DETERMINATION OF GENE COPY NUMBER STATUS IN *TERT* AND *TERC* AMONG CHRONIC MYELOID LEUKAEMIA PATIENTS RESISTANT AND RESPONSIVE TO IMATINIB MESYLATE TREATMENT

by

ZAIDATUL SHAKILA BT MOHAMAD ASHARI

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PENENTUAN STATUS BILANGAN SALINAN GEN DALAM *TERT* DAN *TERC* DI KALANGAN PESAKIT LEUKEMIA MIELOID KRONIK RINTANG DAN RESPONSIF TERHADAP RAWATAN IMATINIB MESYLATE

oleh

ZAIDATUL SHAKILA BT MOHAMAD ASHARI

Tesis yang diserahkan untuk
memenuhi keperluan bagi
Ijazah Sarjana Sains

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DEDICATION

...To my late father and mother, Mohamad Ashari Sabtu and Khatijah Ahmad ~ your words of inspiration and encouragement in pursuit of excellent, still linger on...

...To my sister, Shima who always believed that I can do anything she puts her mind to. And for Khairol, for giving me hope...

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A	absorbance
ALL	adult lymphoid leukaemia, acute lymphoblastic leukaemia
ABL	Ableson proto-oncogene
aCGH	array comparative genome hybridization
AML	acute myeloid leukaemia
AP	accelerated phase
APL	acute promyelocytic leukaemia
ARG	Abelson-related gene
ATP	adenosine triphosphate
ATP10A	ATPase class V type 10A
BACT	β-actin
BCR	breakpoint cluster region
BFM 95	Berlin-Frankfurt-Munster ALL Study Group
BLAST	basic local alignment search tool
bp	base pair
BP	blast phase
CCA	clonal chromosome abnormalities
CCyR	complete cytogenetic response
CGH	comparative genome hybridization
CHR	complete haematological response
CIN	cervical intraepithelial neoplasia
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CML-IM resistant	chronic myeloid leukaemia-imatinib mesylate resistant patients
CML-IM responsive	chronic myeloid leukaemia-imatinib mesylate respond patients
CN	copy number
CNC	copy number calculated
CNP	copy number predicted
CMR	complete molecular response
CP	chronic phase

Cq	quantification cycle or threshold cycle
CR4/5/7	conserved regions 4/5/7
CTE	C-terminal extension
DAPI	4,6-diamidino-2-phenylindole
df	degrees of freedom
dNTP	dinucleotide triphospate
DNA	deoxyribonucleic acid
DN-*hTERT*	dominant-negative-human telomerase reverse transcriptase
DM	double minutes
DMSO	dimethyl sulphoxide
E	efficiency
EDTA	ethylenediamine tetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
ELN	European LeukemiaNet
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3
F	forward, or testing of hypothesis value
FAM	6-carboxyfluorescein
FDA	Food and Drug Administration
FISH	fluorescence *in situ* hybridization
g	gravity
gDNA	genomic DNA
G	guanosine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GIPAP™	Glivec® International Patient Assistant Program
HCl	hydrochloric acid
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HSR	homogeneously staining regions
HLA	human leucocyte antigen
HPV	human papillomavirus
IM	imatinib mesylate
IFN-α	interferon alfa
kDA	kilodaltons

kb	kilo-base
KIT	receptor for stem cell factor
L	litre
m	slope
MAP	mitogen-activated protein
Mb	megabase
mCyR	major cytogenetic response
MDM2	MDM2 oncogene, E3 ubiquitin protein ligase
MDS	myelodysplastic syndrome
MET	met proto-oncogene
m^2	square meter
mg	milligram
$MgCl_2$	magnesium chloride
MGB	dihydrocylopyrroloindole tripeptide *minor groove binder*
minCyR	minor cytogenetic response
mL	mililitre
mM	milimolar
MLPA	multiplex ligation-probe amplification
MMR	major molecular response
mRNA	messenger ribonucleic acid
n	sample size
N	level of amplification
NGS	next-generation sequencing
ng	nanogram
nm	nanometre
noCyR	no cytogenetic response
NSCLC	non-small-cell lung cancer
nt	nucleotide
NTC	no-template control
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCyR	partial cytogenetic response
PDFGRA/B	platelet-derived growth factor receptors
Ph	Philadelphia

Ph+	Philadelphia positive
PI3K	phosphatidylinositol 3-kinase
PNA	peptide nucleic acid
POT1	protection of telomeres 1
PTC	papillary thyroid cancer
qPCR	quantitative real-time polymerase chain reaction
R	reverse
r^2	coefficients of correlation
r.p.m	revolutions per minute
rRNA	18S ribosomal RNA
Rap1	repressor/activator protein 1
RBC	red blood cell
RID	RNA-interaction domain
Rn	fluorescence of reporter dye divided by fluorescence of reference dye
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
scaRNA	small Cajal body-specific RNA
SCT	stem cell transplantation
SD	standard deviation
SNPA	single nucleotide polymorphism array
SKY	spectral karyotyping
SPSS	statistical package for social sciences
SRC	steroid receptor co-activator
SSC	saline-sodium citrate
STAT	signal transducers and activators of transcription
TAMRA	6-carboxytetramethylrhodamine
Taq	*Thermophilus aquaticus*
TBE	template boundary element, or tris-borate EDTA
TCCSG 95-14	Tokyo Children's Cancer Study Group
TEN	telomerase essential N-terminal
TERC	telomerase RNA component
TERT/hTERT	telomerase reverse transcriptase

