APPLICATION OF HIGH RESOLUTION MELTING (HRM) ANALYSIS IN DETECTION OF PDGFRA GENE MUTATIONS AMONG CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH IMATINIB MESYLATE

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by

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%	Percent
^{o}C	Degree celcius
>	Change to for SNPs and mutation
X^2	Chi-square
=	Equal to
±	Plus minus
’	Prime
-	Range
:	Ratio
ATP	Adenosine triphosphate
BCR	Breakpoint cluster region
BCR-ABL	BCR-ABL gene
BLAST	Basic Local Aligment Search Tool
BP	Blast phase
Bp	Base pair
CCyR	Complete cytogenetic response
CI	Confidence interval
CML	Chronic myeloid leukemia
CP	Chronic phase
Cq	Quantification cycle
CyR	Cytogenetic response
ddH_2O	Deionized distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
dsDNA	Double stranded dinucleotide triphosphate
EDTA	Ethylenediamine tetraacetic acid
G	Gram
GIST	Gastrointestinal stromal tumour
HCl	Hydrochloride
HES	Hypereosinophilic syndrome

HRM	High resolution melting analysi s
HPP	Hospital Pulau Pinang
HUSM	Hospital Universiti Sains Malaysia
IM	Imatinib mesylate
KIT	Stem cell growth factor receptor gene
$MgCl_2$	Magnesium chloride
mg/Dl	Milligram per decilitre
min	Minutes
mL	Millilitre
mM	Milimolar
MPN	Myeloproliferative neoplasms
NCBI	National Centre for Biotechnology Information
nm	Nanomolar
NTC	Non template control
P (Pro)	Proline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFRA	Platelet derived growth factor receptor alpha gene
PDGFRA	Platelet derived growth factor receptor alpha protein
PDGFRB	Platelet derived growth factor receptor beta protein
Ph	Philadelphia chromosome
S (Ser)	Serine
Taq	*Thermophilus aquaticus*
TBE	Tris-Boric Acid-EDTA
TKI	Tyrosine kinase inhibitor
T_m	Melting temperature
UK	United Kigdom
USA	United State of America
UV	Ultra violet
V	Volt
WBC	White blood cell
WHO	World Health Organization

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APLIKASI ANALISIS PELEBURAN RESOLUSI TINGGI DALAM PENGESANAN MUTASI GEN PDGFRA DI KALANGAN PESAKIT-PESAKIT LEUKEMIA MEILOID KRONIK YANG DIRAWAT DENGAN IMATINIB MESYLATE

ABSTRAK

Pengesanan molekul bagi varian *PDGFRA* adalah sangat relevan untuk meramal prognosis dan terapi untuk pesakit leukemia meiloid kronik (CML) yang dirawat dengan Imatinib mesylate (IM). Walaupun IM telah menunjukkan keberkesanan dalam rawatan utama CML, namun kemunculan rintangan kepada IM telah menjadi masalah utama yang membimbangkan. Kemunculan rintangan terhadap IM boleh disebabkan oleh sama ada mekanisma bersandar *BCR-ABL* atau mekanisma tidak bersandar *BCR-ABL*. Mekanisma tidak bersandar *BCR-ABL* terdiri daripada beberapa faktor termasuk perubahan dalam pharmakokinetik IM seperti penyerapan dan pengedaran metabolism. Walau bagaimanapun, kerintangan terhadap IM juga boleh berlaku dengan penglibatan tirosina kinase selain daripada BCR-ABL yang juga terlibat di dalam kerintangan terhadap IM. Sebagai tindak balas kepada permintaan yang semakin meningkat untuk kaedah yang boleh dipercayai, cepat dan lebih sensitif, kajian ini menggunakan analisa peleburan resolusi tinggi untuk menjelaskan mekanisma yang tidak bersandar *BCR-ABL* yang melibatkan *PDGFRA* tirosina kinase pada pesakit CML di Malaysia yang menerima rawatan IM. Sejumlah 86 pesakit CML yang menjalani rawatan IM (43 rintang terhadap IM dan 43 respon terhadap IM) telah menyertai kajian ini. Menggunakan analisis peleburan resolusi tinggi, 86 pesakit CML telah disaring untuk *PDGFRA* ekson 10 (c.1432 T>C), ekson 12 (c.1701 A>G), ekson 14 (c.1977 C>A) and ekson 18 (c.2525 A>T). Sampel yang

mempunyai corak lengkung lebur yang berbeza daripada sampel rujukan telah diteruskan untuk proses penjujukan DNA bagi mengesahkan genotip. Daripada analisis data, terdapat dua *PDGFRA* variasi yang dikenalpasti iaitu *PDGFRA* ekson 10 dan 12. Profil HRM yang telah ditubuhkan melalui kajian ini menunjukkan 100% sensitiviti dan spesifisiti jika dibandingkan dengan penjujukan DNA. *PDGFRA* ekson 10 menunjukkan faktor risiko yang signifikan untuk pembentukan rintangan kepada IM (OR 3.797; 95% CI: 1.502 – 9.591; p = 0.005). *PDGFRA* ekson 12 pula adalah faktor risiko tidak signifikan (OR 1.597; 95% CI: 0.681 – 3.745; p = 0.281) untuk kerintangan terhadap IM. Walau bagaimanapun, tiada variasi *PDGFRA* dikesan untuk kedua-dua ekson 14 c.1977 C>G dan ekson 18 c.2525 A>T. Keputusan keseluruhan menunjukkan bahawa *PDGFRA* mempunyai pengaruh dalam menentukan kerintangan terhadap IM dalam kalangan pesakit CML dan analisis selanjutnya perlu dijalankan untuk mengesahkan peranan *PDGFRA* dalam mekanisma kerintangan IM pada pesakit CML.

