MOLECULAR GENETIC AND EPIGENETIC MECHANISMS OF PRIMARY AND SECONDARY RESISTANCE TO IMATINIB MESYLATE TREATMENT IN Ph CHROMOSOME POSITIVE CHRONIC MYELOID LEUKEMIA PATIENTS

by

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LIST OF ABREVIATIONS

˚C	Degree Celsius
5’ UTR	5’ Untranslated region
A_{260}/A_{280}	Ratio of 260 absorbance over 280 absorbance
ABL1	Abelson Murine Leukemia Viral Oncogene Homolog 1
ALL	Acute Lymphocytic leukemia
AML	Acute myeloid leukemia
AP	Accelerated phase
ASR	Age-standardized incidence rates
ATP	Adenosine triphosphate
BCR	breakpoint cluster region
bp	Base pair
BP	Blast phase
CCA	Clonal chromosomal abnormalities
CCyR	Complete cytogenetic response
cDNA	Complimentary Deoxyribonucleic acid.
CHR	Complete haematological
CI	Confident interval
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CMR	Complete molecular response
CP	Chronic phase
ddH_20	Double distilled water
DEPC	Diethylpyrocarbonate
D-FISH	Double fusion Fluorescent In Situ Hybridization
dHPLC	Denaturing High Performance Liquid Chromatography.
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FAB	French-American-British subtype classification

FBS	Fetal Bovine Serum
FISH	Fluorescent In Situ Hybridization
HCl	Hydrochloric acid
HRM	High Resolution Melt Analysis
IFN- α	Interferon- α
IM	Imatinib Mesylate
IRIS	International Randomized Study of Interferon and STI571
Kb	Kilo base
KCl	Potassium Chloride
kDa	Kilo dalton
LB	Lithium Borate
MCyR	Major cytogenetic response
mCyR	Minor cytogenetic response
$MgCl_2$	Magnesium Chloride
min	Minute
minCyR	Minimal cytogenetic response
ml	Milliliter
mM	Millimolar
MMR	Major molecular response
mRNA	Messenger ribonucleic acid
MS-HRM	Methylation Specific High Resolution Melt Analysis
N/A	Not available
Na_2HSO_3	Sodium bisulfite
NaOH	Sodium Hydroxide
ng/µl	Nanogram per microliter
nM	Nano molar
noCyR	No cytogenetic response
OD	Optical density
OR	Odds ratio
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction

PCyR	Partial cytogenetic response
pH	Puissance de Hydrogen
Ph+	Philadelphia positive
PHA	Phytohaemagglutinin
pmol	Pico mole
qPCR	Quantitative Polyerase Chain Reaction
RBC	Red blood cell
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
ROC curve	Receiver operating characteristics curve
rpm	Rotation per minute
RT	Room temperature
RT-PCR	Real Time Polymerase Chain Reaction
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
sec	Second
Taq	*Thermuphilus aquaticus*
TBE	Tris Base EDTA
TEAA	Triethylammonium acetate
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
T_m	Melting temperature
TSGs	Tumour suppressor genes
TSS	Transcriptional start site
U	Unit
UV	Ultra-violet
V	Voltage
WBC	White blood cell
WHO	World Health Organization

ABSTRAK

Imatinib mesylate (IM) adalah ubat perencat khusus untuk *BCR-ABL* tirosina kinase yang digunakan sebagai terapi utama pada pesakit leukemia mieloid kronik (CML). IM adalah sangat berkesan dan dianggap sebagai penjagaan piawai dalam pengurusan CML. Walaupun IM telah menjadi piawai utama dalam rawatan CML, rintangan kepada IM telah muncul sebagai masalah utama yang membimbangkan. Hampir 33% daripada pesakit CML yang menjalani terapi IM mengalami rintangan yang disebabkan oleh sama ada mekanisma bersandar *BCR-ABL* atau mekanisma tak bersandar *BCR-ABL*. Mekanisma bersandar *BCR-ABL* melibatkan mutasi titik dalam domain *BCR-ABL* tirosina kinase dan amplifikasi gen *BCR-ABL*. Mekanisma tak bersandar *BCR-ABL* terdiri daripada beberapa faktor termasuk perubahan dalam farmakokinetik IM yang bergantung kepada penyerapan, pengedaran metabolisma serta perubahan epigenetik. Kajian ini telah dijalankan untuk menjelaskan mekanisma yang bersandar kepada *BCR-ABL* dan mekanisma tak bersandar *BCR-ABL* yang melibatkan perubahan epigenetik, pada pesakit CML di Malaysia yang menjalani terapi IM. Sejumlah 205 pesakit CML yang menjalani terapi IM (122 rintang terhadap IM dan 83 mempunyai respon yang baik terhadap IM) menyertai kajian ini. Menggunakan Penyahaslian Kromatografi Cecair Prestasi Tinggi (dHPLC) diikuti oleh penjujukan DNA, 122 pesakit CML yang rintang terhadap IM telah disaring untuk mutasi *BCR-ABL*. Sembilan puluh dua pesakit CML yang rintang terhadap IM dan yang tidak menunjukkan mutasi *BCR-ABL* (*BCR-ABL* tidak termutasi) telah dikaji buat mengenalpasti kehadiran amplifikasi gen *BCR-ABL*. Sebagai sebahagian daripada pendekatan epigenetik, 175 pesakit CML yang terdiri daripada 83 pesakit respon baik

