DETECTION OF *PDGFRA* MUTATIONS AT EXONS 12 AND 18 AMONG CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH IMATINIB MESYLATE

By

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Dissertation Submitted In
Partial Fulfilment Of The Requirements For The Degree Of
Master Of Pathology
(Medical Genetics)

UNIVERSITI SAINS MALAYSIA

2015

ACKNOWLEDGEMENT

First of all I would like to thank my supervisor which is also the Director of Human Genome Centre, Dr Sarina Sulong and my co supervisor Professor Rosline Hassan. I owe them a huge debt of gratitude for providing me with a wealth of knowledge in medical genetics and for their guidance and support throughout the study.

My gratitude also goes to Professor Dr Zilfalil Alwi for his assistance and guidance in getting my post graduate career started on the right path and providing me with the foundation for becoming a medical geneticist.

To our most respected 'guru' Professor Ravindran Ankathil who offered ample encouragement and understanding, a thousand thank you.

I have been privileged to have shared working area with Dr Hashima Hashim and Dr Rose during the course of my thesis. I have learnt so many things from them and highly indebted to them. Tremendous supports, cooperation and selfless help extended by them during various stages of my work have been extremely valuable to me.

I would like to extend my sincere gratitude to the senior member of Human Genome Centre; Ms Amira, Ms Syakila, Ms Najla, Mrs Shafawati, Mrs Hayati, Mr Aizat, Ms Hasnah, Ms Sathiya, Ms Fatin, Ms Fazreen, Dr Nazihah, Puan Atifah. Thank you for spending your valuable time in sharing a vast input regarding laboratory techniques and tasks.

To a panel of the most admired and expert technologists; Mrs Hidayah, Mrs Hafizah, Mrs Alia, Mrs Sima, Mrs Shimah, Mr Nik, Mr Qais and Ms Ika your valuable friendship and thoughtful help would be fondly remembered and cherish.

Most importantly, I would like to thank my husband and daughter for their tolerance, encouragement, quiet patience and unwavering love. I thank my parents, for their faith in me and it was under their watchful eyes that I have gained so much drive and an ability to tackle challenges head on.

Finally I would like to acknowledge the Ministry of Education and Universiti Technology Mara for providing me the scholarship to fulfil my career goals and to Universiti Sains Malaysia for supporting this study under one of the grants.

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LIST OF ABBREVIATIONS

%	percent
µl	microlitre
µM	micromolar
A (Ala)	alanine
A260/280	optical spectrometer measurement of absorbance at the wavelengths of 260 nm over 280 nm
ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
AP	accelerated phase
ATP	adenosine triphosphate
BCR-ABL	BCR-ABL gene
BCR-ABL	BCR-ABL protein
BLAST	Basic Local Alignment Search Tool
BP	blastic phase
bp	base pair
CCyR	complete cytogenetic response
CML	chronic myeloid leukemia
CP	chronic phase
CyR	cytogenetic response
D (Asp)	aspartic acid
ddH_2O	deionized distilled water
DMSA	dimethyl sulphoxide

DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphotase
EDTA	ethylenediamine tetraacetic acid
FGFR1	fibroblast growth factor receptor 1 gene
g	gram
gDNA	genomic deoxyribonucleic acid
GIST	gastrointestinal stromal tumour
HCL	hydrochloride
HES	hypereosinophilic syndrome
HPP	Hospital Pulau Pinang
HRM	high-resolution melting
HRPB	Hospital Raja Permaisuri Bainun
HSA	Hospital Sultanah Aminah
HUKM	Hospital Universiti Kebangsaan Malaysia
HUSM	Hospital Universiti Sains Malaysia
I (Ile)	isoleucine
IM	imatinib mesylate
IRIS	International Randomized Study of Interferon versus STI571
K (Lys)	Lysine
KIT	SCFR stem cell factor receptor gene
L (Leu)	leucine
LNs	lymphoid neoplasms
MCyR	major cytogenetic response
MDS	myelodysplastic syndrome

$MgCl_2$	magnesium chloride
ml	mililiter
mM	milimolar
MNs	myeloid neoplasms
MPN	myeloproliferative neoplasms
N (Asn)	Asparagine
NCBI	National Center for Biotechnology Information
NGS	next generation sequencing
NK	natural killer
nm	nanometer
^oC	degree celsius
P (Pro)	proline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFA	platelet-derived growth factor alpha protein
PDGFR	platelet-derived growth factor receptor
PDGFRA	platelet-derived growth factor receptor alpha protein
PDGFRA	platlet-derived growth factor receptor alpha gene
PDGFRB	platelet-derived growth factor receptor beta protein
PDGFRB	platelet-derived growth factor receptor beta gene
Ph	Philadelphia
S (Ser)	serine
T (Thr)	threonine
taq	*Thermophilus aquaticus*
TBE	Tris-Boric Acid-EDTA

TERT	Telomerase reverse transcriptase gene
TKI	tyrosine kinase inhibitor
UK	United Kingdom
USA	United States of America
UV	ultra violet
V	volt
V (Val)	valine
WHO	World Health Organisation

ABSTRAK

PENGESANAN MUTASI DI *PDGFRA* PADA EKSON 12 DAN 18 DI KALANGAN PESAKIT-PESAKIT LEUKEMIA MEILOID KRONIK YANG TELAH DIRAWAT DENGAN IMATINIB MESYLATE