TET	tetrachlorofluorescei
TIN2	TRF2- and TRF1-interacting nuclear protein 2
TPP1	TIN2 and POT1 interacting protein 1
TRAP	telomeric repeat amplification protocol
TRBD	telomerase RNA-binding domain
TRF1	telomeric repeat binding factor 1
TRF2	telomeric repeat-binding factor 2
UKALL-97/99	United Kingdom Medical Research Council Working Party on Childhood ALL-97/99
US	United States of America
UV	ultraviolet
WBC	white blood cell
WCP	whole chromosome paints
WT-*hTERT*	wild-type *hTERT*
WHO	World Health Organization
X	time
°C	degree celcius
µL	microlitre
µM	micromolar
%	percent
+/-	plus minus
∞	infinity
Δ	delta

PENENTUAN STATUS BILANGAN SALINAN GEN DALAM *TERT* DAN *TERC* DI KALANGAN PESAKIT LEUKEMIA MIELOID KRONIK RINTANG DAN RESPONSIF TERHADAP RAWATAN IMATINIB MESYLATE

ABSTRAK

Rawatan barisan hadapan untuk pesakit-pesakit fasa kronik (CP) leukemia mielod kronik (CML) ialah penggunaan Imatinib Mesylate (IM). Terdapat pesakit-pesakit CP CML (1/3) gagal untuk mencapai mana-mana tahap respon yang baik terhadap rawatan IM. Sel-sel kanser mengekpreskan aktiviti telomerase lebih tinggi berbanding dengan sel-sel normal dalam pelbagai jenis kanser; amplifikasi komponen-komponen teras telomerase, gen *TERT* dan *TERC*, adalah salah satu mekanisma yang bertanggungjawab mengawal atur aktiviti telomerase. Kami andaikan bahawa amplifikasi gen-gen telomerase adalah salah satu mekanisma onkogenesis dalam perkembangan penyakit CML dan terlibat dalam kerintangan terhadap IM. Kajian ini bertujuan untuk menentukan status bilangan salinan gen-gen *TERT* dan *TERC* di kalangan pesakit CML samada rintang atau responsif terhadap rawatan IM.

Sebanyak 63 pesakit CML-IM rintang, 63 pesakit CML-IM responsif, 30 kawalan normal dan 2 titisan sel terlibat di dalam kajian ini. DNA diekstrak daripada darah periferi dan juga pelet sel. Amplifikasi salinan gen-gen *TERT* dan *TERC* dianalisis oleh asai qPCR iaitu secara konvensional (lengkungan piawai) dan asai komersial (Taqman® copy number). Analisis FISH dijalankan untuk mengesahkan kehadiran

amplifikasi gen-gen *TERT* dan *TERC*. Kami juga menilai hubungan antara umur pesakit, jantina dan status rawatan mereka dengan bilangan salinan gen-gen *TERT/TERC*.

Terdapat sedikit peningkatan purata nilai N (kaedah lengkungan piawai) *TERT* dan *TERC* dalam kedua-dua kumpulan pesakit-pesakit CML (CML-IM rintang 0.75 dan 0.82; CML-IM responsive 0.77 dan 0.93) apabila dibandingkan dengan kawalan normal (0.62 dan 0.60). Purata nilai N *TERT* dan *TERC* di antara dua kumpulan pesakit-pesakit CML signifikannya berbeza berbanding kawalan normal. Namun, tiada perbezaan yang signifikan antara purata nilai N *TERT* dan *TERC* di antara pesakit-pesakit CML-IM rintang dan pesakit CML-IM responsif. Hanya seorang pesakit (CML-IM responsif) mempunyai amplifikasi gen *TERT* manakala 5 orang pesakit mempunyai amplifikasi gen *TERC* (2 CML-IM rintang dan 3 CML-IM responsif). Apabila dibandingkan dengan kaedah lengkungan piawai, asai 'Taqman® copy number' mengesan 6 CML-IM rintang dan 11 CML-IM responsif mempunyai amplifikasi *TERT* gene, manakala 5 CML-IM rintang dan 4 CML-IM responsif mempunyai amplifikasi *TERC* gene. Hampir 80% daripada status amplifikasi gen-gen *TERT* dan *TERC* di dalam sampel-sampel CML yang dianalisis melalui teknik lengkungan piawai mempunyai hasil yang sama dengan FISH. Tiada hubungan yang jelas antara amplifikasi gen-gen *TERT/TERC* dengan umur pesakit, jantina dan status rawatan mereka.

Amplifikasi tahap rendah gen-gen *TERT/TERC* dan kekurangan perbezaan statistik yang signifikan antara amplifikasi *TERT/TERC* dalam pesakit-pesakit CML-IM rintang dan CML-IM responsif mungkin tidak kukuh untuk menyatakan bahawa

amplifikasi gen-gen *TERT/TERC* adalah salah satu rintangan molekular terhadap IM. Kami mencadangkan bahawa kenaikan aktiviti telomerase melalui amplifikasi gen-gen telomerase mungkin tidak mempunyai impak yang besar terutamanya dalam rintangan ubat.

DETERMINATION OF GENE COPY NUMBER STATUS IN *TERT* AND *TERC* AMONG CHRONIC MYELOID LEUKAEMIA PATIENTS RESISTANT AND RESPONSIVE TO IMATINIB MESYLATE TREATMENT

ABSTRACT

The frontline treatment of chronic phase (CP) chronic myeloid leukaemia (CML) patients is the use of Imatinib Mesylate (IM). A remarkable CP CML (1/3) patients failed to achieve any degree of good response towards IM treatment. Cancer cells express a higher telomerase activity compared to normal cells in various type of cancers; thus amplification of telomerase core components *TERT* and *TERC* gene, is a mechanism responsible for up regulation of telomerase activity. We postulate amplification of telomerase genes might be one of the oncogenesis mechanisms in CP CML development and involved in resistance to IM. The aim of this study was to determine the gene copy number status of *TERT* and *TERC* among CML patients who were either resistant or responsive to IM.

