INFLUENCE OF TERT GENE EXPRESSION, TELOMERASE ACTIVITY AND TELOMERE LENGTH IN RELATION TO IMATINIB MESYLATE RESISTANCE IN MALAYSIAN CHRONIC MYELOID LEUKAEMIA PATIENTS

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by

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LIST OF SYMBOLS

<	Less than
>	More than
α	Alpha
A	Absorbance
°C	Degree Celcius
Δ	Delta
C_T	Cycle threshold
g	Gram
min	Minute
ml	mililtre
mm	Milimeter
mM	Milimolar
ul	Microlitre
%	Percentage
U	Unit

LIST OF ABBREVIATIONS

aTL	Absolute Telomere Length
ABL	Abelson Leukaemia Virus Oncogene
ALL	Acute Lymphocytic Leukaemia
AML	Acute Myleloid Leukaemia
AW1	Washing Buffer1
AW2	Washing Buffer 2
BCR	Breakpoint Cluster Region
bp	Base pair
CBA	Chromosome Binding Analysis
CBC	Complete Blood Count
CCyR	Complete Cytogenetic Response
CHR	Complete Hematologic Response
CML	Chronic Myeloid Leukaemia
CMR	Complete Molecular Response
CyR	Cytogenetic Response
DDR	DNA Damage Response
ddH_2O	Deionized Distilled Water
del	Deletion
DMSO	Dimetyl sulfoxide
DNA	Dioxyribonucleic Acid
dNTPs	Dinucleotide Triphosphates
EDTA	Ethlene Diamine Tetraacetic Acid

FBC	Full Blood Count
FISH	Fluoroscene In Situ Hybridisation
hOCT1	Human Organic Cation Transporter Type 1
HR	Hematologic Response
hTERC	Human Telomerase RNA Component
hTERT	Human Telomerase Reverse Transcriptase
HPP	Hospital Pulau Pinang
HRPB	Hospital Raja Permaisuri Bainun
HSA	Hospital Sutanah Aminah
IFN	Interferon
IM	Imatinib Mesylate
KPP	Klinik Pakar Perubatan
$MgCl_2$	Magnesium Chloride
mRNA	Messenger Ribonucleic Acid
MMR	Major Molecular Response
nm	Nanometer
NTC	Non Template Control
PAGE	Polyacrylamide Gel Electrophoresis
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction

Ph	Philadephia
PO	Phosphoprotein
POT1	Protection of Telomerase 1
POS	Positive Control
PPUKM	Pusat Perubatan Universiti Kebangsaan Malaysia
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RBC	Red Blood Cell
RNA	Ribonucleic Acid
STELLA	Single Telomere Length ANalysis
Taq	*Thermophilus Aquaticus*
TBE	Tris-Borate-EDTA
TEL STD	Telomere Standard
TeSLA	Telomere Shortest Length Assay
TKD	Tyrosine Kinase Domain
TKI	Tyrosine Kinase Inhibitor
TRAP	Telomeric Repeat Amplification Protocol
TRF	Terminal Restriction Fragmentation
T/S	Telomere to Single Gene Copy Ratio
USM	Universiti Sains Malaysia
UV	Ultraviolet

PENGARUH PENGEKSPRESAN GEN TERT, AKTIVITI TELOMERASE DAN PANJANGAN TELOMERE TERHADAP KERINTANGAN IMATINIB MESYLATE DALAM PESAKIT LEUKEMIA MIELOID KRONIK DI MALAYSIA

ABSTRAK

Leukemia mieloid kronik adalah neoplasma myeloproliferatif yang didiagnosa dengan kehadiran gen gabungan *BCR-ABL* yang menghasilkan tirosin kinase yang memberikan isyarat proliferasi sel darah putih. Pengenalan imatinib mesylate pada awal tahun 2000, telah meningkatkan kelangsungan hidup pesakit yang terjejas dan mengubah pengurusan penyakit secara dramatik. Walau bagaimanapun, kegagalan membasmi sel-sel leukaemia sepenuhnya dan pelepasan sel-sel ini dari kawalan sebelumnya telah menyebabkan pencarian secara intensif dijalankan bagi mekanisme rintangan dan rawatan seterusnya untuk mengatasi rintangan ini. Sementara itu, pengawalaturan telomerase dan penyelenggaraan telomere adalah faktor penting dalam percambahan sel dan kelangsungan hidup yang memainkan peranan penting dalam perkembangan kanser. Anggapan bahawa telomerase sebagai komponen penting dalam pengawalaturan sel, ia mungkin merupakan salah satu mekanisme tercetusnya kanser dalam penghasilan CML dan mungkin juga menyumbang dalam mekanisme rintangan terhadap rawatan imatinib mesylate. Sebanyak 98 pesakit CML direkrut dari empat hospital yang bekerjasama di seluruh negara iaitu Hospital Pulau Pinang, Hospital Sultanah Aminah, Hospital Raja Permaisuri Bainun dan Pusat Perubatan Universiti Kebangsaan Malaysia. Sampel juga dikumpul dari Hospital USM yang merupakan pusat penyelidikan bagi