dan 92 pesakit rintang terhadap IM yang tidak termutasi di uji menggunakan Analisis Pencairan Resolusi Tinggi Spesifik kepada Metilasi (MS-HRM). Dalam analisis mutasi *BCR-ABL*, mutasi dikesan pada 30/122 pesakit (24.6%) dengan dua daripada pesakit CML menunjukkan mutasi berganda. Tujuh belas jenis mutasi (T315I, G250E, E255K, E255V, M351T, Y253H, V289F, E355G, F359V, L387M, H396R, E355A, D276G, A397P dan E281K) termasuk dua mutasi novel (G251E dan N368S) telah dikenal pasti. Oleh kerana mutasi yang berbeza memberikan tahap kerintangan yang berbeza, pengesanan dan pencirian mutasi *BCR-ABL* adalah sangat relevan pada pesakit CML untuk menjadi panduan dalam memilih dos IM yang paling sesuai atau menukar kepada terapi TKI lain. Walau bagaimanapun, 92 pesakit CML yang rintang terhadap IM yang mempunyai BCR-ABL tidak termutasi, tidak menunjukkan amplifikasi gen *BCR-ABL*. Berhubung dengan mekanisma tak bersandar *BCR-ABL*, tahap metilasi *HOXA4* dan *HOXA5*, tetapi bukan *SOCS1*, didapati lebih tinggi pada pesakit CML yang menunjukkan kerintangan. Pesakit CML yang dirawat dengan IM yang mempunyai tahap metilasi *HOXA4* dan *HOXA5* lebih tinggi daripada 62.5% didapati berkait dengan dengan risiko yang lebih tinggi (OR, 4.71; 95% CI, 2.46, 9.03; P <0.001 dan OR, 4.26; 95% CI, 2.22, 8.17; masing-masing P<0.001) untuk mengalami kerintangan terhadap IM berbanding dengan kumpulan yang bertidak balas secara optimum terhadap IM. Hipermetilasi pada pengalak gen *HOXA4* dan *HOXA5* boleh dianggap sebagai salah satu daripada mekanisma tak bersandar *BCR-ABL* yang menyebabkan kerintangan terhadap IM dan berpotensi menjadi penanda bio epigenetik, sebagai tambahan kepada mutasi gen *BCR-ABL* dalam meramalkan tindak balas terhadap rawatan IM di kalangan pesakit CML. Dalam lima tahun analisis kemandirian, kehadiran mutasi *BCR-ABL* terutama mutasi Y253H dan E355G (masing-masing P=0.005 dan P=0.025) didapati berkaitan

dengan prognosis dan kemandirian pesakit CML yang dirawat dengan IM. Walau bagaimanapun, selepas pelarasan bagi pembolehubah lain dalam analisis regresi Cox berganda, peringkat CML telah muncul sebagai satu-satunya faktor ramalan yang penting (HR: 27.04, P<0.001 untuk BP dan HR: 9.58, P<0.001 untuk AP). Keputusan keseluruhan menunjukkan bahawa kerintangan terhadap IM bukan disebabkan oleh mekanisma tunggal atau mudah, tetapi disebabkan fenomena multi-faktorial. Mutasi *BCR-ABL* boleh dianggap sebagai penanda molekul untuk meramalkan tindak balas serta prognosis pesakit CML dalam rawatan IM manakala tahap metilasi pada penggalak *HOXA4* dan *HOXA5* boleh dianggap sebagai penanda epigenetik untuk meramalkan tindak balas kepada IM.

ABSTRACT

Imatinib mesylate (IM) is a *BCR-ABL* targeted tyrosine kinase inhibitor drug used for frontline therapy in patients with chronic myeloid leukemia (CML). IM is highly effective and is considered the standard of care in CML management. Even though IM has become the gold standard frontline treatment of CML, resistance to IM has emerged as a major problem of concern. Nearly 33% of CML patients on IM therapy develop resistance which can be either due to *BCR-ABL* dependent or *BCR-ABL* independent mechanisms. *BCR-ABL* dependent mechanism involves point mutation in the *BCR-ABL* tyrosine kinase domain and amplification of the *BCR-ABL* gene. *BCR-ABL* independent mechanisms include several factors including alteration in pharmacokinetics of IM with respect to absorption, distribution of metabolism as well as epigenetic alterations. The present study was undertaken to elucidate the *BCR-ABL* dependent mechanism and *BCR–ABL* independent mechanism involving epigenetic alterations, in Malaysian CML patients undergoing IM therapy. A total of 205 CML patients on IM therapy (122 IM resistant and 83 IM good response) were included in this study. Using denaturing High Performance Liquid Chromatography (dHPLC) followed by DNA sequencing, 122 IM resistant CML patients were screened for *BCR-ABL* mutations. Ninety two IM resistant CML patients who did not show *BCR-ABL* mutations (BCR-ABL non-mutated) were investigated for *BCR-ABL* gene amplification. As part of epigenetic approach, 175 CML patients comprising of 83 good response and 92 IM resistant *BCR-ABL* non-mutated CML patients were subjected to Methylation Specific High Resolution Melt Analysis (MS-HRM). In *BCR-ABL* mutation analysis, mutations

were detected in 30/122 patients (24.6%) with two of the CML patients showing double mutations. Seventeen different types of mutations (T315I, G250E, E255K, E255V, M351T, Y253H, V289F, E355G, F359V, L387M, H396R, E355A, D276G, A397P and E281K) including two novel mutations (G251E and N368S) were identified. Since different mutations confer different levels of resistance, detection as well as characterization of *BCR-ABL* mutations is highly relevant in CML patients to guide in selecting the most suitable IM dosage or changing to other Tyrosine kinase inhibitor therapy. However, the 92 IM resistant *BCR-ABL* non-mutated CML patients did not show amplification of the *BCR-ABL* gene. With regard to *BCR-ABL* independent mechanism, methylation levels of *HOXA4* and *HOXA5*, but not of *SOCS1*, were found to be higher in CML patients showing resistance. IM treated CML patients with higher than 62.5% of *HOXA4* and *HOXA5* promoter methylation levels were found to be associated with a higher risk (OR, 4.71; 95% CI, 2.46, 9.03; P<0.001 and OR, 4.26; 95% CI, 2.22, 8.17; P<0.001, respectively) for developing IM resistance compared to the optimal response group. Promoter hypermethylation of *HOXA4* and *HOXA5* genes could be considered as one of the *BCR-ABL* independent mechanisms mediating IM resistance and could be a potential epigenetic biomarker in supplement to the *BCR-ABL* gene mutation in predicting IM treatment response among CML patients. In a five-year survival analysis, the presence of *BCR-ABL* mutations especially Y253H and E355G mutations (p-value =0.005 and p-value =0.025 respectively), were found to be associated with the prognosis and survival of CML patients on IM therapy. However, after adjusting for other variables in multiple Cox regression analysis, CML stage has emerged as the only significant prognostic factor (HR: 27.04, p-value <0.001 for BP and HR: 9.58, p-value <0.001 for AP). The overall results suggest that resistance to IM is not

due to a single or simple mechanism, but is a multi-factorial phenomenon. *BCR-ABL* mutations could be considered as molecular marker for predicting the IM response as well as prognosis of CML patients on IM treatment whereas promoter methylation levels of *HOXA4* and *HOXA5* could be considered as epigenetic markers for predicting the response to IM.

