ELUCIDATING THE MOLECULAR PATHWAYS OF CELLULAR SENESCENCE VIA S-PHASE KINASE ASSOCIATED PROTEIN 2 GENE SUPPRESSION IN ACUTE MYELOID LEUKEMIA CELL LINES

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by

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Five years of hard work, sweat and tears are about to be come to an end with the submission of my precious thesis. God knows it was a rollercoaster ride that had its ups and downs, mostly downs I think. From failed experiments to rethinking my decision to pursue my study, this journey was full of challenges that kept surprising me. Repeating the experiments multiple number of times, troubleshooting the problems, contamination issues, -80℃ freezer problem, cells can't be revived, culture media stolen, Covid-19 restrictions (contributed to almost 6 months delay), lack of funds to purchase items (contributed to another 6 months delay) were probably the highlights of my PhD journey.

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LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage
ng	Nanogram
µl	Microliter
CO_2	Carbon dioxide
ml	Milliliter
xg	Gravitational force
w/v	Weight over volume
V	Volt
ms	Millisecond
rpm	Revolutions per minute
M	Molarity
mg	Milligram
nm	Nanometer
nM	Nanomolar
µg	Microgram
kDa	KiloDalton
β	Beta
A	Ampere
bp	Base pairs
g	Gram

LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
ATRA	All-trans Retinoic Acid
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CKI	Cyclin-dependent kinase inhibitor
CLL	Chronic lymphoblastic leukemia
CML	Chronic myeloid leukemia
CPM	Count-per million
C-terminal	Carboxyl terminal
DCF	Dichlorofluorescein
DCFDA	Dichlorofluorescein Diacetate
DDR	DNA damage response
DEG	Differentially expressed gene
del	Deletion
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FAB	French-American-British
FBS	Fetal bovine serum
FDR	False discovery rate
GO	Gene Ontology
GSEA	Gene set enrichment analysis
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
ICC	International Consensus Classification
inv	Inversion

IMDM	Iscove’s modified Dulbecco's Medium
LSC	Leukemic stem cell
KEGG	Kyoto encyclopedia of genes and genomes
MOPS	3-morpholinopropane-1-sulfonic acid
mRNA	Messenger RNA
miRNA	MicroRNA
NES	Normalized Enrichment Score
NGS	Next-generation sequencing
N-terminal	Amino terminal
OncomiR	Oncogenic microRNA
PBS	Phosphate buffered saline
PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RFW	RNAse-free water
RIPA	Radio-immunoprecipitation Assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SA-βgal^{+}	Senescence-associated beta galactosidase positive
SASP	Senescence-associated secretory phenotype
SC	Senescent cell
SCF^{SKP2}	Skp1-Cullin1-F-box protein ^{SKP2}
SDS	Sodium dodecyl sulphate
siMM	Mismatch siRNA
siRNA	Small-interfering RNA
siSKP2	S-phase kinase associated protein 2 siRNA
SKP2	S-phase kinase associated protein 2
t	Translocation
TAE	Tris-acetate-EDTA
TBST	Tris-buffered saline with Tween 20
TEMED	Tetramethylethylenediamine
TIS	Therapy-induced senescence

TMM	Trimmed Mean of M-values
UPS	Ubiquitin proteosome system
UTR	Untranslated region
WHO	World Health Organization

LIST OF APPENDICES

MERUNGKAI TAPAK JALAN MOLEKULAR PENUAAN SEL MELALUI PENINDASAN GEN S-PHASE KINASE ASSOCIATED PROTEIN 2 DALAM SEL SELANJAR LEUKEMIA MIELOID AKUT

ABSTRAK

Leukemia mieloid akut (AML) ialah sejenis kanser hematologi yang dicirikan dengan perkembangan klon progenitor mieloid dalam sum-sum tulang dan darah periferi. AML adalah isu kesihatan global yang perlu diberi perhatian kerana ia mampu menyumbang kepada beban penyakit keseluruhan. Selaras dengan ini, kajian semasa telah menunjukkan bahawa penuaan selular boleh berfungsi sebagai mekanisme penyekatan dalam pelbagai jenis kanser terutamanya AML. Pengawalaturan proses penuaan selular melibatkan pelbagai komponen dan salah satunya ialah "S-Phase Kinase Associated Protein 2" (SKP2). Tahap ekspresi SKP2 yang berlebihan dalam AML telah didokumentasikan secara meluas, Selain itu. kajian terdahulu juga menekankan penglibatan SKP2 dalam modulasi negatif penuaan selular. Walau bagaimanapun, mekanisme pengawalaturan proses penuaan selular oleh SKP2 dalam AML masih belum dirungkai. Sehubungan dengan itu, kajian ini bertujuan untuk menerokai mekanisme molekul proses penuaan selular pada tahap transkripsi, pascatranskripsi dan translasi melalui penindasan gen *SKP2* dalam sel AML. Hipotesis utama kajian ini ialah SKP2 berupaya untuk menghalang proses penuaan selular dalam sel AML. Hipotesis ini telah diuji melalui kaedah penindasan gen *SKP2* menggunakan siRNA dalam sel AML: Kasumi-1, THP-1 dan HL-60. Hasil kajian menunjukkan bahawa penindasan gen *SKP2* berjaya mengaruh penuaan replikatif yang dicerminkan oleh peningkatan aktiviti β-galactosidase sebanyak dua kali serta peningkatan peratusan sel dalam fasa G1 sebanyak 6% ke 10% dalam sel AML. Tambahan pula,

data daripada penjujukan RNA serta pengesahan melalui eksperimen menunjukkan bahawa penindasan gen *SKP2* mengakibatkan perubahan yang ketara pada tahap transkrip gen yang terlibat dalam tapak jalan penuaan selular seperti gen-gen yang berkaitan dengan kitaran sel (*CDK1* dan *CDKN1A)*, "senescence-associated secretory phenotype" (*CXCL-8),* proliferasi (*MAPK14)* dan tindak balas kerosakan DNA (*RAD9A)*. Selain itu, analisis pascatranskripsi telah mengenal pasti beberapa calon miRNA yang menunjukkan perubahan ketara dalam tahap ekspresi susulan penindasan gen *SKP2* dalam sel AML. Seterusnya, penemuan daripada analisis translasi mencadangkan bahawa SKP2 memodulasi penuaan selular melalui mekanisme yang tidak melibatkan p53 dalam sel selanjar Kasumi-1 dan THP-1. Sebaliknya, dalam sel HL-60, penuaan selular mungkin dipengaruhi oleh kedua-dua tapak jalan yang bergantung kepada p53 serta tapak jalan yang tidak bergantung kepada p53. Secara keseluruhannya, pengawalaturan, penuaan selular yang dimodulasi oleh SKP2 adalah berbeza antara pelbagai jenis AML. Kesimpulannya, hasil daripada kajian ini boleh digunakan untuk membangunkan terapi sasaran alternatif yang berpotensi untuk meningkatkan hasil dan kemandirian pesakit AML