kajian ini. Pesakit CML yang dirawat dengan imatinib mesylate dibahagikan kepada 2 kumpulan iaitu kumpulan yang memberi respon yang baik tehadap rawatan dan kumpulan yang rintang terhadap rawatan. Pada awal pengumpulan sampel, seramai enam puluh enam pesakit terdiri daripada kumpulan yang memberikan respon baik dan 32 pesakit bagi kumpulan yang rintang telah berjaya direkrut. Walau bagaimanapun, 90 sampel dapat diekstrak DNA dan RNA sementara hanya 79 sampel sahaja berjaya digunakan untuk pengekstrakan protein kerana kualiti sampel yang diperoleh tidak baik. Umur pesakit yang terlibat dalam kajian ini adalah antara 71 hingga 77 tahun dan merupakan etnik utama di Malaysia yang terdiri daripada kaum Melayu, Cina dan India. Analisis tahap ekspresi *hTERT*, aktiviti telomerase dan panjang telomere pada sampel telah dilakukan. Tahap ekspresi meningkat dan kehadiran aktiviti telomerase dijumpai dalam 90% daripada jumlah keseluruhan pesakit CML dalam kajian ini tanpa mengambikira perbezaan terhadap kumpulan tindakbalas terhadap rawatan menunjukkan kawalatur telomerase sebangai petunjuk kejadian kanser dalam CML. Walaubagaiman pun, perbandingan antara kumpulan tindakbalas berbeza terhadap rawatan imatinib mesylate tidak menunjukkan sebarang perbezaan yang signifikan ($p=0.463$ dalam pengekspresan *hTERT* dan $p=0.961$ dalam aktiviti telomerase). Perbandingan panjang telomere dalam kedua dua kumpulan juga tidak menunjukkan perbezaan yang signifikasi ($p=0.228$). Hasil kajian menunjukkan pengekspresan *hTERT*, aktiviti telomerase dan panjang telomere berkemungkinan tidak memberi pengaruh secara langsung terhadap kerintangan kepada rawatan imatinib mesylate. Sehingga kini, masih tiada kajian perbandingan berkaitan tahap pengekspresan dan aktiviti telomerase dalam kumpulan pesakit dengan tondakbalas berbeza terhadap rawatan yang diberi. Daripada kajian ini juga dapat dianggapkan bahawa pengekspresan *hTERT* dan telomerase aktiviti berserta

panjang telomere mungkin mempunyai pengaruh lain dalam perbezaan kesan kerintangan berbanding kanser lain. Kajian lanjutan dengan saiz sampel yang lebih besar diperlukan untuk mengesahkan potensi pengaruh komponen telomerase dan telomere dalam leukemogenesis dan kerintangan terhadap imatinib pada pesakit CML di Malaysia.

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ABSTRACT

Chronic myeloid leukaemia (CML) is a myeloproliferative neoplasm diagnosed by the presence of the *BCR-ABL* fusion gene which produces a tyrosine kinase to signal a proliferation of white blood cells. The introduction of imatinib mesylate in early 2000, has dramatically increased the survival of affected patients and changed the disease management. However, failure to completely eradicate leukaemic cells and the escape of these cells from previous control has led to an intensive search for the mechanisms of resistance and subsequent treatments by which to overcome the resistance. Meanwhile, telomerase regulation and telomere maintenance are the critical factors in cell proliferation and survival which plays important roles in development of cancers. Considering telomerase as an important component in cell regulations, it is postulated that telomerase might be one of the oncogenesis mechanism in CML development and might contribute in the resistance mechanism of imatinib mesylate. A total of 98 CML patients were recruited from four collaborated hospitals over the country which are Hospital Pulau Pinang, Hospital Sultanah Aminah, Hospital Raja Permaisuri Bainun and Pusat Perubatan Universiti Kebangsaan Malaysia. Samples were also collected from Hospital USM which is the research center for this study. CML patients treated with imatinib were enrolled into this study and grouped as good response and resistance according to response of the imatinib mesylate treatment. Sixty-six of patients with good response

at the beginning of samples collection and 32 patients with resistance response were managed to recruited. However, only 90 samples were able to proceed with DNA and RNA extraction while 79 samples were successfully applied for protein extraction due to poor quality of samples obtained. Range of age was between 71 to 77 years old and represented of main ethnicity in Malaysia which were Malay, Chinese and Indian. The analysis of *hTERT* expression level, telomerase activity and length of telomere were performed. The up regulated of *hTERT* expression and the presence of telomerase activity were found up to 90% in CML patients recruited in this study regardless of response group. This indicated that telomerase regulations as the indicator of cancer events in CML. However the telomerase regulations might not directly influenced the response and resistance group of treated patients. Comparison between good response and resistance group showed no significant difference ($p=0.463$ in *hTERT* expression and and $p=0.961$ in telomerase activity). Comparison of telomere length in both groups were also showed no significant difference ($p=0.228$). The findings indicated that *hTERT* expression, telomerase activity and telomere length may not directly influnced the resistance to imatinib mesylate treatment. To the best of our knowledge, there is no study comparing the expression and activity of telomerase in group of difference response to imatinib treatment in CML patients. This could consider that *hTERT* and the activitiy of telomerese together with telomere length in CML might have different contributions in resistance development compared to other solid tumours. Further more, investigations with a larger number of samples is warranted to confirm the potential influence of the telomerase and telomere components in the leukaemogenesis and imatinib resistance in Malaysian CML patients.

