REGULATION OF TELOMERASE REVERSE TRANSCRIPTASE (TERT) BY THE LEUKAEMIC FUSION GENE AML1/ETO

EMMANUEL JAIRAJ MOSES

UNIVERSITI SAINS MALAYSIA

2015

REGULATION OF TELOMERASE REVERSE TRANSCRIPTASE (TERT) BY THE LEUKAEMIC FUSION GENE AML1/ETO

by

EMMANUEL JAIRAJ MOSES

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

November 2015

ACKNOWLEDGEMENT

I would like to take this opportunity to thank everyone who has made it possible for me to complete this enormous and gigantic task of completing a PhD thesis. First and foremost, I would like to thank Universiti Sains Malaysia (USM) and the Ministry of Higher Education Malaysia for sponsoring my studies through the Academic Staff Training Scheme (ASTS).

I would also like to thank my supervisor Professor Narazah Mohd Yusoff for her encouragement, support, advice and financial support through her research grants. A round of applause for Prof Olaf Heidenreich my field supervisor for his excellent supervision and technical expertise. I would like to also thank him for his generosity in sharing his vast knowledge and willingness to collaborate with AMDI, USM. I would like to thank my co-supervisor Dr Azman Seeni for his guidance especially at the initial phase.

I would also like to appreciate all scientific officers, medical laboratory technicians, administrative staff, post-doctoral fellows and all other staff at AMDI, USM who have helped in one way or another.

A big thank you to all my lab-mates who have been a part of my life for this past four years or so. I will not forget the tears of frustrations and the light moments that we shared together.

Next, a very big thank you to my father Mr Jacob Moses, mothers Katherine Moses and Irene Moses, siblings and loved ones for their constant care, support, love, concern and many prayers. I would also like to appreciate Dr Priscilla Moses for her encouragement, prayers and help in formatting the reference list.

Most importantly, I would like to thank the Almighty God for His providence and abundant grace without which I would have not been able to complete this endeavour. I would like to thank Him for bestowing upon me the extra strength to get up and carry on each time I felt like giving up.

TABLE OF CONTENTS

CHAPTER 3- MATERIALS AND METHODS

LIST OF TABLES **PAGE**

LIST OF FIGURES **PAGE**

LIST OF ABBREVIATIONS

A (nucleotide)- Adenine

ANOVA- Analysis of variance between groups

C (Amino acid)- Cysteine

C (nucleotide) – Cytosine

CD markers-Cell differentiation markers

cDNA- complimentary DNA

CHAPS- 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

C-terminal-Carboxyl terminal

DICER- helicase with RNase motif

DNA- Deoxyribonucleic acid

DTT-Dithiothreitol

EDTA- ethylenediaminetetraacetic acid.

G (Amino acid)-Glycine

G (nucleotide) - Guanine

H-Histidine

I-Isoleucine

ITD- Internal tandem duplication

K-Lysine

L-Leucine

M-Methionine

MOPS- 3-(N-morpholino)propanesulfonic acid

mRNA- messenger RNA

N-terminal-Amino terminal

PBS- phosphate buffer saline

PMSF-phenylmethylsulfonyl fluoride

Q-Glutamic Acid

R- Arginine

RIPA-Radioimmunoprecipitation assay

RNA- Ribonucleic acid

RT- reverse transcription

SDS-PAGE- SDS polyacrylamide gel electrophoresis

S-Serine

T (nucleotide)- Thymine

TAR- *trans*-activation response

TBS- Tris-buffered saline

TBST- Tris- buffered saline with TWEEN 20

TEMED-Tetramethylethylenediamine

TGS- Tris-Glycine-SDS

TKD- Tyrosine kinase domain

V- Valine

W-Tryptophan

LIST OF SYMBOLS

ºC- degree celcius

%- percentage

ng- nanogram

µl- microliters

CO_2- carbon dioxide

ml- milliliters

g- Gravitational force

w/v- weight over volume

V- volt

ms- miliseconds

rpm- revolutions per minute

M- molarity

mg- miligrams

nm- nanometer

nM- nanomolar

µg- microgram

kD- kilodalton

β- beta

KB- kilobase

A- Ampere

PENGAWALATURAN TELOMERASE TRANSKRIPTASE BERBALIK (TERT) OLEH GEN GABUNGAN LEUKEMIA AML1/ETO

ABSTRAK

Translokasi kromosom t (8; 21) adalah suatu aberasi kromosom yang biasa berlaku dalam penyakit akut mieloid leukaemia (AML) *de novo*. Kebarangkalian translokasi ini berlaku dalam AML adalah sebanyak 15%. Translokasi ini menghasilkan gen bergabung onko-protein AML1-ETO (juga dikenali sebagai AML1 / MTG8, RUNX1 / ETO atau RUNX1 / RUNX1T1). Penyelidikan mengenai translokasi ini giat dijalankan. Pesakit dengan translokasi kromosom ini pada kebiasaan dilaporkan mempunyai prognosis klinikal yang lebih baik. Kajian terkini menunjukkan bahawa supresi AML-ETO akan menyebabkan pengurangan expresi enzim telomerase transkriptase berbalik (TERT). Walau bagaimanapun, mekanisme bagaimana supresi ini berlaku masih tidak dapat dijelaskan secara mendalam hingga kini. Oleh itu, tujuan kajian ini adalah untuk mengkaji peranan AML-ETO dalam pengawalaturan TERT. Kajian ini mengemukakan hipotesis bahawa AML-ETO mengawalatur TERT melalui tapak jalan CDKN1B (p27) / RB-E2F l. Hipotesis ini telah diuji secara eksperimen melalui kaedah perencatan gen yang menggunakan siRNA. Tiga siRNA yang berbeza iaitu siAGF1, si*CDKN1B* dan si*SKP2* serta kombinasinya telah digunakan. Eksperimen dengan siAGF1 menunjukkan bahawa tahap AML-ETO dan TERT berkurang pada peringkat RNA dan protein. Protein lain yang menunjukkan pengurangan termasuk RB, E2F1 dan SKP2. Walau bagaimanapun, terdapat peningkatan protein CDKN1B (p27). Jumlah sel di fasa G1 dalam kitaran sel juga bertambah. Pemerhatian lain yang boleh dikaitkan dengan kehilangan TERT seperti pengurangan keupayaan klonogenik dan peningkatan kadar