APPLICATION OF HIGH RESOLUTION MELTING (HRM) ANALYSIS IN DETECTION OF PDGFRA GENE MUTATIONS AMONG CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH IMATINIB MESYLATE

ABSTRACT

A molecular detection of *PDGFRA* variants is highly relevant for prognosis and therapy prediction in chronic myeloid leukemia (CML) patients treated with Imatinib mesylate (IM). Even though IM has shown an excellent efficacy as a frontline treatment in CML, emerging of resistance towards IM in some CML patients has become a major concern. The development of resistance can be either due to *BCR-ABL* dependent mechanism or *BCR-ABL* independent mechanism. The *BCR-ABL* independent mechanisms include factor in pharmacokinetics of IM such as absorption and distribution of metabolism. However, resistance to IM can also due to the involvement of others IM targeted tyrosine kinases that may play a role in the IM resistance. In response to growing demand for reliable, faster and more sensitive methods, the present study used a High resolution melting (HRM) analysis to elucidate the *BCR-ABL* independent mechanism involving *PDGFRA* tyrosine kinase, in Malaysian CML patients undergoing IM therapy. A total of 86 CML patients on IM therapy (43 IM resistances and 43 IM responses) were included in this study. Using HRM analysis, 86 CML patients were screened for *PDGFRA* variants of exon 10 (c.1432 T>C), exon 12 (c.1701 A>G), exon 14 (c.1977 C>A) and exon 18 (c.2525 A>T). The selected samples with showing different melting curve profile from the reference genotype was subjected to DNA sequencing analysis to validate the genotype. From the data analysed, two *PDGFRA* variants were detected in exons 10

and 12. The established HRM profile has demonstrated 100% sensitivity and specificity when compared to DNA sequencing. *PDGFRA* exon 10 (c.1432 T>C) showed a significant risk factor (OR 3.797; 95% CI: 1.502 – 9.591; $p = 0.005$) for the development of IM resistance. *PDGFRA* exon 12 (c.1701 A>G) was not a significant risk factor (OR 1.597; 95% CI: 0.681 – 3.745; $p = 0.281$) for IM resistance. However there was no *PDGFRA* heterogeneity detected for both exon 14 c.1977 C>G and exon 18 c.2525 A>T. The results suggested that *PDGFRA* influenced IM resistance in CML patients and further analysis are warranted to confirm the role of *PDGFRA* in IM resistance mechanism in CML patient.

CHAPTER 1

LITERATURE REVIEW

1.1 Study overview

Leukemia is defined as the uncontrolled proliferation or expansion of haematopoietic cells that do not retain the capacity to differentiate normally to mature white blood cells. In 2010, the incidence of leukemia was reported to be increased over the years from 30,800 cases in year 2000 to 44,790 in year 2009 worldwide and it was estimated that by 2018, 60,300 people diagnosed with leukemia in United States (Panno, 2005; Murphy *et al.*, 2018). In Malaysia population, leukemia was reported to be the sixth most frequent cancer from 2007 to 2011, after lymphoma and cancer of nasopharynx, and it accounted for 4.4% of all types of cancer (Azizah *et al.*, 2016).

According to World Health Organisation (WHO) leukemia is divided into two main classes according to their progression. It can be either acute leukemia or chronic leukemia. In acute leukemia, the abnormal blasts remain very immature and the number of blasts increases rapidly. While for chronic leukemia, the cells are more mature with presence of some blasts cells and will progress slowly and gradually than in acute leukemia.

This leukemia can arise either of the two types of white blood cells – lymphoid cells or myeloid cells. Based on the cells affected, there are four types of leukemia; acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML).

Acute lymphocytic leukemia (ALL) is marked by neoplasia of precursor B- or precursor T-lymphoid cells in blood. It was reported to be the most common cancer among children with highest incidence among aged 1 to 10 years. In ALL, the accumulation of leukemic cell in the bone marrow and extramedullary site occur due to abnormal proliferation and differentiation of the leukemic cells which may lead to anemia, splenomegaly, hepatomegaly, lymphadenophaty and other manifestation of organ infiltration (Kebriaei *et al.*, 2002).

Acute myeloid leukemia (AML) is a type of leukemia that affects the myeloid cells. AML grows rapidly which is characterized by accumulation of immature blastic cells that proliferate abundantly with lack of normal differentiation (Mendelsohn *et al.*, 2008).

Chronic lymphoid leukemia (CLL) is reported to be the most common form of leukemia in adults with more than 10,000 people diagnosed every year in the United State with approximately 75% of survival rate (Bozzone, 2009). CLL affects the lymphoid cells which progress slowly over times.

In CML, the myeloid cells undergone uncontrolled expansion of mature and maturing granulocytic cells without loss their capacity to differentiate. The incidence were about 1 to 2 cases per 100,000 adults and classified as the most common chronic myeloproliferative disorder (Jemal *et al.*, 2010). CML commonly affect older patients with a median age of about 65 years and rarely diagnosed in children (Zhang and Rowley, 2011). In Malaysia, the occurance of CML in 2007 to 2011 was reported as 14.1% of all type of leukemia and 29.9% of myeloid leukemia (Azizah *et al.*, 2016). Even though the incidence rate in Asian countries is lower compared to

Western counteries, the occurrence of CML in Asians tends to affect younger population with median age 36 years old (Au *et al.*, 2009).

CML is associated with the presence of the Philadelphia (Ph) chromosome and it was the first cancer that shows a consistent genetic abnormality (Sherbenou and Druker, 2007). The presence of Ph chromosome has become a hallmark in CML identification. The Ph chromosome was first discovered by Nowell and Hungerford in 1960 and later Janet Rowley that characterized the Ph chromosome to be the results of reciprocal translocation between chromosome 9 and 22, t(9;22)(q34;q11).