CHAPTER 1

INTRODUCTION

1.1 Research background

Cancer is a malignant growth that can spread to almost any part of the body. The growths often attack surrounding tissue and potentially metastasize to other parts of body. Early detected cancers might be cured by medical procedure such as surgery, radiotherapy or chemotherapy. Nowadays cancers are responsible for the majority of global death. According to World Health Organization (WHO), cancer is the second leading cause of death globally and responsible for an estimated 9.6 million deaths in 2018 with 18.1 million of cases and expected to reach 2.4 million cases in 2040 (WHO, 2020). Global Cancer Statistics 2018 reported that three leading cause of cancer death are lung cancer as the most frequent followed by female breast cancer and prostate cancer (Bray *et al*., 2018). The formation and development of cancer is caused by the accumulation of genetic mutations in cells. An increasing life expectancy extends the period over which oncogenes act on cells and increases the risk of cancer development.

In Malaysia, report on incidence of cancer cases was latest updated for the year period of 2012-2016 by Malaysia National Cancer Registry (MNCR). A total of 115,238 cancer cases were diagnosed during that period. Three most common cancers among Malaysian residents were breast cancer followed by colorectal and lung. Of these 51,505 (44.7%) were reported in males and 63,733 (55.3%) in females. The most common cancer among male and female residents was colorectal (14.8 in 100,000) and breast (34.1 in 100,000) respectively. Leukaemia is among ten

most common cancer in Malaysia (Table 1.1) being the seventh most common in males and eighth most common in females (Department of Statistics, 2019).

Table 1.1Number of cases in most common cancers in Malaysia.

Types of cancer	**Number of cases (%)**
Breast	21,925 (19.0)
Colorectal	15,515 (13.5)
Trachea, Bronchus, Lung	11,256 (9.8)
Lymphoma	5,830 (5.1)
Nasopharynx	4,957 (4.0)
Leukaemia	4,273 (3.7)
Prostate	4,189 (3.6)
Liver	4,033 (3.5)
Uteri	3,981 (3.5)
Ovary	3,575 (3.1)
Others	36,064 (31.3)
Total Number	**115,238 (100.0)**

*Data from Malaysia National Cancer Registry Report 2012-2016.

1.2 Problem statement

Chronic myeloid leukaemia is characterized by the presence of Philadephia (Ph) chromosome which occurs from the fusion of Abelson (ABL) gene on the chromosome 9 with the break-point cluster region (BCR) gene on chromosome 22. This results in expression of hybrid protein termed BCR-ABL1; a constitutively active tyrosine kinase that gives signal for the production of unregulated cell division thus contributing to leukaemogenesis. Starting 2001, United States Food and Drug Administration (US FDA) has proven the use of imatinib mesylate as first-line treatment to CML patients (Cohen *et al*., 2002). Imatinib is an orally administered protein-tyrosine kinase inhibitor of the BCR-ABL protein tyrosine kinase which blocks proliferation and induces apoptosis of BCR-ABL1. Since the year of introduction of imatinib mesylate, the annual mortality in CML has decreased from 10-20% down to 1-2% (Huang, Cortes & Kantarjian, 2012). Therefore, imatinib mesylate has unquestionably revolutionized the therapy of CML by lowering the rates and duration of remission of the disease. However, there was still a subset of patients who developed suboptimal response or fail to response upon this treatment. Drug resistance in this disease remains a major problem and raised up clinical challenges in treating CML patients.

1.3 Rationale of the study

The emergence of imatinib resistance in CML patients highlights the importance of understanding the molecular mechanisms associated with imatinib mesylate resistance. The molecular mechanisms implicated in this resistance include those that involve upregulation or mutation of BCR-ABL1 kinase and those that are BCR-ABL1 independent. The *BCR-ABL1* dependent is generally associated with point mutations in ABL-kinase domain which is the main reason for imatinib resistance in CML. While in *BCR-ABL1* independent, the BCR-ABL1 tyrosine kinase remains inhibited by imatinib mesylate, but there might be post-translational activation of another key enzyme such as telomerase that activates alternative signalling pathways leading to uncontrolled cell growth.

In this study, we focused on the *BCR-ABL1* independent mechanism in particular, factors related with telomerase in the resistance of imatinib mesylate treatment. Accessing the telomerase expression and activity together with the length of telomere in CML patients treated with imatinib might give better understanding on the molecular level whether these factors has any influnce on the resistance of the treatment.

1.4 Research hypotheses

1. There will be higher expression of *hTERT* and relatively higher frequency of patients with telomerase activity in resistance group of patients compare with good response group.
2. There will be higher telomerase activity in resistance group of patients
3. There will be more patients with shorter telomere length in response group.

1.5 Research questions

1. Is *hTERT* expression level different and relatively higher in resistance group of CML patients?
2. Is telomerase activity different and relatively higher in resistance group of CML patients?
3. Is telomere length relatively longer in resistance group of CML patients?

1.6 Aims of study

General objective:

This study focused on *hTERT* expression and telomere length in chronic myeloid leukaemia patients and the potential activation of telomerase among treated patients that may influence the resistance to imatinib mesylate.

Specific objectives:

1. To determine the expression profile of *hTERT* in good response and resistance chronic myeloid leukaemia patients treated with imatinib mesylate.
2. To identify the telomerase activity in good response and resistance chronic myeloid leukaemia patients treated with imatinib mesylate.
3. To measure the average telomere length in good response and resistance chronic myeloid leukaemia patients treated with imatinib mesylate.
4. To associate the *hTERT* expression level, telomerase activity and telomere length with clinical and demographic data of both groups of patients response and resistance to imatinib mesylate treatment.