A total of 63 CML-IM resistant, 63 CML-IM responsive, 30 normal controls and 2 cell lines were enrolled in this study. The DNAs were extracted from peripheral blood and cell pellets. *TERT* and *TERC* genes copy number amplification were analysed by qPCR assay by conventional (standard curve) and commercialized assay (Taqman® copy number). FISH analysis was done to validate the presence of *TERT* and *TERC* genes amplification. We also evaluated the association between patients' age, gender, treatment status and copy number of *TERT*/*TERC* genes.

There was an increase in *TERT* and *TERC* mean N values (standard curve method) in both groups of CML (CML-IM resistant 0.75 and 0.82; CML-IM responsive 0.77 and 0.93) compared to normal controls (0.62 and 0.60). Mean N of *TERT* and *TERC* values between the two groups of CML patients were significantly different from normal controls. There was however no significant difference of mean N *TERT* and *TERC* values between CML-IM resistant patients and CML-IM responsive patients. Only one patient (CML-IM responsive) had *TERT* gene amplification whereas 5 patients had *TERC* gene amplification (2 CML-IM resistant and 3 CML-IM responsive). Comparing to standard curve method, Taqman® copy number assay detected 6 CML-IM resistant and 11 CML-IM responsive had amplified *TERT* gene, whilst 5 CML-IM resistant and 4 CML-IM responsive had amplified *TERC* gene. Almost 80% of *TERT* and *TERC* genes amplification status in CML samples analysed by standard curve method have similar outcomes with FISH. There was no clear association between *TERT/TERC* genes amplification and patients' age, gender and their treatment status.

Our results showed a low level of *TERT/TERC* genes amplification and lack of statistical significant difference between *TERT/TERC* amplification in CML-IM resistant and CML-IM responsive patients may not strongly to show that *TERT/TERC* genes amplification were one of the molecular resistance towards IM. We postulate that increment of telomerase activity via amplification of telomerase genes might not have a huge impact on CML patients particularly in drug resistance.

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a terminology used to describe diseases characterized by abnormal and uncontrolled proliferative growth of cells in parts of the body that leads to death. The basic or primary characteristic of cancer cells is the formation of tumour cells in which the cells is capable to divide rapidly and results in the accumulation of cancer cells. It is categorized as benign if the tumour is growing but unable to invade the surrounding tissues cells whereas it is classified as malignant if the tumour cells spread to nearby or distant tissues. Migration and transformation of the malignant tumours to the other locations or parts of the body from the original tumour site forming the secondary tumour is known as metastasis (Almeida and Barry, 2010).

Cancer in principal is a genetic disease that usually begins when the three types of genes responsible for carcinogenesis (the oncogenes, tumour-suppressor genes or stability genes) are altered or precisely mutated (Fearon, 1997; Vogelstein and Kinzler, 2004). A mutated gene can transform a normal cell into a cancerous cell and uncontrollably divides and finally disperse widely throughout the body. The mutated genes associated with cancer may include germ line mutations which can be inherited from one to the next generation or may acquire and develop through somatic mutations (Chial, 2008).

Cancer is a world burden disease and major health problem as increasing of its incidence is related to aging, growing of the population and lifestyle in economical and developing countries. It is estimated about 12.7 million incidence of cancer (56% of all diseases) and 7.6 million mortality (64% of death) due to cancer occurred in 2008 worldwide (Jemal *et al.*, 2011). In Malaysia, cancer is the third (11.28%) leading cause of mortality after heart diseases (16.49%) and septicaemia (13.38%) in the year of 2007. It is estimated a total of 18 219 newly diagnosed cancer which constitute 8 123 males and 10 096 females. It was reported that there are ten most commonly cancer has been diagnosed among Malaysian population including breast, colorectal, lung, nasopharynx, cervixr, lymphoma, leukaemia, ovary, stomach and liver cancers (Zainal and Nor Saleha, 2011).

Human cancers can be differentiated from one to another based on the origin of the cell in the part of body. Some of the tumours are based on the type of tissues from where they initiate for instance hepatoma is a liver cancer (*hepato* = liver), myeloma is a bone marrow tumour (*myelos* = marrow) or melanoma is a cancer of melanocytes. There are four major classification of human cancer which is carcinomas, sarcomas, lymphomas and leukaemias. Carcinomas arise in the skin or in epithelium of internal organs, glands and body cavities such as in breast, skin, colorectal, prostate or lung. Sarcomas originate in connective tissues for example cartilage, bone, muscle or fat. Both carcinomas and sarcomas are in the form of solid tumours. Lymphomas and leukaemias are types of cancer which do not form solid tumours. Lymphomas originate from the lymphatic system which is a component of the body's immune system such as lymph nodes and lymph vessels whereas

leukaemias are cancers that arise from bone marrow that cause accumulation of immature leucocytes in blood (Almeida and Barry, 2010).

1.2 Haematological malignancies

Haematological malignancies describe a scope of cancers that develop and affect bone marrow, blood and lymph nodes. It allows us to differentiate this type of cancers with cancer in tissues or in solid tumours. If there are abnormal functions in haemopoietic process, it can result in the failure to produce the blood cells, increase cellular proliferation, accumulation of cells at a particular stage of maturation (maturation arrest) or failure of apoptosis (Peixoto *et al.*, 2011). Haemopoiesis is the process responsible in the development of red blood cells (RBC), white blood cells (WBC which is the neutrophils, eosinophils, basophils, monocytes and lymphocytes) and platelets (Gordon-Smith, 2013). There are four broad divisions of haematological malignancies: myeloid neoplasms, lymphoid neoplasms, lymphoproliferative disorders and histiocytic/dendritic cell neoplasms. Leukaemia defines the presence of cancer cells either from myeloid cell lines (RBC, WBC or platelets) or lymphoid cell lines (B cell, T cell, plasma cells or natural killer cells) and can be divided into acute and chronic leukaemia. While lymphoma is a lymphoid malignancy that involved lymph nodes (Lewis and Lee, 2007; Moore *et al.*, 2010).