In early 1980s, with the enhancement of molecular biology has led to the discovery of human homologue of the murine Abelson1 gene (*ABL1*gene) which located on chromosome 9 was found to be translocated to the chromosome 22 in CML patients. Later in 1984, the breakpoint cluster region (*BCR* gene) on chromosome 22 was explored (Goldman and Mughal, 2008). The translocation has resulted in a BCR-ABL fusion gene (**Figure 1.1**). this fusion gene codes for BCR-ABL transcript and fusion protein with a constitutively active tyrosine kinase activity and increased activation of multiple signal transduction pathways (Kurzrock *et al.*, 1988). These, later will leads to a massive production of myeloid cell that induce leukemia. The Ph chromosome has been observed in approximately 95% among CML patients (Faderl *et al.*, 1999).

The presence of well-defined pathogenetic of CML has led to the development of IM which is a tyrosine kinase inhibitor that inhibit both ABL and BCR-ABL protein. Since the BCR-ABL fusion protein is a constitutively activated tyrosine kinase that causes CML, IM has been one of the treatment options in treating newly diagnosed patients or patient who have not respond to previous treatment (Druker *et al.*, 2001).

The clinical use of IM has resulted in significantly improve prognosis and survival rate where 80% to 90% of patients in early chronic phase have shown major cytogenetic responses toward IM (Buchdunger *et al.*, 2001).

In addition to BCR-ABL, IM also inhibits other tyrosine kinases of c-KIT and platelet derived growth factor alpha (PDGFRA). Platelet derived growth factor (PDGF) receptor is belong to the receptor tyrosine kinase family of type III group. The genetic alteration of this receptor was reported mostly in myeloproliferative disorder and solid tumor such as in myelomonocytic leukemia, gastrointestinal stromal tumors (GISTs) and gliomas (Arora and Scholar, 2005; Demoulin and Montano-Almendras, 2012). The discovery that IM also blocks PDGF receptor was a major breakthrough. Where, it have shown to inhibit PDGFRA at the concentration similar to the inhibition concentration for BCR-ABL (Heinrich *et al.*, 2000). In addition, several studies also showed a significant response of IM to the treatment of GIST patients which involved the PDGFRA kinase (Druker, 2004).

However, until today some patients in both CML and GIST has become resistant towards IM therapy. The emergence of resistance has prompted intense research on wider prospects of mechanism involve in IM resistant. Previous studies have showed that PDGFRA exon 10, 12, 14 and 18 were the most common site for PDGFRA mutation that associated with IM treatment response (Holtkamp *et al.*, 2006). Therefore, this present study focused on application of High Resolution Melting (HRM) analysis in detection of *PDGFRA* variant in exon 10, 12, 14 and 18 and its association with IM resistant in CML patients.

HRM analysis is gaining more demands upon today's laboratories to generate a great need for rapid and cost-effective analytical methods. This technique was first

introduced in 2002 by a collaboration between academics at the University of Utah (UT,USA) and company Idaho Technology (UT,USA) (Montgomery *et al.*, 2010). The flexibility of HRM analysis has allowed it to be applied in wide range of disciplines for a variety of applications including gene scanning, genotyping, sequence matching and also in methylation analysis (Erali *et al.*, 2008). Perhaps, this technique would be able to discriminate even small sequence variations of single base substitution in PDGFA gene.

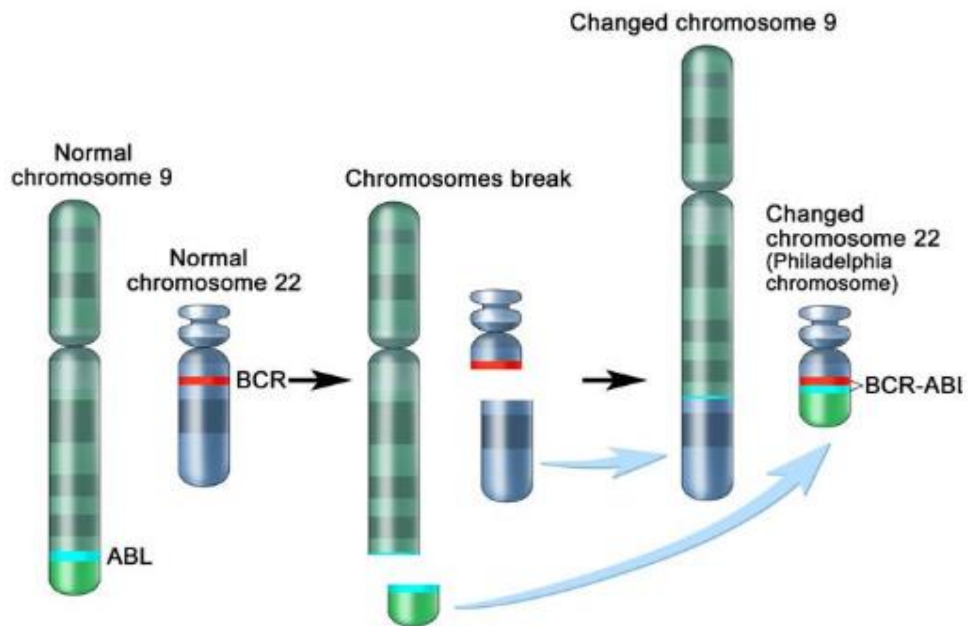

Figure 1.1 The reciprocal translocation of *ABL1* gene on chromosome 9 into the *BCR* region on chromosome 22 that resulted in the formation of Philadelphia (Ph) chromosome (Adapted from (Ali, 2016).