CHAPTER 2 LITERATURE REVIEW

2.1 Hematological malignancies

Hematological malignancies are cancers that affect blood, bone marrow and/or lymph nodes. The pluripotent hematopeitic stem cells in bone marrow mature and differentiate into 3 types of blood cells; white blood cells, red blood cells and platelets. White blood cells are mainly fight for infection, red blood cells facilitate tissue oxygenation while platelets are to maintain the integrity of vascular system and blood clotting. The disruption of normal differentiation in hematopoietic stem cells causes the hematological malignancies. The resulting disease depends on how mutations affect the differentiation pathway of the stem cells. Hematological malignancies includes multiple myelomas, lymphomas (Hodgkin's and non-Hodgkin's) and various type of leukaemias.

2.2 Leukaemia

There are four major types of leukaemias; acute lymphocytic leukaemia (ALL), acute myeloid leukaemia (AML), chronic lymphocytic leukaemia (CLL) and chronic myeloid leukaemia (CML). Leukaemias are generally categorized into acute or chronic based on the percentage of blast cells in bone marrow or blood. The acute and chronic forms differ in cell maturity on onset. In acute leukaemias, the number of immature blood cells are rapidly increased. The crowding that results from such cells causes the bone marrow to be unable to produce healthy blood cells. Later, resulting in low production of hemoglobin and platelets in the body. Rapid progression and accumulation of the malignant cells could spills over into the peripheral blood and spread to other organs of the body. Therefore, an immediate

treatment is required in acute leukaemias. Acute forms may occur in both adults and children but most common in children.

Chronic leukaemias are distinguished from acute leukaemias by the slower progression with matured abnormal cells. Normal cell production may occurs as well for a long period of time but later in late stages of chronic disease, the abnormal cells interfere with normal cell productions. The abnormal cells eventually suppress the production of normal white blood cells causes the body to expose on infection. Onset for chronic is much slower that normally over months or years. Chronic forms mostly occurs in older people and rare in children (de la Fuente *et al*., 2014). The acute and chronic leukaemias are divided into myeloid or lymphoid based on the lineage of the blast cells.

The biological centrum of leukaemia is the formation and development of blood cells called hematopoeisis. All blood cells are derived from hematopoeitic stem cells which are produced in bone marrow. These cells can self-renew and differentiate into precursor called myeloid and lymphoid lineages. The myeloid lineages lead to the mature fully differentiated cells including megakaryocyte, which form thrombocytes, erythrocytes and various of granulocytes (eosinofil, basofil, neutrophil and monocytes). The matured lymphoid lineages of hematopoeisis form B-lymphocytes, T-lymphocytes and natural killer cell (Figure 2.1).

The classification of leukaemia is according to World Health Organization (WHO) and French-American-British (FAB) system. The WHO classification is based on a combination of clinical, morphologic, immunophenotypic, and genetic features (Arber *et al*., 2016). The FAB system is less commonly used and the classification is based on the morphology of the abnormal leukocytes.

Higher numbers of immature white blood cells in leukaemia patients result in lack of blood platelets and easily become bruised, excessively bleed or develop petechiae. Suppressed or dysfunctional white blood cells cause the patient's immune system to unable to fight off even a mild infection. Some patients experience frequent infection ranging from infected tonsils, sores in the mouth or diarrhea to life-threatening pneumonia or opportunistic infections. The red blood cell deficiency leads to anemia. Some patients experience other symptoms such as night sweats, chills, feeling sick, fatigue, having fever, nausea or a feeling of fullness due to an enlarged liver and spleen.

Several risk factors have been identified for leukaemia including ionizing radiation and exposure to chemotherapeutic drugs or chemical such as benzene (Bloemen *et al*., 2004). Risk of developing leukaemia is increased in patients with history of autologous stem cell transplantation. Like other cancers, leukaemia results from somatic mutations in the DNA. Therefore, activating oncogenes or deactivating tumor suppressor genes can cause distruption in differentiation or regulation of cell death. The use of tobacco is also associated with a small increase in the risk of developing acute myeloid leukaemia in adults (Fircanis *et al*., 2014). Cohort and case-control studies have linked exposure to some petrochemicals and hair dyes to the development of some forms of leukaemia (Clapp, Jacobs & Loechler, 2008). A few cases of maternal-fetal transmission have also been reported (Hassanzadeh *et al*., 2011). Diet has very limited or no effect, although eating more vegetables may confer a small protective benefit. Viruses have also been linked to some forms of leukaemia. Experiments on mice and other mammals have demonstrated the relevance of retroviruses in leukaemia, and human retroviruses have also been identified (Dalirsani *et al*., 2015).