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ABSTRACT

Acute myeloid leukemia (AML) is a type of hematological malignancy characterized by clonal expansion of myeloid progenitors that primarily occurs in bone marrow and/or peripheral blood. AML represents a significant global health issue that warrants attention due to its contribution to overall disease burden. In accordance with this, contemporary studies suggest that cellular senescence could function as a tumour suppressive mechanism in a variety of cancers, notably AML. The regulation of cellular senescence involves several components, including S-phase kinase-associated protein 2 (SKP2). Overexpression of SKP2 has been extensively documented in AML, with prior studies underscoring its involvement in the negative modulation of cellular senescence. Nonetheless, the precise mechanism through which SKP2 governs cellular senescence in AML remains to be clarified. Therefore, this study aims to explore the mechanisms involved in the senescence response at the transcriptional, post-transcriptional, and translational levels as a consequence of *SKP2* suppression in AML cells. It was hypothesized that SKP2 prevents cellular senescence in AML cells. This hypothesis was tested through siRNA-mediated *SKP2* knockdown in Kasumi-1, THP-1 and HL-60 AML cell lines. Results obtained showed that *SKP2* suppression led to the successful induction of replicative senescence, reflected by up to two-fold increase in β-galactosidase activity and a 6 to 10% increase in the percentages of cells in G1 phase of cell cycle in each of the three cell lines studied. Moreover, RNA-seq data and its experimental validation indicated that *SKP2* knockdown resulted in pronounced

modifications at transcript level to genes involved in the cellular senescence pathway. This was especially true for genes pertinent to the cell cycle (*CDK1* and *CDKN1A)*, the senescence-associated secretory phenotype (*CXCL-8)*, proliferation (*MAPK14)*, DNA damage response (*RAD9A)*. Furthermore, analysis of post-transcriptional regulation of SKP2-mediated cellular senescence has identified several candidate miRNAs that exhibited notable changes in their expression in AML cells. The findings from translational analyses suggest that SKP2 modulates cellular senescence via p53-independent mechanisms in both Kasumi-1 and THP-1 cell lines. Conversely, in HL-60 cells, cellular senescence may be influenced by both p53-dependent and p53-independent pathways. Overall, it can be concluded that SKP2-mediated cellular senescence differs among various subtypes of AML. The findings from this study paves the way for the development of novel targeted therapeutic agents that could significantly improve AML patients' outcomes and overall survival.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Cellular senescence refers to a state of stable cell cycle arrest in response to stress signals whilst remaining metabolically active (Roger, Tomas & Gire, 2021). Calcinotto and group described that cellular senescence can be broadly categorized into two groups: replicative senescence which occurs as a result of telomeric signal and premature senescence which occurs as a result of non-telomeric signal (Calcinotto *et al.*, 2019). Cellular senescence has been hailed as an effective tumour suppressive mechanism since the early 2000 due to the ability of damaged cells to enter proliferative arrest as means to prevent the propagation of acquired mutations which could potentially contribute to oncogenesis (Hara, 2004; Dimri, 2005). Cellular senescence is regulated by various players and one such player is S-phase kinase-associated protein 2 (SKP2), an F-box protein that positively regulates G1 to S phase transition (Wang *et al.*, 2014). Interestingly, SKP2 is overexpressed in various types of cancers resulting in abrogation of senescence response which inadvertently contributes to tumorigenesis, suggesting that SKP2 acts as an oncogene (Ewald and Jarrard, 2012; Lee *et al.*, 2014; Liu *et al.*, 2022; Wang *et al.*, 2014). Furthermore, a number of studies have demonstrated that SKP2 is frequently overexpressed in acute myeloid leukemia (AML), a type of hematological malignancy, and has been used as a biomarker to predict prognosis and overall survival in AML patients (Dan *et al.*, 2022).

Our research group has previously established that SKP2 negatively regulates cellular senescence in AML. It was observed that upon *SKP2* suppression in AML cell line Kasumi-1, the cells displayed an increase in senescence-associated beta

galactosidase (SA-βgal) activity (Moses *et al.*, 2023). It was postulated that RUNX1/RUNX1T1 fusion protein contributes to SKP2 protein stabilization. This finding was further supported by the observation that *RUNX1/RUNX1T1* suppression results in the significant downregulation of SKP2. It was also reported that senescence-associated beta galactosidase (SA-βgal) activity was upregulated upon *RUNX1/RUNX1T1* suppression accompanied by G1 cell cycle arrest which is a hallmark of cellular senescence. Cyclin-dependent kinase inhibitor 1B (CDKN1B), also known as p27 is a cell cycle regulator involved in inducing G1 cell cycle arrest, a hallmark of cellular senescence. This study also demonstrated that p27 protein level was upregulated significantly following *SKP2* knockdown. Similar observation was reported in AML THP-1 cells whereby prolonged *SKP2* suppression for six days results in the significant upregulation of p27 at both transcript and protein level (Rosli, 2023). It is also worth noting that underphosphorylated Rb, a negative cell cycle regulator that takes part in halting S phase entry is significantly upregulated following *SKP2* depletion in THP-1 cells (Rosli, 2023). These findings collectively suggests that SKP2 negatively regulates cellular senescence, possibly through mediating the cell cycle players.

Given the above discussion, it is evident that cellular senescence is indeed regulated by SKP2 in AML. As the argument above points out, SKP2 can negatively regulate cellular senescence and potentially contributes to AML progression. Again, it is for this reason that this study aims to elucidate the mechanism of senescence response upon *SKP2* suppression in Acute Myeloid Leukemia (AML) cell lines.