CHAPTER 1

INTRODUCTION

1.1 Cancer – An Overview

Worldwide, cancer is responsible for several million annual deaths. In 2008, cancer accounted for 7.6 million deaths, which was around 13% of all deaths (WHO, 2008). Cancer was reported to be the third common cause of death in hospitals under the Ministry of Health, Malaysia in 2007 (11.28%), after heart diseases and diseases of pulmonary circulation and septicaemia. According to the Malaysian National Cancer Registry report, a total of 18,219 new cancer cases were registered with the National Cancer Registry in 2007, which comprised of 8,123 (44.6%) males and 10,096 (55.4%) females (Omar and Tamin, 2011).

However, the trend of cancer incidence rate in Malaysia was reported to be decreasing. The age-standardised incidence rates (ASR) for all cancers in the year 2003 was 134.3 per 100,000 males and 154.2 per 100,000 females (Lim and Halimah, 2003). In the following three years, the ASR among males was 128.6 per 100,000 population and among females was 135.7 per 100,000 population (Omar *et al.*, 2006). The trend was further decreased in 2007 when the ASR were reported as 85.1 per 100.000 in males and 94.4 per 100,000 in females (Omar and Tamin, 2011). In all of these three studies, age-standardized incidence was identified rather than the incidence rate as age-

standardized incidence is more reliable. This is because, age-standardized incidence was calculated with the reference population being the World Standard Population.

Cancer is not one single disease, but a complex group of around 200 diseases, in which there is a diminution in control over cell proliferation and cell death in the affected tissues. Cancer, also known as malignant neoplasm, originates from a single abnormal cell which grows and divides without obeying normal cell cycle regulatory mechanisms and acquires the ability to invade local tissue and spread distantly to other parts of the body (metastasize).

There are eight hallmarks of cancer which are 1) sustaining proliferative signalling, 2) evading growth suppressors, 3) resisting cell death, 4) enabling replicative immortality, 5) inducing angiogenesis, 6) activating invasion and metastasis, 7) reprogramming of energy metabolism and 8) evading immune destruction (Hanahan and Weinberg, 2011). These changes in cellular behaviour are the result of alterations in the function or levels of the proteins that control these processes. And these alterations are, in turn, usually caused by mutations, or changes in expression of genes encoding the proteins.

1.1.1 Cancer Development

Development of cancer is a complex, multistep process and involves several mechanisms. Basically, cancer arises from the accumulation of multiple genetic alterations in responsible genes that are involved in many molecular pathways. There are two major classes of genes, oncogenes and tumour suppressor genes (TSGs), that are

responsible for cancer formation. The products of all these genes are all part of a network that work collectively in controlling cell proliferation, differentiation and survival (Boyle *et al.*, 2008).

In general, oncogenes (called proto-oncogenes in their normal, non-mutated form) promote cell proliferation and survival, whereas tumour suppressor genes inhibit cell growth. Cells proliferate only when required, as a result of delicate balance between growth promoting and growth inhibiting mechanisms that are controlled by an intricate network of intra- and extra cellular molecules. But cancer cells, in stark contrast, override these controlling mechanisms and follow their own internal program for timing their reproduction.

Another group of genes that are responsible in cancer formation is the DNA repair genes. There are increasing evidence and reports which demonstrate that breakdown of DNA repair genes can cause development of human cancers (Mendelsohn *et al.*, 2008).

1.1.2 General Classification of Cancer

Generally, cancers are classified in two ways, which are based on the histological type of tissue in which the cancer originates (histological type) or the location in the body where the cancer first develops (primary site). Based on the histological perspective, there are hundreds of different cancers. However, according to U.S. National Institutes of Health, cancer can be grouped into six major categories, which are the carcinoma, sarcoma, myeloma, leukemia, lymphoma and mixed type.

Carcinoma is a type of cancer that originates at the internal or external epithelial lining of the body. Malignancies of epithelial tissue account for 80 to 90 percent of all cancer cases. Carcinomas are divided into two major subtypes which are adenocarcinoma and squamous cell carcinoma. The major difference between them is, adenocarcinoma develops in an organ or gland but squamous cell carcinoma originates in the squamous epithelium.

Sarcoma is a type of cancer that originates in supportive and connective tissues such as bones, tendons, cartilage, muscle, and fat. Examples for sarcomas are Osteosarcoma which occurs in bone, Chondrosarcoma that occurs in cartilage and Liposarcoma that develops in adipose tissue.

Myeloma is a type of cancer that originates in the plasma cells of bone marrow. The plasma cells produce some of the proteins found in blood. Thus, myeloma can lead to excess of protein in blood plasma. Because plasma cells are part of the immune system and produce antibodies, the development of myeloma results in antibody overproduction, thus impairing the immune system.

The leukaemias are neoplastic proliferations of cells of the haematopoietic (blood forming) lineage. Leukemia, also known as “blood cancer”, develops in the bone marrow or blood often leading to the overproduction of immature white blood cells which can travel to any part of the body. These immature white blood cells do not perform what they are supposed to do normally. Consequently, the patient is often prone to infection. Leukemia also can affect red blood cells and can cause series of bleeding due to poor blood clotting and fatigue due to anemia.

