DOWNREGULATION OF *SKP2* VIA siRNA MEDIATED GENE KNOCKDOWN AND ITS EFFECTS ON *FOXO3* AND *c-MYC* EXPRESSION IN AML t(8,21) CELLS

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DECLARATION

Here, I declare that this research has been sent to Universiti Sains Malaysia for degree of Master of Science. It is also not be send to any other Universities. With that, this research might be used for consultation and can be photocopied as reference.

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LIST OF ABBREVIATION

The following abbreviations have been used commonly throughout this thesis:

AKT	Protein kinase B
ALL	Acute Lymphoid Leukemia
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
cdk	Cyclin dependent kinase
cHL	Classical Hodgkin Lymphoma
c-MYC	Cellular MYC
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E1	Ubiquitin-activating enzyme
E2/UBC	Ubiquitin-conjugating enzyme
E2F1	E2F transcription factor 1
ER	Estrogen receptors
EZH2	Enhancer of zeste homolog 2
FAB	French American British
FOXO	Forkhead Box O
G1	Gap 1
G2	Gap 2
HBXIP	Hepatitis B virus X-interacting
IGF-I	Insulin-like growth factor I

IKK	IκB kinase
MLL	Myeloid/Lymphoid or mixed-lineage leukemia
M phase	Mitosis Phase
mRNA	Messenger RNA
p21	Cyclin-dependent kinase inhibitor 1
p57	Cyclin-dependent kinase inhibitor 1C
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcription
Ser	Serine
SKP2	S-phase-kinase-associated protein 2
S phase	Synthesis phase
siRNA	Small interference RNA
SIRT1	Sirtuin 1
SIRT2	Sirtuin 2
STAT3	Signal transducer and activator of transcription 3
TERT	Telomerase reverse transcriptase
Thr	Threonine
WHO	World Health Organization

LIST OF SYMBOL

°C	Degree celcius
%	Percentage
CO_2	Carbon dioxide
ml	Milliliters
nm	Nanometer
ng	Nanogram
µl	Microliters
RPM	Revolutions per minute
α	Alpha
β	Beta
g	Gravitational force
w/v	Weight over volume
msec	Millisecond

ABSTRAK

Leukemia Myeloid Akut (AML) adalah gangguan sel tunjang hematopoietik yang berlaku disebabkan ketidakupayaan sel-sel untuk berbeza dan berkembang secara normal. Fasa-S kinase bersekutu protein 2 (SKP2) ialah protein yang amat bertanggungjawab dalam pengiktirafan dan penentuan degradasi protein sasaran dan dikenali sebagai onkogen. Hal ini menyebabkan protein ini seringkali menjadi sasaran terapeutik dalam pelbagai kanser. Protein FOXO3a telah dikenal pasti dalam AML dan bertindak sebagai gen penindas tumor. c-MYC memainkan peranan penting sebagai proto-onkogen dan dikenal pasti untuk mendorong AML. Pelbagai eksperimen telah menunjukkan hubungan antara SKP2 dan FOXO3a serta SKP2 dan c-MYC. Walau bagaimanapun, masih tiada maklumat yang boleh didapati mengenai hubungan antara SKP2, FOXO3a dan gen c-MYC dalam translokasi t (8; 21) AML. Dalam kajian ini, hubungan antara tahap ungkapan *SKP2, FOXO3*a dan gen *c-MYC* telah dikenal pasti. Sel kasumi-1 telah digunakan sebagai model AML. Perencatan gen *SKP2* melalui gen pengantara siRNA telah dijalankan untuk mengenalpasti kesannya ke atas gen *FOXO3a* dan *c-MYC* pada tiga titik masa yang berbeza iaitu 24, 48 dan 72 jam selepas perencatan gen *SKP2*. siRNA telah diperkenalkan ke dalam Kasumi-1 melalui electroporasi dan tahap ungkapan setiap gen telah dikenal pasti menggunakan real-time PCR. Hasilnya menunjukkan penjatuhan tahap *SKP2* pada semua titik masa dengan lebih daripada 50% ($p < 0.05$). Walau bagaimanapun tidak ada perbezaan yang signifikan antara kedua-dua tahap ungkapan *FOXO3a* dan *c-MYC* dengan atau tanpa perencatan gen *SKP2*. Namun, ungkapan *FOXO3a* gen telah dikenal pasti meningkat secara sedikit dari 24 jam kepada 72 jam selepas perencatan gen *SKP2*. Dalam usaha untuk

mendapatkan nilai yang signifikan, pemanjangan perencatan gen *SKP2* adalah penting.