1.2 Chronic Myeloid Leukemia and clinical phases

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 their early phases (Goldman and Mughal, 2008). The disease is neither preventable nor inherited and the cause for its formation is unknown in majority of the patients (Quintás-Cardama and Cortes, 2006).

CML consists of three phases based on clinical features and laboratory findings: the chronic phase (CP), the accelerated phase (AP) and the blast phase (BP) or blast crisis. Most cases of CML are diagnosed during the CP with a few clinical sign and symptoms that vary among individuals include fatigue, anemia, progressive splenomegaly and leucocytosis. CML patients at this phase usually have mild symptoms and usually asymptomatic. Approximately 90% of patients are diagnosed in the CP (Cortes *et al.*, 2006). According to World Health Organization (WHO), CP is characterized by < 10% blasts in the blood which can last for several years. Without effective treatment, CML can progress to the AP or BP which usually associated with additional chromosomal and molecular alterations (Calabretta and Perrotti, 2004).

In AP, patients may show a sign of 10 to 19% of blasts in peripheral blood, presence of ≥ 20% basophils, persistent thrombocytopenia and increasing spleen size (Baccarani *et al.*, 2013). According to Cortes et al, in 2006, two-third of patients go through the AP before develop into the BP which is the terminal phase of CML. It is characterized by more than 20% of leukemic cells in blood with extramedullary involvement (Kantarjian *et al.*, 1988; Baccarani *et al.*, 2013). At this blast phase,

immature cells proliferate rapidly and progression of disease considerably fast within 5 years of diagnosis.

1.3 Diagnosis of CML

CML is diagnosed by mean of haematological testing, cytogenetic analysis and molecular diagnosis. Complete blood count is the first indicator of CML diagnosis. The typical haematologic findings are anemia with haemoglobin level less than 7 gm/dl to 11 gm/dl as well as marked leucocytosis with elevation of total leukocyte count from 100 to 500 x 10^9/L. The peripheral blood smear demonstrated immature white cells of all stages including neutrophils, metamyelocytes, myelocytes, promyelocytes and blast cells. The abnormal results in blood screening are followed with bone marrow aspirate analysis and trephine biopsy. The bone marrow aspirate usually shows a marked myeloid hyperplasia together with markedly increased of myeloid to erythroid precursor (M:E) ratio from 20:1 to 49:1 (Rodgers and Young, 2013).

The cytogenetics analysis is performed by mean of chromosome banding analysis (CBA) of marrow cell metaphases (Shaffer *et al.*, 2013) to detect additional chromosomal abnormalities which determined by the presence of Ph chromosome. If marrow cells cannot be obtained, CBA can be replaced by interphase fluorescence in situ hybridization (I-FISH) using dual colour fusion probes that allow detection of BCR-ABL + nuclei to detect some variant translocation (Testoni *et al.*, 2009).

More recently, molecular diagnostic methods including 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-ABL* positive cells. The qRT-PCR analysis identifies the transcript types of either e14a2 or 13a2 (also known as b3a2 and b2a2) and rarely recognizes the transcript of e19a2 or e1a2 which indicate the BCR-ABL protein weight (Baccarani *et al.*, 2012).

1.4 Treatment and management

Treatment of the CML in last century was based on radiotherapy, busulfan, and hydroxyurea (Pavlovsky *et al.*, 2009). Those treatments were able to control the clinical manifestation of the disease, but rarely of capable to supress the progression of the disease. Following this, interferon-alpha and allogenic stem cell transplantation was introduced and showed to induce cytogenetic response among the patients (Goldman, 2009; Pavlovsky *et al.*, 2009).

Increase understanding on the molecular pathogenesis of CML and discovery of the Philadelphia chromosome has led to the development of IM; the tyrosine kinase inhibitor (TKI) that target the BCR-ABL protein (Pavlovsky *et al.*, 2009). Since then, IM has become a gold standard treatment that results in dramatically improved clinical management and outcome in CML patients (Manley *et al.*, 2002).

1.5 Imatinib mesylate

Imatinib mesylate also known as Gleevec or formely referred as STI571 (Norvatis, Switzerland), is a specific inhibitor of tyrosine kinases BCR-ABL and platelet-derived growth factor (PDGF) and stem cell factor (Savage and Antman, 2002). IM

was designated chemically as 4-[[(4-methyl-1-1piperazinyl)methyl]-*N*-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]aminophenyl] benzamide nethanesulfonate with a molecular formula of $C_{29}H_{31}N_7O.CH_4SO_3$ and relative molecular mass of 589.7 (Cohen *et al.*, 2002). IM has been approved by the Food and Drug Administration (FDA) for treatment of CML and gastrointestinal stromal tumors (GISTs) (Savage and Antman, 2002; O'brien *et al.*, 2003). The initial standard doses of IM for a newly diagnosed Ph+ CML patients is 400 mg daily and 600 mg daily for CML patients in the accelerated and blasts phases (Talpaz *et al.*, 2002; Druker, 2004; Pavlovsky *et al.*, 2009). **Figure 1.2** shows the chemical structure of IM.

Administration of IM to CML patients has resulted in marked clinical response. An *in vitro* and *in vivo* study, has shown the ability of IM to inhibit proliferation of cells expressing *BCR-ABL* and have reduced the formation of the *BCR-ABL* positive colonies by 95% (Buchdunger *et al.*, 2001; Savage and Antman, 2002). About 65% to 90% CML patient who have failed interferon-α and 80% to 90% of untreated CML patients have showed to induce complete cytogenetic response (CCyR) from the IM treatment which demonstrated by the complete absence of Ph chromosome in CML cell metaphases (Druker *et al.*, 2001; Kantarjian *et al.*, 2002). IM therapy specifically inhibit proliferating myeloid cell with minimal side effects including neutropenia, thrombocytopenic anemia and non-hematologic adverse effects including nausea, skin rash, peripheral edema and muscle cramps which only can be observed in 10 to 20% of patients (Arora and Scholar, 2005).