Lymphoma is a solid cancer that develops in the glands or nodes of the lymphatic system, a network of vessels, nodes, and associated organs (specifically the spleen, tonsils, and thymus) that purify bodily fluids and produce infection-fighting white blood cells (lymphocytes).

Cancer also can present in mixed type, in which the type components may be within one category or from different categories. A few examples of mixed type of cancer are adenosquamous carcinoma (contains two types of cells: squamous cells and gland-like cells) and carcinosarcoma (malignant tumor that is a mixture of carcinoma and sarcoma).

According to the U.S. National Institutes of Health, the international standard for the classification and nomenclature of histology is the International Classification of Diseases for Oncology, Third Edition (ICD-O-3) (WHO, 2000).

1.2 Overview of Leukemia

Leukemia is usually defined as the uncontrolled proliferation or expansion of haematopoietic cells that do not retain the capacity to differentiate normally to mature white blood cells. As white blood cells are part of body's immune system, the disorder in the white blood cells production will impair body's defence against infection (Panno, 2010). In 2010, the incidence of leukemia was reported to be increasing over the years, from 30,800 cases in year 2000 to 44,790 in the year 2009 worldwide (Panno, 2010). In another report, it was estimated that by 2012, 47,150 people would be diagnosed with and 23,540 people would die of leukemia in United States (Howlader *et al.*, 2012).

In Malaysia, leukemia was reported to be the seventh most frequent cancer in 2007 (Table 1.1), after lymphoma and cancer of the uterine cervix, and it accounted for 4.1% of all types of cancer (Omar and Tamin, 2011). In 2003, the age-standardised incidence rates (ASR) for leukemia was reported to be 7.2 per 100,000 males and 5.3 per 100,000 females (Lim and Halimah, 2003). In the following three years, the ASR among leukemia patients decreased on a large scale wherein the ASR for male leukemia patients was 4 per 100,000 population and for female leukemia patients was 2.4 per 100,000 population (Omar *et al.*, 2006). However, the trend fluctuated in 2007, when the ASR was reported as 3.5 per 100.000 in males and 2.7 per 100,000 in females (Omar and Tamin, 2011). Due to the inconsistent ASR trend of CML patients, it is crucial to study on the factors that may involve and on how to overcome fluctuation in the ASR of Malaysian CML patients.

Table 1.1: Ten most frequent cancers in Malaysia (2007), with leukemia as the seventh most frequent cancer (adapted from Omar & Tamin, 2011).

Type of Cancer	No. Of patients	Percentage (%)
Breast	3292	18.1
Colorectal	2246	12.3
Trachea, Bronchus, Lung	1865	10.2
Nasopharynx	940	5.2
Cervix Uteri	847	4.6
Lymphoma	776	4.3
Leukaemia	741	4.1
Ovary	656	3.6
Stomach	630	3.5
Liver	605	3.3

1.2.1 Types of Leukemia

Usually, leukemia is divided into two main classes; acute and chronic leukemia. The terms acute and chronic refer to the rate at which the disease progresses, in which acute refers to rapid progression and chronic refers to slow progression (Bozzone, 2009). Acute leukemia shows a fast clinical pattern and can result in death in a relatively short period of time whereas chronic leukemia is generally less aggressive and patients might live with this disease for several years if treated. Both acute and chronic leukemia are subdivided into different categories based on the lineage and cell type involved. Leukemia that arises from lymphoid cells is categorized into lymphocytic leukemia and leukemia that arises from myeloid cells is categorized into myelogenous leukemia (Panno, 2010).

There are four main types of leukemia which are the acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML).

Acute lymphoblastic leukemia (ALL) is a type of leukemia, marked by neoplasia of precursor B- or precursor T-lymphoid cells in blood. ALL is reported to be the most common cancer among children (Gurbuxani and Anastasi, 2011) and it accounts for 75 percent of childhood leukemia with highest incidence among children aged 1 to 10 years. In ALL, accumulation of leukemic lymphoblasts in the bone marrow and extramedullary site such as thymus occur due to abnormal proliferation, differentiation arrest as well as apoptosis resistance of the leukemic cells (Gurbuxani and Anastasi, 2011). Thus, normal hematopoiesis will progressively decline and may lead to anemia, infection, bleeding, splenomegaly, hepatomegaly, lymphadenophaty and other manifestation of organ infiltration (Gurbuxani and Anastasi, 2011). However, ALL presentation is usually acute with non-specific signs and symptoms.

Chronic lymphoid leukemia (CLL) is a type of leukemia that affects lymphoid cells (lymphoproliferative disorder of the B lymphocytes) and usually this disease grows slowly. It mostly affects people more than 55 years old and most likely never affects children (Panno, 2010). In western countries, CLL is reported to be the most common form of leukemia in adults and more than 10,000 people are diagnosed each year in the United States alone and the survival rate is approximately 75% (Bozzone, 2009).

AML is a type of leukemia that affects the myeloid cells. However, unlike CML, AML usually grows rapidly and affects both children and adults. Clinically, AML is

characterized by accumulation of immature blastic cells that proliferate abundantly with lack of normal differentiation (Mendelsohn *et al.*, 2008). AML involves various morphologic, immunologic and genetic subtypes that are associated to a very diverse clinical profiles and treatment results, leading to an increasing role of the prognostic parameters (Kern *et al.*, 2011).

The fourth type of leukemia is chronic myeloid leukemia (CML) of which the present study is being focused on.

1.3 Chronic Myeloid Leukemia – Molecular Pathogenesis

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disease, originating from an abnormal pluripotent bone marrow stem cell. The progression of CML is slow during the early stage. CML is very rarely found in children and its incidence increases in older ages. Making it more complex, there are no known hereditary, familial, geographic, ethnic, or economic factors that can be associated with CML. Therefore, the disease is neither preventable nor inherited (Quintas-Cardama and Cortes, 2006) and the cause for its formation is unknown in the majority of patients (Yong and Melo, 2011).