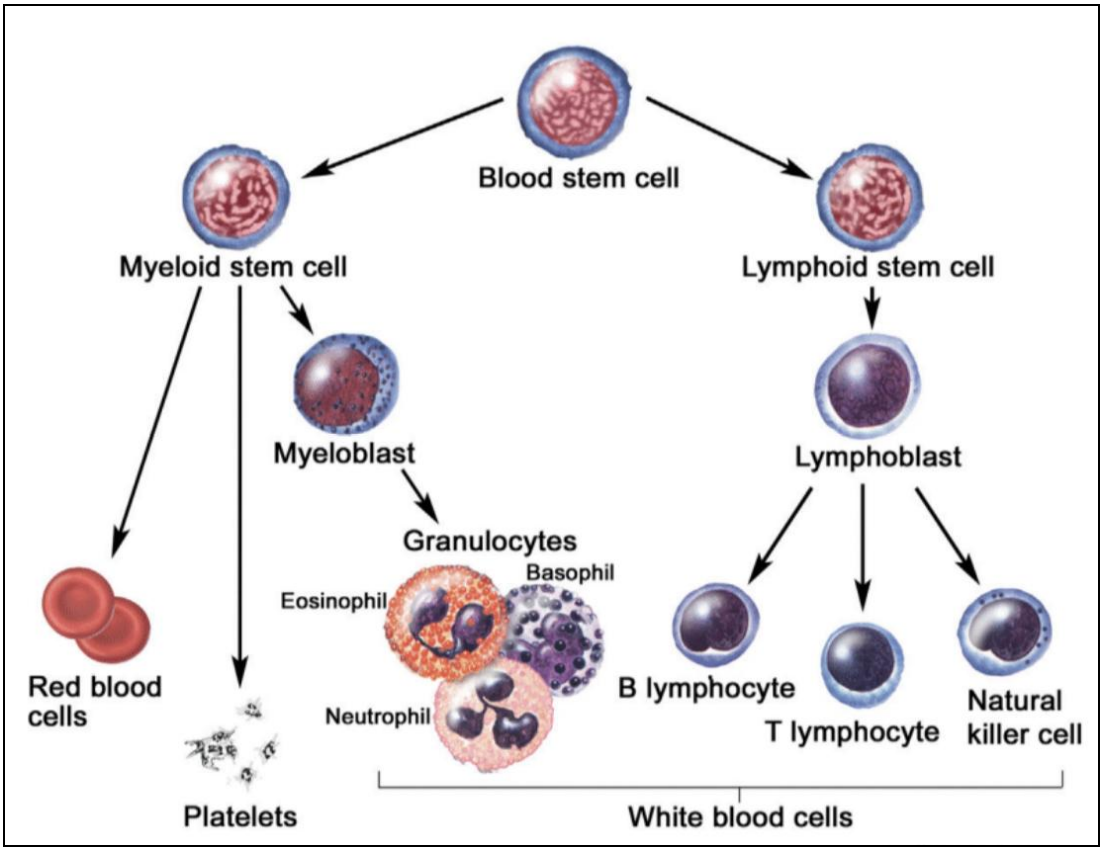

Figure 2.1 Diagram shows hematopoetic stem cells differentiation. The blood stem cells differentiate into pluripotent stem cells which are lymphoid lineage and myeloid lineage. The myeloid stem cell then differentiates into red blood cells, or a type of white blood cell, a neutrophil while the lymphoid stem cell differentiates into T cells, B cells, and NK cells (Adapted from Brody, 2016).

2.3 Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is a myeloproliferative neoplasm characterized by dysregulated production and uncontrolled proliferation of mature and maturing granulocytes in the bone marrow**.** CML accounts for 15–20% of all cases of leukaemia in adults (Soverini *et al*., 2016). Clinically, peripheral blood cell profile shows an increased number of granulocytes and blast cells. Patients are often asymptomatic at the early stage with onset of nonspecific symptoms such as fatigue, weakness, anorexia, weight loss, night sweats, a sense of abdominal fullness, gouty arthritis and symptoms of leucostasis which may prompt evaluation.

CML is a slow progressing white blood cancer of myeloid cell lineage. There is an elevated white blood cell count including granulocytes especially neutrophils. Other than chronic myeloid leukaemia, CML also known as chronic myelogenous leukaemia, chronic myelocytic leukaemia or chronic granulocytic leukaemia. CML is the third most common type of leukaemia. Currently CML is treated with imatinib mesylate as a standard first-line treatment.

2.3.1 Molecular pathogenesis of chronic myeloid leukaemia

Philadephia chromosome discovered by Nowell and Hungerford in 1960 is the hallmark of genetic abnormality in CML. It was named after the city it was discovered. Peter Nowell was an investigative pathologist in the Department of Pathology at the University of Pennsylvania, and David Hungerford was a cytogeneticist at the Institute for Cancer Research in Philadelphia. They found a

short chromosome in patients with granulocytic leukaemia and speculated a direct involvement of this truncated chromosome in the genesis of the disease (Nowell, 1962). This was the first proof that the disease results in some changes to DNA. After the establishment of chromosomal banding techniques, shortened chromosome 22 resulting from a reciprocal translocation of chromosome 9 and 22 were explained by Janet Rowley and known as Philadephia positive (Ph^+) (Rowley, 1973) (Mughal *et al*., 2016).

Majority of CML patients (90-95%) exhibited this abnormality. However, the Philadelphia translocation is not specific for CML since it can also be detected in other entities. It was observed in 11-29% of acute lymphoid leukaemia (ALL) patients (Pullarkat *et al*., 2008) and relatively rare in acute myeloid leukaemia (AML) patients with less than 1.5% (Soupir *et al*., 2007). To date, the mechanism of this single aberration that give results in different clinical phenotypes is still not well understood (Mughal *et al*., 2016).

The shortened chromosome 22 at band q11.21 and elongated chromosome 9 at band q34.1 was referred as t(9;22)(q34;q11) (Goldman, 2008). The translocation involved a large segment of Abelson (ABL) proto-oncogene from the long arm of chromosome 9 and the breakpoint cluster region (BCR) genes on the long arm of chromosome 22 producing a fusion gene, *BCR-ABL1* (Goldman and Mughal, 2005) (Figure 2.1).