1.2 Importance and rationale of study

SKP2 has been established as a major contributor to leukemogenesis and is frequently overexpressed in AML as evidenced by a number of studies (Thacker *et al.*, 2020; Dan *et al.*, 2022). It has recently come to light that prolonged suppression of *SKP2* leads to cellular senescence in AML (Moses *et al.*, 2023). This observation was accompanied by an upregulation of cyclin-dependent kinase inhibitor, CDKN1B at transcript and protein level. Nevertheless, the mechanism by which SKP2 prevents cellular senescence has yet to be elucidated. Therefore, the primary aim of this study is to elucidate the mechanism of senescence response at transcriptional, post-transcriptional and translational level upon *SKP2* suppression in Acute Myeloid Leukemia (AML) cell lines. It is essential to determine the molecular mechanisms involved in the regulation of cellular senescence via SKP2-mediated pathways because it allows us to have an in-depth understanding of players that could potentially participate in leukemogenesis or even disease progression in AML. Indeed, it is only when we have managed to unravel the molecular mechanism underlying the regulation of cellular senescence via SKP2-mediated pathway specifically in AML, that we will be able to develop novel targeted therapy for the eradication of the disease. It is worth to note that AML is a highly heterogenous disease which makes it even more crucial for the development of novel targeted therapeutic agents that confer higher specificity because ‘one size fits all’ approach is rather impractical for AML treatment.

Cellular senescence has long been a question of great interest exclusively in cancer therapeutic field as it has emerged as a critical tumour barrier mechanism. It is now well established from a variety of studies in AML that cellular senescence exerts beneficial effects primarily by attenuating cancer progression (Bazzar *et al.*, 2021; Peng *et al.*, 2018; Zhang *et al.*, 2012). Consequently, therapy-induced senescence

(TIS) has become a much sought after approach for therapeutic options in cancer therapy. One such study was performed by Gilioli and colleagues whereby they studied the effects of TIS in AML blasts and found that cells expressing high senescent activity post-chemotherapy was able to induce immune recognition for eradication by T-cell (Gilioli *et al.*, 2021).

However, recent developments in the field of cellular senescence have identified new roles of senescence in AML which acts as a double-edged sword. An interesting finding that sheds light on the detrimental effect of senescence has been demonstrated by Abdul-Aziz and group (Abdul-Aziz *et al.*, 2019). This study highlights the fact that AML blasts induce a senescent state in bone marrow stromal cells to benefit from the paracrine secretion of senescence-associated secretory phenotype (SASP) composed of various growth factors, pro-inflammatory chemokines and proteases to promote tumour progression. Hence, this study presents cellular senescence as a mechanism by which AML blasts promotes survival by reshaping the tumour microenvironment. Nevertheless, this type of senescence is distinct from replicative senescence which is characterized by a G1 phase arrest of the cell cycle. Furthermore, another recent study by Duy and colleague have identified a subgroup of primary AML cells that enter senescent-like state post chemotherapy (Duy *et al.*, 2021). These group of cells have been identified to be the cause of disease relapse as they exhibit increased self-renewal potential post-senescence. Hence, this study presents cellular senescence as a mechanism by which leukemic blasts withstand chemotherapy-mediated cell death. These two studies have pointed out that cellular senescence indeed contributes to AML disease relapse and subsequently therapy resistance. While senescence does provide advantageous effects, it cannot be denied

that senescence also poses adverse effect by giving rise to leukemogenesis and disease relapse.

This study is crucial as it decipher the molecular mechanism of cellular senescence in AML which could give us insights into the possible role of AML blasts. Unravelling the network of players involved in the regulation of cellular senescence would increase our understanding with regards to the induction and mediators of cellular senescence response. This would enable potential drug targets to be identified for wider therapeutic options that could significantly improve AML patients' outcomes and overall survival.

1.3 Research Hypothesis

1.3.1 Study hypothesis

SKP2 prevents cellular senescence in AML.

1.3.2 Null hypothesis

SKP2 does not prevent cellular senescence in AML.

1.4 Research Objectives

1.4.1 General Objective

To elucidate the mechanism of senescence response upon *SKP2* suppression in Kasumi-1, THP-1 and HL-60 Acute Myeloid Leukemia (AML) cell lines at transcriptional, post-transcriptional and translational levels.

1.4.2 Specific Objectives

1. To assess the capacity of AML cells to enter senescence upon *SKP2* suppression.
2. To decipher the transcriptional regulation of senescence post *SKP2* silencing in AML cells via whole transcriptome sequencing and qRT-PCR analysis.
3. To identify microRNAs (miRNAs) that are implicated in post-transcriptional regulation of SKP2-mediated cellular senescence pathway via bioinformatics tools and qRT-PCR analysis.
4. To analyse the changes in senescent-related proteins at translational level post *SKP2* suppression in AML cells via immunoblotting.

CHAPTER 2

LITERATURE REVIEW

2.1 Leukemia

Leukemia is defined as the excessive accumulation of immature white blood cells also known as ‘blasts’ in the bone marrow and/or peripheral blood. It can be categorized based on the clinical course (acute or chronic) and cell of origin (lymphoid or myeloid) (Fiegl, 2016).

Leukemia can be categorized into acute or chronic. Acute leukemia refers to a type of hematological malignancy in which the malignant cells proliferate uncontrollably in a rapid state while chronic leukemia is characterized by an increase in malignant cells but at a slower rate as compared to acute leukemia. Acute and chronic leukemia can be further divided into lymphoid or myeloid origins. Acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) are of myeloid origin whereas acute lymphoblastic leukemia (ALL) and chronic lymphoblastic leukemia (CLL) are of lymphoid origin (Hoffbrand and Moss, 2016).

2.2 Leukemia Epidemiology

Recent global cancer statistical report, Global Cancer Observatory (GLOBOCAN) has been published by International Agency for Research on Cancer (IARC) in 2020. In Malaysia, it was done in collaboration with National Cancer Patient Registry (NCPR). NCPR is a hospital-based database which is responsible for collecting data on Malaysian patients that have been officially diagnosed with cancer having visited any of the participating facilities. A total of 48639 new cancer cases and 29530 death cases have been recorded in Malaysia in the year 2020. Leukemia is classified as the ninth most common cancer among Malaysian population, registering

a total number of 1905 new cases. Table 2.1 shows ten most common new cancer cases recorded in Malaysia in 2020. Leukemia is a1so classified as the fifth most common death from cancer cases, registering a total number of 1481 death cases. Table 2.2 shows ten most common death of cancer cases recorded in Malaysia in 2020.