Rintangan terhadap rawatan imatinib mesylate (IM) merupakan cabaran baru dan utama dalam merawat pesakit leukemia mieloid kronik (CML). Rintangan terhadap IM boleh dibahagikan kepada dua laluan iaitu laluan yang bergantung kepada *BCR-ABL* dan laluan yang tidak bergantung kepada *BCR-ABL*. Mekanisma yang berada di laluan yang tidak bergantung kepada *BCR-ABL* diuji dalam projek ini; yang melibatkan mutasi di *PDGFRA* sebagai mekanisma rintangan pada pesakit -pesakit CML positif Philadelphia yang dirawat dengan IM. *PDGFRA* adalah tergolong dalam kumpulan tyrosine kinase Kelas III; bukan sahaja menyumbang kepada pembentukan haematopoiesis tetapi juga telah dikaitkan dengan kanser. Mutasi *PDGFRA* membawa kepada pengaktifan kendirian menyebabkan penambahan spontan sel. Analisis penjujukan PCR-DNA telah dijalankan untuk mengesan mutasi *PDGFRA* pada ekson 12 dan 18. Lapan puluh enam pesakit CML positif Philadelphia dari lima hospital rujukan di seluruh Semenanjung Malaysia yang berada dalam fasa penyakit yang berbeza dirawat dengan IM dari tahun 2010 sehingga 2013 telah dinilai (pesakit yang

responsif, n= 43 ; pesakit yang rintang, n=43). Sampel pesakit-pesakit ini diperoleh daripada kajian projek berkaitan CML terdahulu. Daripada kesemua pesakit-pesakit dalam kumpulan yang rintang terhadap IM, 32 pesakit berada dalam fasa CML kronik; 7 pesakit dalam fasa percepatan CML; dan 4 pesakit dalam fasa blastik CML. Pesakit yang berumur dari 20 hingga 73 tahun telah dikategorikan ke dalam pelbagai kumpulan umur dari pesakit yang lebih muda (umur yang lebih muda daripada 60 tahun) sehingga pesakit yang lebih tua (umur 60 tahun atau lebih tua). Amplifikasi tindak balas rantai polimerase di lokasi yang terpilih telah dijalankan iaitu pada ekson 12 dan 18 diikuti oleh saringan mutasi oleh penjujukan langsung untuk kesemua sampel. Keputusan penjujukan telah sejajar dengan menggunakan Asas Tempatan Penjajaran *Search Tool* (BLAST) untuk membandingkan urutan pertanyaan dengan rujukan urutan. Kebanyakan pesakit yang tidak memberi respon terhadap rawatan IM adalah wanita. Median umur ialah 43 tahun. Sebanyak 74 % daripada pesakit CML yang tidak memberi respon terhadap IM berada dalam fasa kronik. Namun begitu, tiada pesakit CML dalam kajian ini menunjukkan mempunyai mutasi pada ekson potensi; ekson 12 dan 18 dalam *PDGFRA*. Kajian ini menunjukkan wanita mempunyai kekerapan rintang yang lebih tinggi terhadap rawatan IM dan berlaku terutamanya di kalangan pesakit yang lebih muda. Ketiadaan mutasi *PDGFRA* di ekson 12 dan 18 kemungkinan kerana mutasi berada di lokasi-lokasi lain pada gen ini. Majoriti sampel kajian adalah dari pesakit fasa kronik mungkin menyumbang kepada mutasi negatif. Kajian pada masa depan haruslah berpandukan ke arah mencari ekson-ekson lain yang berpotensi dengan memilih lebih pesakit dalam peringkat berbahaya untuk meperolehi kekerapan mutasi yang lebih tinggi. Kajian klinikal dan tambahan *'pathogenetic'* diperlukan untuk memahami kaitan antara *PDGFRA* dan IM rintang CML.

ABSTRACT

DETECTION OF *PDGFRA* MUTATIONS AT EXONS 12 AND 18 AMONG CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH IMATINIB MESYLATE

Imatinib mesylate (IM) resistance is an emerging and major challenge in the treatment of patients with chronic myeloid leukaemia (CML). Resistance toward IM can be divided into *BCR-ABL* dependent pathways and *BCR-ABL* independent pathway. In this study, the BCR-ABL independent pathway was investigated; the involvement of *PDGFRA* mutation as a mechanism of resistance in Philadelphia positive CML patients treated with IM. The PDGFRA belongs to the tyrosine kinase Class III; not only contribute to haematopoiesis development but also has been implicated in cancers. Mutation of *PDGFRA* leads to constitutive activation causing spontaneous proliferation. PCR-DNA sequencing analyses were carried out to detect exons 12 and 18 of *PDGFRA* mutations. Eighty-six patients from five tertiary hospitals around peninsular Malaysia in different phases of Philadelphia-positive CML who were treated with IM from 2010 until 2013 were evaluated (IM-responsive

patients, n= 43; IM-resistant patients, n=43). These samples were archived from the earlier CML project. Of all the patients in resistant group, 32 patients in chronic phase CML; 7 patients in accelerated phase CML; and 4 patients in blastic phase CML. Patients aged from 20 until 73 years were categorized into a range of age group from younger patients (age younger than 60 years) till older patients (age 60 years or older). Polymerase chain reaction amplifications were performed on the selected hotspots; exons 12 and exon 18 followed by screening for mutations by direct sequencing in 43 resistant and 43 responsive CML samples. Sequencing results were aligned by using Basic Local Alignment Search Tool (BLAST) to compare a query sequence with a reference sequences. Resistant patients predominate by female and the median age was 43. There were 74% of the resistant CML in chronic phase. None of the CML patients in this study exhibit any mutation on the hotspot exons 12 and 18 in *PDGFRA*. This study shows higher frequency of IM resistance notable in female and occurring mainly in younger age population. The absence of *PDGFRA* mutation at exons 12 and 18 may suggest that other regions of this gene could be involved. Majority of the study samples were from chronic phase patients might contribute to the negative mutation finding. Future study should be directed towards finding more potential exons by selecting more patients in advance stage in order to yield higher mutation frequency. Additional clinical and pathogenetic studies are needed to understand the association between *PDGFRA* and IM-resistant CML.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