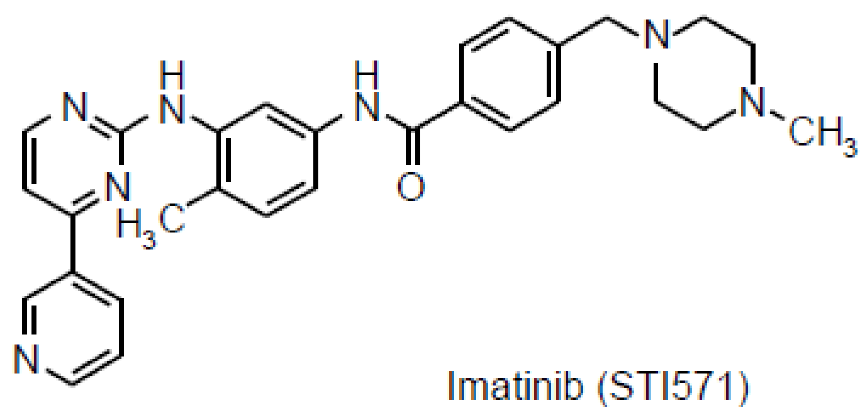

Figure 1.2 Chemical structure of IM (Adapted from (Manley *et al.*, 2002)).

1.5.1 Mechanism of action

IM was developed as a specific inhibitor of the BCR-ABL protein tyrosine kinase. IM exhibits potent inhibitory activity against ABL kinase and the chimeric BCR-ABL fusion oncoprotein, KIT, and PDGFR (Quek and George, 2010).

At the cellular level, IM binds to the inactive form of ABL kinase and function as a competitive inhibitor of the adenosine triphosphate (ATP) binding site of the BCR-ABL protein. The main purpose of the IM binding is to block the autophosphorylation of the ABL kinase, which responsible for kinase activation and signal transduction (Buchdunger *et al.*, 2002). In BCR-ABL expressing cells, IM has shown to produce three related effects; inhibition of BCR-ABL autophosphorylation, inhibition of proliferation and induction of apoptosis (Druker *et al.*, 1996; Gambacorti-Passerini *et al.*, 1997).

The BCR-ABL protein targets were reported to include members of Ras, Phosphatidylinositol-3 kinase (PI3K)/Akt and Jak/Stat signalling pathway that play a role in regulating cell proliferation and apoptosis. BCR-ABL also act by repealing the cell dependence on external growth factors by up-regulating the production of interleukin-3 as well as altering the cell adhesion properties by expression and activation of the focal adhesion kinase and associated proteins. Therefore, IM prevented the BCR-ABL tyrosine kinase enzyme from phosphorylating subsequent proteins and initiating the signalling cascade (Kantarjian *et al.*, 2006). The schematic diagrams of how IM works as competitive inhibitor of ATP was shown in **Figure 1.3.**

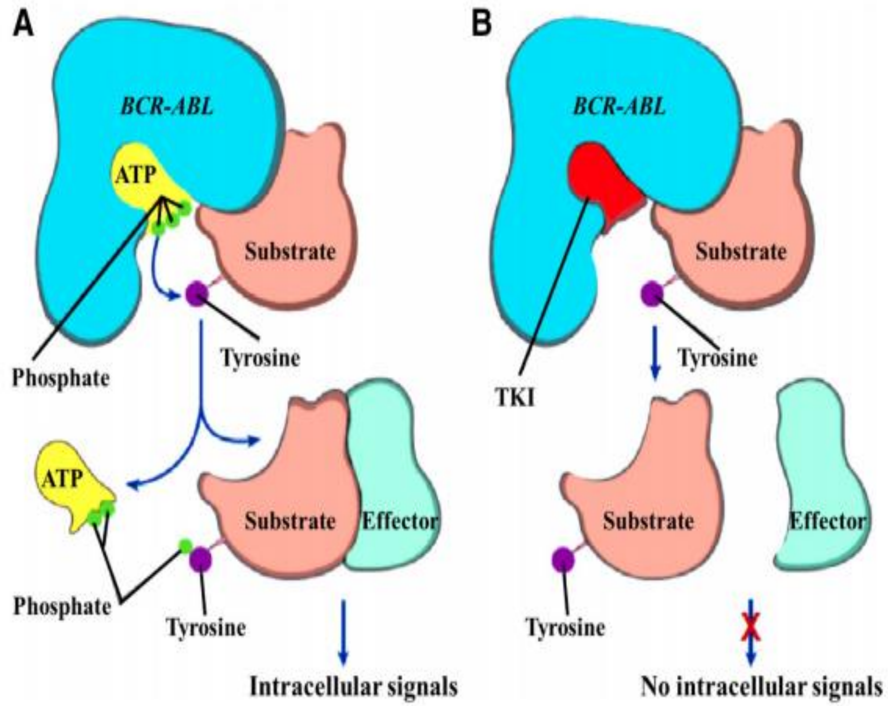

Figure 1.3 Schematic diagrams on mechanism of action of IM. (**A**) Activation of BCR-ABL that led to autophosphorylation of ABL kinase (**B**) Binding of IM to the BCR-ABL that block the autophosphorylation of ABL kinase thus inhibit intracellular signals (Adapted from (Ali, 2016).