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	1481	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

According to Malaysian National Cancer Registry Report 2012 to 2016, leukemia is the sixth most common cancer among Malaysian population (Figure 2.1). It is the seventh most common cancer in male and ninth in female (Figure 2.2). As for the types of leukemia, myeloid leukemia shows the highest incidence rates among both male (Figure 2.3) and female (Figure 2.4) between the ages of 15 to 75 and above as compared to other types of leukemia (Azizah *et al.*, 2019).

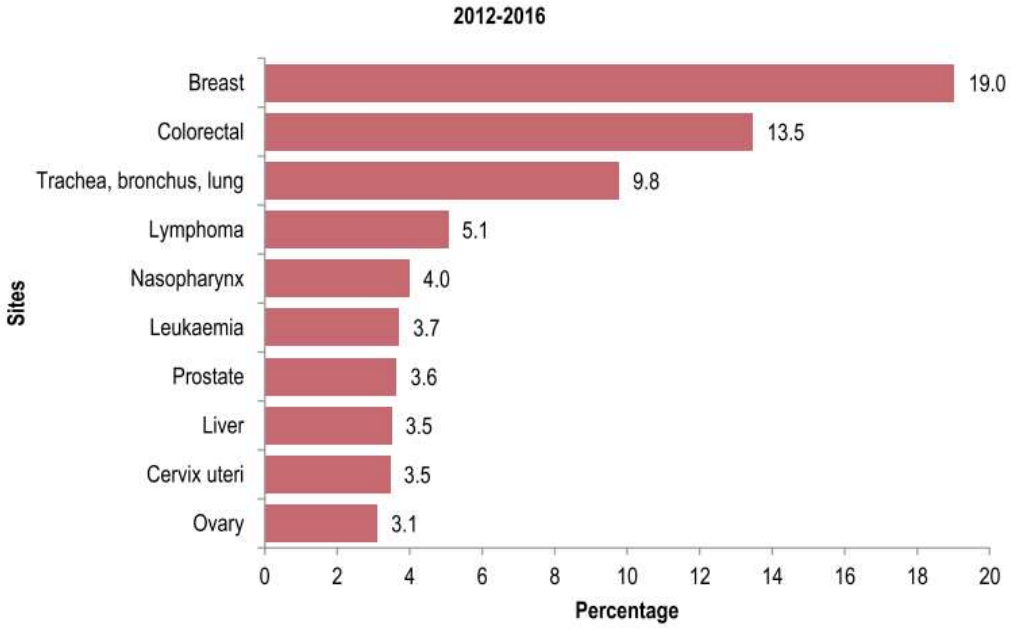

Figure 2.1 Ten most common cancer among Malaysian residents from year 2012 to 2016. Figure was adapted from Azizah *et al.*, 2019

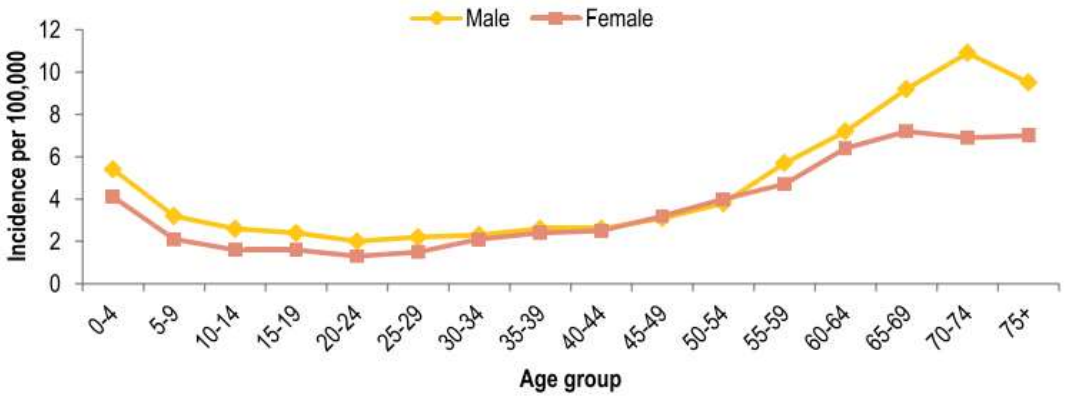

Figure 2.2 Age-specific incidence rate of leukemia by sex among Malaysian residents from year 2012 to 2016. Figure was adapted from Azizah *et al,*. 2019

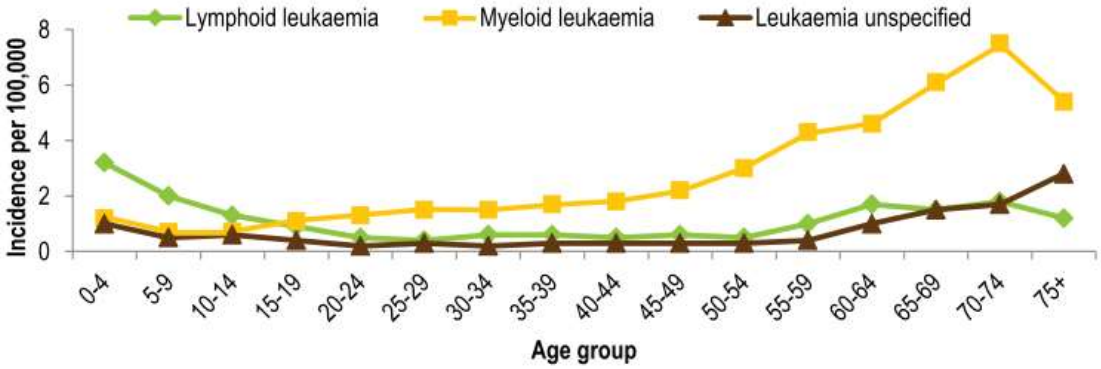

Figure 2.3 Age-specific incidence rate of leukemia types among male Malaysian residents from year 2012 to 2016. Figure was adapted from Azizah *et al.*, 2019