penuaan sel telah diperhatikan dengan jelas. Eksperimen menggunakan si*CDKN1B* menujukkan penurunan tahap CDKN1B (p27) pada peringkat RNA dan protein. Jumlah protein TERT, AML/ETO, SKP2, RB dan E2F1 protein meningkat dengan ketara. Keupayaan klonogenik sel-sel tidak terjejas dan peratusan sel uzur berkurang. Eksperimen menggunakan si*SKP2* menujukkan hasil yang memberangsangkan dimana kesan experimen ini adalah selaras dengan kesan yang ditunjukkan semasa siAGF1 digunakan Walau bagaimanapun, kesannya tidak mencapai tahap yang ditunjukkan oleh experimen yang menggunakan siAGF1. Kesan pengurangan TERT, AML-ETO dan SKP2 serta peningkatan paras CDKN1B dapat diperhatikan. Pengurangan keupayaan klonogenik serta peningkatan kadar penuaan sekali lagi boleh dikaitkan dengan kehilangan TERT. Secara rumusan, terdapat bukti jelas yang menunjukkan bahawa AML-ETO mengawalatur TERT melalui paksi SKP2/p27/ RB-E2F.

REGULATION OF TELOMERASE REVERSE TRANSCRIPTASE (TERT) BY THE LEUKAEMIC FUSION GENE AML1/ETO

ABSTRACT

The chromosomal translocation t(8;21) is a common chromosomal aberration that occurs in up to about 15% of all *de novo* acute myeloid leukaemia (AML) cases. This translocation results in the formation of a fusion onco-protein known as AML1-ETO (also known as AML1/MTG8, RUNX1/ETO or RUNX1/RUNX1T1). Much research has been done regarding this chromosomal aberration. Patients with this chromosomal translocation tend to have better clinical prognosis. Recent studies have shown that suppression of AML/ETO caused a reduction in telomerase reverse transcriptase (TERT) levels. Nevertheless, the molecular mechanism by which this fusion onco-protein governs TERT regulation is still unknown. Therefore, the aim of this study was to investigate the role of AML/ETO in TERT regulation. It was hypothesized that AML-ETO regulates TERT via the CDKN1B (p27)/RB-E2F pathway. This hypothesis was experimentally tested using a siRNA mediated gene knockdown approach. Three different siRNA's namely siAGF1, siCDKN1B and siSKP2 and combinations thereof were utilized. Gene knockdown experiments using siAGF1 showed that AML-ETO and TERT levels were reduced at the RNA and protein level. Other proteins that showed reductions include RB, E2F1 and SKP2. There was also an accumulation of CDKN1B (p27) protein. A G1 phase arrest in the cell cycle was observed too. Other consequential observations linked to TERT loss such as reduced clonogenicity and increased senescence were evident as well. Knockdown experiments utilizing siCDKN1B exhibited decreased levels of CDKN1B

(p27) at the RNA and protein level. Levels of TERT, AML-ETO, SKP2, RB and E2F1 proteins showed marked increase. Clonogenicity of the cells were not affected and percentages of senescent cells were reduced. Interestingly, experiments with siSKP2 mirrored the knockdown effects of siAGF1 albeit to a lower extent. This includes reductions in TERT, AML-ETO and SKP2 levels, increase in CDKN1B levels, reduced clonogenicity and increased senescence. This again could be attributed to TERT loss. Overall, it is clearly shown that AML-ETO regulates TERT via the SKP2/p27/RB-E2F axis.

Chapter 1: INTRODUCTION

1.1 Research Background

Telomeres are guanine rich DNA tandem repeats formed by special chromatin structures at the ends of all eukaryotes with linear chromosomes. This is to protect chromosomes from fusion, recombination and degradation (Blackburn, 2001; Hiyama & Hiyama, 2009). Telomeres are about 10-15 Kb in length in human somatic cells and can be up to 40 Kb in mice (Wright & Shay, 2005; Blasco, 2005). Due to the semi conservative nature of DNA replication, telomeres gradually shorten after each cell cycle. This replicative process which eventually causes cell aging and senescence is commonly known as the 'end replication' problem. A unique enzyme known as telomerase overcomes this problem by providing a template and catalytic subunit to add telomeric repeat sequences thus maintaining telomere lengths (Depcrynski *et al.,* 2009).

Telomerase is a large ribonucleoprotein that is composed of at least two important components; the catalytic subunit (TERT) which has reverse transcriptase activity and the RNA component (TERC- also termed hTR) which acts as template for the synthesis of telomeric tandem repeats by TERT (Parkinson *et al.,* 2008). The other component is a RNA binding protein known as dyskerin which is encoded by the *DKC1* gene (Mitchell *et al.,* 1999; Cao *et al.,* 2008). TERC is ubiquitously expressed in most cells (Feng *et al.,* 1995; Avilion *et al.,* 1996) while TERT expression is low in normal somatic cells but up-regulated in tumor cells (Killian *et al.,* 1997; Nakamura *et al.* 1997).

Telomerase has been drawing much attention as it has been implicated in over 90% of all human cancers thus making it an ideal candidate for diagnostic and therapeutic purposes (Shay & Bacchetti, 197; Depcrynski *et al.,* 2009). Note worthily, various studies have shown that telomerase is elevated in Acute Myelogenous leukemia (AML) as well (Counter *et al.,* 1995; Ohyashiki *et al.,* 1997; Engelhardt *et al.,* 2000).

Acute Myeloid Leukemia (AML) is a clonal disorder arising from the genetic abnormalities that occur in hematopoietic stem cells and hampers the ability of these cells to differentiate into erythrocytes, granulocytes and platelets. This eventually leads to the accumulation of abnormal leukemic cells known as blasts. AML is highly aggressive and heterogeneous in nature (Fialkow *et al.,* 1981, Sawyers *et al.,* 1991, Vardiman *et al.,* 2002). In Malaysia, the age standardized incidence for AML is 2.9 per 100,000 of the population (Zainal & Nor Saleha, 2011; Meng *et al.,* 2013).