1.1.1 Blood malignancies

Blood malignancies or haematological neoplasms are comparatively common, accounting for around 9% of all cancers and being the fourth most frequently diagnosed cancer in both men (after prostate, lung, and colorectum) and women (after breast, lung, and colorectum) in economically developed regions of the world (Smith *et al.*, 2011).

These neoplasms are forms of cancer that begin in the cells of blood-forming tissue, such as the bone marrow, or in the cells of the immune system. It may derive from either of the two major blood cell lineages: myeloid and lymphoid cell lines. The myeloid cell line normally produces granulocytes, erythrocytes, thrombocytes, macrophages and mast cells; the lymphoid cell line produces B, T, natural killer (NK) and plasma cells. Malignancies such as lymphomas, lymphocytic leukemias, and myeloma are from the lymphoid line, while acute and chronic myeloid leukemia (CML), myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPNs) are myeloid in origin.

Unlike many other cancers, haematological neoplasms are diagnosed using multiple parameters; such as morphology, cytochemistry, immunophenotype, genetics and clinical features in order to define clinically significant disease entities. The current classification system was established by the World Health Organisation (WHO) in 2001 and more recently modified in 2008 to refine the diagnosis (Swerdllow *et al.*, 2008). This classification stratifies neoplasms according to their lineage (myeloid, lymphoid, histiocytic/dendritic) and distinguishes neoplasms of precursor cells from those comprised of functionally mature cells.

Brief overviews on lymphoid neoplasms (LN), LNs are derived from the clonal expansion and proliferation of B- and T-lymphocytes. They encompass a heterogeneous group of lymphomas and leukemias including B-cell, T-cell, and NK-cell disorders. Whereas for myeloid neoplasm (MNs), five major subgroups of MNs are recognized based mainly on their degree of maturation and biologic properties: MPNs which are comprised primarily of mature cells with effective proliferation; myeloid (and lymphoid) neoplasms with eosinophilia and abnormalities of platelet-derived growth factor alpha gene (*PDGFRA),* platelet-derived growth factor beta gene *(PDGFRB)* and fibroblast growth factor receptor 1 gene (*FGFR1)*, defined largely by the finding of significant eosinophilia and specific genetic abnormalities; MDS/MPN comprised mainly of mature cells with both effective and ineffective proliferation of various lineages; MDS, in which immature and mature cells are found with abnormal, dysplastic and ineffective maturation, and acute myeloid leukemia (AML), comprised of precursor cells with impaired maturation (Vardiman, 2010).

According to the revised WHO Classification Scheme in year 2008, CML was assigned under MPN category together with polycythemia vera, essential thrombocytopenia, primary myelofibrosis, chronic neutrophilic leukemia, chronic eosinophilic leukemia (not otherwise specified), mast cell disease and MPN unclassifiable (Tefferi *et al.*, 2009).

1.1.2 Chronic Myeloid Leukemia and its genetic basis

CML is characterized by uncontrolled expansion of myeloid cells particularly the granulocytic cell line without the loss of their capacity to differentiate. The incidence of CML were 1 to 2 cases per 100,000 adults, and accounts for 15% of newly diagnosed cases of leukemia in adults (Jemal *et al.*, 2010) .

This is the first human cancer in which a consistent genetic abnormality was demonstrated to cause the disease (Sherbenou and Druker, 2007). CML is associated with the presence of the Philadelphia (Ph) chromosome leading to a *BCR-ABL* fusion. The Ph chromosome is the result of a balanced t(9;22)(q34;q11) translocation, and is observed in more than 90% of CML cases, with variant Ph translocations being observed in the remainder (O'Brien *et al.*, 1997).

This oncogenic fusion *BCR-ABL*, produce a constitutively active tyrosine kinase and is important in the pathogenesis and expression of CML. CML ensues when an abnormal pluripotent hematopoietic progenitor cell initiates excessive production of granulocytes, primarily in the bone marrow but also manifested in extramedullary sites (eg. spleen, liver). Although granulocyte production predominates, the neoplastic clones include red blood cells, megakaryocytes, monocytes, and even some T and B cells.

With a rapid expansion of granulocytes and advance disease ensues, metastasis may come to the picture which later progress to organ failure and death. Epidemiologic data indicates that almost 5000 new cases are reported every year and 10% of these patients eventually succumb to the disease (An *et al.*, 2010).