ABSTRACT

Acute Myeloid Leukemia (AML) is a hematopoitic stem cell disorder causes by inability of the cells to differentiate and proliferate in normal manner. S-phase kinase–associated protein 2 (SKP2) protein is highly responsible in recognizing and determining the degradation of target protein and is well known for its oncogene property as well as for its therapeutic target in various cancers. FOXO3a protein was identified to present in AML and acts as tumor suppressor gene. *c-MYC* plays a key role as proto-oncogene and is recognized to induce AML. Numerous experiments showed correlation between *SKP2* and *FOXO3a* as well *SKP2* and *c-MYC*. However, there is no information available yet on relationship between *SKP2*, *FOXO3a* and *c-MYC* gene in AML with t(8;21) translocation. In this study, the correlation between expression level of *SKP2*, *FOXO3a* and *c-MYC* gene were identified. Kasumi-1 cell line was used as a model of AML with t(8;21). *SKP2* downregulation via siRNA mediated gene knockdown was carried out to identify its effect on *FOXO3a* and *c-MYC* gene at three different time points which were 24, 48 and 72 hours. siRNAs were introduced into Kasumi-1 cell line via electroporation and the expression level of each gene were identified using real-time PCR. The result showed a successful knockdown of *SKP2* at all different time points with more than 50% ($p<0.05$). However there was no significance difference between both *FOXO3a* and *c-MYC* expression levels with or without *SKP2* knockdown. Yet, the expression of *FOXO3a* gene was identified to increase slightly from 24 hours to 72 hours after *SKP2* knockdown. In order to obtain significant value, prolonged gene knockdown is essential.

CHAPTER 1: Introduction

1.1 Research Background

Acute Myeloid Leukemia (AML) is a hematopoietic stem cell disorder caused by inability of the cells to differentiate and proliferate in a normal manner, thus contributing to accumulation of myeloblasts,(Stone *et al*., 2004, Estey and Döhner, 2006). In AML, t (8;21)(q22;q22) is the most frequently observed translocation which leads to the fusion of *RUNX1* (AML1) with *RUNX1T1* (ETO).

Based on an experiment conducted in AML cells, there are various genes and proteins that affect AML such as S-phase-kinase-associated protein 2 (SKP2), MYC, CDKN1B (p27Kip1) and Forkhead Box O (FOXO3) proteins (Min *et al*., 2004, Chapuis *et al*., 2010, Brondfield *et al*., 2015, Ewerth *et al*., 2016).

SKP2 plays its main function in recognizing and determining the degradation of a particular target protein. This includes cell cycle regulatory proteins such as cyclin-dependent kinase inhibitor 1 (p21) , cyclin-dependent kinase inhibitor 1C (p57), E2F-1 and c-MYC (Wang *et al*., 2012b, Chan *et al*., 2014) . Overexpression of *SKP2* is abundant in various cancers such as breast, ovarian and gastric cancer (Lu *et al*., 2012, Wei *et al*., 2013b, Xu *et al*., 2013). This gene identified to plays role in proliferation, inhibit cell apoptosis, enhance cell invasion as well promote cell migration (Yin *et al*., 2016). Therefore, *SKP2* was suggested to act as proto-oncogene and downregulation of this gene may be a therapeutic target (Wang *et al*., 2012d). Similarly, overexpression of *SKP2* and its role as an independent prognostic factor in AML has been identified (Min *et al*., 2004). However, not many studies have been conducted in identifying the relationship between *SKP2* and AML.

In AML, *FOXO3* gene exhibits (6;11)(q21;q23) chromosomal translocation (Kornblau *et al*., 2010). Nuclear localization of FOXO3 is essential to induce apoptosis and suppress proliferation in AML (Chapuis *et al*., 2010). Various studies have been reported about the relationship between *SKP2* and *FOXO3*. For an example, tissues collected from ovarian cancer patient shows high expression of *SKP2* correlated with low expression of *FOXO3a* and vice versa (Lu *et al*., 2012). SKP2 were identified to play a key role in FOXO3 degradation (Wang *et al*., 2012a), thus ceasing the function of FOXO. However, the relationship between SKP2 and FOXO3 in AML has not been established to date.

c-MYC is recognized to act as a proto-oncogene as overexpression of this particular gene is crucial to induce tumorigenesis in association with other tumor inducing event. SKP2 were reported to enhance the transcriptional activity of c-MYC (Yada *et al*., 2004). But some studies also emphasizes that SKP2 mediated degradation takes places for c-MYC (Von Der Lehr *et al*., 2003). Research also demonstrated that *MYC* gene bind both *in-vivo* and *in-vitro* to SKP2 F-box protein (Von Der Lehr *et al*., 2003) contributing to the statement that these genes are linked.

Although the expression level of *SKP2*, *MYC*, and *FOXO3* affect AML, the exact mechanism behind this event in AML with t (8;21) translocation remains unclear.

Therefore identifying the relationship between *SKP2*, *FOXO3a* and c-*MYC* gene in AML is an interesting research question. In this experiment, Kasumi-1 cells were cultured as a model for AML with t (8;21) translocation and siRNA introduced via electroporation in order to down-regulate *SKP2* gene. The effect of *SKP2*

downregulation on both FOXO3a and c-MYC were determined by identifying the expression level of *FOXO3* and *MYC* gene using real-time PCR. SPSS software was used for statistical analysis and the significant values were obtained by two tailed paired T test. The main and specific objectives of this study include:

1.2 Main objective

To study the relationship between *SKP2*, *FOXO3*, and *c-MYC* in leukemia.