Historically, CML is the first type of leukemia to be recognized (Goldman, 2010) and is the first human cancer to be associated with a consistent chromosomal abnormality (Yong and Melo, 2011). The hallmark of CML identification is the presence of a minute chromosome known as Philadelphia chromosome in the bone marrow cells.

The Philadelphia chromosome was first discovered by Nowell and Hungerford in 1960. With improvements in techniques, the formation of Ph chromosome was characterized as to be the result of reciprocal translocation between chromosome 9 and 22, t(9;22)(q34;q11) by Rowley (1973).

In early 1980s, the enhancement of molecular biology led to the discovery of human homologue of the murine Abelson1 gene (*ABL1* gene) which is normally located on human chromosome 9 but was then found to be translocated to the chromosome 22 (Ph chromosome) in most of the CML patients (Goldman, 2010). Later, in 1984, description of the breakpoint cluster region (*BCR* gene) on chromosome 22 was explored and consequently, a theory of possibility that the *BCR* and *ABL1* might be linked in some way that led to activation of *ABL1* was raised (Goldman, 2010).

The Philadelphia chromosome translocation results in a *BCR-ABL* fusion gene that codes for fusion proteins with unusual tyrosine kinase activity. The fused BCR-ABL proteins are capable of interacting with the interleukin-3 beta receptor and activating a cascade of proteins which speed up cell division. Also, BCR-ABL tyrosine kinase activity does not require activation by cell signalling molecules; it is constitutively active unlike normal kinases. Hence white blood cells containing the fusion protein are able to proliferate without the presence of growth factors. In addition, BCR-ABL fusion proteins inhibit the DNA repair, leaving the cell more susceptible to genetic abnormalities. Cumulative effect of these molecular mechanisms makes CML a progressive disease that develops in three stages.

1.3.1 Incidence of Chronic Myeloid Leukemia

CML is not a common disease worldwide (Quintas-Cardama and Cortes, 2006; Rodgers and Young, 2010). Its annual incidence is 1–2 per 100,000 people, and slightly more men than women are affected (1.5:1) (Rodgers and Young, 2010). CML generally occurs in adults and occurs less than three percent in individuals aged from infancy to 19 years old. Thus CML is very rare in children, with less than 50 cases per year diagnosed in the United States (Bozzone, 2009).

In the Surveillance Epidemiology and End Results (SEER) report from United Kingdom, the age-adjusted incidence rate of CML for the period 2005 to 2009 was reported as 1.6 per 100,000 men and women per year (Howlader *et al.*, 2012). It was reported that the incidence rate of CML in Asian Pacific countries are lower than the western countries. The annual incidence of CML per 100,000 populations in Asia pacific region, was reported to be ranging from 0.4 in China up to 1.0 in Japan (Kim *et al.*, 2010a).

It was reported that CML represents about 15–20% of all cases of adult leukemia in Western populations. In Malaysia, occurrence of CML in 2007 was reported as 14.1% of all types of leukemia and 21.9% of myeloid group of leukemia (Omar and Tamin, 2011).

Even though incidence rate in Asia is lower than in Western countries, it is devastating that the occurrence of CML in Asia pacific countries tends to afflict younger population with median age as low as 36 years (Au *et al.*, 2009). In United State

population, the median age of CML diagnosis was reported as approximately 50 years (Bozzone, 2009).

1.3.2 Clinical Features of Chronic Myeloid Leukemia

Almost 20% of CML patients are asymptomatic (Alagappan, 2011). However, classical presentation of CML includes fever, rapid weight loss, lassitude, night sweats, gout and splenic pain, which refer to substantial splenomegaly (Alagappan, 2011). Leucostasis with leucocyte counts greater than 300,000/ uL accompanied with headache and focal neurologic deficits may also be present (Rodgers and Young, 2010). However, explicit signs and symptoms are rarely encountered among the CML patients nowadays, as the diagnosis is usually made earlier. Consequent to early diagnosis, commonly, patients only present with fatigue, with or without weight loss, abdominal discomfort or simply an observation of elevated leukocyte count in routine blood test (Rodgers and Young, 2010).

Even though many CML patients are diagnosed in an early stage, rare presentations of CML may also be observed among the patients including chloroma, petechiae and bruising, suggesting the progression of CML to an accelerated or blast phase. Ironically, unlike other types of leukemia, CML seldom presents with bacterial or fungal infection due to the preservation of neutrophil functions (Rodgers and Young, 2010).

1.3.3 Diagnosis of Chronic Myeloid Leukemia

In western countries, most patients tend to be diagnosed in the chronic phase where diagnosis is often made on the basis of routine blood testing in which 85% of patients are diagnosed in the chronic phase and half are asymptomatic at their early period of presentation (Laneuville *et al.*, 2006). In Asian Pacific countries, approximately 84% of cases are diagnosed in chronic phase (CP). Minorities of the CML patients are diagnosed in accelerated phase (AP) (~11%) or blast crisis (BC) (~5%) (Kim *et al.*, 2010a). However, in Malaysia, the percentage of CML patients diagnosed in AP and BC has been reported as quite high compared to other countries, with approximately 30% and 10% of CML patients being diagnosed in AP and BC, respectively (Kim *et al.*, 2010a).

The diagnosis of CML is usually based on blood count, blood film, bone marrow aspirate, cytogenetic analysis and molecular diagnosis. Complete blood count is usually applied as the first indicator of CML diagnosis. Usually, leukocyte number in CML patient varies from 200,000 to 700,000 per cubic millimetre with normal or elevated platelet counts. Mild normochromic normocytic anemia may also be present (Rodgers and Young, 2010; Alagappan, 2011).

Consequent to abnormal blood count, blood film screening is done. CML has a unique blood film, with left shift circulating myeloblasts, myelocytes, metamyelocytes and band forms (Figure 1.1). Uniquely in CML cases, basophil counts usually exceed 1000 per cubic millimetre and is a hallmark in CML diagnosis (Rodgers and Young,

2010). Abnormal result in blood film screening is usually followed with bone marrow aspirate analysis which is a more invasive procedure.