1.1.2.1 Classification and clinical phases

CML is classified into three phases based on clinical features and laboratory findings; chronic, accelerated, and blast phase. Approximately 90% of patients are diagnosed in the chronic phase (CP) and, historically have a median survival close to 5 years (Cortes *et al.*, 2006). In the CP of disease, mature cells proliferate exceeding the normal rate. Patients at this phase are asymptomatic but CML progression is insidious, with a nonspecific "benign" stage (malaise, anorexia, weight loss). Unless the disease is treated, CML evolves from a CP characterized by the Ph chromosome as the sole genetic abnormality into blast crisis or blast phase (BP), which is often associated with additional chromosomal and molecular secondary changes (Calabretta and Perrotti, 2004). This is considered as a natural progression of disease.

During this phase, patient manifests ominous signs such as splenomegaly, pallor, easy bruising and bleeding, fever, lymphadenopathy and skin changes. Two-thirds of patients who develop BP go through an intermediate accelerated phase (AP) with median survival of patient in AP is 1 to 2 years (Cortes *et al.*, 2006). The BP is the last stage or blast crisis where immature cells rapidly proliferate and more or less resembling the acute leukemia and at this point of progression the patient deteriorates considerably fast leading to mortality.

The term "advanced phase" is sometimes used to describe both the AP and BP. There are two distinct types of blast phases defined by the types of blast cells present. In most patients the blast cells (immature white cells) resemble those seen in AML, whereas in about a quarter of patients, the blast cells look more like those seen in acute lymphoblastic

leukaemia (ALL). This latter form of the disease is known as lymphoid blast crisis and tends to have a poorer response to treatment.

CML cases were account for 20% of all adult leukemias worldwide (Singer *et al.*, 2011). It typically affects middle-aged individuals. Uncommonly, the disease occurs in younger individuals. Younger patients may present with a more aggressive form of CML, such as in accelerated phase or blast crisis. Uncommonly, CML may appear as a disease of new onset in elderly individuals.

1.1.2.2 Treatment and management

The treatment of CML and the survival rate largely depends on the stage where the diagnosis is made. The time of diagnosis does contribute to the prognosis of the patient with this disease and directly related to the outcome of the patient as in chronic stage the progression can be stopped. Most of the patients seen in AP transition to blast crisis, it is imminent and the outcome is poor.

The elucidation of the molecular pathogenesis of CML led to the development of its targeted therapy. This was unprecedented until CML was regarded as a life-threatening disease with a median life expectancy of around six to seven years with the only exception being the minority of patients who could receive a compatible stem cell transplant. Previous years, cytotoxic drugs are the main modality as therapeutic option for CML before the discovery of *BCR-ABL*. Nevertheless, effective chemotherapy does not change the natural history of CML thus most patients would ultimately progress to blast phase.

The drugs that have been used by clinician to treat CML were hydroxyurea, busulfan and interferon. Hydroxyurea is the easiest therapy to be managed and has the fewest adverse effects. Unfortunately cytogenetic responses are rare and the onset to blast crisis is not delayed, with transformation occurring within a median of 4 to 6 years (Salesse and Verfaillie, 2002).

Busulfan has been known to cause unexpected general myelosuppression, and interferon causes a flu-like syndrome that frequently is unacceptable to patients. In contrary, these therapies alleviate the distressing splenomegaly and adenopathy and help in control of the tumor burden to reduce the incidence of tumor lysis and gout. Too unfortunate, none of these therapies prolongs median survival more than 1 year compared with untreated patients; thus, reduction in symptoms is the major goal, and therapy is not continued when patients have significant toxic symptoms (Porter *et al.*, 2006).

1.1.2.2 (a) Imatinib mesylate (IM)

A very essential lesson learned from IM is that the discovery of the primary genetic abnormality in a malignancy together with the development of an agent that targets that abnormality, can lead to therapeutic success. IM is a 2-phenylaminopyrimidine derivative developed originally as a general tyrosine kinase inhibitor (TKI) that was modified chemically so as to compete with adenosine triphosphate (ATP) for the ATP-binding site or P-loop in the ABL protein and thereby block the deregulated enzymatic function of the BCR-ABL oncoprotein (Goldman, 2009).

This BCR-ABL TKI (Glivec®, formerly STI571, Novartis Pharma AG, Basel, Switzerland) prevent tyrosine kinase turning into its active form thus inhibits cellular proliferation without the induction of apoptosis. An *in vitro* study reported that IM produced a 92% to 98% decrease in CML colony growth without significantly inhibiting normal colony growth (An *et al.*, 2010). It has been proven to produces complete hematologic and cytogenetic responses in a substantial percentage of CML patients and it is effective in CP, AP and blast crisis, with lower response rates in patients with more advanced disease (Buchdunger *et al.*, 2002).

The clinical use of IM has resulted in a significantly improved prognosis, response rate, overall survival, and patient outcome in CML patients compared to previous therapeutic regimens by busulfan, hydroxyurea and interferon. Results from the International Randomized Study of Interferon and STI571 (IRIS) trial in newly diagnosed CML-CP showed that, cumulatively, 98% of patients who received IM as initial therapy achieved a complete haematological remission and 87% achieved a complete cytogenetic remission (Assouline and Lipton, 2011). The above phases of disease progression have changed dramatically in the IM era. For most patients, the CP lasts at least ten years, possibly much longer, and only a minority of patients who start treatment in the CP fail to respond well to IM.