One of the hallmarks of AML are cytogenetic abnormalities. Common cytogenetic abnormalities include AML with t(8;21) (q22;q22.3); RUNX1/RUNX1T1 (AML1/ETO or RUNX1/MTG8) which occurs in 8-12% of all *de novo* AMLs (Swansbury *et al.,*1994), AML inv(16) (p13.1q22) or t(16;16) (p13.1;q22); CBFb/MYH11 which occurs in 5-13% of all de novo AMLs (Kalwinsky *et al.,*1990, Liu *et al.,*1995) and Acute Promyelocytic Leukemia with t(15;17) (q22;q21); PML/RARA which occurs in 5-13% of all *de novo* AMLs (Douer *et al.,*1996).

Translocation AML t(8;21) (q22;q22.3) fuses the AML1[RUNX1, PEBP2αB, CBFα2] gene on chromosome 21 to the almost complete open reading frame of MTG8 [ETO, CBF2T1] on chromosome 8 resulting in a fusion protein AML1/ETO also known as AML1/ETO, RUNX1/RUNX1T1, RUNX1/MTG8, or RUNX1-CBFA2T1

(Heidenreich *et al.,* 2003; Martinez *et al.,* 2004). This fusion protein has been reported in up to 40% of all AML subtype M2 (FAB classification) and 12-15% of *de novo* AML in general. Smaller proportions have been reported in AML subtype M0, M1 and M4 as well (Peterson & Zhang, 2004). AML1/ETO has been known to directly bind to transcription factors such as SMAD3, C/EBPα or vitamin D receptor thus affecting signal transduction pathway that govern cell proliferation and differentiation (Westendorf *et al.,*1998; Jakubowiak *et al.,* 2000; Pabst *et al.,* 2001b; Puccetti *et al.,* 2002; Vangala *et al*., 2003). This translocation merits further investigation as patients with this translocation tend to have a better prognosis and complete remission rates (Ferrara & Vecchio, 2002).

One interesting fact to note is that AML1/ETO by itself cannot cause leukemia as further secondary mutations are required for leukemia development (Rhoades *et al.,* 2000; Buchholz *et al.,* 2000; Yuan *et al.,* 2001; Schwieger *et al.,* 2002; Grisolano *et al.,* 2003). Furthermore, the interaction of AML1/ETO in molecular pathways associated with leukemogenesis is not fully understood despite numerous studies (Peterson & Zhang, 2004).

1.2 Rationale and importance of the study.

Recent studies by Gessner et al., 2010 and Ptasinska et al. 2012 have shown that *AML1/ETO* gene knockdown caused a reduction of *TERT* transcript levels in AML t(8;21) positive cell lines. A decrease in TERT protein levels and telomere shortening was observed as well. These observations show that AML/ETO regulates telomerase reverse transcriptase (TERT); the catalytic subunit of telomerase which is a key component for self-renewal. Nevertheless, the mechanism by which this fusion protein mediates TERT regulation is still unknown. Therefore, the aim of this study will be to elucidate the role of AML1/ETO in TERT regulation.

Owing to the fact that TERT expression and AML1/ETO are both tightly linked to the cell cycle (Martinez *et al.,* 2004, Gizard *et al.,* 2008), it is highly plausible that AML1/ETO interacts with genes such as retinoblastoma (RB), E2F transcription factors and cyclin dependent kinase (CDK) inhibitors which are involved in cell cyle regulation (Harbour & Dean, 2001; Sherr & Roberts, 2004; Blasco, 2005). Besides AML1/ETO, another gene that will be the focal point of this study is the CDK inhibitor $p27^{KIP1}$. This gene was chosen because $p27^{KIP1}$ levels are influenced by AML1/ETO (Martinez *et al.,* 2004) and it has also been postulated to be involved in TERT regulation through interactions with Rb and E2F (Depcrynski *et al.,*2009). Therefore, it is possible that AML1/ETO interacts with $p27^{KIP1}$ to regulate TERT expression. The postulation above is presented in a nutshell in Figure 1.1

Another important aspect of this study would be to look at the consequences of TERT loss on AML t(8;21) cell lines. This is because events related to cell cycle growth arrest and cellular senescence that were observed in AML/ETO knockdowns (Martinez *et al.,* 2004; Gessner *et al.* 2010) could have been caused by the low levels of TERT.

This study is important as it encompasses a key question in the self-renewal capability of leukemic stem cells and cancer stem cells in general. It is hoped that some insights would be gained in relation to the molecular events that govern self- renewal mechanisms in leukemic cancer stem cells through this study. Furthermore, understanding these mechanisms could lead to the discovery of other regulatory networks that interact with AML1/ETO to regulate TERT and provide some insights regarding the molecular pathogenesis of Acute Myeloid Leukaemia. New potential drug targets for Acute Myeloid Leukaemia could be discovered from these regulatory networks. This in turn could certainly open new doors for new therapeutic approaches and cell based therapies which would greatly improve the clinical outcome of leukaemic patients.