1.3 Specific objective

1. To downregulate *SKP2* gene expression levels in Kasumi-1 cells via siRNA-mediated gene knockdown
2. To analyze the expression level of *FOXO3* and *c-MYC* genes after suppression of *SKP2*

1.4 Hypothesis

1. There is a correlation between *SKP2*, *FOXO3a* and *c-MYC* gene in Acute Myeloid Leukemia.

CHAPTER 2: Literature Review

2.1 Acute Myeloid Leukemia

2.1.1 Types of Acute Myeloid Leukemia

Acute Myeloid Leukemia (AML) is a hematopoietic stem cell disorder caused by the inability of cells to differentiate and proliferate in the normal manner (Döhner *et al*., 2015). Due to abnormal differentiation of myeloid cells, abundance of immature malignant cells accumulate in bone marrow and thus fewer differentiated erythrocytes, thrombocytes, monocytes, and granulocytes include basophils, eosinophils and neutrophils (Johansson and Harrison, 2015). In the majority of AML cases, clonal chromosomal abnormalities were identified via chromosome banding analysis. Risk factors for AML include age, exposure to ionizing radiation, petrochemicals, benzene and pesticides (O'Donnell *et al*., 2012).

In adults, out of all acute leukemia, 90% was identified as AML in the United States year 2015. The similar study indicates risk factor of AML were higher from age range 30 to 65 with an approximate increase of 10 fold (Smith and Stein, 2016). Malaysian National Cancer Registry Report (MNCR) 2007-2011 indicates that out of all cancer cases which were diagnosed in Malaysia from 2007 to 2011, the percentage of leukemia cases were accounted for 4.4% with 5.4% in male and 3.6% in female as well ranked as sixth most common cancer among Malaysia population.

To understand in detail on the disease biology, prognosis and for therapeutic importance, effort on the classification of AML have been made. French American British (FAB) initially have a key role in the classification of AML by including morphological differences, cytochemical and immunophenotypic analysis as

classification factor. The World Health Organization (WHO) classification in AML is more comprehensive as compared to FAB classification as WHO includes morphological, immunophenotypic, cytochemical, genetics and clinical features as AML classification factors (Johansson and Harrison, 2015).

Table 2.1 shows Acute Myeloid Leukemia and related neoplasms classification by (WHO). Adapted from (Arber *et al*., 2016)

1. **Acute Myeloid Leukemia with recurrent genetic abnormalities**
    - AML with t(8;21)(q22;q22)
    - AML with with t(6;9)(p23;q34.1)
    - AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)
    - APL with t(15;17)(q22;q21)
    - AML with t(9;11)(p21.3;q23.3);
    - AML (megakaryoblastic) with t(1;22)(p13.3;q13.3)
    - AML with inv(3)(q21.3q;q26.2) or t(3;3)(q21.3;q26.2)
    - AML with mutated *NPM1*
    - AML with biallelic mutations of *CEBPA*
2. **Acute Myeloid Leukemia with myelodysplasia-related changes**
3. **Therapy-related myeloid neoplasms**
4. **Acute Myeloid Leukemia, *not otherwise specified***
    - AML with minimal differentiation
    - AML without maturation
    - Acute myelomonocytic leukemia
    - AML with maturation
    - Acute monobasic/monocytic leukemia
    - Pure erythroid leukemia
    - Acute megakaryoblastic leukemia
    - Acute basophilic leukemia
    - Acute panmyelosis with myelofibrosis

2.1.2 AML with t (8;21) translocation and Kasumi-1 cell line as a model for Acute Myeloid Leukemia

In AML, t (8;21) is the most frequent translocation with 12-15% of general AML and up to 40% out of all AML subtype M2 based on FAB classification (Peterson and Zhang, 2004). This translocation leads to the fusion of *RUNX1* gene also known as AML1 present on chromosome 21 with *RUNX1T1* also known as ETO on chromosome 8 (Figure 2.1). This translocation is also reported to present in lymphoid and biphenotypic acute leukemia (Forster *et al*., 2016). AML1 complex plays a role in regulating a large number of genes that are responsible in hematopoiesis. The fusion of *RUNX1- RUNX1T1* also known as *AML1-ETO* leads to repression of *RUNX1* gene thus blocking the differentiation of myeloid lineage (Kurokawa and Hirai, 2003, Johansson and Harrison, 2015). This types of translocation in leukemic cells were identified with a high frequency of Auer rod and granulocytic line maturation (Berger *et al*., 1982, Miyoshi *et al*., 1993). Translocation of t(8;21) alone is not solely responsible for inducing AML, instead, an additional mutation such as co-operating point mutation is required for leukemogenesis. The exact mechanism in the development of particular mutation is not well defined, however, *AML-ETO* were reported to play role in promoting co-operating mutation (Kurokawa and Hirai, 2003, Forster *et al*., 2016).