Relapses have been common in blast crisis associated with reactivation of BCR-ABL kinase activity. Approximately 33% of patients with CML treated with IM do not achieve a complete cytogenetic response (CCyR), while others have drug resistance or cannot tolerate drug-related toxicities (Bixby and Talpaz, 2009; Hochhaus *et al.*, 2009). Two categories of IM resistance have been characterized: primary resistance is the failure to achieve any of the

landmark responses established by the European LeukemiaNet (ELN) (Baccarani *et al.*, 2006). Primary resistance also known as intrinsic resistance can be further divided into primary hematologic resistance and primary cytogenetic resistance. Primary hematologic resistance accounts for 2 to 4% of cases who fail to normalize peripheral counts within 3 to 6 months of initiation of treatment whereas primary cytogenetic resistance, which is more common, observed in approximately 15 to 25% of patients who fail to achieve any level of cytogenetic response (CyR) at 6 months, a major CyR (MCyR) at 12 months or a complete CyR (CCyR) at 18 months (Shah, 2007). Secondary resistance occurs in those who have previously achieved and subsequently lost their response in accordance with the guideline (Bhamidipati *et al.*, 2013). The mechanisms of resistance to IM can be either *BCR-ABL* dependent (gene amplification or point mutations) or *BCR-ABL* independent (La Rosée and Deininger, 2010). The present study was designed to investigate the mechanisms of resistance involving *BCR-ABL* independent pathway.

The use of pre-imatinib-era treatment strategies such as hydroxyurea, busulfan or interferon by some physicians as salvage treatment after IM failure and unsuitability of stem cell transplantation still occurs despite the growing availability of newer TKIs (Rohrbacher and Hasford, 2009). This has reflected that not all CML cases are caused by solely Ph chromosome translocation, some other molecular mechanism must have contributed to its pathogenesis and disease progression making treatment with the most effective drug turned into a complicated non effective effort.

1.1.3 Role of tyrosine kinases (TKs) in cancer

TKs are a subclass of protein kinase. Protein TKs are enzymes that transfer phosphate groups to tyrosine residues on protein substrates. Phosphorylation of proteins cause changes in their function and/or enzymatic activity resulting in specific biological responses (Gocek *et al.*, 2014). It functions as a switch in many cellular functions and is the key role in diverse biological processes like growth, differentiation, metabolism and apoptosis in response to external and internal stimuli.

As the sequencing of the human genome is completed by the Human Genome Project, 90 unique kinase genes can be identified. Of the 90 TKs, 58 are receptor type, receptor tyrosine kinases (RTKs), distributed into 20 subfamilies and 32 non-receptor tyrosine kinases (NRTKs) can be placed in 10 subfamilies (Robinson *et al.*, 2000) . RTKs are part of the larger family of protein tyrosine kinases which contain a transmembrane domain, whereas the NRTKs do not possess transmembrane domains. Haematopoiesis is controlled by a number of growth factors and cytokines, a number of which act through binding to high-affinity RTKs (Reilly, 2003). RTKs are activated by ligand binding to the extracellular domain followed by dimerization of receptors facilitating trans-phosphorylation in the cytoplasmic domain (Paul and Mukhopadhyay, 2004). The most important downstream signalling cascades activated by RTKs include the Ras–extracellular regulated kinase (ERK)–mitogen activated protein kinase (MAPK) pathways, the phosphoinositide 3-kinase (PI 3-kinase)–Akt and the Janus kinase and Signal Transducer and Activator of Transcription (JAK/STAT) pathway leading either to a complex activation or repression of various subsets of genes for running biological functions.

TKs have been implicated in the pathophysiology of cancer. Though their activity is tightly regulated in normal cells, deregulation may result in deregulated TK activity with constitutive or strongly enhanced signalling capacity, leading to malignancy. The most important mechanisms leading to constitutive RTK signalling include: overexpression and/or gene amplification of RTKs, genetic alterations such as deletions and mutations within the extracellular domain as well as alterations of the catalytic site, or autocrine–paracrine stimulation through aberrant growth factor loops (Zwick *et al.*, 2002).

In CML, the important and related TK gene involves directly is *ABL* which is classified under NRTK whereas the gene in this study is *PDGFRA* which is under class III RTK. Class III RTK is arguably one of the most intriguing RTK classes in terms of evolutionary, functional and clinical considerations (Grassot *et al.*, 2006; Verstraete and Savvides, 2012). Characterized by present of five immunoglobulin-like domains in the extracellular ligand-binding region, this class of RTK has grown to harbour another four members include PDGFRB (platelet-derived growth factor receptor beta), c-KIT (cellular component-stem cell factor receptor, CD117), FLT3 (FMS-like tyrosine kinase, CD135) and c-FMS (colony-stimulating factor-1 receptor).

1.1.4 Platelet derived growth factor receptor alpha (PDGFRA)

Platelet-derived growth factor receptors (PDGFRs) and their ligands, platelet-derived growth factor (PDGFs) play critical roles in mesenchymal cell migration and proliferation (Jones and Cross, 2004). These growth factors are mitogens for cells of mesenchymal origin. Two types of PDGFRs have been identified: alpha-type and beta-type.

The PDGFRA belongs to the sub-family of proteins within the family of receptor tyrosine kinases which is class III. The structure of the PDGFA receptor comprises an extracellular domain (the portion extending outside of the cell membrane), a juxtamembrane domain (the portion just inside of the cell membrane), and two sections inside the cell known as the two catalytic parts of the kinase domain. (Refer **Figure 1.1**) One part of the kinase domain is essential for the binding of ATP and the other part is needed for the transfer of a phosphate group that leads to kinase activation. PDGFRA can forms homo- or heterodimers by binding to the endogenous PDGFR ligands; PDGF-A, -B, -C and –D. Upon binding of the receptor and its ligand, this will induce receptor dimerization and transphosphorylation at specific tyrosine residues thus activate the intracellular kinase activity, initiating intracellular signalling.