1.3 Background of the research study

Chronic myeloid leukaemia (CML) is a type of leukaemia that arises from myeloid cell lineage and affects the development of granulocytes, neutrophils, eosonophils and basophils. CML does not cause arrested granulocytes maturation but failure of apoptosis. Increased proliferation of the granulocyte rate as well as apoptotic failure results in increased amount of white cell count in CML. The key feature of CML is the presence of Philadelphia (Ph) chromosome or the fusion product of chromosomal translocation of 9^{th} and 22^{nd} chromosome, the *BCR-ABL* gene (Hughes-Jones *et al.*, 2008).

The introduction of Imatinib Mesylate (IM) in the treatment of CML leads to the recommendation of IM as the initial therapy for the majority of CML patients diagnosed with chronic phase and used in a minority of those diagnosed in advanced phase (accelerated and blast phase) (Breccia *et al.*, 2010). In Malaysia, IM has been used 2001 and widely available and cost-free under the Glivec® International Patient Assistant Program (GIPAP™) to patients who are not insured or unable to afford the treatment (Kim *et al.*, 2010). However, the emergence in resistance to IM observed in some CML patients infers that all patients not respond to the same degree of treatment. In addition, the use of second generation of CML treatment such as dasatinib and nilotinib, even though these drugs are the promising agents to substitute IM and overcoming the resistance in patients (Deininger, 2006), has evoked refinement of patient's supervision and adjustment of cost for treatment. The National insurance scheme in Malaysia does not cover the advanced treatment and IM remains the backbone of treatment for the majority of patients.

There are three known clinical mechanisms of IM resistance: (i) gene mutations in tyrosine kinase domain (*BCR-ABL*), (ii) overexpression of the BCR-ABL protein due to the amplification of *BCR-ABL* gene (the first and second mechanisms are the *BCR-ABL* dependent) and (iii) other signalling pathway, the independent *BCR-ABL* phenomena. In the third mechanism of resistance, the *BCR-ABL* tyrosine kinase remains inhibited by IM but other alternative pathway become activated due to another key enzyme responsible for cell proliferation and immortalization (Melo and Chuah, 2007; Turgeon, 2012).

Telomerase is a ribonucleoprotein enzyme which functions by adding the DNA sequence (TTAGGG) onto the 3' end of telomere sequences in DNA chromosome. The DNA-protein complexes, telomeres, are a protective covering end of chromosome (Meyerson, 2000) which act as a defender to chromosomes from damage, degradation and control the regulation towards cell senescence (Hahn, 2003). The two core components of telomerase ribonucleoprotein enzyme are telomerase reverse transcriptase (TERT) encoded by *TERT* gene which is a catalytic protein and functional telomerase RNA component, TERC encoded by *TERC* gene (Cong *et al.*, 2002).

In most cases of cancer, telomerase activity are up regulated and its level are higher than in normal cell (Kim *et al.*, 1994), and because of this reason telomerase becomes a key marker in cancer and therapy management. Increase in telomerase activity is considered to play a significant role in maintenance and development of cancer cells by sustaining the telomere length since telomerase helps to synthesize telomeric DNA, contributes to the telomeres stability which in turn able to cause

cancer cells to escape its normal limits of proliferation (Meyerson, 2000; Davison, 2007). Tauchi *et al.*, (2002) showed the inhibition of the telomerase activity increased the effect of the IM and reduce the life span of leukaemic cells. Moreover, increased copy number of telomerase core components, *TERT* and *TERC* genes, have been found to be prevalent in many cases of the human cancers (Cao *et al.*, 2008). Gain in the gene dosage of telomerase core components might have an important role in up regulation of telomerase activity in cancer cells thus might contribute in part to resistance of treatment management of CML.

1.4 Importance of the study

In spite of the successful outcome of IM as a standard drug therapy against CML, there are several reports of resistance to IM treatment (Donato *et al.*, 2004). The rationale of this study focuses on the potential activation of the two core telomerase components as a marker in possible drug resistance after treatment with IM. Yamada *et al.*, (2008) found that the presence of telomerase activity together with expression of *TERT* in leukaemic cell line developed IM resistance by escaping the imatinib-induced apoptosis. Based on the evidence that the copy number of *TERT* and/or *TERC* genes are frequently increased in human cancers by gaining of chromosome or amplification of gene (Cao *et al.*, 2008), clearly that the *TERT* and *TERC* genes are important in human cancers. To date, there are no studies on the involvement of *TERT* and *TERC* genes copy number amplification status in CML patients who are resistant or have good respond to IM both on a global platform or in Malaysia. Therefore, detection of *TERT* and *TERC* genes copy number amplification may contribute to the usefulness in CML diagnosis and prognosis. Since an increase in

number of genes often relate to poor prognosis, revelation of pathway resistance to treatment is important. Thus detection of amplification of *TERT* and *TERC* genes may serve as a marker or an indicator in the development of CML disease.

Application of the specific detection method using Taqman chemistries of quantitative real-time polymerase chain reaction (qPCR) (conventional standard curve method and commercialized Taqman® copy number assay) and validation method using fluorescence *in situ* hybridization (FISH) of copy number status *TERT* and *TERC* genes will provide valuable data to other researchers. The results will emphasize the importance of genes copy number status of *TERT* and *TERC* in the pathological process of this malignancy and highlight the essential challenges in order to create an eligible drug against this cancer. The findings may contribute in the association between patients' clinical characteristics and presence of genes copy number of the two core components of telomerase enzyme.