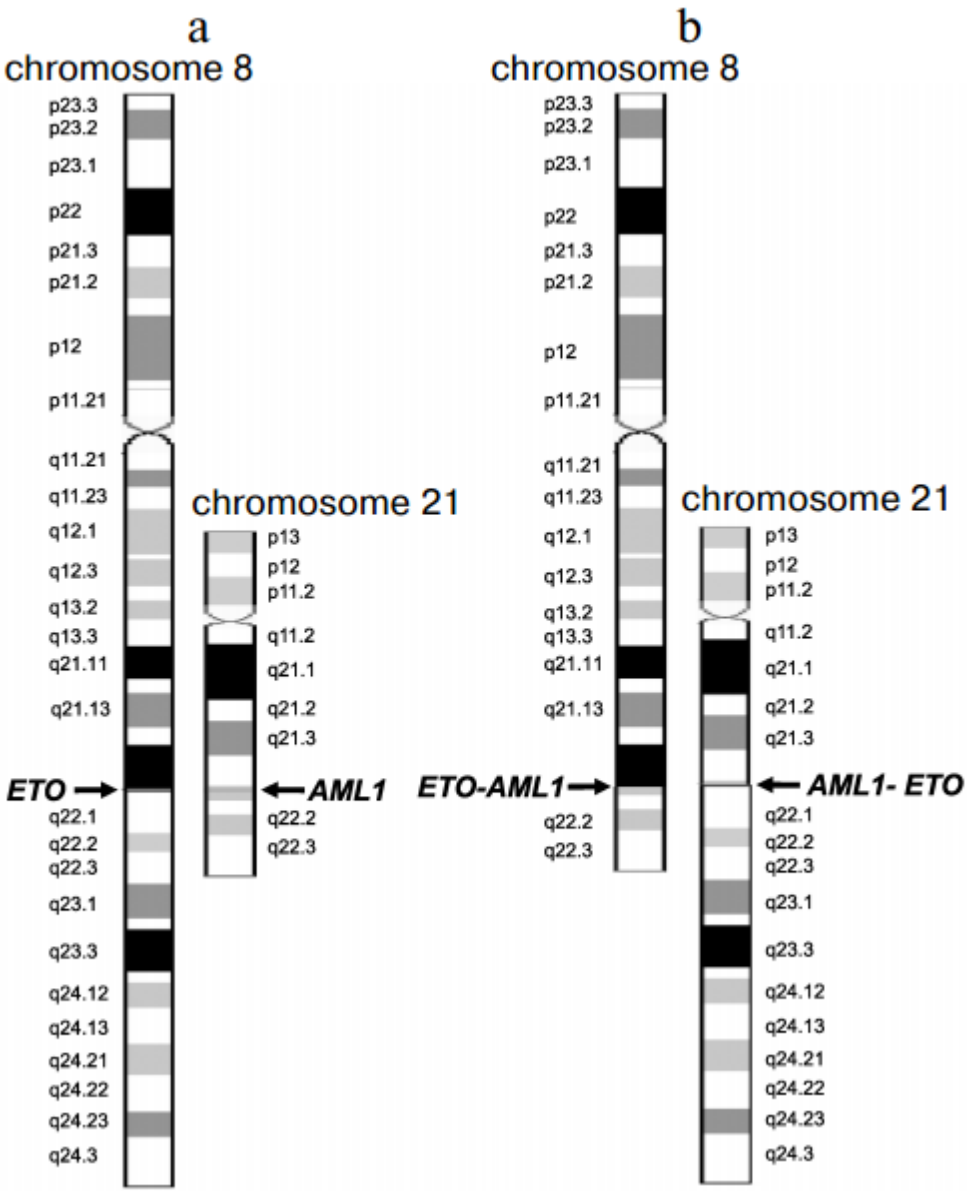

Figure 2.1: Schemes shows before (a) and after (b) translocation of t (8,21) (q22; q22) . Arrows indicate breakpoints. Usually, *AML1-ETO* mRNA is assayed only in single out of the two possible chimeric transcript type. Adapted from (Chetverina and Chetverin, 2010).

Kasumi-1 cell line is a novel t (8;21) (q22;q22) chromosomal translocation leukemic cell line (Asou *et al*., 1991). Peripheral blood of a 7 years old Japanese child with AML after bone marrow transplantation was used to design Kasumi-1 cell line (Asou *et al*., 1991). The second class of mutation in Kasumi-1 was identified with a c-kit gene mutation in the tyrosine kinase domain. Development of Kasumi-1 cell line has contributed to the various finding of t(8;21) chromosomal translocation including morphologic, molecular level, immunophenotypic and the effect of the fusion as well useful in identifying its therapeutic target (Beghini *et al*., 2002, Larizza *et al*., 2005). For an example, studies on Kasumi-1 cell have shown that with *AML1-ETO* gene expression drastically reduced, expression level of *LAT1* and *RUNX3* genes increased accordingly (Sawney *et al*., 2015).

2.2 SKP2

2.2.1 SKP2 and its role

SKP2 (S-phase kinase–associated protein 2) is a component and substrate-recognition subunit of SCF E3 ubiquitin ligase complex and this SKP2 is a member of second class F-box protein (Zhang and Wang, 2006, Chan *et al*., 2010). Other components of SCF complex include Skp1, Cullin1 and Rbx (Figure 2.2) which plays a role in proteasomal degradation. Skp2 consist of the N-terminal domain, F-box domain through which SKP2 interacts with SKP1, and C-terminal leucine-rich (Zhang and Wang, 2006, Chan *et al*., 2010, Wang *et al*., 2012c, Chan *et al*., 2014).