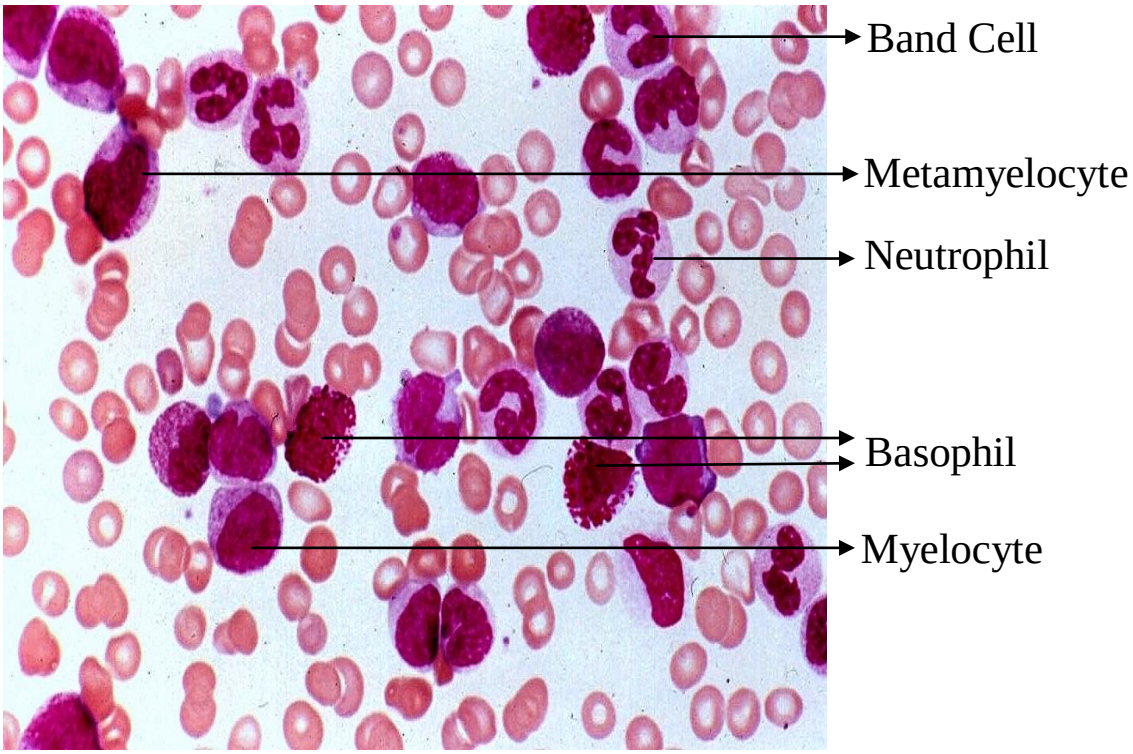

Figure 1.1: Blood smear of CML showing neutrophilic leukocytosis with left shift and basophilia (adapted from Krafts, 2009).

CML bone marrow aspirate usually shows cellular spicules, hypercellularity, and almost complete effacement of the fat spaces. The cell morphology shows granulocytic hyperplasia of neutrophil, eosinophil and basophil series as a feature of myeloid predominance in marrow (Naeim, 2001; Rodgers and Young, 2010). Megakaryocytes are abundant in CML marrow and show marked variety of morphology ranging from small size to large, bizarre, multilobulated forms (Naeim, 2001). Progression of CML to accelerated phase is featured by an increase of blast cells up to 15% and blastic phase is featured by an increase of blast cells more than 20%.

1.3.4 Haematological and Bone Marrow Abnormalities in Chronic Myeloid Leukemia

Patients with CML are usually presented with several haematological abnormalities. The typical haematologic findings are anaemia with haemoglobin level less than 7 gm/dl to 11 gm/dl as well as marked leukocytosis with elevation of total leukocyte count from 100 to 500 $X10^9$ /L (Singh, 2008). In peripheral blood smear, CML patients demonstrate immature white cells of all stages including neutrophils, metamyelocytes, myelocytes, promyelocytes and blast cells. Basophils and eosinophils may also increase up to 5% to 15%.

Apart from those haematologic findings, CML patients also show a decline in neutrophil alkaline phosphatase score to as low as 0 to 20. However, platelet count is higher in CML patients (thrombocytosis), at the range of 300 to 500 X 10^9 /L (Singh, 2008).

Bone marrow smear of CML patients are usually presented with marked myeloid hyperplasia together with a markedly increase of myeloid to erythroid precursor (M:E) ratio from 20:1 to 49:1 (Singh, 2008). Thus, erythriod cells gradually diminish, resulting in anaemia consequential to myeloid hyperplasia. CML patients in chronic phase are usually presented with 2% to 5% blast cells.

Further, cytogenetic analysis for CML diagnosis is also crucial for absolute confirmation of the disease. More than 95% patients that present with clinical and morphologic features of CML have Philadelphia (Ph) chromosome in the marrow with typical karyotype of the reciprocal translocation t(9;22) (q34;q11). Variant Ph translocation also may be present in 5% of CML patients. Variant Ph translocation include three-way translocations involving chromosomes 9, 22 and any one of the 22 pairs of autosomes (Rodgers and Young, 2010).

More recently, molecular diagnostic methods including fluorescence *in situ* hybridization (FISH) and quantitative reverse transcription polymerase chain reaction (qRT-PCR) have been introduced and were reported to be more sensitive than conventional cytogenetic analysis for the detection of *BCR-ABL1* positive cells. In the qRT-PCR, the *BCR-ABL* transcript in the peripheral blood sample is measured in relation to the housekeeping gene glucose-6-phosphate dehydrogenase (*G6PDH*) (Gadzicki *et al.*, 2005). Molecular diagnosis of CML is based on the detection of *BCR-ABL* gene fusion transcripts using the qRT-PCR (Goh *et al.*, 2006).