The next chapter, will focus on CML and the involvement of telomerase core components in disease progression and IM treatment resistance.

1.5 Objectives of the study

1.5.1 General objective

To determine the gene copy number status of *TERT* and *TERC* among chronic myeloid leukaemia (CML) patients resistant and responsive to Imatinib Mesylate (IM) treatment.

1.5.2 Specific objectives

1. To compare the status of *TERT* and *TERC* gene copy number amplification between two groups of CML patients (resistant and responsive) using Taqman chemistry of quantitative real-time polymerase chain reaction (qPCR) analysis (standard curve method and Taqman® copy number assay).

2. To validate the gene copy number amplification of *TERT* and *TERC* in CML patients with high copy number using fluorescence *in situ* hybridization (FISH) analysis.

3. To correlate between the gene copy number amplification of *TERT* and *TERC* with clinical characteristics of patients with CML who are IM resistant and IM responsive.

CHAPTER 2

LITERATURE REVIEW

2.1 Leukaemia

Leukaemia is a group of malignant blood cell disease or cancer of white blood cells (WBCs) characterized by the excessive accumulation of abnormal or immature WBCs (blast) in the bone marrow and peripheral blood. The immature WBCs develop in an uncontrolled manner and suppress the production of healthy and normal blood cells. The uncontrolled cell proliferation maybe due to the decrease apoptosis rate and also inadequate differentiation that result in displacement of normal blood cells (Hanahan and Weinberg, 2000; Testa and Riccioni, 2007). There are several types of WBCs based on the morphological appearance. Granulocytes derived from the myeloid stem cell can be divided into neutrophil, eosinophil and basophil. The most common granulocyte in the blood is neutrophil as it acts to recognize and kill bacteria and other microorganisms. Another type of WBC is the non-granulocyte such as monocyte and lymphocyte. Monocyte cells are also derived from the myeloid stem cell and it will turn into macrophage once it is mature and able to ingest the foreign antigen. Lymphocytes are derived from the lymphoid stem cell. Lymphocytes mature and differentiate into either B lymphocytes or T lymphocytes. Lymphocytes are involved in immune protection as it produces antibodies against the foreign cells (Ball and Kagan, 2009).

2.1.1 Subtypes of leukaemia

There are several types and subtypes of leukaemia and it can be divided either acute form or chronic form. Acute leukaemia usually develops in a short duration and has a rapid onset. In acute leukaemia, the leukaemic cells are capable to divide but the daughter cells are blocked from maturing and differentiating during early cell development. This arrest in maturation leads to the accumulation of blast cells in bone marrow which replaces the normal cells and release into the blood circulation as malignant cells. Whereas in chronic leukaemia, the disease has an insidious onset. The differences between chronic and acute leukaemia is that the chronic leukaemic cells is capable to proliferate without arrestment of cell maturation yet it still grows an unregulated manner (Winkelstein *et al.*, 1998; Szczepański *et al.*, 2003).

The four major subtypes of leukaemia are acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), acute lymphoblastic leukaemia (ALL) and chronic lymphocytic leukaemia (**Table 2.1**). Other types of leukaemia which are rare, include hairy cell leukaemia, prolymphocytic leukaemia and leukaemic phase of lymphoma (Ball and Kagan, 2009). The classification of different subtypes of leukaemia is based on the cell's origin such as those malignant cells derived from myeloid stem cell are classified as myeloid neoplasm whereas other cells derived from lymphoid stem cell are classified as lymphoid neoplasms. The World Health Organization (WHO) proposed several criteria in classifying the different haematological disorder which is based on the histopathological (morphology and immunophenotype), clinical features, molecular and genetic features (Mughal *et al.*, 2006). WHO 2008 classified five major lymphoid neoplasms with more than 100 subtypes of lymphoid

neoplasms under the five major types; and eight major myeloid neoplasms with more than 100 myeloid neoplasm subtypes (reviewed in Vardiman *et al.*, 2009; Campo *et al.*, 2011).

2.1.2 Incidence and prevalence of leukaemia

Leukaemia amongst the ten most common cancers worldwide. The incidence and mortality of leukaemia is approximately 100 per 100 000 population (the number of new cases of leukaemia occurred during certain period) and 60/100 000 incidence and mortality in 2008 worldwide (Boyle and Levin, 2008). In 2012, the incidence of leukaemia was estimated 26 830 in males and 20 320 in females per 1 638 910 new cases of cancers and mortality was estimated 13 500 in males and 10 040 in females per 577 190 deaths occurred in United States (Siegel *et al.*, 2012).

In Asia, leukaemia is the seventh most common cancer after stomach, lung, liver, colorectal, oesophagus and breast cancers. It is estimated in 2008 there were more than fifty thousand incidence of leukaemia (out of 2 213 000 cases of all cancers) in males and less than fifty thousand incidence of leukaemia (out of 1 476 000 deaths of all cancers) in females. There were approximately fifty thousand deaths (out of 1 629 000 deaths of all cancers) reported in males and thirty thousand deaths (out of 946 000 deaths of all cancers) reported in females (Boyle and Levin, 2008).

Based on the Malaysian National Cancer Registry data (2003), leukaemia is the fourth most common cancer in males and seventh most common among females in Peninsular Malaysia (Chang and Ong, 2008). In Peninsular Malaysia there was

estimated 3.6% per 100 000 of haematological disorders occurred in men and 2.2% per 100 000 occurred in women. Malay ethnic was accounted a higher percentage (3.6%) having leukaemia compared to other ethnic groups, 3.2% in Chinese and 2% in Indian (Zainal *et al.*, 2006). The survival rate for leukaemia depend on accessibility to treatment, the cost of drugs, the socioeconomic status of the country and varies in different regions of the world. The rates for all types of leukaemia in USA and Western Europe were the highest (43% in men and 45% in women), Eastern Europe (29% both in men and women), Japan (25% among men and 29% among women), India (19% in both genders), South America (24% in both genders), Thailand (15% both), Sub-Saharan Africa (14% in men, 17% in women) were reported (Boyle and Levin, 2008).