For SKP2 mediated ubiquitination of a particular substrate, ubiquitin-activating enzyme (E1) first attaches with ubiquitin and the ubiquitin is then transferred to an ubiquitin-conjugating enzyme (UBC/E2). The interaction of E2 with E3 leads to the transportation of ubiquitin into the substrate which is recognized by SKP2 and this process is repeated in order to add more ubiquitin to the substrate (Figure 2.2), thus leading to the formation of a polyubiquitin chain which is then degraded by 26S proteasome (Chan *et al*., 2010, Wang *et al*., 2012c, Chan *et al*., 2014).

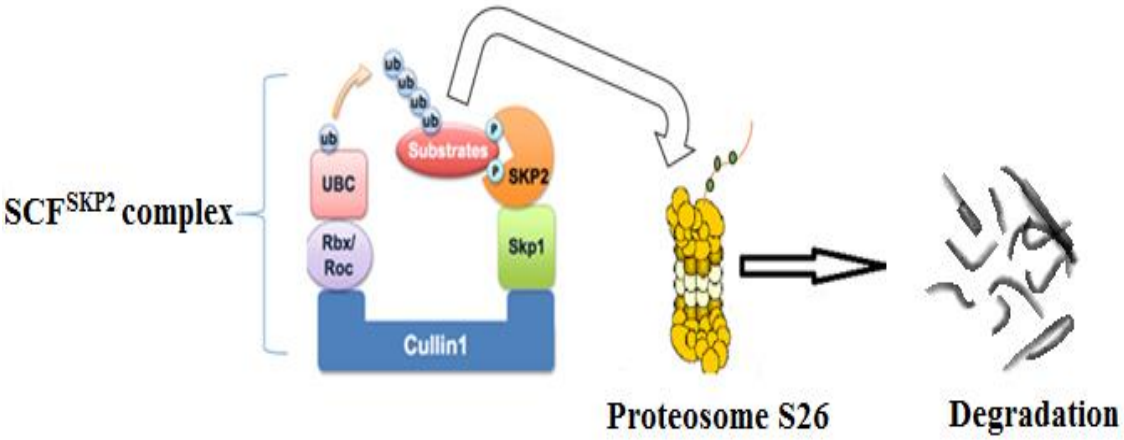

Figure 2.2: The SCF^{skp2} E3 ubiquitin ligase complex. In this complex, SKP2 is the F-box protein component and function on recognizing the substrate protein. UBC/E2 in association with E3 transfer ubiquitin to the substrates protein which is recognized by SKP2. Then the substrates will be degraded by proteasome S26. Adapted from (Kukan, 2004, Wang *et al*., 2012c)

SKP2 with the mechanism mentioned above plays its main function in recognizing and determining the degradation of particular or target protein which includes cell cycle regulatory proteins (Chan *et al*., 2014). Example of proteins targeted by SKP2 are shown in table 2.2. This suggest that SKP2 affects cell cycle activity and also acts as an oncogene (Chan *et al*., 2010, Chan *et al*., 2012).

Table 2.2: SKP2 target protein respective to its function (Wang *et al*., 2012b).

Function of proteins	**SKP2 target proteins**
Cell cycle regulators	Cyclin-dependent kinase inhibitor 1, p21 Cyclin-dependent kinase inhibitor 1C, p57 E2F-1 ETS-related transcription factor Elf-4, MEF Retinoblastoma-like protein 2, p130 Tob1 Cyclin D Cyclin E 1 SMAD family member 4, Smad4 Myc B-Myb Ras association domain-containing protein 1, RASSF1A
Apoptosis regulators	Myc Forkhead box O, FOXO
Diverse functions for degradation	Origin recognition protein, Orc1p Chromatin licensing and DNA replication factor 1,Cdt1 Recombination activating gene 2, Rag-2 BRCA2 Cyclin-dependent kinase 9, Cdk9 Dual specificity phosphatase 1, MKP1 Ubiquitin specific peptidase 18, UBP43

Besides all the proteins listed in table 2.2, SKP2 is also known for its role in the degradation of p27. p27 acts as a tumor suppressor and negatively regulates the G1 cell cycle (Wang *et al*., 2012d). Thus, it is evident that SKP2 plays a vital role in

cycle progression, apoptosis and a wide range of other biological processes (Wang *et al*., 2012b).

SKP2 cDNA was transfected in MCF-7 and MDA-MB-231, breast cancer cell line in order to overexpress *SKP2*. The result showed that the upregulation of *SKP2* levels lead to cell proliferation, inhibition of cell apoptosis, enhancement of cell invasion and promotion of cell migration. Overexpression of *SKP2* also affects the activity of rottlerin, a phenolic compound which was known to induce apoptosis, inhibit cell proliferation and inhibit cell migration (Yin *et al*., 2016). Research on breast cancer cell line which was transfected with Hepatitis B virus X-interacting (MCF-7-HBXIP) suggested that SKP2 plays role in HBXIP mediated breast cancer proliferation (Xu *et al*., 2013). In Hela cell, *SKP2* was downregulated and the result shows an increase in cell death (Wang *et al*., 2012a).