1.3.5 Stages of Chronic Myeloid Leukemia

CML is a triphasic disease that progress from a chronic phase (CP) to accelerated phase (AP) and finally worsens to Blast Phase (BP). Chronic phase can be distinguished from the other advanced phases by looking at several factors. Patients at CP have less than 15% of blasts in blood and less than 20% in bone marrow (Rodgers and Young, 2010). In CP, Ph chromosome can also be detected through cytogenetic analysis. Duration of CP in CML patients is highly variable before progression to more advance phases. CP can last as short as from months to as long as years with commonly from three to five years (Bozzone, 2009). However, median time to progression from CP to AP is slowly increasing due to better treatment and earlier diagnosis of CML patients.

AP takes place after the progression from the CP. AP is defined as having one or more of the following criteria; increase in number of blasts in peripheral blood (15% to 30%) and in bone marrow (20% to 50%) , Leukocytosis (>50 $x10^9$ leukocytes per liter), anemia (hematocrit, <25%), thrombocytopenia (<100 $x10^9$ thrombocytes per liter) that is not controlled with antileukemic therapy, marked thrombocytosis (>1000 $x10^9$ thrombocytes per liter) or myelofibrosis with teardrop cells in blood smear and increased marrow reticulin (Faderl *et al.*, 1999; Rodgers and Young, 2010).

During AP, more genetic errors are accumulated in cells and the production of abnormal cells are markedly increased. Distraughtly, AP can only last from three to nine months before it progresses into even worse stage if the CML patients are not treated well enough (Bozzone, 2009).

The third and last stage of CML is the blastic phase. The signs and symptoms of BP resemble acute leukemia, which consist of bone pain, weight loss and B symptoms (systemic symptoms of fever, night sweats, and unintentional weight loss of >10% of normal body weight over a period of six months or less). Blasts cells are usually increased to more than 30% in peripheral blood and more than 50% in bone marrow. Marrow failure can also be observed, marked by decreasing red blood cell and platelets count. Cytogenetically, further additional chromosomal abnormalities are also frequently observed in BP CML patients. Usually, CP can last from three to six months (Bozzone, 2009).

During BP, even more genetic errors are accumulated in the leukemic cells, especially in tumor suppressor genes. Thus, the cells tend to proliferate very swiftly and are unresponsive to signals that initiate apoptosis and die (Bozzone, 2009). Consequently, if patients in BP are left untreated, the blast crisis can be life threatening.

1.4 Treatment of Chronic Myeloid Leukemia

Treatment of CML was previously focused on normalizing the number of peripheral blood granulocytes. Before 2001, hydroxyurea was used and was considered as the standard treatment for newly diagnosed CML patients (Tahir *et al.*, 2007). Hydroxyurea works by inhibiting one of the enzymes involved in DNA replication, and thus suppressing the excessive multiplication of the myeloid peripheral cells. Hydroxyurea was reported to help relieve symptoms with few adverse effects and

produce haematological remission in over 90% of CML patients without affecting the expression of the Ph chromosome phenotype (Au *et al.*, 2009).

After the introduction of Interferon- α (IFN- α), this treatment was used widely and was sometimes combined with hydroxyurea when the treatment with IFN- α failed, or when IFN- α was not tolerated, or in very elderly or frail people (Tahir *et al.*, 2007).

However, only small group of the IFN-α treated CML patients reach complete cytogenetic remission (CCyR). IFN-α acts by down-regulating *BCR-ABL1* gene expression, activating several transcriptional factors that control cell proliferation, maturation, and apoptosis as well as promoting the cycling of normal dormant hematopoietic stem cells thereby exposing leukemic stem cell to the action of chemotherapy agents (Kreutzman *et al.*, 2011).

The discovery of *BCR-ABL* mediated pathogenesis of CML has provided the rationale for designing a drug that targets the kinase activity of *BCR-ABL* fusion protein. Imatinib mesylate (also known as Glivec/Gleevec) is the molecularly targeted drug that acts on the molecular mechanisms that leads to CML- the unusual tyrosine kinase protein coded for by *BCR-ABL1* fusion gene.

In 2001, imatinib mesylate (IM), the first tyrosine kinase inhibitor (TKI), was approved by the United States Food and Drug Administration for the treatment of CML (Cohen *et al.*, 2002). A high percentage (81%) of CML patients who received IM as the frontline treatment was reported to achieve complete cytogenetic response (Roy *et al.*, 2006). Since then, IM has been widely used as the frontline treatment for CML patients. Thus, IM is now accepted worldwide in the treatment and management of CML patients.

The efficiency of CML treatment is usually monitored and measured based on the remission status of the disease condition which is evaluated on three main criteria. The earliest monitoring of IM response is on the haematologic remission of the CML patients. The monitoring should be done every 15 days until complete haematologic response is achieved or at least every three months after the initiation of IM therapy. Later, at three months, six months and 12 months, Cytogenetic analysis is carried out for cytogenetic response monitoring of the CML patients, aiming for complete cytogenetic response. After every six months, or at least after 18 months of IM initiation, molecular monitoring is carried out, aiming for undetectable *BCR-ABL1* mRNA transcripts by real time quantitative PCR and this is termed as complete molecular response. More details of treatment response monitoring is described in Chapter Two.

1.5 The Present Study: Rationale and Importance

Even though IM has become the gold standard frontline treatment of CML, resistance to this drug has emerged as an increasing problem of concern. Development of resistance to IM has been a setback for patients and physicians facing this clinical crisis and remains a daunting situation. Resistance to IM is a major problem because it can develop at any time and lead to disease progression. In an International Randomized Study of Interferon and SST1571 (IRIS) study, it was reported that, 15% of CML patients never even achieved partial cytogenetic response (PCyR) and loss of response was observed in 11% of patients with IM treatment (Roy *et al.*, 2006).