1.6 IM resistance

The responses toward IM treatment are assessed based on three types of response, which are the haematological response, cytogenetic response and molecular response. These responses were assessed on the third month of continuous IM treatment followed by sixth month, twelfth month and each subsequent three months later. According to LeukemiaNet 2013 recommendation (**Table 1.1**), IM response groups were divided into three major groups, which are the optimal, warning and failure. The response criteria were mostly based on the molecular response.

However, some CML patients has experience an unsatisfactory therapeutic effects with IM. The main reason is because of the development of resistance to IM. The resistance to IM can be divided into primary resistance and secondary resistance. Where, both groups have led to less favourable prognosis to CML patients.

The primary resistance or also known as intrinsic resistance is defined as having less than complete cytogenetic response within 12 months after IM treatment initiation and/or having more than 1% of *BCR-ABL1* transcript when tested with (RT-qPCR) (Baccarani *et al.*, 2013).

While, secondary resistance which known as acquired resistance was characterized by loss of complete haematological response, complete cytogenetic response, loss of major molecular response confirm with two constitutive test to show the presence of *BCR-ABL1* transcripts level of more or equal to 1% and may presented with mutations and/or clonal chromosome abnormalities (Baccarani *et al.*, 2013)

Approximately about 33% of CML patients treated with IM do not achieve complete cytogenetic response (CCyR) (Bixby and Talpaz, 2009; Hochhaus *et al.*, 2009). The

mechanisms of resistance to IM have been extensively studied and can be divided into two main types which are BCR-ABL dependent and BCR-ABL independent (**Figure 1.4**).

Table 1.1 Definition of response to tyrosine kinase inhibitor as first line treatment in LeukemiaNet 2013 recommendation.

Time	Optimal response	Warning	Failure
Baseline	N/A	High risk Major route CCA/Ph+	N/A
3 months	*BCR-ABL1* ≤ 10% and/or Ph+ ≤ 35% (PCyR)	*BCR-ABL1*> 10% and/or Ph+ 36-95%	No CHR and/or Ph+ > 95%
6 months	*BCR-ABL1* < 1% and/or Ph+ 0% (CCyR)	*BCR-ABL1* 1-10% and/or Ph+ 1-35%	*BCR-ABL1* > 10% and/or Ph+ > 35%
12 months	*BCR-ABL1* ≤ 0.1% (MMR)	*BCR-ABL1* 0.1-1%	*BCR-ABL1* > 1% and/or Ph+ > 0%
Then, and at any time	MMR or better	CCA/Ph- (-7 or 7q-)	Loss of CHR Loss of CCyR Loss of MMR* Mutations CCA/Ph+

N/A: Not available; MMR: ***BCR-ABL1*** ≤0.1%=MR or better; CCA/Ph+: Clonal chromosomal abnormalities in Ph+ cells; CCA/Ph-: Clonal chromosomal abnormalities in Ph- cells; *: in 2 consecutive test of which ***BCR-ABL1*** transcript level ≥1%

1.6.1 *BCR-ABL* dependent

There are two main mechanisms involved under the BCR-ABL dependent which are amplification of *BCR-ABL* gene and point mutation in the tyrosine kinase domain of the *ABL* gene (Gambacorti-Passerini *et al.*, 2003).

The amplification of *BCR-ABL* gene in IM resistant leukemic cells was first described in a LAMA84R cell line generated by culturing cells with increasing IM concentrations where it express about five times more BCR-ABL protein compared to the parent LAMA84 cells. Further analysis using marker chromosome labelled by *BCR-ABL* specific probe confirmed that the overexpression of the protein was because of the amplification of the *BCR-ABL* gene (le Coutre *et al.*, 2000). While, the fluorescence *in situ* hybridization (FISH) of CML blast phase patients following treatment with IM discovered the same finding of *BCR-ABL* amplification (Melo and Chuah, 2007).

As IM was a competitive inhibitor of the ATP binding site of ABL, mutations affecting IM binding were likely to produce non-functioning enzymes. However, later studies showed that IM binds and stabilise ABL in its inactive conformation where no ATP binds to the kinase rather than competing with ATP for binding. Consequently, mutations in the kinase domain of *BCR-ABL* may be able to disrupt IM binding without interfering its enzymatic activity (Tauchi and Ohyashiki, 2004). Investigation of mechanism of IM resistance then has led to the discovery of T315I mutation in the ABL kinase domain of *BCR-ABL* that confers resistant toward IM via blocking IM binding to ABL where has subsequently been found in at least one *BCR-ABL* positive cell (Gorre *et al.*, 2001; Ricci *et al.*, 2002).

1.6.2 *BCR-ABL* independent

BCR-ABL independent mechanism mainly involves several factors and can be due to alterations in their pharmacokinetics for example drug efflux/influx and binding of IM in the plasma by α-1 acid glycoprotein. Overexpression of adenosine triphosphate binding cascade (ABC) transporter in the cell of blast phase CML patients has been linked to the development of IM resistance. This ABC transporter is a transmembrane protein that regulate efflux of numerous chemotherapeutic agents (Mahon *et al.*, 2000). Whereas, influx inhibition through human organic cation transporter (hOCT1) was also an important factor that affecting regulation intracellular IM and any polymorphism in these transporter have the ability to alter the entry of IM into the cell which may contribute to development of resistance (Crossman *et al.*, 2005; Quintas-Cardama and Cortes, 2009).