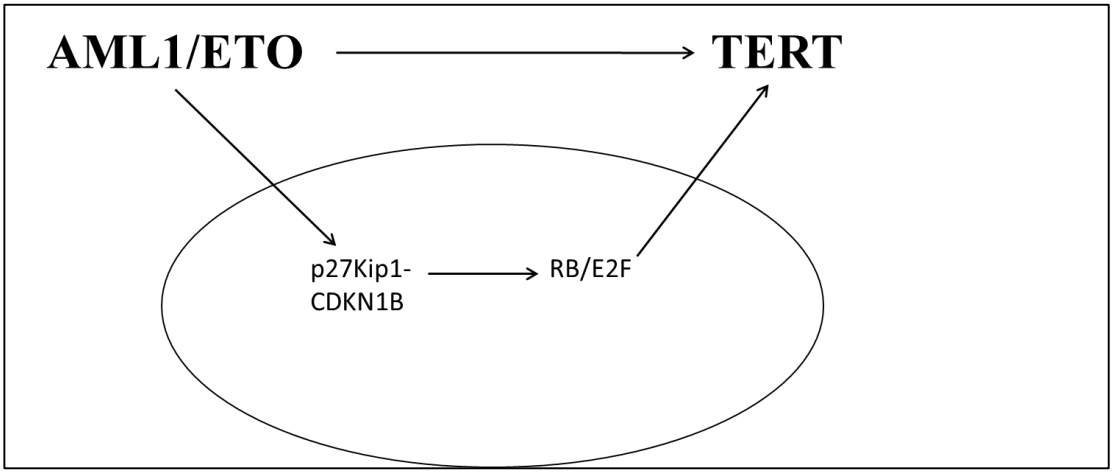

Figure 1.1: Postulation of TERT regulation by AML1/ETO. It is postulated that AML/ETO regulates TERT via the p27- RB/E2F axis

1.3 Hypothesis and objective of the study

1.3.1 Hypothesis

Null Hypothesis

AML/ETO does not regulate TERT via the p27-RB/E2F axis

Alternative Hypothesis (Hypothesis of this study)

AML1/ETO regulates TERT via the p27-RB/E2F axis

1.3.2 Objective of the study

The main objective of this study is to elucidate the mechanism of TERT regulation by the leukaemic fusion gene AML1/ETO. The specific objectives are as follows;

1. To suppress AML1/ETO, *CDKN1B* and *SKP2* genes in t (8;21) AML cell lines using small interfering RNAs (siRNAs) in order to understand the underlying mechanism of TERT regulation by AML1/ETO

2. To investigate the effects of *AML1/ETO*, *CDKN1B* and *SKP2* suppression on AML/ETO, TERT, p27, E2F, RB and SKP2 genes and proteins

3. To investigate the effects of TERT loss on cell cycle, clonogenecity and cellular senescence after *AML1/ETO*, *CDKN1B* and *SKP2* supression

4. To study interactions between AML1/ETO, TERT, p27, E2F, RB and SKP2 and develop a model to explain the mechanism of TERT regulation by AML1/ETO.

CHAPTER 2: LITERATURE REVIEW

2.1 Acute Myeloid Leukaemia (AML)

Acute myeloid leukaemia (AML) is a malignant disease which stems from clonal disorder of haematopoietic stem cells. It usually causes a differentiation block which results in accumulation of immature blood cells (blasts) in the bone marrow. Production of healthy haemopoietic elements are greatly reduced and haematopoiesis is hampered (Ferarra & Schifer, 2013). The abnormal accumulation of blast is caused by various inherited and acquired genetic mutations which disrupt normal mechanisms of self-renewal, proliferation and differentiation (Gilliland, 1998; Dash & Gilliland 2001; Gilliland & Tallman, 2002).

Several predisposing environmental factors and inherited genetic disorders have been associated with the development of AML (Rubnitz *et al.,* 2010). Environmental factors include exposure to chemotherapy and ionizing radiations, petroleum products, benzene (organic solvents), pesticides (organophosphates) and herbicides (Yin, *et al.,* 1996; McBride, 1997; Korte *et al.,* 2000; Mills & Zahm, 2001). Inherited genetic disorders that have been implicated in AML development include Down syndrome, Fanconi anaemia, Schwachman-Diamond Syndrome, Diamond-Blackfan syndrome, neurofibromatosis syndrome, noonan syndrome and *dyskeratosis congenita* among others (Bader & Miller, 1978; Bader-Meunier *et al.,* 1997; Rosenberg *et al.,* 2003).

AML afflicts about 3.8 per 100,000 people among the whites and about 3.2 per 100 000 people among the Asian populations (Howlader *et al.,* 2014). It is the most common form of acute leukaemia and is more prevalent in males than female amongst adults (Meng *et al.,* 2013). Due to its heterogeneity, AML produces variable clinical outcomes or prognosis (Marcucci *et al.,* 2011).

2.1.1 Classification of AML

Two systems of AML classification are widely accepted globally. The first system is the French-American-British (FAB) system. This system is primarily based on the morphology of cells and status of cell maturation as well as differentiation (Bennett *et al.,* 1985; Cheson, 1990).

The second system was introduced by the World Health Organization (WHO) in 2001. This system takes into account the morphology, cytogenetics, molecular genetics and immunologic properties of AML thus giving it more accuracy and prognostic validity (Vardiman *et al.,* 2002). The WHO classification was last updated in 2008. In this latest update of the classification, AML was broadly classified into four different categories namely AML with recurrent genetic abnormalities, AML with myelodysplasia related changes, therapy related neoplasms and AML not otherwise specified-NOS (Vardiman *et al.,* 2009).

AML classifications according to FAB and WHO systems are presented in Tables 2.1 and 2.2.

Table 2.1: AML classification according to French-American-British system. Table is taken from Tenen, 2003

Table 2 | **French–American–British (FAB) classification of AML**

FAB subtype	Description	Comments
M0	Undifferentiated	Myeloperoxidase negative; myeloid markers 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(15;17) PML–RARα or another translocation involving RARα
M4	Myelomonocytic	
$M4_{Eo}$	Myelomonocytic with bone-marrow eosinophilia	Characterized by inversion of chomosome 16 involving CBFβ, which normally forms a heterodimer with AML1
M5	Monocytic	
M6	Erythroleukaemia	
M7	Megakaryoblastic	GATA1 mutations in those associated with Down's syndrome

AML1, acute myeloid leukaemia 1; APL, acute promyelocytic leukaemia; PML, promyelocytic leukaemia; RARα, retinoic-acid receptor-α. Modified from REF. 65.