PDGFRA is the approved gene symbol according to the HUGO Gene Nomenclature Committee (HGNC). The other names that also encodes for this gene were *CD140A* and *PDGFR2*. The full length *PDGFRA* is encoded by a 6.4 kb transcript but there is also evidence for a 3.0 kb transcript as a result of alternative splicing, potentially encoding a truncated, dominant-negative receptor isoform (Mosselman *et al.*, 1996). Both these transcripts are generated from a promoter upstream of exon 1 (P1 promoter). This gene can be found at chromosome 4 located at 4q12. This gene has 23 exons and 1089 amino acid residues.

PDGFRA and other members in the same class were originally grouped under the PDGFR family. They are crucially important in the development and homeostasis of the cellular repertoire of the haematopoietic and immune systems but both PDGFR subtypes have primary roles in mesenchymal processes such as angiogenesis, fibroblast proliferation, bone

formation and tissue repair (Verstraete and Savvides, 2012). Recently in Frenette`s group, *PDGFRA* was shown to be one of the markers of hematopoiesis-supporting nestin-expressing multipotent stromal cells in the human system (Pinho *et al.*, 2013).

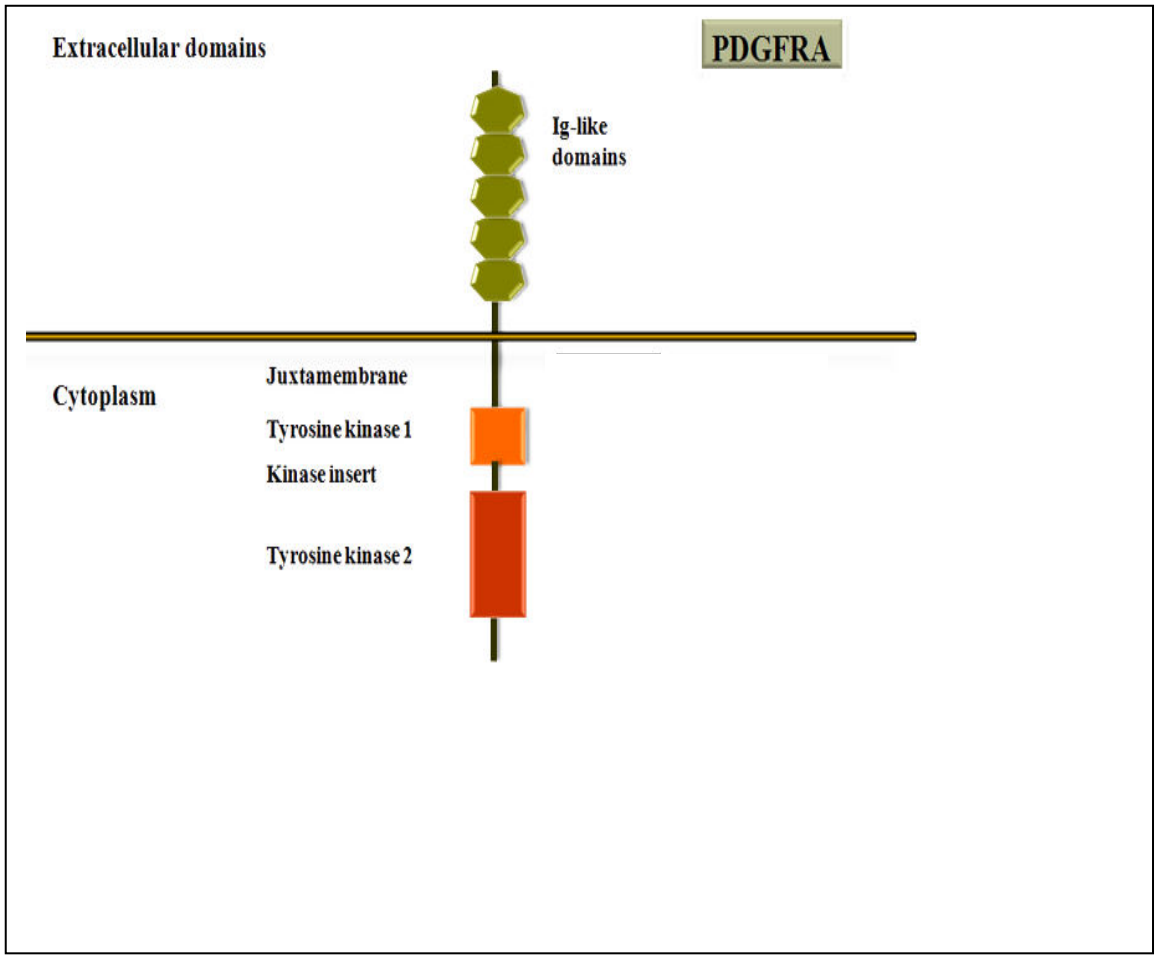

Figure 1.1 Structure of PDGFA receptor is comprised of five extracellular immunoglobulin- like domains, a juxtamembrane domain and two catalytic part of kinase domains. (Adapted from Pierotti *et.al.,* 2011).