SKP2 was also identified abundantly in gastric cancer cell line such as MGC803, SGC7901, and NCI-N87. Downregulation of *SKP2* was identified to suppress cell proliferation, inhibit cell migration and invasion, reduce tumorigenesis and tumor metastasis, and induce cell apoptosis. *In vivo* studies in mice showed that *SKP2* downregulation inhibits tumor growth (Wei *et al*., 2013a). In prostate cancer, *SKP2* was suggested to play a role as a proto-oncogene and a potential therapeutic target (Wang *et al*., 2012d).

2.2.2 SKP2: Expression and Regulation

Various compounds are responsible for suppressing or upregulating *SKP2* expression level. One of the main protein that is responsible in *SKP2* downregulation is *FOXO3* and was explained in next section.

In MCF-7 breast cancer cell line, Hepatitis B virus X-interacting protein (HBXIP) was identified to upregulate *SKP2* expression levels and HBXIP binds with E2F transcription factor 1 (E2F1) to activate SKP2 promoter activity (Xu *et al*., 2013).

MCF-7 and MDA-MB-231 cells which were treated with rottlerin showed lower expression level of *SKP2* when quantified with real time PCR (Yin *et al*., 2016). Besides, downregulation of signal transducer and activator of transcription 3 (STAT3) in human gastric cancer cell line, SGC-7901 showed suppression of *SKP2* (Wei *et al*., 2013b).

Bortezomib which plays an essential role as proteasome inhibitor identified induces *SKP2* suppression (Li *et al*., 2012, Tian *et al*., 2013). In chronic myeloid leukemia cell line, AR230 and K562, bortezomib induces apoptosis by downregulating *SKP2* expression level and causes accumulation of p27Kip1 (Iskandarani *et al*., 2016). A similar result was also identified in colorectal cancer cell line and epithelial ovarian cancer (Uddin *et al*., 2008, Uddin *et al*., 2009).

Compound 25 also known as SZL-P1-41 was identified via structure-based high-throughput virtual screening technologies for its property as SKP2 inhibitor (Chan *et al*., 2013, Yin and Wang, 2015). Compound 25 inhibit SKP2-SKP1 interaction, thus suppressing SKP2 SCF E3 ligase activity via binding of a particular

compound with SKP2. Compound 25 basically was identified not to downregulate SKP2 mRNA levels, instead, suppression of SKP2 at protein levels. By inhibiting SKP2 protein level, compound 25 stabilized expression levels of p21 and p27, induced cell-cycle arrest in G2/M phase and enhanced p53 mediated senescent response (Chan *et al*., 2013, Yin and Wang, 2015).

In pancreatic cancer cells, Arsenic trioxide was reported to suppress SKP2 mRNA levels besides stabilizing FOXO1 and p53. Similarly, Arsenic trioxide was also identified to downregulate SKP2 in mesothelioma cancer as well with c-myc. Androgen suppresses the activity of Cdk2 in phosphorylating SKP2 (Kokontis *et al*., 2014). Another experiment showed that in prostate cancer cell, SKP2 suppression by androgen-mediated p107 dependent and independent pathways(Jiang *et al*., 2012, Wang *et al*., 2012d).

Besides, there are many compounds that directly interacts or indirectly plays role in affecting SKP2 expression level. Due to the role of *SKP2* as a proto-oncogene, many cancer research are being conducted to identify compounds that can responsible in downregulating *SKP2* and may serve as potential therapeutic target in attenuating cancer.

2.2.3 *SKP2* and Acute Myeloid Leukemia

Higher expression of SKP2 and its role as an independent prognostic factor for AML was first demonstrated from a sample of AML patients (Min *et al*., 2004). First, AML samples were collected, the expression of SKP2 was identified using

western blot analysis and grouped into higher SKP2 expression and lower SKP2 expression group. Compare to lower SKP2 expression group, groups with higher expression of SKP2 was identified to have lower overall survival rate. Besides, SKP2 expression was reported to positively correlate with cytoplasmic localization of the p27Kip1 protein. However, this study does not demonstrate the exact mechanism for the higher SKP2 expression in AML (Min *et al*., 2004, Ewerth *et al*., 2016). Till date, very few resource are available on studies conducted in AML and SKP2 expression level.

Experiment conducted in Kasumi-1 cell line and SKNO1 showed prolonged downregulation of AML1/ETO inhibit Telomerase reverse transcriptase (*TERT*) expression (Ptasinska *et al*., 2012). Based on (Moses, 2015), downregulation of AML1/ETO and TERT in protein and RNA level is mentioned to be associated with reduced in SKP2 protein expression level. It is also stated that SKP2/ RB-E2F/p27 axis plays key role in regulation of TERT by AML/ETO.

2.3 FOXO3a

2.3.1 Role of FOXO as a tumor suppressor gene

Forkhead Box O (FOXO) is one of a family member in winged-helix/forkhead transcriptional factor. This forkhead family is characterized by highly conserved 100 amino acid DNA binding domain (Clark *et al*., 1993, Kaufmann and Knöchel, 1996, Kaestner *et al*., 2000, Fu and Tindall, 2008). FOX genes are grouped into 19 subclasses which are FOXA to FOXS based on the homology within the region. (Kaestner *et al*., 2000, Fu and Tindall, 2008) . Family members of subclass FOXO in mammals are further classified into four members: FOXO1, FOXO3, FOXO4 and FOXO6 and play a key role as tumor suppressor gene (Calnan and Brunet, 2008, Xie *et al*., 2012).