Table 2.2: AML classification according to WHO system. Table is based on Vardiman *et al.,* 2009**.**

1	AML with recurrent genetic abnormalities
	AML with t(8;21)(q22;q22)
	RUNX1-RUNX1T1
	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;p22); CBFB-MYH11
	Acute promyelocytic leukemia with t(15;17)(q22;q12); PML-RARA
	AML with t(9;11)(p22;q23)MLLT3-MLL
	AML with t(6:9)(p23;q34); DEK-NUP214
	AML with inv(3)(q21q26.2) or t(3.3)(q21;q26.2); RPN1-EVl1
	AML (megakaryoblastic) with t(1:22)(p13;q13); RBM15-MKL1
	Provisional AML with mutated NPM1
	Provisional AML with mutated CEBPA
2	AML with myelodysplasia-related changes
3	Therapy-related myeloid neoplasms
4	Acute myeloid leukemia, NOS
	AML with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic and monocytic leukemia
	Acute erythroid leukemia
	Acute megakaryoblastic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis
	Myeloid sarcoma
	Myeloid proliferations related to Down syndrome
	Transient abnormal myelopoiesis
	Myeloid leukemia associated with Down syndrome
	Blastic plasmacytoid dendritic cell neoplasm

2.2 Cytogenetic Abnormalities in AML

One prominent feature of AML is the accumulation of cytogenetic abnormalities caused by chromosomal aberrations. In a large cohort multinational study carried out by United Kingdom Medical Research Council, over 54 types of cytogenetic abnormalities were discovered in a group of 5876 patients with AML. The age group of the patients ranged from 16-59 years of age. About 2432 patients (41%) exhibited normal karyotype. The rest had various types of chromosomal abberations. This included monosomy (e.g monosomy 3, 5, 7 and 9), trisomy (e.g trisomy 4, 6, 8, 11, 21), deletions (e.g del 5q, del 7q, del 13q), additions (e.g add 5q, add 7q), and translocations [e.g t(8;21), t(15;17), t(9;11)] (Grimwade *et al.,* 2010). Cytogenetic abnormalities serve as an important prognostic tool and can be widely classified into three different subgroups. This include those with favourable clinical outcome, intermediate clinical outcome and adverse or poor clinical outcome (Shipley & Butera, 2009).

Chromosomal abnormalities t(8;21), t(15;17) and t(16;16) are usually associated with good clinical prognosis (Grimwade & Hills, 2009). Patients with t(8;21) and t(16;16) who have undergone chemotherapy with high doses of cytarabine had cure rates of more than 60% (Byrd *et al.,* 2004; Bloomfield *et al.,* 2004) while patients with t(15;17) who had undergone treatment with all-trans retinoic acid (ATRA) had curable rates of up to 90% (Wang & Chen, 2008).

Patients with normal karyotype, t(9;11), del 7q, del 9q and other non-complex karyotype are usually placed in the intermediate group (Grimwade *et al.,* 1998; Shipley & Butera, 2009). Patients with these abnormalities have a worse prognosis than the first group but better if compared to the third group.

Patients with chromosomal aberrations t(9;22), t(6;9), abnormal 3q26, del 5q, t(11;17), t(3,3) and complex karyotype among others have the worst prognosis among all the groups (Ferrara & Schifer, 2013; Grimwade *et al.,*1998; Shipley and Butera, 2009). Complex karyotype are defined as patients with 4 or more unrelated types of chromosomal abnormalities (Grimwade *et al.,* 2010). In a study done by Slovak et al 2006, patients with t(6;9) responded very poorly to chemotherapy. In another separate study, chemotherapy was found to have an adverse effect on patients with abnormal 3q26 (Lugthart *et al.,* 2008).

There are several limitations of using karyotyping as a prognostic tool. Unsuccessful cytogenetic analysis and presence of cryptic aberrations are some of the limitations (Grimwade & Hills, 2009). Another factor to be taken into consideration is the fact that 40-50% of patients with AML have normal karyotype (Grimwade *et al*; 2010, Lin &Smith, 2011; Meng *et al.,* 2013). Therefore, mutations in other genes such as Nucleophosmin 1 gene (*NPM1*), CCAAT/enhancer binding protein alpha (CEBPA) and FMS-related Tyrosine Kinase 3 gene (FLT3) have to be taken into account before a more comprehensive risk stratification of AML patients could be made (Lin & Smith, 2011).

2.3 Mutations in AML.

AML is also represented by a plethora of other genetic mutations. Some of these mutations have been documented well and serve as important prognostic indicators especially when a normal karyotype is present or the cytogenetic analysis is unsuccessful (Lin & Smith, 2011; Marcucci *et al.,* 2011)

2.3.1 Nucleophosmin 1 (*NPM1*) mutations

Nucleophosmin 1 is a nuclear cytoplasmic shuttling phosphoprotein (Gianfelici *et al.,* 2012). Nucleophosmin 1 mutation was first discovered by using immunohistochemical analysis to observe the abnormal cytoplasmic localization of NMP1 protein (Falini *et al.,* 2005). Ever since, more than 50 mutations in exon 12 of the *NPM1* gene have been reported (Falini *et al.,* 2007). The main function of *NPM1* is to transport pre-ribosomal particles from the nucleus to the cytoplasm. Mutation of *NPM1* causes this transport pathway to be defective (Lin & Smith, 2011). NPM1 has also been shown to interact with CDK2-cyclin E and regulate the ARF-p53 tumor suppressor pathway (Falini *et al.,* 2007).

This gene mutation is by far the most common mutation in AML and is present in almost one third of all patients with *de novo* AML. This mutation is usually found in 45%-64% of patients with normal karyotype and 35-40% of patients with trisomy 8. Other mutations are usually present with *NPM1* as well. Over 40% of patients with *NPM1* mutations have *FLT3-ITD* mutations as well, 10% have *FLT3-TKD* mutations and 25% have *IDH* mutations (Marcucci *et al.,* 2011; Gianfelici *et al;* 2012).

Patients with *NPM1* mutations have good clinical outlook in general (Schnittger *et al.,* 2005). However those with accompanying *FLT3-ITD* mutations have a poor prognosis (Dohner *et al.,* 2005; Thiede *et al.,* 2006).