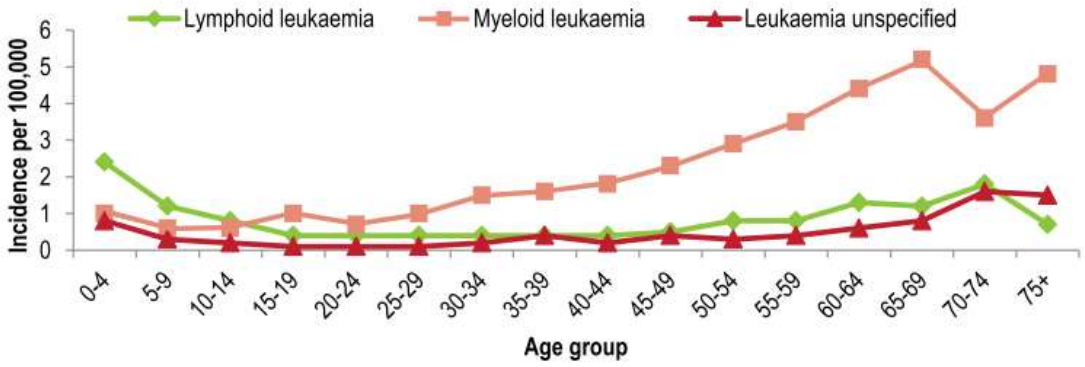

Figure 2.4 Age-specific incidence rate of leukemia types among female Malaysian residents from year 2012 to 2016. Figure was adapted from Azizah *et al,*. 2019

2.3 Acute myeloid leukemia (AML)

Acute myeloid leukemia (AML) is a hematological malignancy that results in impaired differentiation, proliferation, and accumulation of myeloid progenitor cells in the bone marrow and/or extramedullary sites (Hoffbrand & Moss, 2016). Normal hematopoiesis process is significantly hampered due to excessive myeloid blasts accumulation in the bone marrow, subsequently leading to reduced production of healthy and fully functional mature blood cells (Ferrara & Schiffer, 2013).

Clinical features of AML include fatigue, shortness of breath, recurrent infections, easy bruising and bleeding, hepatosplenomegaly and lymphadenopathy. Other features includes anemia (low count of red blood cells), leukopenia (low count of white blood cells) and thrombocytopenia (low level of platelets) (Hoffbrand & Moss, 2016; Khwaja *et al.*, 2016).

AML onset could be contributed by either genetic or environmental factors. Inherited genetic disorders such as Down syndrome, Bloom syndrome, congenital neutropenia, ataxia telangiectasia, Fanconi anemia and others could act as predisposing factors to AML development. Germline mutations in *CEBPA*, *AML1*, *TP53*, *ANKRD26*, *DDX41*, *ETV6*, *GATA2*, *SRP72*, *TERC* and *TERT* genes may lead to AML onset (Godley, 2014; Gruszka, Valli & Alcalay, 2017). Myelodysplastic syndrome (MDS) and myeloproliferative neoplasms acts as a predisposing factor for AML development (Khwaja *et al.*, 2016). Environmental factors include exposure to chemotherapy (alkylating agents or topoisomerase inhibitors), ionizing radiation (as a result of primary malignancy) and chemicals such as benzene and vinyl chloride (Gruszka, Valli & Alcalay, 2017; Short, Rytting & Cortes, 2018).

2.4 Classification of Acute myeloid leukemia

Three classification systems are utilized to categorize AML into subtypes. The classification systems are French-American-British (FAB) classification, 2022 World Health Organization (WHO) classification and International Consensus Classification (ICC) 2022.

2.4.1 French-American-British (FAB) classification

FAB classification relies mainly on morphology and cytochemical staining of blasts. It classifies AML into nine groups: M0, M1, M2, M3, M4, $M4_{EO}$, M5, M6 and M7. The principle used for classification is based on the degree of maturation and differentiation of the cells (Bennett *et al.*, 1976, 1985, 1991; Tenen, 2003; Abdul-Hamid, 2011). It should be noted that FAB classification is still widely used despite its simplicity in certain developing countries. Table 2.3 illustrates AML FAB classification system.

Table 2.3 French-American-British (FAB) classification system of AML. Table is adapted from Tenen, 2003

FAB Subtype	Description	Comments
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	-
$M4_{EO}$	Myelomonocytic with bone marrow eosinophilia	Characterized by inversion of chromosome 16 involving CBFβ, which normally forms a heterodimer with AML1
M5	Monocytic	-

Table 2.3 (Continued)

M6	Erythroleukemia	-
M7	Megakaryoblastic	GATA1 mutations in those associated with Down's syndrome

2.4.2 World Health Organization (WHO) 2022 classification

World Health Organization (WHO) published the first edition of a whole new classification system back in 2002 to provide a more accurate diagnosis of myeloid neoplasms. Since then, this classification system has been revised continuously to accommodate the latest clinical findings and diagnostic technology that aims for better patient diagnosis. Recently, WHO has published the latest edition of the classification in 2022 integrated with clinical, morphologic, immunophenotypic, cytogenetics and molecular features. AML was broadly classified into two categories: AML with defining genetic abnormalities and AML defined by differentiation (Khoury *et al.*, 2022). WHO 2022 classification of AML is illustrated in Table 2.4.

Table 2.4 WHO 2022 classification system of AML. Table is adapted from Khoury *et al.*, 2022

AML WHO 2022 Classification
Acute myeloid leukemia with defining genetic abnormalities
• Acute promyelocytic leukemia with *PML::RARA* fusion • Acute myeloid leukemia with *RUNX1::RUNX1T1* fusion • Acute myeloid leukemia with *CBFB::MYH11* fusion • Acute myeloid leukemia with *DEK::NUP214* fusion • Acute myeloid leukemia with *RBM15::MRTFA* fusion • Acute myeloid leukemia with *BCR::ABL1* fusion • Acute myeloid leukemia with *KMT2A* rearrangement • Acute myeloid leukemia with *MECOM* rearrangement • Acute myeloid leukemia with *NUP98* rearrangement • Acute myeloid leukemia with *NPM1* mutation • Acute myeloid leukemia with *CEBPA* mutation • Acute myeloid leukemia, myelodysplasia-related • Acute myeloid leukemia with other defined genetic alterations

Table 2.4 (Continued)

Acute myeloid leukaemia, defined by differentiation
• Acute myeloid leukemia with minimal differentiation • Acute myeloid leukemia without maturation • Acute myeloid leukemia with maturation • Acute basophilic leukemia • Acute myelomonocytic leukemia • Acute monocytic leukemia • Acute erythroid leukemia • Acute megakaryoblastic leukemia

2.4.3 International Consensus Classification (ICC) 2022 classification

Previously, AML classification has been based on morphology, cytogenetic abnormalities, gene mutations and immunophenotype. Owing to the current advances in molecular biology and sequencing technologies, a more rigorous and comprehensive diagnosis is made possible. This brought about another classification system for AML known as International Consensus Classification (ICC). ICC was published in 2022 to provide a better diagnosis, prognostication and treatment for AML patients (Arber *et al.*, 2022). Similar to WHO 2022 classification, this system incorporates clinical, molecular/genetic, morphologic and immunophenotypic features as well. Table 2.5 illustrates ICC 2022 classification.