Nearly one third of CML patients undergoing IM therapy, have an inferior response to IM, either failing to respond to primary therapy or demonstrating progression after an initial response. A patient with CML could display either a primary or secondary resistance to IM therapy. Primary resistance, which is also referred as refractoriness or failure, is defined as inability to achieve any landmark response from the very beginning of the treatment (Quintas-Cardama *et al.*, 2009; Goldman, 2010). These patients may never achieve complete haematology response even after three months of IM treatment and/or complete cytogenetic response even after 12 months of IM treatment (Baccarani *et al.*, 2009a; Baccarani *et al.*, 2009b; LeukemiaNet, 2010).

The second group is secondary resistance which is also known as acquired resistance. Patients categorized in secondary resistance group achieve complete cytogenetic response to IM for a period of time but the length of response varies between CML patients. Patients are classified into secondary resistance once an event of losing a major cytogenetic response and complete cytogenetic response occurred (Baccarani *et al.*, 2009a; Baccarani *et al.*, 2009b; Quintas-Cardama *et al.*, 2009; LeukemiaNet, 2010).

Therefore, in clinical practice, haematologic, cytogenetic and molecular response to IM is evaluated at regular periodic intervals to monitor the treatment response and development of either primary or secondary resistance (LeukemiaNet, 2010). In Malaysia, at HUSM and other collaborating centres, there are several CML patients undergoing IM treatment and who show signs of resistance to IM. This trend of CML progression due to the development of resistance towards IM treatment has become an alarming problem in the management of CML patients.

Development of resistance to IM is a multifactorial phenomenon in patients with CML and may be mediated by a diversity of mechanisms. However, there are two broad mechanisms of resistance; *BCR-ABL* dependent and *BCR-ABL* independent pathways (Hochhaus, 2006; Mauro, 2006). So, in these CML patients molecular, molecular cytogenetic and epigenetic studies could be helpful in elucidating the mechanisms of resistance involving both *BCR-ABL* dependent as well as *BCR-ABL* independent pathways. This is because, knowing the pattern of *BCR-ABL1* dependent as well as other possible mechanisms among Malaysian IM resistant CML patients can be very helpful towards designing a better approach in patients' treatment and management.

BCR-ABL dependent pathways mainly consist of *BCR-ABL1* kinase domain mutations and *BCR-ABL1* gene amplification. Both of these parameters were reported to be associated with clinical resistance to IM therapy (Gorre *et al.*, 2001; Virgili and Nacheva, 2010). Researchers have identified nearly 100 different types of *BCR-ABL1* mutations in different population groups worldwide (Hughes *et al.*, 2009). The identification of the type of *BCR-ABL1* mutation is very crucial as different type of mutations is known to be associated with different level of clinical resistance to IM (Bengio *et al.*, 2011; Soverini *et al.*, 2011).

The duplication of the Ph chromosome resulting in two copies of the *BCR-ABL1* fusion gene was reported to be a common abnormality acquired during CML disease progression (Virgili and Nacheva, 2010). There have not been many reports on the prevalence of *BCR-ABL1* gene amplifications among CML patients showing resistance to IM. However, few case studies have reported the association of *BCR-ABL1* gene

amplification in CML patient(s) with resistance to IM treatment (Campbell *et al.*, 2002; Gadzicki *et al.*, 2005; Phan *et al.*, 2008).

Although there are reports on the occurrence of *BCR-ABL* mutations and *BCR-ABL1* gene amplifications, still, no reports are available from Malaysia as no previous studies have been undertaken till date. So, in the present study, it was aimed to elucidate the contribution of mutations in the tyrosine kinase domain and also amplification of the *BCR-ABL1* gene, as *BCR-ABL1* dependent mechanisms mediating resistance to IM in Malaysian CML patients.

However, several CML patients showing IM resistance might not fit into the *BCR-ABL* dependent mechanisms as not all IM resistant CML patients might be having *BCR-ABL1* mutation or *BCR-ABL1* amplification. It was presumed that the mechanisms of IM resistance in such patients might be mediated through *BCR-ABL* independent pathways. As the progression and the development of resistance to IM treatment among CML patients involve a complex and multi factorial mechanism, a combination of *BCR-ABL* dependent and *BCR-ABL* independent mechanisms is implicated.

BCR-ABL independent pathways of IM resistance may include several mechanisms such as pharmacokinetic variability in IM efflux, IM import, IM metabolism, IM binding or IM concentration, as well as activation of alternative signalling pathways and epigenetic modification (Bixby and Talpaz, 2009). Existing knowledge of genetic alterations is inadequate in addressing the issues of *BCR-ABL* independent mediated resistance to IM. Thus, it is very important and prolific to explore novel pathways that contribute to the development of resistance to IM.

It is well known that genetic alterations in the human genome are not the only cause in promoting cancer or resistance to cancer treatment. To date, association between epigenetic alterations of the epigenome with cancer development and cancer therapy response also have attracted the interest of many scientists around the world.

The epigenome which is composed of chromatin, its modifications and DNA methylation, plays a critical role in controlling gene expression, as well as in drug responsiveness (Strathdee *et al.*, 2004; Glasspool *et al.*, 2006). Epigenetic silencing is a phenomenon whereby gene transcription may be suppressed through DNA methylation, resulting in decreased or no protein expression. Abnormal DNA methylation plays a role in inactivation of tumor transcript genes by means of promoter hypermethylation. DNA hypermethylation can provide valuable markers for malignant progression, residual disease and response to treatment.

Few studies (Esteller, 2003; Herman and Baylin, 2003) have suggested that DNA hypermethylation might play a role in disease progression in CML. In CML, increased epigenetic silencing of potential tumour suppressor genes has been reported (Esteller, 2003) to correlate with disease progression in a certain proportion of patients treated with IM. Thus, it was hypothesized that there might be a relationship between epigenetic silencing and development of IM resistance in CML patients.

Methylation of two types of *HOXA* genes, *HOXA4* and *HOXA5*, have been reported to be strongly associated with progression to blast crisis and poor response to IM in CML patients (Strathdee *et al.*, 2007a). Since *HOXA4* and *HOXA5* hypermethylation exhibits frequent correlation with disease progression and poor