2.3.2 CEBPA mutations

The CCAAT/enhancer binding protein alpha (CEBPA) is a key transcription factor in haematopoiesis and is involved in granulocyte maturation (Pabst *et al.,* 2001a; Marcucci *et al.,* 2011). It is found in about 7% of all patients with *de novo* AML. It is also present in about 10-18% patients with normal karyotype and 40% of patients with del 9q (Frohling *et al.,* 2004; Bienz *et al.,* 2005; Ferrara & Schifer, 2013).

Mutations are usually found in both the N-terminal and C-terminal of the protein and are heterozygous (bi-allelic) in nature. Mutations in the N-terminal produce a truncated protein with dominant negative properties. Mutations in the C-terminal occur at the leucine zipper domain and affects DNA binding as well as dimerization properties of the protein (Marcucci *et al.,* 2011).

Patients with this mutation, especially those with double mutations (both N and C terminal) have a good clinical prognosis (Schlenk *et al.,* 2008; Wouters *et al.,* 2009; Dufour *et al.,* 2010).

2.3.3 FMS-related tyrosine kinase 3 (*FLT-3*) mutations

FLT-3 protein is a member of the class 3 receptor tyrosine kinase family also more commonly known as Platelet-derived growth factor receptors (PDGF-R). It is vital for the survival, proliferation and differentiation of hematopoietic progenitor cells **(**Marcucci *et al.,* 2011). There are two types of FLT-3 mutations namely *FLT- ITD* mutations and *FLT-3 TKD* mutations (Ferrara & Schifer; 2013).

FLT-3-ITD mutations occur in about 20-25% of all AML patients. It is present in about 30-35% of patients with normal karyotype (Ferrara & Schifer, 2013). Mutations in FLT-3-ITD occur primarily in the juxta-membrane (JM) region (Lin & Smith, 2011). However, *ITD* mutations have been reported in the tyrosine kinase 1 (TK1) region as well (Breitenbuecher *et al.,* 2009).

FLT-3 TKD mutations are less common and occur in about 5% of all AML patients. It is present in 10-14% patients with normal karyotype and 14-24% of patients with inv(16) (Marcucci *et al.,*2011; Ferrara & Schifer, 2013). *TKD* mutations primarily occur in the carboxyl terminal and mostly affect codons 835 and 836 in the form of point mutations, small insertions and deletions (Frohling *et al.,* 2005; Dohner & Dohner 2008).

FLT-3-ITD mutations generally heralds a poor prognosis for the patients even in the presence of *NPM-1* mutations (Dohner *et al.,* 2005; Dohner & Dohner, 2008; Mrozek *et al.,* 2007). The clinical prognosis of *FLT-3 TKD* mutations are still non conclusive (Li *et al.,* 2012).

2.3.4 *KIT* gene mutations

KIT protein is a PDGF-R as well. It is important for the survival, differentiation and activation of hematopoietic progenitor cells (Marcucci *et al.,* 2011). It is found in 25-30% of patients with core binding factor (CBF) leukaemia (Paschka, 2008). *KIT* is not only mutated but also highly expressed in CBF leukaemia (Bullinger *et al.,* 2004; Valk *et al.,* 2004). *KIT* mutations have been associated with poor clinical outcome (Marcucci *et al.,* 2011).

2.3.5 Isocitrate Dehydrogenase (IDH1/IDH2) mutations

Isocitrate dehydrogenase (IDH1/IDH2) is a metabolic enzyme that converts isocitrate to α-ketoglutarate. IDH1 is localized in the cytosol while IDH2 is localized in the mitochondria. One arginine mutations has been found in IDH1 (R132H/C/L/I/S/G/V) while two arginine mutations have been found in IDH2 (R140Q/W, R172K/M/G/W). IDH 1 R132H amino acid substitution is usually found together with *NPM1* mutations in AML while IDH2 mutations have a distinct gene expression and microRNA profile (Marcucci *et al.,* 2010; Paschka *et al.,* 2010; Boissel *et al.,* 2010, Yang *et al.,* 2012).

IDH mutations were first discovered in brain tumours (Mardis *et al.,* 2009) and only later in AML (Schnittger *et al.,* 2010). *IDH* mutation is found in 15-20% of all AML patients in general. *IDH1* mutations are found in 10-16% patients with normal karyotype while 10-19% of patients with normal karyotype have *IDH2* mutations (Marcucci *et al.,* 2010; Paschka *et al.,* 2010). Patients with a combination of *IDH* and

NPM1 mutations have a poor prognosis whereas patients with *IDH* mutations alone tend to have slightly better clinical remission (CR) rates (Marcucci *et al.,* 2010; Paschka *et al.,* 2010; Boissel *et al.,* 2010).

IDH mutations are distinct in AML as they involve a metabolic enzyme. Mutations in IDH render cells with the ability to convert α-ketoglutarate to 2-hydroxyglutarate (2-HG) which is potential oncogenic substrate (Ward *et al.,* 2010). It inhibits α-ketoglutarate dependent dioxygenases such as lysine histone demethylases and the ten eleven translocation (*Tet*) family of DNA hydroxylases which includes *Tet2*. *Tet2* mutations are commonly found in myeloid malignancies such as myelodysplasia and AML (Mullighan, 2009; Yang *et al.,* 2012)

2.3.6 *WT1* mutations

WT1 is a transcription factor that is associated with the regulation of apoptosis and differentiation in hematopoietic progenitor cells. *WT1* mutations occur in about 10-13% of AML patients with normal karyotype (Paschka *et al.,* 2008; Virappane *et al.,* 2008). Single nucleotide polymorphism (SNP) rs16754 in *WT1* mutations have been shown to be an indicator of poor prognosis (Damm *et al.,* 2010).

There have been conflicting results regarding the prognostic value of this mutation. Some study groups have shown a poor clinical outcome for subgroup of AML patients with this mutation (Paschka *et al.,* 2008; Virappane *et al.,* 2008) while others indicate that there is no difference in the clinical outcome of patients with this mutation (Gaidzik *et al.,* 2009). This discrepancy could be due to differences in post-remission treatment modalities (Marcucci *et al.,* 2011).