FOXO1 known originally as FKHR, one of the members of FOXO family were found to be abundantly present in B-cell lineage compare to other members of the same family. However, the expression level of FOXO1 was comparably less in classical Hodgkin Lymphoma (cHL). Nuclear translocation of FOXO1 was identified to induce apoptosis in five different cells of cHL includes L428, KM-H2, L1236, U-HO1, SUP-HD. FOXO1 was also identified to induce cell cycle arrest and function in pro-apoptotic effect by activating tumor suppressor FOXO1 target gene such as *NOXA*,p27kip1, BCL2L11 and TNFSF10 (Xie *et al*., 2012). Overall survival of cancer cells with the effect of FOXO1 was studied in H1299 cells and the finding clearly indicates that nuclear translocation of FOXO1 induce cell death via apoptosis while cytosolic FOXO1 carry out autophagy in order to induce cell death. *Ex vivo* studies

in nude mice indicate that tumor weight was reduced in FOXO1 expressing cells but in absence of Atg7 or Atg5 the weight of tumor was not affected (Zhao *et al*., 2010).

Experiment suggest that FOXO1a along with FOXO3a (FKHRL1) repress the activity of estrogen receptors- α (ER-α) and estrogen receptors- β (ER-β) proteins in 293T, human embryonic kidney cells. Studies in MCF-7, human breast carcinoma cell line, suggest that FOXO3a interact with ER-α and ER-β to downregulate estrogen receptor (ER) transcriptional activity. *In vivo* studies from a mouse model of estrogen-dependent breast cancer further, indicates that E2-dependent tumor growth is suppressed by overexpression of FOXO3a. FOXO3a was also identified to induce the expression of three CDK inhibitors (p27Kip1, p21Cip1, and p57Kip2) and overexpression of FOXO3a suppress proliferation of MCF-7 cells (Zou *et al*., 2008). In Natural killer (NK)-cell neoplasms, FOXO3a and PRDM1 was suggested to have a role in suppression of cell proliferation compared to other genes located in 6q21-23 (Karube *et al*., 2011). Besides, FOXO3a was also shown to have significance in AML.

Overexpression of FOXO4(AFX) in 786-0, renal cancer cell line shows to induce apoptosis by increasing the expression of *Bim* (Wang *et al*., 2016). Expression level of FOXO4 in gastric cancer patients and gastric cancer cell line includes SGC7901, BGC-823, MKN28, MKN45, 9811 and AGS was identified in relatively lower level compared to its respective control. FOXO4 was detected to inhibit gastric cancer proliferation and have been shown to affect cell cycle activity by G1 arrest. FOXO4 was also identified to suppress gastric cancer migration, invasion, tumorigenesis and metastasis (Su *et al*., 2014).

However, FOXO6 was spotted to upregulate c-MYC and induce tumorigenesis by regulating gastric cancer cell, SNU-16 cell proliferation (Qinyu *et al*., 2013). This suggestion opposes the role of FOXO acting as tumor suppressor gene. Yet, several research shows FOXO6 for its tumor suppression property. For an example, FOXO6 inhibit lung cancer, A549 cell proliferation. During post-transcription or translation level, FOXO6 plays a key role in inducing p53 protein expression (Hu *et al*., 2015).

Overall, FOXO is known for tumor suppression property due to its role in cell cycle arrest, pro-apoptotic effect by activating tumor suppressor gene, suppressing cancer cells migration as well invasion, tumorigenesis, metastasis and inhibiting cancer cell proliferation.

2.3.2 FOXO3a: One of FOXO family present in Acute Myeloid Leukemia

In Acute Myeloid Leukemia, the *FOXO3* gene was identified at (6;11)(q21;q23) chromosomal translocation (Kornblau *et al*., 2010). PCR screening of human leukemic cell line K562 cDNA library shows that in this chromosomal translocation site, *FOX03* gene is located to fuse with *MLL* gene (Figure 2.3) (Hillion *et al*., 1997, Katoh and Katoh, 2004).

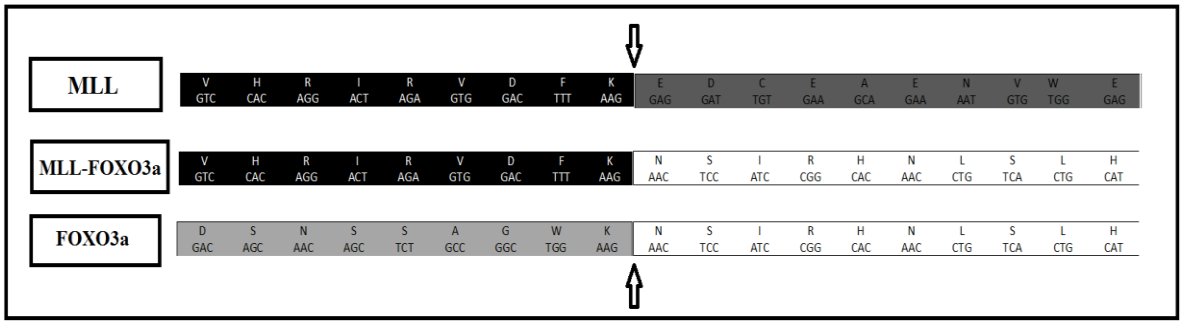

Figure 2.3: Nucleotide and predicted the amino acid sequence of fused *MLL-FOXO3a* cDNA. Adapted from (Hillion *et al*., 1997).