The BCR gene spans 130 kb, contains 23 exons (Miller *et al*., 1988) and situated closer to the centromere (Heisterkamp *et al*., 1985). It is widely expressed in a wide variety of actively proliferating and nonproliferating vertebrate tissues (Collins, Coleman & Groudine, 1987). In chick embryos, as well as in mouse and

human tissues, BCR mRNA levels were found to be highest in the brain and hematopoietic cells (Zhu, Heisterkamp & Groffen, 1990). The gene represents a serine or threonine kinase consisting among others of a coiled-coil oligomerization domain and influences different signalling pathways via interaction with partners such as growth factor receptor bound protein 2 (GRB2) or ABL1 (Ren, 2005).

ABL1 gene, located on chromosome 9 is an oncogene that changes the activity of other proteins by adding a cluster of oxygen and phosphorus atoms (Szczylik *et al*., 1991). It is a member of the non-receptor tyrosine kinase family that transduces signals from growth factor and adhesion receptors from the cell surface to the nucleus (Hernández *et al*., 2004). The native ABL kinase has tightly regulated kinase activity and located in the nucleus while the mutated gene encodes a protein containing a tyrosine kinase activity domain (SH1) in addition to two other regulatory domains (SH2 and SH3) that mediates protein-protein interaction and modulate activation of signal transduction (Vigneri & Wang, 2001). The ABL1 kinase is normally inactive and should be activated to perform its functions. The kinase can be activated by a number of different triggers by adding a phosphate group to other different proteins. The diversity allows ABL1 to be involved in a wide variety of cellular processes including cell proliferation, differentiation and migration (Deininger *et al*., 2005).

The BCR-ABL fusion protein is located in the cytoplasm and has a constitutively activated tyrosine kinase. This chimeric gene functions by binding to ATP and transfers phosphate from ATP to tyrosine residues, activating multiple signal transduction pathways . This event causes excessive cellular proliferation, prevention of apoptosis, and decreasing cellular adhesion. It has been shown that the enhanced tyrosine kinase activity of BCR-ABL1 chimeric protein is essential for the

pathogenesis and the expression of this aberrant transcript is likely to represent the initiating event of CML (Sawyers Charles L, 1999).

Due to varying breakpoints of BCR-ABL1 transcripts containing different exons of ABL1 and BCR, proteins with molecular weights of 190 kDa, 210 kDa, and 230 kDa were translated. The three types of breakpoint in the BCR-ABL region are Major breakpoint cluster region (M-bcr), minor breakpoint cluster region (m-bcr) and micro breakpoint cluster region (μ-bcr). M-bcr is commonly found within 5.8kb region, spanning approximately 5 exons and it encodes a fusion protein called p210 BCR-ABL fusion protein (Melo, 1997). Minor breakpoint cluster region (m-bcr) is detected in two-third of Ph-positive ALL and in rare cases of CML which spans in the long intron (54.4kb) (Chissoe *et al*., 1995) and translated into a smaller fusion protein of 190 kDa ($p190^{BCR-ABL}$). Micro breakpoint cluster region (μ-bcr) is translated into p230 BCR-ABL fusion gene (Melo, 1997). This $p230^{BCR-ABL}$ has additional of 180 amino acids encoded by 540 bp of extra sequence of BCR as compared to the classic $p210^{BCR-ABL}$. All these three type of breakpoints have tyrosine kinase activity (Wada *et al*., 1995).

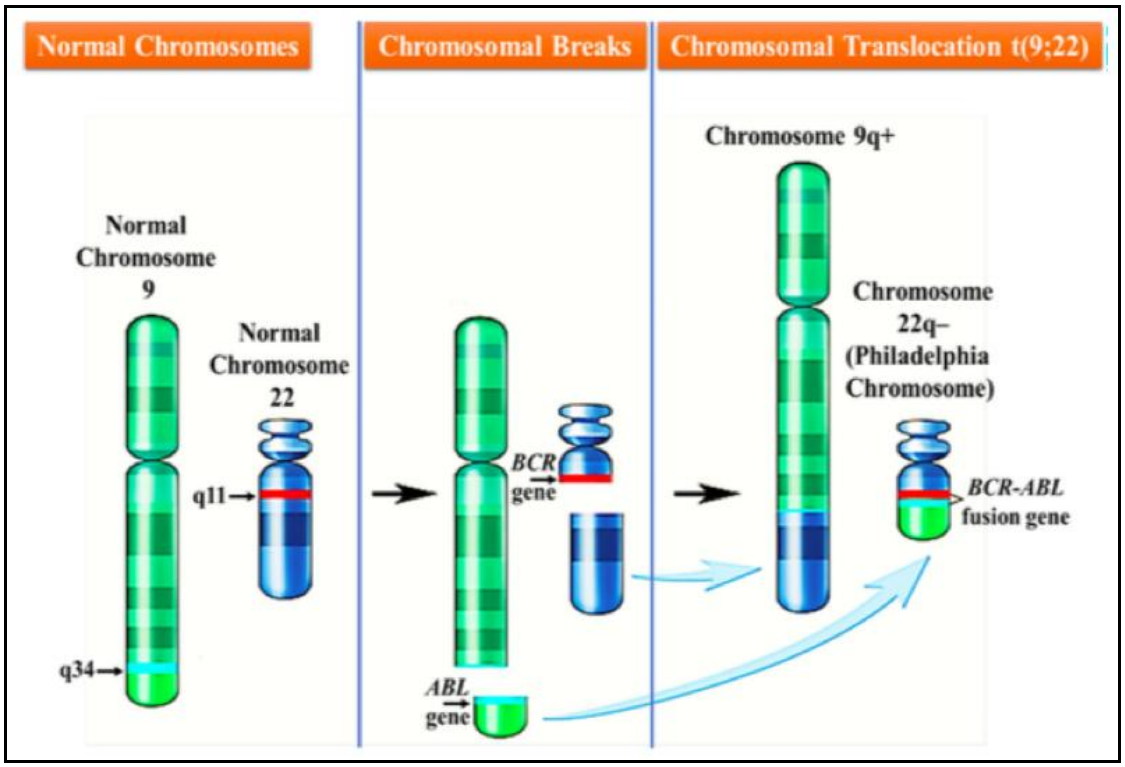

Figure 2.2 Ilustration of Philadephia chromosome formation resulting from reciprocal translocation events between chomosome 9 and 22 which carries the BCR-ABL fusion gene [Adapted from (Ali, 2016)].