2.3.7 MLL mutations

MLL is a DNA binding protein which induces leukaemia via epigenetic regulation of tumor suppressor genes. MLL mutations with partial tandem duplication (MLL-PTD) is present in 5-11% of patients with normal karyotype and up to 90% of patients with trisomy 11. (Mrozek *et al,* 2007; Dohner & Dohner, 2008). DNA methyltransferase and histone deacetylase (HDAC) inhibitors could serve as an alternative treatment for AML patients with this mutation (Marcucci *et al.,* 2011). Generally, patients with these mutations have a poor clinical outcome. However, recent studies have shown that autologous hematopoietic stem cell (HSC) transplant and consolidation cycles of chemotherapy seem to offer a slight benefit to patients with this mutation (Mrozek *et al,* 2007; Dohner & Dohner, 2008).

2.3.8 Other Mutations in AML

There are various other genes that are mutated in AML as well. These include *NRAS*, *TP53, TET2* and *ASXL1* genes. *NRAS* encodes for membrane associated proteins involved in proliferation and differentiation while *TP53* encodes for the tumour suppressor protein p53. *TET2* could possibly be involved in epigenetic mechanisms while *ASXL1* plays an important role in chromatin remodelling (Neubauer *et al.,* 2008; Haferlach *et al.,* 2008; Delhommeau *et al.,* 2009; Langemeijer *et al.,* 2009; Abdel-Wahab *et al.,* 2009).

The mutations mentioned above have not been evaluated thoroughly as a prognostic tool as its frequency is relatively low in AML patients. Nonetheless, it is important to carry out future clinical trials on these potential biomarkers as they could aid in risk stratification of patients with AML (Nibourel *et al.,* 2010; Carbuccia *et al.,* 2010).

2.4 Fusion oncoproteins in AML

The first fusion onco-protein (BCR-ABL) was discovered in chronic myeloid leukaemia (CML) (Druker, 2008). This led to the research of a myriad of other fusion protein in various cancers. The importance of fusion proteins is especially apparent in myeloid malignancies and this includes AML (Gianfelici *et al.,* 2012).

One of the hallmarks of AML is the formation of leukaemic fusion onco-proteins. More than 750 chromosomal translocations have been identified to date (Mitelman *et al.,* 2009). These proteins are a result of gene rearrangements caused by non-random chromosomal translocations (Look, 1997). Leukemic fusion oncoproteins primarily interfere with the nuclear receptor signalling and transcriptional program of the cell. Other changes that are induced include chromatin modifications and structural organization (Scandura *et al.,* 2002; Martens *et al.,* 2010).

The four most common oncofusion proteins in AML are PML/RARα, AML1/ETO, CBFβ/MYH11 and MLL-fusions. There are other fusion proteins such as MOZ-CBP, DEK-CAN and RPN1-EVI1 but these proteins are less prevalent (Martens *et al.,* 2010). A list of AML fusion oncoproteins together with their chromosomal translocation and prognosis is shown in Table 2.3.

Table 2.3: Types of oncofusion proteins in AML (Adapted from Martens *et al.*, 2010).

Translocation	Prognosis	FAB	Oncofusion-protein	Prevalence in AML
t(8;21)	Favourable	M2	AML1/ETO	10%
t(15;17)	Favorable	M3	PML-RARα	10%
inv(16)	Favorable	M4	CBFβMYH11	5%
der(11q23)	Variable	M4/M5	MLL-fusions	4%
t(9;22)	Adverse	M1/M2	BCR-ABL1	2%
t(6;9)	Adverse	M2/M4	DEK-CAN	<1%
t(1;22)	Intermediate	M7	OTT-MAL	<1%
t(8;16)	Adverse	M4/M5	MOZ-CBP	<1%
t(7;11)	Intermediate	M2/M4	NUP98-HOXA9	<1%
t(12;22)	Variable	M4/M7	MN1-TEL	<1%
inv(3)	Adverse	M1/M2/M4/M6/M7	RPN1-EVI1	<1%
t(16;21)	Adverse	M1/M2/M4/M5/M7	FUS-ERG	<1%

2.4.1 PML-RARα t(15;17)

Translocation t(15;17) causes the *RARα* gene from chromosome 17 to fuse with the *PML* gene on chromosome 15. This gene rearrangement causes the formation of the fusion protein PML-RARA. This fusion protein is present in up to 95% of patients with acute promyelocytic leukaemia (APL). On rarer occasions (~2%), other partner genes such as *PLZF*, *NPM1*, *NuMa* and *STAT5b* fuses with the *RARα* gene (Martens *et al.,* 2010; Gianfelici *et al.,* 2012).

RARα is a nuclear receptor which heterodimerizes with retinoid X receptor (RXR) and functions as a ligand inducing transcription factor. The absence of a ligand causes this complex to repress transcription and condense the chromatin via interaction with co-repressors that have histone deacetylase (HDAC) activities. PML-RARα fusion protein interacts with RXR via PML coiled-coil domains to form the PML-RARPML-RARα complex which has a slightly different DNA binding ability. The altered DNA binding properties enables this complex to block differentiation and promote self-renewal (Melnick & Licht, 1999; Dilworth & Chambon, 2001).

AML patients with this subgroup of mutation have been very responsive to chemotherapy treatment with all-*trans*-retinoic acid (ATRA) and arsenic trioxide with over 70% success rate (Sanz & Lo-Coco, 2011; Avvisati *et al.,* 2011; Breccia *et al.,* 2011). This is because ATRA and arsenic has the ability to degrade PML-RARα which in turn causes apoptosis and abolishes the self-renewing capability of the leukemic cells (Ablain & de The, 2011).

2.4.2 AML1/ETO t (8;21)

AML1/ETO fusion protein is formed by the chromosomal translocation t (8;21). This aberration fuses the region coding for the N-terminal of the AML1 (RUNX1) on chromosome 21 to almost the entire open reading frame of ETO (RUNX1T1) gene on chromosome 8 thus creating the fusion protein AML1/ETO. This is the most common chromosomal translocation found in adult AML. The structure and function of this versatile oncoprotein will be described further in section 2.7.