Table 2.5 International Consensus Classification (ICC) 2022

International Consensus Classification (ICC) 2022 Classification
Acute promyelocytic leukemia (APL) with t(15;17)(q24.1;q21.2)/*PML::RARA* ≥ 10%
APL with other *RARA* rearrangements* ≥ 10%
AML with t(8;21)(q22;q22.1)/*RUNX1::RUNX1T1* ≥ 10%
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB::MYH11* ≥ 10%
AML with t(9;11)(p21.3;q23.3)/*MLLT3::KMT2A* ≥ 10%
AML with other *KMT2A* rearrangements† ≥ 10%
AML with t(6;9)(p22.3;q34.1)/*DEK::NUP214* ≥ 10%
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2; MECOM(EVI1*) ≥ 10%
AML with other *MECOM* rearrangements‡ ≥ 10%
AML with other rare recurring translocations (see supplemental Table 5) ≥ 10%
AML with t(9;22)(q34.1;q11.2)/*BCR::ABL1*§ ≥ 20%
AML with mutated *NPM1* ≥ 10%
AML with in-frame bZIP *CEBPA* mutations ≥ 10%
AML and MDS/AML with mutated *TP53*† 10-19% (MDS/AML) and ≥ 20% (AML)
AML and MDS/AML with myelodysplasia-related gene mutations 10-19% (MDS/AML) and ≥ 20% (AML) • Defined by mutations in *ASXL1, BCOR, EZH2, RUNX1, SF3B1*, *SRSF2, STAG2, U2AF1*, or *ZRSR2*
AML with myelodysplasia-related cytogenetic abnormalities 10-19% (MDS/AML) and ≥ 20% (AML) • Defined by detecting a complex karyotype (≥ 3 unrelated clonal chromosomal abnormalities in the absence of other class-defining recurring genetic abnormalities), del(5q)/t(5q)/add(5q), −7/del(7q), +8, del(12p)/t(12p)/add(12p), i(17q), −17/add(17p) or del(17p), del(20q), and/or idic(X)(q13) clonal abnormalities
AML not otherwise specified (NOS) 10-19% (MDS/AML) and ≥ 20% (AML)
Myeloid sarcoma

*Includes AMLs with t(1;17)(q42.3;q21.2)/*IRF2BP2::RARA*; t(5;17)(q35.1;q21.2)/*NPM1::RARA*; t(11;17)(q23.2;q21.2)/*ZBTB16::RARA*; cryptic inv(17q) or del(17) (q21.2q21.2)/*STAT5B::RARA*, *STAT3::RARA*; Other genes rarely rearranged with *RARA:TBL1XR1* (3q26.3), *FIP1L1* (4q12), *BCOR* (Xp11.4).

†Includes AMLs with t(4;11)(q21.3;q23.3)/*AFF1::KMT2A*#; t(6;11)(q27;q23.3)/*AFDN::KMT2A*; t(10;11)(p12.3;q23.3)/*MLLT10::KMT2A*; t(10;11)(q21.3;q23.3)/*TET1::KMT2A*; t(11;19)(q23.3; p13.1)/*KMT2A::ELL*; t(11;19)(q23.3;p13.3)/*KMT2A::MLLT1* (occurs predominantly in infants and children).

‡Includes AMLs with t(2;3)(p11?23;q26.2)/*MECOM*::?; t(3;8)(q26.2;q24.2)/*MYC, MECOM*; t(3;12)(q26.2;p13.2)/*ETV6::MECOM*; t(3;21)(q26.2;q22.1)/*MECOM::RUNX1*.

§The category of MDS/AML will not be used for AML with *BCR::ABL1* due to its overlap with progression of CML, *BCR::ABL1*-positive.

2.5 Genetics of Acute Myeloid Leukemia

The classification systems outlined in the previous section has shown the importance of genetic abnormalities for diagnosis and prognostication of AML. Studies have also shown that genetic abnormalities are one of the main drivers of AML as it is primarily associated with uncontrolled cell proliferation and differentiation block of hematopoietic progenitors (Kirtonia *et al.*, 2020). Heterogeneity of AML is contributed by a myriad of cytogenetic abnormalities and gene mutations which are not mutually exclusive (Kumar, 2011). Thus, it is imperative to understand the genetic abnormalities in AML as it is highly associated with clinical outcome, prognosis and in guiding therapeutic strategies in AML patient.

2.5.1 Cytogenetic abnormalities

Chromosomal aberrations that give rise to cytogenetic abnormalities are one of the primary contributing factors that results in AML onset. Cytogenetic abnormalities can be detected in about 56% in adult and 70-80% pediatric AML patients (Bendari *et al.*, 2020). Karyotyping is vital during AML diagnosis as cytogenetic abnormalities detected offers prognostic value and best treatment course.