Table 2.1 Classification of four major subtypes of leukaemia

Types of leukaemia	**Type of cell involved**	**Disease criteria based on genetic or molecular abnormalities**	
Acute myeloid leukaemia (AML)*	Proliferation of immature blast cells; AML, myeloblast; ALL, lymphoblast	Genetic abnormalities AML with recurrent genetic abnormalities: AML with t(8;21)(q22;q22) AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22) Acute promyelocytic leukemia with t(15;17)(q22;q12) AML with t(9;11)(p22;q23) AML with t(6;9)(p23;q34) AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) AML (megakaryoblastic) with t(1;22)(p13;q13)	Molecular abnormalities *RUNX*1-*RUNX1T*1 *CBFB-MYH*11 *PML-RARA* *MLLT3-MLL DEK-NUP*214 *RPN1-EVI1* *RBM15-MKL*1
Acute lymphoblastic leukaemia (ALL)[i]		Genetic abnormalities B-ALL/LB with t(9;22)(q34;11.2) B-ALL/LBL with t(12;21)(p13;q22) B-ALL/LBL with t(v;11q23) T-ALL/LBL with del (9)(p21) T-ALL/LBL with 1p32 deletion T-ALL/LBL with t(10;14)(q24;q11) or t(7;10)(q35;q24)	Molecular abnormalities *BCR-ABL1* fusion *ETV6-RUNX1 (TELAML1)* fusion MLL rearranged; t(4;11)(q21;q23) *AFF1/AF4-MLL* *CDKN2A (p16)* *SIL/TAL1* *TLX1 (HOX11)-TCRA/D* or *TCRB-TLX1(HOX11)*, respectively
Chronic myeloid leukaemia (CML)	Proliferation of mature cells; CML, myelocytes (neutrophils, eosinophils, basophils); CLL, lymphocytes	Genetic abnormalities t(9;22)(q34;q11)	Molecular abnormality *BCR-ABL*-1-positive
Chronic lymphocytic leukaemia (CLL)[j]		Genetic abnormalities Deletion (13q13), Trisomy 12, deletion (11q), deletion (17p)	

Classification of acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), acute lymphoblastic leukaemia (ALL) and chronic lymphocytic leukaemia (CLL) based on World Health Organization (WHO) classification of tumours of haematopoietic and lymphoid tissues 2008 (reviewed in Jones, 2010; Sabattini *et al.*, 2010; Campo *et al.*, 2011; Grigoropoulos *et al.*, 2013).

* Not all AML subtypes are listed. Only those with genetic abnormalities are included.

[‡] Not all ALL subtypes are listed. Only common ALL subtypes are included.

[ٴ] Commonly found genetic abnormalities in CLL.

2.1.3 Signs and symptoms of leukaemia

The general signs and symptoms of patients with leukaemia include fever or chills, continuously fatigue and weakness, infection, weight loss, easy bleeding, excessive noctural sweat production, bone and joint pain, swollen lymph nodes and enlargement of the liver and spleen (Grigoropoulos *et al.*, 2013). Patients with leukaemia frequently experience signs and symptoms originating from the failure of their bone marrow and organ infiltration by the leukaemic cells. Bone marrow failure will lead to anaemia (too few red blood cells), increased susceptibility to infection (because the patients have too few white blood cells) and bleeding as a results of reduced number of platelet. Patients with anaemia may have pale skin, become easily tired and shortness of breath. Acute leukaemia patients often present in a few weeks to a few months of signs and symptoms. In ALL and CLL patients, they tend to have enlargement of the lymph nodes, liver (hepatomegaly) and spleen (splenomegaly). Children affected with ALL commonly present with bleeding in unusual places such as the gums, intestine or skin. They may not susceptible to the infections, develop abscess or pneumonias or sudden fever and pain in the arms and legs (Mughal *et al.*, 2006).

2.1.4 Causes and risk factors of leukaemia

To date, there is no clear causative factor of leukaemia identified. Haematological disorders can arise from damage or alterations in the DNA which may then affect many genes. Alteration in genes may occur as a result of chromosomal translocation, insertion, inversion, deletion, amplification or point mutation of genetic information

which results in transformation of the normal function of the genes. Certain congenital diseases such as Down syndrome are also associated with an increased incidence of leukaemia. Lifestyle factors may also be causative of leukaemia. Exposure to tobacco smoke, alcohol intake or dietary habits may result in this disease. Certain chemicals such as benzene, ionizing radiation or drugs used to treat other cancer can initiate a growth of leukaemic cells (Faderl and Kantarjian, 2010).

Study by Kasim *et al.*, (2005) evaluated increased risk of smoking and obesity associated with leukaemia. They found approximately 15% risk of AML for those who are actively smoking. They also found smoking AML patients were slightly thinner than non-smoking AML. Overweight and obesity were found approximately 16.5% in AML, CLL and CML patients and there was also a positive association risk between those leukaemias and obesity.

2.1.5 Treatment of leukaemia

The treatment of leukaemia varies and depends on the specific type of leukaemia due to the different course of these diseases. The treatment should be able to reach all parts of the body since leukaemia is a systemic disease. Some of the treatment involve the use of chemotherapy which are drugs that can be taken orally (by mouth), intravenously (injected into the vein) or subcutaneously (injected under the skin). Treatment may also be via immunotherapy which involves use of monoclonal antibodies, a portion of cells or a portion of biological molecules. Another approach is by allogeneic stem cell transplantation (SCT) to patients who are in complete

remission but have a high possibility to relapse (Henderson *et al.*, 2002; Ball and Kagan, 2009).