2.3.2 Diagnosis and classification of chronic myeloid leukaemia

Chronic myeloid leukaemia normally suspected based on an abnormal complete blood count (CBC) obtained incidentally or during evaluation of splenomegaly. A CBC shows the number of different kinds of cells in the blood and is often done as part of a regular medical checkup. The granulocyte count is elevated, usually $\leq 50\times10^9$/L in asymptomatic patients and 200×10^9/L to $1{,}000\times10^9$/L in symptomatic patients. Neutrophilia, basophilia, and eosinophilia are common. The platelet count is normal or moderately increased, and in some patients, thrombocytosis is the presenting manifestation. The hemoglobin level is usually more than 100 g/L. To differentiate CML from leukocytosis of other etiology, peripheral smear is needed. In CML, the peripheral smear frequently shows immature granulocytes as well as absolute eosinophilia and basophilia. However, in patients with white blood cell counts $\leq 50\times10^9$/L and even in some with higher white blood cell counts, immature granulocytes may not be seen. Bone marrow examination should be done to confirm the diagnosis of CML by cytogenetic or molecular analysis. However, the absent of Philadephia abnormality in 5% of CML patients could be confirmed by fluorescence in situ hybridization (FISH) or reverse transcription polymerase chain reaction (RT-PCR).

In defining the disease entities of clinical significance, criteria revised by WHO includes clinical features, morphology, immunophenotyping, cytogenetics, and molecular genetics. These criterias which derived from numerous published clinical and scientific studies from the experience of more than 100 worldwide pathologists, hematologists, oncologists, geneticists and clinicians were regularly updated for the

improvement of the diagnostic criteria as well as the prognostic relevance of current entities (Arber *et al*., 2016).

2.3.3 Clinical phases and stages in CML

The next stage of diagnosis is to determine the phase of the disease. Most patients at the point of diagnosis are in a chronic phase (CP). However, there are also patients who are diagnosed with an acceleration phase (AP) or a blastic phase (BP). Determination of the phase of the disease is important for the appropriate treatment option. There are triphasic clinical phases of CML which are chronic, accelerate and blastic. In Sweden, the WHO classifications are mainly used while the European LeukaemiaNet (ELN) classifications are used in most clinical studies. The diagnostic criteria are summarized in Table 2.1. Since WHO recognizes the practical importance of the blast count in categorizing myeloid diseases and in predicting prognosis, the phases are based mainly on the number of blast cells in the blood or bone marrow (Vardiman *et al*., 2014).

Table 2.1 Classification of CP, AP and BC according to World Health Organization (WHO) and European LeukaemiaNet (ELN), adapted from Gunnarsson, 2017

World Health Organization (WHO)	**European LeukaemiaNet (ELN)**
Chronic phase	
• Blasts in bone marrow <10 %	• Blasts in bone marrow <15 %
Accelerated phase	
• Blasts in bone marrow 10-19 % • Basophils in blood ≥20 • Persistent thrombocytopenia (<100 x 109/L) unrelated to therapy • Clonal chromosome abnormalities in Ph+ cells (CCA/Ph+), major route, at diagnosis • CCA/Ph+ on treatment • Thrombocytosis (≥1000 x 10^9/L) unresponsive to therapy • Increasing spleen size and increasing white blood cell count unresponsive to therapy	• Blasts in blood or marrow 15-29%, or blasts plus promyelocytes in blood or marrow >30 %, with blasts <30 % • Basophils in blood ≥ 20 % • Persistent thrombocytopenia (<100 x 109/L) unrelated to therapy • CCA/Ph+ on treatment
Blast crisis	
• Blasts in blood or marrow ≥20 % • Extramedullary blast proliferation, apart from spleen • Large foci or clusters of blasts in the bone marrow biopsy	• Blasts in blood or marrow ≥30 % • Extramedullary blast proliferation, apart from spleen

2.3.3(a) Chronic phase

Chronic phase is the earliest phase of CML. Patients in the chronic phase typically have less than 10% blasts in their blood or bone marrow samples (Vardiman *et al*., 2014). Most patients were diagnosed in the chronic phase with 85% of patients are asymptomatic. Diagnosis is usually incidentally when the patient has a full blood count or peripheral blood smear showing an elevated white blood count including basophilia or platelet. A bone marrow aspiration is then needed for further confirmation. Patients with chronic phase may have fatigue, weight loss, low-grade fever, loss of energy, decreased exercise tolerance and excessive sweating due to hypermetabolism. Elevated white blood cell count or splenomegaly found on routine assessment. Others such as early satiety and decreased food intake from encroachment on stomach by enlarged spleen.