1.1.4.1 IM resistance in *PDGFRA* mutation

The expanding understanding of the basis of IM-mediated tyrosine kinase inhibition has revealed a spectrum of potential new antitumor applications apart from its excellence capability in the treatment of CML. IM is a potent inhibitor of both receptors, PDGFRA and PDGFRB and has shown activity in vivo against platelet-derived growth factor (PDGF)-driven tumor models including gastrointestinal stromal tumours (GISTs), glioblastoma, dermatofibrosarcoma protuberans and chronic myelomonocytic leukemia (Buchdunger *et al.*, 2002) .

GIST commonly harbours oncogenic mutations of the KIT or PDGFRA kinases, which are targets for IM. *(Heinrich et al., 2003b; Hirota et al., 2003)*. The PDGFRA is very similar to the KIT (both are the member of same RTK family) and the genes coding for KIT and PDGFA receptors are found in close proximity on chromosome 4q12 (Spritz *et al.*, 1994). Mutations of *PDGFRA* lead to independent activation of the receptor in the absence of ligand; this is called constitutive activation (Heinrich *et al.*, 2003b). This means the cell is carrying out spontaneous proliferation without the normal signalling ligand telling the cell to grow. The result is uncontrolled growth of a tumour. The "downstream" pathways that come into play in the cell following activation of mutant *PDGFRA* are thought to be fairly similar with *KIT* in GIST (Duensing *et al.*, 2004). However, different sets of genes are expressed depending on which gene is mutant (Antonescu *et al.*, 2004; Subramanian *et al.*, 2004; Kang *et al.*, 2006).

Approximately 80% of GISTs express the mutated receptor tyrosine kinase KIT. However about 8% of GISTs have a normal *KIT* (wildtype) but 5% show mutations in the gene for *PDGFRA* (Joensuu, 2006). *PDGFRA* mutations in GIST have been found in exons 12, 14 and 18 (corresponding to exons 11, 13 and 17 of *KIT*) **(**Refer **Figure 1.2)**.