Two major prognostic factors related to treatment are time response to complete remission and minimal residual disease following induction therapy (Faderl and Kantarjian, 2010). For example, the treatment of CML using IM, the patient's bone marrow cytogenetics should be monitored every six months until complete remission and BCR-ABL transcript levels should be measured at least three months until major molecular response (discussed in **Section 2.2.7**). **Table 2.2** lists some of the common treatment used in leukaemic diseases. Example of common treatment for CLL is use of Fludarabine which is a front line treatment for treating CLL. It is a water soluble drug and it inhibits DNA and RNA synthesis and affects the DNA repair thus mediates the induction of leukaemic cells apoptosis (Faguet, 2004). B-cell ALL patients are treated with intensive chemotherapy (high dose of methotrexate, cytarabine and cyclophosphamide) protocols. Whereas other ALL patients are treated with other specific therapy which emphasizes the induction of remission, eliminate residual leukaemia, prevent central nervous system leukaemia and continued treatment for durable remission. There have been several clinical trials conducted to recognize the best treatment regimen for childhood ALL for instance Tokyo Children's Cancer Study Group (TCCSG 95-14), Berlin-Frankfurt-Munster ALL Study Group (BFM 95) and the United Kingdom Medical Research Council Working Party on Childhood ALL-97/99 (UKALL-97/99). In the UKALL-97/99 trial, the use of dexamethasone at 6.5 mg/m^2 was shown to be superior to prednisolone at 40 mg/m^2 and the use of 6-thioguanine at 40 mg/m^2 resulted in less relapses but

increased the incidence of deaths in remission patients compared with 6-mercaptopurine at 75 mg/m^2 (as reviewed in Hon Pui, 2012).

2.2 Chronic myeloid leukaemia (CML)

Chronic myeloid leukaemia (CML) is a myeloproliferative disorder of stem cells characterized by the increased growth of myeloid cells in bone marrow and the accumulation of these cells in blood. It is also known as chronic myelogenous leukaemia or chronic granulocytic leukaemia. CML was the first malignancy discovered to have a unique and specific chromosome abnormality, the Philadelphia chromosome (Provan and Gribben, 2008).

2.2.1 Epidemiology of CML

Chronic myeloid leukaemia (CML) is the most common haematology disease reported in Asian regions. CML appears about 20% of adult leukaemia diagnoses in the West however a higher percentage reported in the Far East region (Goldman, 2008). Comparing Asian to the US data with average annual incidence (year of 2006 to 2010), it is estimated 1.6 per 100 000 in both genders, incidence rate of CML is 1.6 per 100 000 in men and 0.7 per 100 000 in women which is lower than the Caucasians with 1.7 to 2.1 per 100 000 in men and 1.1 to 1.3 in women. Mortality in US estimated 0.3 per 100 000 in both genders, which is 0.3 to 0.5 in men and 0.2 to 0.3 in women whereas in Asian, it is estimated 0.2 per 100 000 in both genders. In Asia CML seems to occur at a younger age with median age at diagnosis between 35 to 55 years old, lower than US median age at 65 years old and it is rare below 20 of

age. Men reported more commonly affected than women in Asia as well as in the US (Au *et al.*, 2009). **Table 2.3** shows some of the Asia-Pacific countries and their incidence data of CML compared to the US annual incidence data. Difference in life expectancy, unidentified variations of genetic, family planning for female patients and requirement for long-term medication were the reasons lower median age diagnosed in Asian (Kim *et al.*, 2010).

2.2.2 A historical perspective of CML

Between 1841 till 1845, David Craigie, Hughes Bennett and Rudolph Virchow discovered patients with fever, anaemia, enlargement of the spleen and leucocytosis but they were in argumentation of disease interpretation based on their personal findings. Bennet claimed that "the presence of purulent matter in the blood" which is the infection that cause death of his patient. While Rudolph Virchow found that there was an additional of colourless particles and he then introduced a term of "Leukamie", means the white blood. In 1872, Ernst Neumann determined the existence of leukaemic cells originated from bone marrow. Eighty eight years later in 1960, Peter Nowel and David Hungerford revealed the cytogenetic abnormality known as the Philadelphia (Ph) chromosome. The discovery of the Ph chromosome by Janet Rowley in 1973, that it was a translocation between chromosomes nine and 22 led to a better understanding of the causative factor of this disease. The presence of *BCR* (breakpoint cluster region) and *ABL* (Ableson proto-oncogen) fusion oncogene between chromosomes 9 and 22 that produced a functional translated protein ($p210^{BCR\text{-}ABL}$) was described in 1980s. During 1990s, Daley and colleagues reported the ability of the *BCR-ABL* fusion gene to induce CML-like disease in the

mice model and proved its vital role in the origin and development of CML (Deininger, 2008; Goldman 2010).