When leukemic cells from patients were analysed after initial treatment with IM, only partial inhibition was observed although BCR-ABL could completely blocked when exposed to the equivalent plasma IM concentrations (Druker *et al.*, 2001; Gambacorti-Passerini *et al.*, 2002). These can be explained that approximately 95% of IM is bound to the plasma protein which is α-1-acid glycoprotein (AGP) and albumin. The binding of AGP can inhibit the activity of IM (Gambacorti-Passerini *et al.*, 2000; Larghero *et al.*, 2001; Le Coutre *et al.*, 2002). Therefore, IM that present in the plasma could be mostly bound to the AGP and thus biologically inactive.

The resistance that involving *BCR-ABL* independent is not only due to the mechanism mentioned above. Several studies had reported that a significant portion of IM resistance in CML patients could not be associated with *BCR-ABL* fusion gene. Therefore it is presumed that the mechanism of IM resistance in such patients

might be via additional mechanisms involving other IM targeted tyrosine kinases for example PDGFRA which could also affecting the response of IM

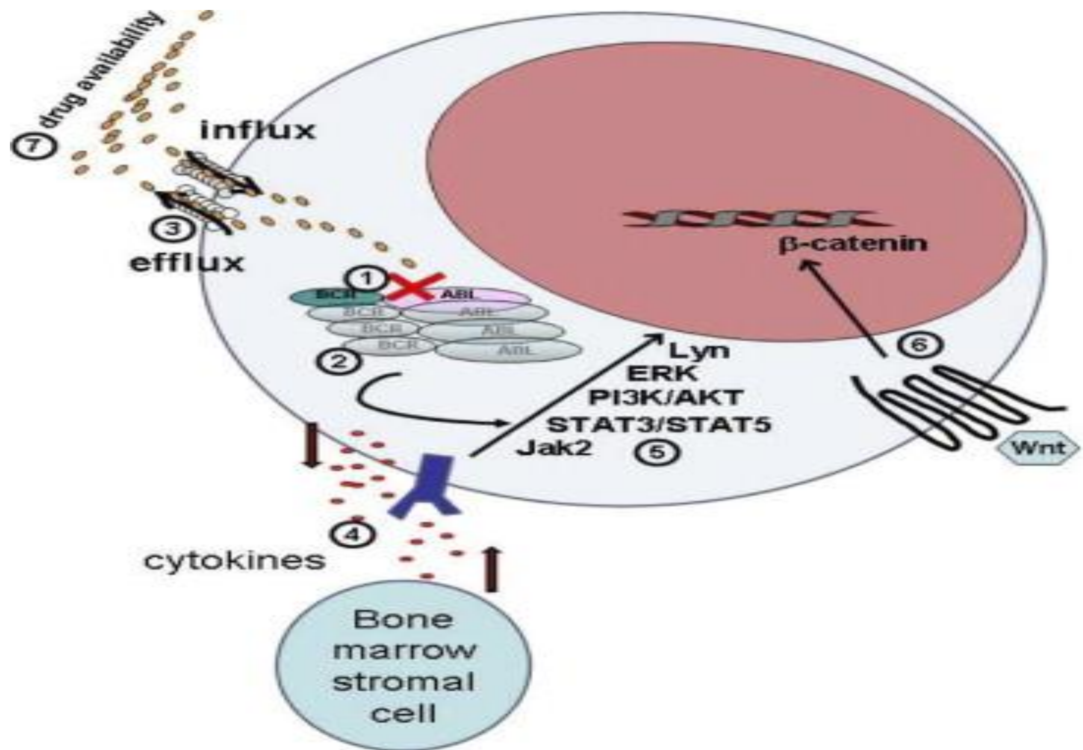

Figure 1.4 Mechanisms of resistance against IM. (1) Mutation in the BCR-ABL gene (2) Amplification of BCR-ABL (3) Drug influx/efflux mechanisms (4) Autocrine cytokine secretion (5) Activation of alternative signalling pathway (6) Wnt/β-catenin pathway (7) low intracellular drug availability (Adapted from (La Rosée and Deininger, 2010)).

1.7 Tyrosine kinase

Tyrosine kinases are enzymes that catalyse the transfer of the gamma (ϒ) phosphate group from adenosine triphosphate to target protein. It plays a role in diverse regulatory processes including the modulation of growth factor signalling, proliferation and differentiation. Tyrosine kinases can be classified into receptor protein kinase and non-receptor protein kinase. There are approximately 60 RTKs that have been identified and further divided into 20 subfamilies (Pawson, 2002)

The receptor tyrosine kinase are activated by ligand binding which then induce dimerization of these receptor resulting in autophosphorylation of their cytoplasmic domain and activation of the tyrosine kinase activity (Paul and Mukhopadhyay, 2004). The most important downstream signalling pathways activated by receptor tyrosine kinase including the Ras/Raf mitogen-activated protein kinase pathway, the phosphoinositol 3'-kinase/AKT pathway, the signal transducer and activator of transcription 3 pathway (JAK/STAT), the protein kinase C pathway and scaffolding protein (Schlessinger, 2000). These intracellular mediators then effects the biological processes including cell growth, migration, differentiation and death (Blume-Jensen and Hunter, 2001).

Even there are multiple levels of regulation of tyrosine kinase, dysregulation may also occur which lead to malignancy through several mechanisms including amplification of receptor tyrosine kinase gene, genetic alterations and autocrine-paracrine stimulation through aberrant growth factor loops (Zwick *et al.*, 2002). In CML, the related tyrosine kinase involve is the *ABL* gene which classified under non receptor tyrosine kinase. However in this study we are exploring others tyrosine kinase which is PDGFRA that classified under type III receptor tyrosine kinase, since

the receptor in this class is one of the intriguing receptor tyrosine kinase in terms of evolutionary, functional and clinical considerations (Grassot *et al.*, 2006; Verstraete and Savvides, 2012).

