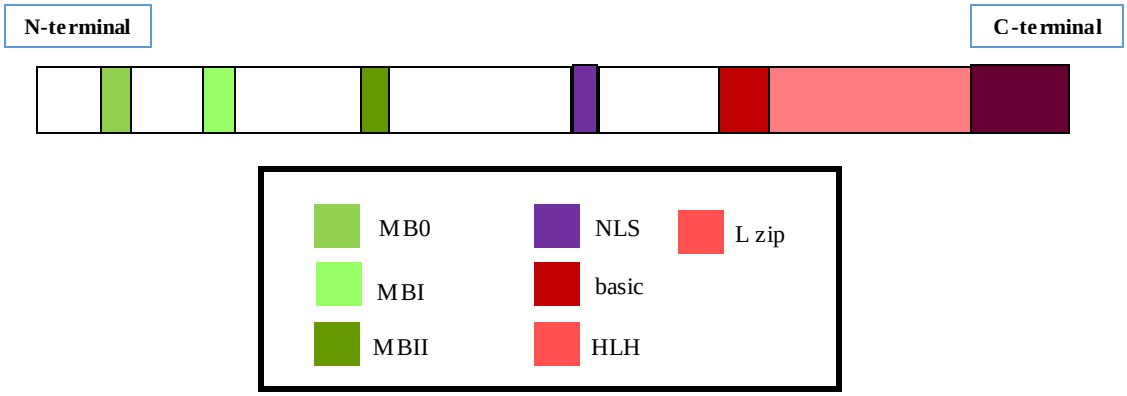

Figure 2.2 Domain structure of c-Myc

2.7.2 c-Myc dysregulation in cancer and acute myeloid leukemia

Myc is one of the most extensively researched cancer-causing genes, and correlate with tumor formation and aggressiveness. It is known as global transcription factor that contributes to various cellular processes including leukemogenesis (Ahmadi *et al.*, 2021; Mudgapalli *et al.*, 2019). Naturally, c-Myc expression is tightly regulated at multiple levels, including transcription (initiation and elongation), mRNA stability, translation, and post-translational modification protein stability (Ahmadi *et al.*, 2021; Madden *et al.*, 2021). In AML, c-Myc plays a significant role in AML maintenance by promoting RNA synthesis and translation, cell proliferation, leukemic stem cell self-renewal, and chemotherapy resistance (Ahmadi *et al.*, 2021).

The in vitro and in vivo studies suggested that c- Myc overexpression is present in most human hematopoietic malignancies and correlates with AML progression (Call *et al.*, 2020; Gao *et al.*, 2021; Mudgapalli *et al.*, 2019). c-Myc instability and overexpression can occur due to retroviral integration, chromosomal rearrangements, activation of super-enhancers of its gene, and mutations in signaling pathways. In particular, activating

mutations of the FLT3 receptor tyrosine kinase as well as the AML-associated fusion proteins AML1-ETO and PML/RARA could also lead to c-Myc overexpression (Leon *et al.*, 2015; Salvatori *et al.*, 2011). Overexpression of c-Myc in AML is linked to poor overall survival and often correlates with a worse prognosis (Leon *et al.*, 2015). In fact, c-Myc is a highly short-lived protein with a half-life of around 20–30 minutes.

In addition, study shows a complex and comprehensive inverse relationship between the functions of FOXO and c-Myc (Eijkelenboom *et al.*, 2013; Peck *et al.*, 2013). c-Myc and FOXO3 are becoming more widely recognized as essential regulatory genes in the initiation and progression of leukemia (Yik *et al.*, 2020).

The c-Myc also competes with FOXO3 for the binding of promoter regions and this interaction inhibits FOXO3-mediated activation of the p27 promoter (Ahmadi et al., 2021; Jiramongkol and Lam, 2020; Liu *et al.*, 2018). The inverse patterns of FOXO3 as a tumour suppressor, whereas c-Myc is a proto-oncogene, has shown in various human malignancies including liver cancer, leukemia and MDS (Yik *et al.*, 2020; Zhou *et al.*, 2022).