2.4.3 CBFβ–MYH11 inv(16)

Inv(16) gene rearrangement causes the first 165 amino acid residues of core binding factor beta (CBFβ) to fuse with the C-terminal coiled-coil region of a smooth muscle myosin heavy chain (MYH11) thus forming the fusion protein CBFβ–MYH11 (Liu *et al.,*1993). This fusion protein can cause monocytic and eosinophilic differentiation (Martens *et al.,* 2010; Gianfelici *et al.,* 2012).

CBFβ–MYH11 contains AML1 binding sites in both the N and C terminal. Transcriptional repression is achieved via interactions of AML1 with mSinA and Groucho co-repressors. The C-terminal of MYH11 also plays an important role in repression by recruiting SIN3A and HDAC8 (Lutterbach *et al.,* 1999; Lutterbach & Hiebert, 2000, Lukasik *et al.,* 2002). Subgroup of patients with this mutation have a relatively good prognosis (Marcucci *et al.,* 2011; Ferrara & Schifer, 2013).

2.4.4 MLL rearrangements

MLL fusions are present in about 10% of all acute leukaemias. This include acute lymphoblastic leukaemia (ALL), biphenotypic acute leukaemia, infant leukaemia and acute myeloid leukaemia (AML) treatment related leukaemia (Eguchi *et al.,* 2005). Patients with this subgroup of mutations have a relatively poor clinical outlook in AML

MLL is important for hematopoiesis and has methyltransferase activity. It regulates gene expression via chromatin acetylation, methylation and nucleosome modifications (Druker, 2008).

The N-terminal of MLL has two DNA binding domains, namely the AT-hook region and the CXXC domain. AT- hook domains interacts with menin (MEN1) while the CXXC domains recruit HDAC components by binding to CpG dinucleotides thus acting as a transcriptional repressor (Zeleznik-Le *et al.,* 1994).

The C-terminal region (SET, TAD and PHD fingers) of the *MLL* gene is replaced with a fusion partner of whom more than 70 have been identified to date. The most common MLL fusion in AML is MLLT3 (AF9). These fusion partners can either be nuclear proteins or cytoplasmic proteins. However, in most cases, the MLL fusion partner is a nuclear protein. MLL fusions with cytoplasmic proteins as fusion partners have less transforming capabilities and the mechanism of its action is not fully understood (Gianfelici *et al.,* 2012).

In MLL fusions, the amino terminal (N-terminal) acts as a targeting unit to direct the oncoprotein complex while the fusion partner acts as an effector and is responsible for transactivation of the complex (Slany *et al.,* 1998; Ayton *et al.,* 2004).

Potential treatment of MLL fusions could involve the disruption of MLL protein-protein interactions by targeting menin (MEN1). This could be achieved by using peptides and small molecule inhibitors (Grembecka *et al.,* 2010). DNA demethylating agents and specific methyltransferase inhibitors such as Dot1L inhibitors are some other potentially attractive alternatives (Liedtke & Clearly, 2009; Daigle *et al.,* 2011, Daigle *et al.,* 2013).

2.5 AML1: The N-terminal fusion partner of AML1-ETO

AML1 is a member of the runt related group of transcription factors (RUNXs). Other members of this group include the RUNX2 and RUNX3 proteins. Other names for AML1 include RUNX1, CBFA2 and PEBP2αB (Van Wijnen *et al.,* 2004). This protein was discovered when studying the chromosomal breakpoint caused by t(8;21) chromosomal aberration in AML (Miyoshi *et al.,*1991). This protein has also been proven to be an indispensable component of *Drosophila* embryogenesis (Nusslein-Volhard *et al.,* 1984; Kania *et al.,* 1990; Kagoshima *et al.,* 1993). AML1 has also been detected as serine and threonine phosphorylated protein (Erickson *et al.,* 1996).

2.5.1 Structure of AML1

AML1 consist of three major domains namely the RUNT domain, the transactivation domain and the negative regulatory elements (Meyers *et al.,* 1993; Levanon *et al.,* 1998; Petrovick *et al.,* 1998).

The RUNT domain occupies 128 amino acid residues on the N-terminal (Exons 2, 3 and 4). The primary function of this domain is to mediate binding of DNA to core binding factor beta; CBF-β (Ogawa *et al.,* 1993; Wang *et al.,* 1993).

The C-terminal of RUNX-1 has three very important regions namely the transactivation domain (TD), the NMTS domain and the VWRPY motif. These domains act as negative regulatory elements. The transactivation domain is located on exon 6 and facilitates protein-protein interactions. Nuclear matrix targeting signal (NMTS) domain is a 31 amino acid region which is involved in transcriptional activation of target genes (Zeng *et al.,* 1998), while the VWRPY motif which is located at the very end of the C-terminal exhibits Groucho and TLE dependent transcriptional repressor activity (Aronson *et al.,* 1997; Levanon *et al.,* 1998). Both NTMS domains and VWRPY motifs have been shown to be important for T-cell development (Telfer *et al.,* 2004).

Over 12 mRNA isoforms of *AML1* have been described (Levanon *et al.,* 2001). However, there are predominantly three major isoforms; *AML1a, AML1b* and *AML1c*. These isoforms are a consequence of alternative splicing (Miyoshi *et al.,* 1995). *AML1a* is significantly shorter than the rest as it lacks the C-terminal. The C-terminal of *AML1* has been specifically shown to be important for haematopoiesis as the absence of it caused abnormal haematopoiesis (Lam & Zhang, 2012). *AML1c* is transcribed by a promoter situated on the distal region of the *AML1* locus while *AML1a* and *AML1b* is transcribed by a promoter on the proximal region (Tsuzuki *et al.,* 2007; Liu *et al.,* 2009). The promoter on the proximal side plays an important role in the initial events of hematopoiesis while the distal promoter regulates more mature progenitor cells (Sroczynska *et al.,* 2009).