1.7.1 Platelet-derived growth factor receptor alpha (PDGFRA)

Platelet derived growth factor receptor (PDGFR) or also known as CD140A and PDGFR2 is belongs to the class III of tyrosine kinases have shown to play a role in mesenchymal cell migration and proliferation (Jones and Cross, 2004). These growth factors are mitogens for the cells of mesenchymal origin. Two type of PDGFRs have been identified which are alpha type (PDGFRA) and beta type (PDGFRB). These two types of subunit are differently expressed on the cell surface of different cell types.

The full length PDGFRA is encoded by a 6.4 kb transcript generated from a promoter upstream of exon 1 (P1 promoter). It is located at chromosome 4 in the 4q12 and has 23 exons and 1089 amino acid residues (Mosselman *et al.*, 1996). The structure of PDGFRA receptor comprises an extracellular domain with five immunoglobulin (Ig), a juxtamembrane domain inside the cell membrane and two cytoplasmic tyrosine kinase domain (**Figure 1.5**) (Claesson-Welsh *et al.*, 1989; Matsui *et al.*, 1989). PDGFRA bind to its ligand (PDGF-A, -B, -C and –D) and induce receptor dimerization that lead to autophosphorylation of their cytoplasmic tyrosine residue thus activate inter/intracellular kinase activity, initiating the intracellular signalling (Kawagishi *et al.*, 1995).

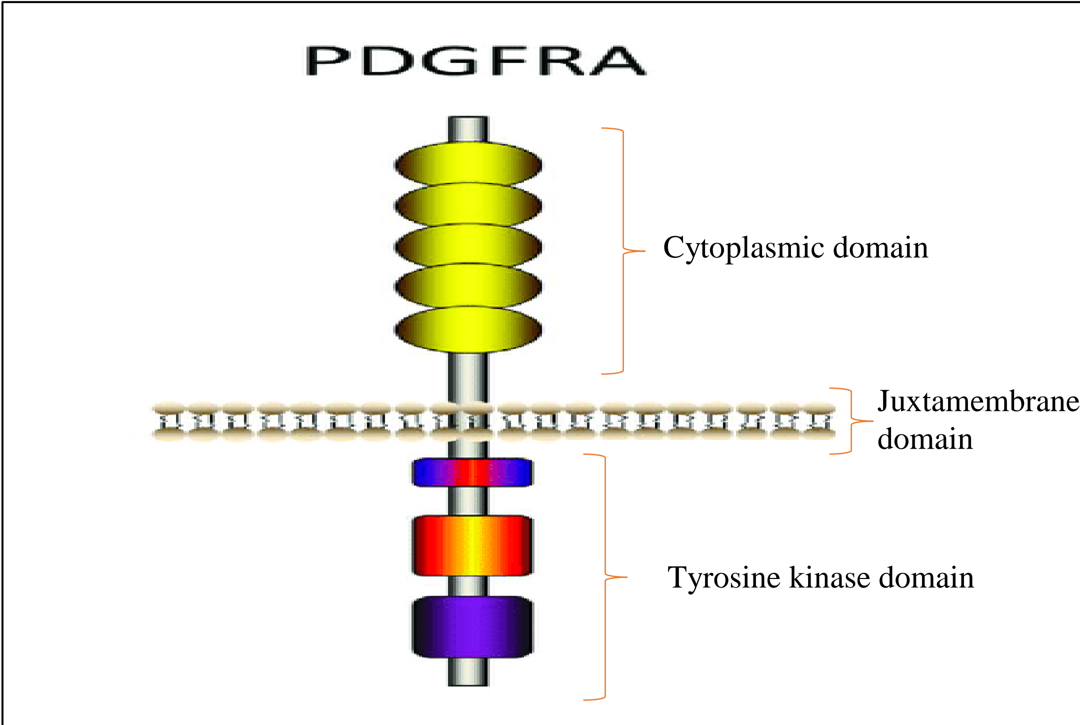

Figure 1.5 Schematic diagram of PDGFRA structure (Adapted from (Li *et al.*, 2017).

1.7.2 *PDGFRA* and IM resistance

In addition to *BCR-ABL*, IM is also a potent inhibitor for c-*KIT* and *PDGFRA* in gastrointestinal stromal tumour (GISTs), and also has shown marked response as a treatment in glioblastoma, dermatofibrosarcoma protuberans, malignant peripheral nerve sheet tumour (MPNST) and chronic myelomonocytic leukemia (Buchdunger *et al.*, 2002). Several clinical trials have demonstrated a significant response to IM in advanced GISTs patients and have shown to inhibit the *PDGFRA* tyrosine kinase at the concentration similar to the concentration required for the inhibition of *BCR-ABL* in CML (Heinrich et al., 2003a).

Mutations of *PDGFRA* lead to ligand-independent activation of the receptor. Approximately about 80% of GISTs patients expressed the mutated KIT receptor. However about 8% of the patients had a normal KIT and 5% of the patients demonstrated to have mutation in the PDGFRA receptor (Joensuu, 2006). Since, *PDGFRA* is very similar to the *KIT* and found in close proximity on chromosome 4q12, the mutation of the receptor seen to be related (Spritz *et al.*, 1994).

Most of the *PDGFRA* mutations occur in exons 10, 12, 14, 18 in GISTs and MPNT. The site of mutations may influence response to tyrosine kinase inhibitors such as IM (Holtkamp et al., 2006). Mutation in exon 18 of *PDGFRA* (D842V) has shown to be resistant toward IM therapy which account for about 89.6% of cases in GISTs (Corless *et al.*, 2005). It is suggested that a similar mutational status of *PDGFRA* might also occur in CML patients that causes development of resistance towards IM treatment.