Chromosomal aberrations include balanced structural rearrangement and unbalanced abnormalities. Balanced structural rearrangement refers to translocations or inversions of the chromosomes. Both of these structural chromosome arrangements result in the formation of novel fusion genes which may drive AML pathogenesis. Chromosomal translocations such as t(8;21), t(15;17) and t(9;11) are typically found in AML cases. Chromosomal translocation involving runt-related transcription factor 1 (*RUNX1*) on chromosome 21 and RUNXT1 partner transcription co-repressor 1 (*RUNXT1*) gene on chromosome 8 gives rise to fusion gene *RUNX1-RUNXT1*. The protein product of this fusion gene is crucial in AML development as it blocks myeloid

differentiation and induces self-renewal ability (Snaith *et al.*, 2024). This translocation is commonly observed in children diagnosed with AML and it makes up about 13% of all AML cases (Rosli *et al.*, 2023). AML patients carrying t(8;21) chromosomal abnormality typically have a good prognosis (Wilde *et al.*, 2019)

Chromosomal translocation involving promyelocytic leukemia gene (*PML*) on chromosome 15 and the retinoic acid receptor alpha (*RARα*) gene on chromosome 17 gives rise to fusion gene *PML-RARα.* PML-RARα fusion protein acts as an initiating factor in leukemogenesis by inhibiting differentiation at promyelocyte stage of hematopoiesis (De Braekeleer, Douet-Guilbert & De Braekeleer, 2014). This translocation is present in about 10% of AML cases and is associated with favourable prognosis (Lagunas-Rangel *et al.*, 2017; Snaith *et al.*, 2024).

Chromosomal translocation involving lysine methyltransferase 2A (*KMT2A*) gene on chromosome 11 and super elongation complex subunit (*MLLT3*) on chromosome 9 gives rise to fusion gene *KMT2A-MLLT3* which ultimately leads to leukemogenesis through upregulation of transcriptional activity (Fleischmann *et al.*, 2014). This translocation is present in about 10% of pediatric AML cases and about 4-5% in *de novo* adult AML cases (Rosli *et al.*, 2023). AML patients carrying t(9;11) chromosomal abnormality typically have an intermediate prognosis (Lagunas-Rangel *et al.*, 2017).

Moreover, inv(16) (p13;q22) or t(16;16)(p13;q22) results in the formation of core-binding factor β subunit gene (*CBFβ*) and myosin, heavy chain 11, smooth muscle gene (*MYH11*) fusion gene. The protein product of this fusion gene disrupts the differentiation of myeloid progenitors during hematopoiesis and thereby contributes to AML onset (Yi *et al.*, 2019). This chromosomal inversion is present in

about 5-8% of AML cases and is associated with intermediate prognosis (Lagunas-Rangel *et al.*, 2017; Rosli *et al.*, 2023).

Unbalanced abnormalities in AML includes monosomies, trisomies, deletions and isochromosomes. Chromosomal aneuploidies such as monosomy 7 or partial deletion of chromosome 7, trisomy 8 and trisomy 21 are some of the most frequently observed cytogenetic abnormalities in AML. Furthermore, chromosomal deletions including del 20q, del 7q, del 9q are some examples of recurrent abnormalities found in AML cases (Pedersen-Bjergaard and Rowley, 1994; Pourrajab *et al.*, 2020; Shi *et al.*, 2017). Some of the other examples of recurrent chromosomal abnormalities in AML are listed in Table 2.6.

Table 2.6 Additional chromosomal abnormalities in AML

Chromosomal abnormalities	**Cytogenetic biomarkers**	**Description**	**References**
t(6;9)(p23;q34)	DEK-NUP214	• Common in young patients • Usually accompanied with *FLT3-ITD* mutation • Present in about 1% of AML cases • Poor outcome	(Sandén *et al.*, 2013)
t(8;16)(p11.2;p13.3)	KAT6A-CREBBP	• Common in neonates, occurring at a frequency of less than 1% • Poor outcome	(Quessada *et al.*, 2021)
t(1;22)(p13;q13)	RBM15-MKL1	• Occurs only in infants and toddlers at a frequency of 0.3% • Intermediate outcome	(Quessada *et al.*, 2021)
t(11;17)(q23;q21)	PLZF-RARα	• Present in about 1% of APL cases • Poor prognosis • Insensitive to ATRA treatment	(Duan *et al.*, 2023; Zhang *et al.*, 2021)

Table 2.6 (Continued)

Inv (3) (q21q26.2);	RPN1-EVI1	• Common in adults • Poor prognosis	(Raya *et al.*, 2015)
Deletion 5q	-	• Adverse prognosis • Low rate of complete remission	(Rosli *et al.*, 2023)

Cytogenetic testing is crucial as it is used to classify AML patients into favourable, intermediate, or unfavorable risk groups. However, it should also be noted that approximately 40-50% of AML patients exhibit cytogenetically normal karyotype upon testing. Thus, it is imperative to look into gene mutations that could potentially affect the risk stratification of the patients (Kumar, 2011).

2.5.2 Gene mutations

Recurrent gene mutations that are widely reported in AML includes FMS-like tyrosine kinase 3 (*FLT3*), nucleophosmin 1 (*NPM1*), CCAAT/enhancer binding protein alpha (*C/EBPα*), Runt-related transcription factor 1 (*RUNX1*), isocitrate dehydrogenase (*IDH1/IDH2*), DNA methyltransferase 3A (*DNMT3A*) and tumor protein p53 (*TP53*) mutations (Blau, 2015; Yu *et al.*, 2020).

FMS-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase (RTK) expressed on immature hematopoietic progenitor cells and is implicated in the survival, proliferation and differentiation of hematopoietic cells. Accumulating evidence have shown that *FLT3* is mutated in about 30% of all AML cases. Recurrent *FLT3* mutation in AML occur either as a duplication of a part of its coding region forming internal tandem duplication (*FLT3-ITD*) or as missense mutation in its tyrosine kinase domain (*FLT3-TKD*) (Grafone *et al.*, 2012; Kiyoi, Kawashima & Ishikawa, 2020). Both of these mutations culminate in the constitutive activation of the otherwise auto-inhibitory FLT3 receptor, leading to aberrant cell growth (Staudt *et al.*, 2018). AML patients carrying *FLT3-ITD* mutations have poor prognosis whereas

AML patients carrying *FLT3-TKD* mutations have adverse prognosis (Daver *et al.*, 2019).

Nucleophosmin 1 (NPM1) is a nuclear-cytoplasmic shuttling protein and is primarily localized in the nucleus. NPM1 wildtype protein works together with PU.1, a master hematopoiesis transcription factor responsible for myeloid differentiation (Gu *et al.*, 2018; Zhou *et al.*, 2015). Accumulating evidences have shown that *NPM1* is mutated in about 30% of all AML cases (Falini *et al.*, 2020). Mutant *NPM1* causes NPM1 to be aberrantly accumulated in cytoplasm together with PU.1. This phenomena inadvertently results in the repression of granulocyte and monocyte terminal differentiation genes and hence contribute to AML onset (Gu *et al.*, 2018). AML patients with *NPM1* mutations without accompanying *FLT3-ITD* mutation have good prognosis (Blau, 2015).