2.2.3 Pathophysiology of CML

The hallmark of genetic abnormality in CML is the presence of Philadelphia (Ph) chromosome which is the event of balanced reciprocal chromosomal translocation between chromosomes 9 and 22 that results in one shortened chromosome 22 at band q11.21 and an elongated chromosome 9 at band q34.1 referred to as t(9;22)(q34;q11) (Goldman, 2008). The majority (90% to 95%) of CML patients exhibited this Ph chromosome. This translocation is also present in 15% to 30% of adult lymphoid leukaemia (ALL) and in 2% of newly diagnosed AML patients. The balanced translocation in this malignancy described as the reciprocal translocation of a large segment of 'Ableson proto-oncogene' (*ABL*) from the long arm of chromosome nine to the 'breakpoint cluster region' (*BCR*) genes on the long arm of chromosome 22 resulting *BCR-ABL* fusion gene on the Ph chromosome (der22q-). Whereas on the 9q+ derivative chromosome, the reciprocal translocation resulting of the *ABL-BCR* fusion gene. The *BCR-ABL* fusion gene can actually form several active tyrosine kinases that vary in size from 185 kDA to 230 kDA (Goldman and Mughal, 2005). The location of the *ABL* gene breakpoint commonly occurs in exon a2 whereas the location of the *BCR* gene breakpoint commonly occurs in intron between exons e13 and e14 or in the intron between exons e14 and e15 (e13-e15 formerly known as b2-b4). The *BCR* gene breakpoint also occurs in the first intron and results in fusion of the first exon of *BCR* gene (e1) with second exon (a2) in *ABL* gene thus produces e1a2 mRNA molecule which encode the 190 kDa protein ($p190^{BCR-ABL}$). Some may

form e19a2 mRNA and produces a $p230^{BCR\text{-}ABL}$ oncoprotein. CML patients rarely have the p190 and p230 protein, and these proteins may be detected in chronic neutrophilic leukaemia. The fusion gene *BCR-ABL* is an active tyrosine kinase component expressed as an 8.5 Kb with e13a2 or e14a2 mRNA molecule which then encodes a cytoplasmic oncoprotein with a molecular weight of 210 kDa, known as $p210^{BCR\text{-}ABL}$ in CML patients (Shteper and Ben-Yehuda, 2001; Goldman and Mughal, 2005; Quintás-Cardama and Cortes, 2009). The mechanism of *BCR-ABL* fusion gene involved in the development of CML disease is that this gene can phosphorylate various intracellular proteins which in turn induce signals involved in abnormal cell growth, increase survival of cell, unregulated cell differentiation, abnormal cell adhesion or migration and death. The uncommon phosphorylated intracellular proteins may act by activating the signal transducers and activators of transcription (STAT), mitogen-activated protein (MAP) kinases or phosphatidylinositol 3-kinase (PI3K) (Ren, 2005). This molecular mechanism contributes to the prognosis of this blood cancer.

Table 2.2 Treatment and drugs commonly used in different types of leukaemia

Types of Leukaemia	General Name	Trade Name
AML	Cytarabine Idarubicin Daunorubicin Mitoxantrone VP-16 Gemtuzumab ozogamicin	Cytosar-U Idamycin Cerubidine Novantrone Etoposide Mylotarg
ALL	Cyclophosphamide Vincristine Prednisolone Daunorubicin L-asparaginase Cytarabine Methotrexate, amethopterin 6-mercaptopurine 6-thioguanine	Cytoxan Oncovin Deltasone Cerubidine Oncaspar Cytosar-U Folex Purinethol Tabloid
CML	STI-571, Imatinib mesylate Hydroxyurea Interferon alpha Dasatinib	Gleevec, Glivec Hydrea Intron A Sprycel
CLL	Fludarabine Cyclophosphamide Prednisolone Chlorambucil Alemtuzumab Rituxumab	Fludara Cytoxan Deltasone Leukeran Campath Rituxan

This table shows a list of some common drugs used to treat different types of leukaemia. AML-acute myeloid leukaemia; ALL-acute lymphoblastic leukaemia; CML-chronic myeloid leukaemia; CLL-chronic lymphocytic leukaemia. Some of the drugs have its own trade names and general name in order to avoid confusion of variety of the medications (Ball and Kagan, 2009; Faderl and Kantarjian, 2010).

Table 2.3 Incidence of Chronic Myeloid Leukaemia (CML) in ten Asia-Pacific countries and the US

Countries	**Annual CML Incidence (per 100 000 population)**
China	0.39 - 0.55[a]
Hong Kong	0.9[a]
India	0.8 - 2.2 (M)[a] 0.6 - 1.6 (F)
Singapore	0.7[a]
Philippines	0.7 - 0.9[a]
Thailand	0.5[a]
South Korea	0.8[a]
Taiwan	0.6 (M)[a] 0.39 (F)
Japan	1.0[b]
New Zealand	0.8[b]
US	1.48[a] 1.94 (M) 1.13 (F)

M; Male, F; Female.
[a] Reported by Au *et al.*, 2009. [b] Reported by Kim *et al.*, 2010.

2.2.4 Clinical characteristics of CML

Almost one-half of CML patients have absence of symptoms upon onset of this disease. Incidence of finding on routine blood investigation is not uncommon. Symptoms include lethargy, weight loss, susceptibility towards infection, fatigue and pallor, swollen lymph glands, abnormal bleeding, abnormal discomfort, poor appetite, night sweating, headache and also bone pain. Abdominal discomfort due to the enlarged and swollen spleen is also seen. Approximately 50% to 70% of patients exhibit splenomegaly and can be detected by palpation. Liver enlargement is frequently seen in CML patient but it is difficult to define. Sweating often occurs at night; increased sweating is also a characteristic in patients who have CML. CML patients who are in the advanced phase of the disease may have some of the clinical features as mentioned. The spleen and the liver are frequently enlarged and painful. Patients may develop fever, bone tenderness or frequently have signs of infection (Goldman and Mughal, 2005).

2.2.5 Staging of CML

CML has a tri-phasic clinical course, i) chronic phase, ii) accelerated phase and iii) blast phase. These criteria of different phases are based on World Health Organization (WHO) 2001 (reviewed in Goldman and Mughal, 2005; Cortes *et al.*, 2006 and Quintas-Cardama and Cortes, 2006).