2.8 Transcription factor forkhead box O-3 (FOXO3)

Transcription factor forkhead box O-3 (FOXO3) is one of the members of forkhead box proteins of class O subgroup (FOXO). The FOXOs family is characterized by a highly conserved 100 amino acid DNA binding domain (Hedrick *et al.*, 2012). The mammalian system of FOXOs has four separate unlinked genes regulated by the phosphoinositol-3-kinase (PI3K)-PKB signaling pathway. FOXOs gene are known as FOXO1, FOXO3, FOXO4, and FOXO6 (Liu *et al.*, 2018; Wang *et al.*, 2014; Yik *et al.*,

2020). The FOXO1, FOXO3 and FOXO4 are widely expressed in nearly all tissues and the encoded proteins have many of the same post-translational regulatory mechanisms (Hedrick *et al.*, 2012; Jiramongkol and Lam, 2020). In particular, FOXO4 is abundantly expressed in muscle, kidney, and colorectal tissue, whereas FOXO6 is primarily expressed in the brain and liver (Bielka and Przezak, 2021).

2.8.1 FOXO3 structure

FOXO3 is located on chromosome 6q21 with 71 kDa in size and is made up of five domains including a forkhead winged helix-turn-helix DNA binding domain (DBD), two nuclear localization sequences (NLS), a nuclear export sequence (NES), and a C-terminal transactivation domain (TAD) (Jiramongkol and Lam, 2020; Liu *et al.*, 2018).

The DBD has 100 amino acids and is structured resembling butterfly wings with three α-helices and two loops-like-wing (Beretta *et al.*, 2019; Gurnari *et al.*, 2019). The helix-winged bind directly specific DNA consensus sequence (5′-GTAAA(C/T)A-3′) to mediate gene expression (Beretta *et al.*, 2019; Farhan *et al.*, 2020; Jiramongkol and Lam, 2020; Katarina *et al.*, 2019). Figure 2.3 demonstrated structure of FOXO3.

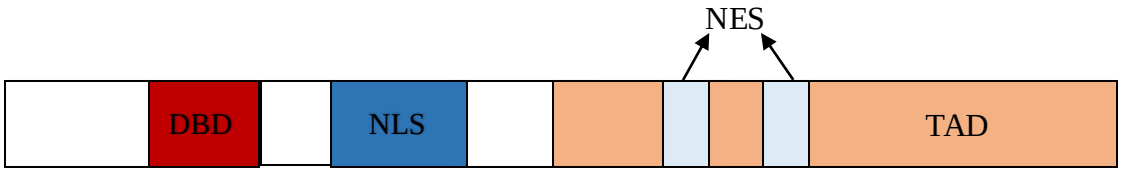

Figure 2.3 Domain structure of FOXO3 proteins

2.8.2 FOXO3 dysregulation in cancer and acute myeloid leukemia

FOXO3 is a transcription factor that has been considered tumor suppressor because of its established functions in differentiation, growth arrest, apoptosis, senescence and DNA damage repair (Hedrick *et al.*, 2012; Yang and Wu, 2019). Post-translational modification regulated FOXO3 activity. Multiple post-translational modifications (PTMs) include phosphorylation, acetylation, methylation, ubiquitination and glycosylation (Beretta *et al.*, 2019; Farhan *et al.*, 2017; Jiramongkol and Lam, 2020; Liu *et al.*, 2018; Wang *et al.*, 2017). Hence, dysregulation of FOXO3 expression has been implicated in cancer predominantly at the post-translational level.

FOXO3 is targeted for phosphorylation by numerous protein kinases such as Akt, ERK, SGK, IKKβ and IKBKE and leads to its translocation from nucleus to cytoplasm (Liu *et al.*, 2018; Wang *et al.*, 2017; Webb and Brunet, 2014; Yik *et al.*, 2020).

FOXO3 undergoes acetylation which impacts DNA binding and transcriptional activity. In contrast, in oxidative-stress-mediated FOXO deacetylation, FOXO3 help in DNA binding capability and transcriptional activity. Study observed that SIRT1 and SIRT2 mediate deacetylation of FOXO3 to promote FOXO3 ubiquitination and degradation in SKP2 dependent manner (Wang *et al.*, 2011).

The dysfunction of FOXO3 has been demonstrated to be involved in various types of cancer, including leukemogenesis (Gurnari *et al.*, 2019b; Menon *et al.*, 2020; Odemis *et al.*, 2021; Yang and Wu, 2019). FOXO3 gene expression was statistically lower in all AML patients than in the healthy control group (Odemis *et al.*, 2021). A recent study reported that down-regulation of FOXO3 expression accelerated tumor formation, leading to a speculation that FOXO3 acts as a tumor suppressor gene (Zhou *et al.*, 2019). The