The chronic phase is usually relatively stable and benign phase of CML and generally lasts for 3–5 years from diagnosis. During this period, malignant progenitor cells proliferate rapidly but retain their ability to differentiate. Progression of CML is due to the gradual loss of differentiation potential of malignant cells.

2.3.3(b) Accelerated phase

Patients who do not response well to treatment during their chronic phase are prone to develop cancer that are more aggressive with noticeable symptoms which is accelerated phase. The accelerated phase is diagnosed in the presence 10-19% blast in peripheral blood or bone marrow. The criteria for accelerated phase by WHO has been revised in 2016 with the additional of reponse to tyrosine kinase inhibitor instead of hematologic or cytogenetic criterias (Table 2.2) (Arber *et al*., 2016). In comparison with the previous editions of the WHO classification, new parameter is the presence of which justifies the diagnosis of AP. These include the persistent leukocytosis (10×10^9/L) not responding to treatment, persistent splenomegaly not responding to treatment and additional clonal chromosomal aberrations in Ph+ cells present at the point of diagnosis. Despite these changes, the criterias are still differ significantly to those described by European LeukaemiaNet (ELN) guidelines, International Bone Marrow Transplant Registry and MD Anderson Cancer Center (MDACC). According to WHO, the AP should be diagnosed in the presence of blasts in the bone marrow or peripheral blood at a percentage of 10%–19%, while according to ELN this range is higher which is 15%-29%.

Table 2.2 Additional criteria for accelerated phase CML updated by WHO (Source: Arber *et al*., 2016)

Any 1 or more of the following hematologic/cytogenetic criteria or response-to-TKI criteria:	
Persistent or increasing WBC (>10 x 10^9/L), unresponsive to therapy	"Provisional" response-to-TKI criteria
Persistent or increasing splenomegaly, unresponsive to therapy	Hematologic resistance to the first TKI (or failure to achieve a complete hematologic response* to the first TKI) or
Persistent thrombocytosis (>1000 x 10^9/L), unresponsive to therapy	Any hematological, cytogenetic, or molecular indications of resistance to 2 sequential TKIs or
Persistent thrombocytopenia (>100 x 10^9/L) unrelated to therapy	Occurrence of 2 or more mutations in *BCR-ABL1* during TKI therapy
20% or more basophils in the PB	
10%-19% blasts$^{\Psi}$ in the PB and/or BM	
Additional clonal chromosomal abnormalities in Ph^+ cells at diagnosis that include "major route" abnormalities (second Ph, trisomy 8, isochromosome 17q, trisomy 19), complex karyotype, or abnormalities of 3q26.2	
Any new clonal chromosomal abnormality in Ph^+ cells that occurs during therapy	

Large clusters or sheets of small, abnormal megakaryocytes, associated with marked reticulin or collagen fibrosis in biopsy specimens may be considered as presumptive evidence of AP, although these findings are usually associated with 1 or more of the criteria listed above.

*Complete hematologic response: WBC, <10x10^9/L; platelet count, <450x10^9/L, no immature granulocytes in the differential, and spleen nonpalpable.

2.3.3(c) Blastic phase

The most aggressive stage of CML is blastic phase. The definition of blastic phase is slightly simpler, when the percentage of blasts in the peripheral blood or bone marrow is 30% or more according to ELN and 20% or more according to WHO. It is also diagnose with the case of extramedullary blast proliferation (Flis & Chojnacki, 2019). At this stage, haematopoietic differentiation has become arrested and immature blasts accumulate in the bone marrow and spill into the circulation system. Symptoms are similar to those of acute myeloid leukaemia. The blast phase is usually fatal within 3–6 months of onset. Transition between the phases may be gradual or rapid. Typically, the annual progression from chronic to blast phase is 5–10% in the first 2 years and 20% in subsequent years (Vardiman *et al*., 2014). During this crisis, almost 80% of patients are observed with additional chromosomal abnormalities such as double Ph chromosome, trisomy 8, trisomy 19, i(17q) and also loss of rearrangment involving 11p (Barnes & Melo, 2002).

2.3.4 Epidemiology

In Western countries, population-based registries such as Surveilance, Epidemiology and End Results (SEER), Leukaemia Research Fund, British Haematological Malignancy Research Network (HMRN), European Treatment and Outcome Study (EUTOS), Swedish CML Registry has been established in detailed the data collection on demographics epidemiology of chronic myeloid leukaemia at regional or national level. CML in Western countries accounts for 15–25% of all adult leukaemias and 14% of leukaemias overall including the pediatric population . The incidence was reported between 0.4-2 cases per 100,000 per year while the prevalence expected between 3-15 cases per 100,000 in 2017. The median age of CML was between 57–60 years and a male to female ratio was 1.2–1.7. (Tadwalkar, 2017), (Noone *et al*., 2017).

To date, global prevalence of CML was seen increasing with approximately 10-12 per 100,000 per year affecting subjects of all age groups. This steadily increasing number was due to prolongation of survival in patients that has been achieved with the efficacy of tyrosine kinase inhibitors as targeted therapy in treating the disease (Höglund, Sandin & Simonsson, 2015), (Rohrbacher & Hasford, 2009).

In Asia, incidence of CML reported lower (0.-2.2 per 100,000 population) than in Western countries with median age at diagnosis within range of 37-55 years old (Au *et al*., 2009). Data on incidence and prevalence of CML in Malaysia was very limited and currently only reported for the area of Southern Sarawak with the incidence of 0.8 per 100,000 population per year for the year 2011-2016 and estimated prevalence of 6.9 per 100,000 population at the year 2016. Median age of