In order to identify the FOXO1, FOXO3a and FOXO4 expression pattern in AML, primary AML cells of patients and human Acute Monocytic Leukemia cell line, MV4-11 were analyzed. The result clearly shows an abundance of FOXO3a mRNA level. When western blot was carried out, there was the detection of FOXO1 level in only some patients and absent of FOXO4. However, all three proteins were expressed abundantly in human leukemia cell lines, MOLM-14 (Chapuis *et al*., 2010). This experiment also emphasizes that in AML blast cells, FOXO3 was constantly expressed compare to other FOXO family member.

Nuclear localization of FOXO3a is essential to induce apoptosis and suppress proliferation in AML (Chapuis *et al*., 2010). However, FOXO3a phosphorylation induces FOXO3a translocation from nucleus to the cytoplasm, thus suppressing FOXO3a activity (Brunet *et al*., 1999, Yang and Hung, 2009, Chapuis *et al*., 2010, Thépot *et al*., 2011).

Experiment from AML blast cells and MV4-11/FOXO3a-GFP cells shows presence of inactive FOXO3 due to its translocation from nucleus to cytoplasm and both AKT and ERK are not responsible for this translocation. When Nemo-antagonistic peptide 29, an inhibitor of IKK signaling pathway is introduced in both

AML cells and MV4-11/FOXO3a-GFP cells, nuclear translocation of FOXO3a was strongly induced. This statement clarifies that IKK plays a key role in FOXO3a cytoplasmic translocation and experiment shows that phosphorylation on S^{644} is essential for this particular function of IKK (Chapuis *et al*., 2010).

Hypomethylation agents such as azacitidine are identified to induce nuclear translocation of FOXO3a. Both azacitidine and decitabine were identified to induce FOXO3a dephosphorylation. When SKM-1 cell line which was established from Myelomonocytic Leukemia in Myelodysplastic Syndrome, were treated with azacitidine, the level of FOXO3a mRNA increases. The experiment was then proceeded using patient-derived AML cells to determine the FOXO3a translocation and apoptosis properties after treatment with hypomethylation agent. The result clearly shows a correlation between azacitidine-treated cells' nuclear FOXO3a translocation and apoptosis. However, when the cells were treated with decitabine, there was no significant correlation between nuclear FOXO3a translocation and apoptosis. This experiment clearly indicates the application of hypomethylation agents to induce FOXO3a dephosphorylation or nuclear translocation in AML (Thépot *et al*., 2011).

Although it was stated FOXO3a nuclear translocation is essential to induce apoptosis in AML cells (Chapuis *et al.,* 2010), some experiment indicates an opposing result. In both MOLM-14 and MM6 cells, knockdown of FOXO3 is shown to increase its phagocytic property. Besides, the rate of apoptosis in CD11+ cells expressing FOXO3 shRNA increased. Yet, the experiment also indicates that ablation of FOXO1, FOXO3, and FOXO4 in MLL-AF9 leukemic bone marrow cells from mice shows an increase in expression of CD11b and Gr-1 (both are mature

myeloid cellular markers) and morphological changes indicates myeloid maturation (Sykes *et al*., 2011).

The experiment was conducted in order to identify the expression pattern of FOXO3a and its adverse effect in cytogenetically normal AML patients. The result shows that patient with a higher level of FOXO3a gene expression have a shorter overall survival (the period from diagnosis to death/last follow-up) and relapse-free survival (period of complete remission to hematological relapse/last follow-up) than patient showing lower *FOXO3* gene expression (Santamaría *et al*., 2009). Similarly, FOXO1, FOXO3 AND FOXO4 ablation in mice with MLL-AF9 positive AML shows a longer latency (Sykes *et al*., 2011).

2.3.3 Gene regulation of FOXO: Phosphorylation and Ubiquitination

The major mechanisms that affect FOXO transcriptional factor are phosphorylation and ubiquitination. Research have shown that various factors are responsible for these two mechanisms. The factors involved include AKT, 14-3-3, SKP2 and MDM2. Phosphorylation of FOXO at different sites leads to localization of FOXO from nucleus to the cytoplasm. This localization pattern affects FOXO transcriptional activity as FOXO is shown to be inactive during its cytoplasmic localization state. It is also suggested that the rate of phosphate group being removed from the phosphorylation site in FOXO by phosphatase may influence the localization pattern (Calnan and Brunet, 2008).

Although (Chapuis *et al*., 2010) shows that IκB kinase(IKK) was identified to overcome the Protein kinase B (AKT) activity in cytoplasmic localization of FOXO,