CCAAT/enhancer binding protein alpha (C/EBPα) is a transcription factor involved in the regulation of hematopoietic progenitor lineage commitment. Accumulating evidences have shown that *C/EBPα* is mutated in about 5-14% of all AML cases (Kurzer & Weinberg, 2023). Mutant C/EBPα plays a role in AML initiation. *C/EBPα* mutation either occurs as a single allele or double allele mutation. Of this, double mutated *C/EBPα* exhibits favorable prognosis (Su *et al.*, 2022).

Runt-related transcription factor 1 (RUNX1) is a transcription factor crucial for hematopoietic development. *RUNX1* mutation occurs about 10% in AML cases, depending on various factors. AML patients harboring *RUNX1* mutation exhibits poor prognosis (Blau, 2015; Mill *et al.*, 2022). Some of the other examples of recurrent gene mutations in AML are listed in Table 2.7.

Table 2.7 Additional gene mutations in AML

Gene mutations	**Description**	**References**
Isocitrate dehydrogenases (*IDH*) 1 and 2	• Prevent myeloid differentiation • Common in older patients • Poor outcome for *IDH1* mutation; Favorable outcome for *IDH2* mutation	(Issa & DiNardo, 2021)
DNA (cytosine-5)-methyltransferase 3A *(DNMT3A)*	• Involved in epigenetic regulation • High relapse rate • Poor prognosis	(Hou *et al.*, 2012)
Ten–eleven translocation 2 (*TET2)*	• Involved in epigenetic regulation • Unfavorable prognosis	(Wang *et al.*, 2019)
Additional sex comb-like 1 (*ASXL1*)	• Tumour suppressive in AML • Adverse prognosis	(Paschka *et al.*, 2015)
KIT	• Common in AML with inv(16) and t(8;21) • High relapse risk	(Blau, 2015; Ayatollahi *et al.*, 2017)

With great advances in diagnostic technologies, novel genetic abnormalities that could potentially play a role in leukemogenesis are being discovered. Hence, it cannot be denied that understanding genetic abnormalities is crucial for guiding therapeutic strategies in AML patients.

2.6 Current therapies for Acute Myeloid Leukemia

The standard protocol for AML patients eligible for intensive chemotherapy begins with induction therapy. This therapy also known as 7+3 consists of cytarabine infusion for seven days continuously in combination with first three days infusion of an anthracycline, typically daunorubicin. This is followed by post-remission therapy whereby the patients undergoes chemotherapy with higher doses of drugs with or without targeted therapies and/or hematopoietic stem cell transplantation (HSCT) (Pelcovits & Niroula, 2020). Targeted therapeutic agents that have been approved by US Food and Drug Administration (FDA) are incorporated into AML treatment regimen. Some of the examples are FLT3 inhibitors which block the signalling pathways that promote the proliferation and survival of leukemic cells (Imatinib, Midostaurin, Gilteritinib), IDH inhibitors which block the signalling pathways that disrupts cellular metabolism and epigenetic regulation (Ivosidenib, Enasidenib), anti-CD33 antibody which targets surface receptor CD33 expressed on AML blasts (Gemtuzumab ozogamicin) and BCL-2 inhibitor that functions to promote apoptosis (Venetoclax) (Kantarjian *et al.*, 2021; Talati and Sweet, 2018; Yu *et al.*, 2020). As for patients ineligible for intensive chemotherapy, they are opted for low dose cytarabine, hypo-methylating agents such as azacitidine or decitabine or participation in clinical trials for novel therapeutic agents (Döhner *et al.*, 2022). However, it is important to note that AML is highly heterogenous and despite the wide availability of therapeutic options for AML patients, it remains a fact that ‘one size fits all’ approach is rather impractical as it is only advantageous to a certain subgroup of patients. Thus, it is critical for the development of targeted therapies that could confer higher specificity in AML.

The biological mechanism of therapeutic agents used for treatment in AML remains to be poorly elucidated. Cytarabine is nucleotide-analog chemotherapeutic agent that functions by inhibiting DNA and RNA synthesis which eventually leads to cell death (Li *et al.*, 2017). Similarly, daunorubicin intercalates between DNA strands and thereby interfere with DNA replication process (Pourrajab *et al.*, 2020). These two drugs have been reported to elicit DNA damage response that ultimately leads to cell death primarily via apoptosis and autophagy (Du *et al.*, 2021; Gamazon *et al.*, 2013). Interestingly enough, it has recently come to light that these agents may also be able to exert tumour suppressive effects through cellular senescence (Gilioli *et al.*, 2021). Hence, cellular senescence may act as a potential mechanism to reverse or inhibit AML progression which will be elaborated further in the next section.

2.7 Cellular senescence

Cellular senescence refers to a state of irreversible cell cycle arrest induced by various stress stimuli. It is a form of physiological response to stress with an ultimate end goal to prevent aberrant changes (Calcinotto *et al.*, 2019). Hayflick and Moorhead discovered that somatic human diploid fibroblasts undergo cell division until they reach a certain limit where they are unable to divide anymore. This phenomena is known as Hayflick limit and it occurs due to progressive shortening of telomeres following each cell division (Hayflick & Moorhead, 1961). However, telomere-dependent signal is not the sole contributor of senescence. Cellular senescence can also be caused by non-telomeric signals such as DNA damage stimuli, oncogenic stimuli and various other undefined stress stimuli (Dimri, 2005; Gorgoulis *et al.*, 2019).

Cellular senescence can be categorized into two groups mainly: replicative senescence which are induced by telomeric signals and premature senescence which are induced by non-telomeric signals (Dimri, 2005; Calcinotto *et al.*, 2019). Replicative senescence occurs as a result of telomere shortening that takes place after each round of cell division, thus exposing chromosome ends and ultimately activating the DNA damage response (Victorelli & Passos, 2017). Premature senescence also known as stress-induced senescence occurs as a result of exposure to various stress stimuli such as oncogenes expression, loss of tumour suppressor, activation of DNA damage response, oxidative stress and many more. Premature senescence is not dependent on telomeric activity and the replicative ‘age’ of the cells (Mendelsohn *et al.*, 2014).