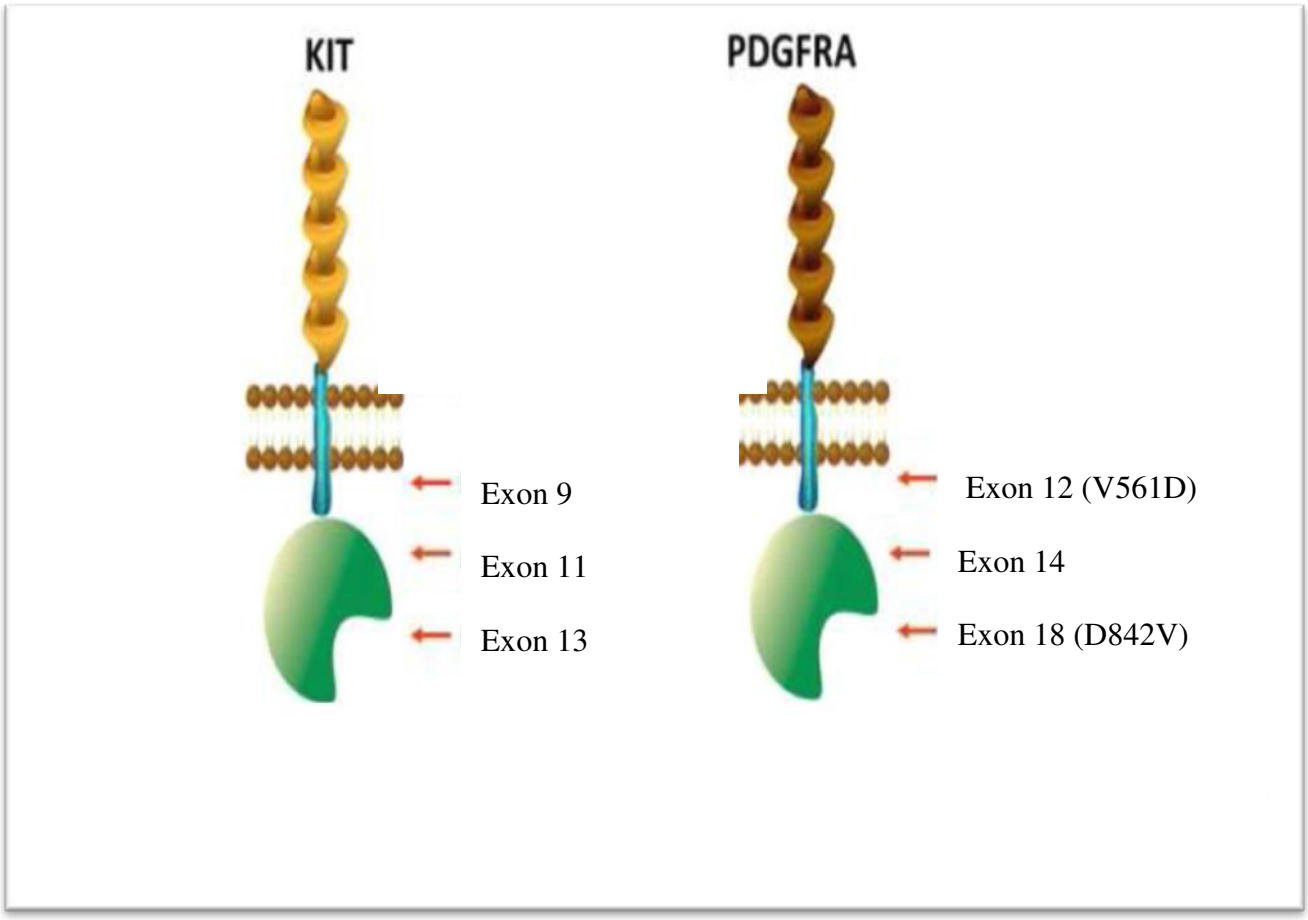

Figure 1.2 Diagrammatic representation of the structure of the KIT and PDGFRA showing the corresponding identified mutations of both genes in GIST. The most frequent activating mutations of the homologous PDGFRA kinase in patients with GIST are in exon 18, such as the D842V substituition that shows resistance to IM. Mutation in the juxtamembrane domain (exon 12; V561D most common) and exon 14 of tyrosine kinase 1 (TK1) domain (e.g., N659K) are less common. (Adapted from Heinrich,2006).

According to a study done by Corless *et.al.,* the leading mutation of all GIST caused by *PDGFRA* activating mutations, is found on exon 18 which accounted for 89.6% followed by 9.3% in exon 12 (Corless *et al.*, 2005). Of all the mutations in exon 18 of *PDGFRA*, the D842V mutation is the most common and has been proven to be IM resistant (Heinrich *et al.*, 2003a). The similar mutational status might also happen in CML that leads to the resistant to the treatment given.

1.2 Rationale of the study

This study may generate new data on potential involvement of *PDGFRA* mutations among CML patients treated with IM. Since many data from previous studies have implicated a subset of *PDGFRA* mutation has caused the IM resistance in GIST cases, this has instigate the curiosity of knowing whether the same mechanism play a role in the resistance cases of IM in CML patients. Thus in this research, since the most common sites of mutations in GIST cases which resistant to IM occur at exons 12 and 18, we adapted the facts as hypotheses to be tested in CML cases treated with IM that manifest similar behaviour.

Identifying key components involved in the CML pathogenesis may lead to the exploration of new approaches that might eventually overcome the resistance of this particular TKI. This may shed some light on how to treat resistance cases and might also reflect a better prognosis of CML patients.

1.3 Objectives of the study

1.3.1 General objective

To determine whether there are *PDGFRA* mutations in exons 12 and exon 18 in CML patients and any association with the resistance the patients experience on the treatment of IM.

1.3.2 Specific objectives

- To perform the PCR amplification of selected hot spots (exons 12 and 18) of *PDGFRA*.
- To sequence the PCR product to determine the presence of any *PDGFRA* mutations.
- To relate frequency of any *PDGFRA* mutations with CML status in association with IM treatment.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study design and study subject

This is a comparative cross-sectional study conducted in the Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, Health Campus. This study was approved by the Research Committee, School of Medical Sciences, Universiti Sains Malaysia (Reference no:USMKK/PPP/JEPem(221.3[9]). The blood samples used in this study were archival from the previous study entitled "Influence of TERT gene copy number status and its mRNA expression level in association with telomerase activity in Chronic Myeloid Leukemia patients resistance to Imatinib Mesylate treatment".

Total of 86 samples from two different groups of patients enrolled by random sampling included in this study which comprised of 43 samples from each group; resistant group and responsive group. The samples were obtained from patients who were clinically confirmed diagnosis of CML.

2.2 Sample size

Power and sample size (PS) software was employed to calculate sample size. A study of independent cases and controls with 1 control(s) per case was designed. Prior data from a study done in a group of patients with rearrangement of *PDGFRA* treated with IM indicate that the failure rate among controls is 0.5 (Cools *et al.*, 2003). If the true failure rate for experimental subjects is 0.214, we will need to study 43 experimental subjects and 43 control subject (which in this situation refers to response group) to be able to reject the null

hypothesis that the failure rates for experimental and control subjects are equal with probability (power) 0.8. The Type 1 error probability associated with this test of this null hypothesis is 0.05. An uncorrected chi-squared statistic was constructed to evaluate this null hypothesis. Therefore, the total sample size is 86; 43 for the resistant and response group.

2.3 Flow of the study

The CML patients were categorized into two groups, the CML-IM resistant labeled as "Resistant" (R) group and the CML-IM responsive labeled as "Good response" (G) group based on their response towards three monitoring parameters; cytogenetic, molecular and haematological. Commercial deoxyribonucleic acid (DNA) extraction kit, GeneAll Exgene Blood SV mini was used to extract genomic DNA from blood. Polymerase Chain Reaction (PCR) for *PDGFRA* on exon 12 and exon 18 were carried out to amplify the amplicons. To detect the presence of DNA fragments from PCR products, the products were loaded to agarose gel for electrophoresis and the stained gel was visualized under ultra violet (UV) transilluminator. This followed by purification of the amplicons using Illustra ™ Exoprostar ™ Enzymatic PCR and Sequencing Clean-Up. The purified PCR products then were sent to First Base Labarotory for DNA sequencing service. The sequencing results were screened by using Basic Local Alignment Search Tool (BLAST) software from National Center for Biotechnology Information (NCBI) by comparing with the sequence from the reference journal; Holtkamp *et al.,* 2006. To the final touch, data from the results were converted by statistical analysis into the form of graphs and charts as shown in the Result chapter. The flow chart of the study is shown in **Figure 2.1**.

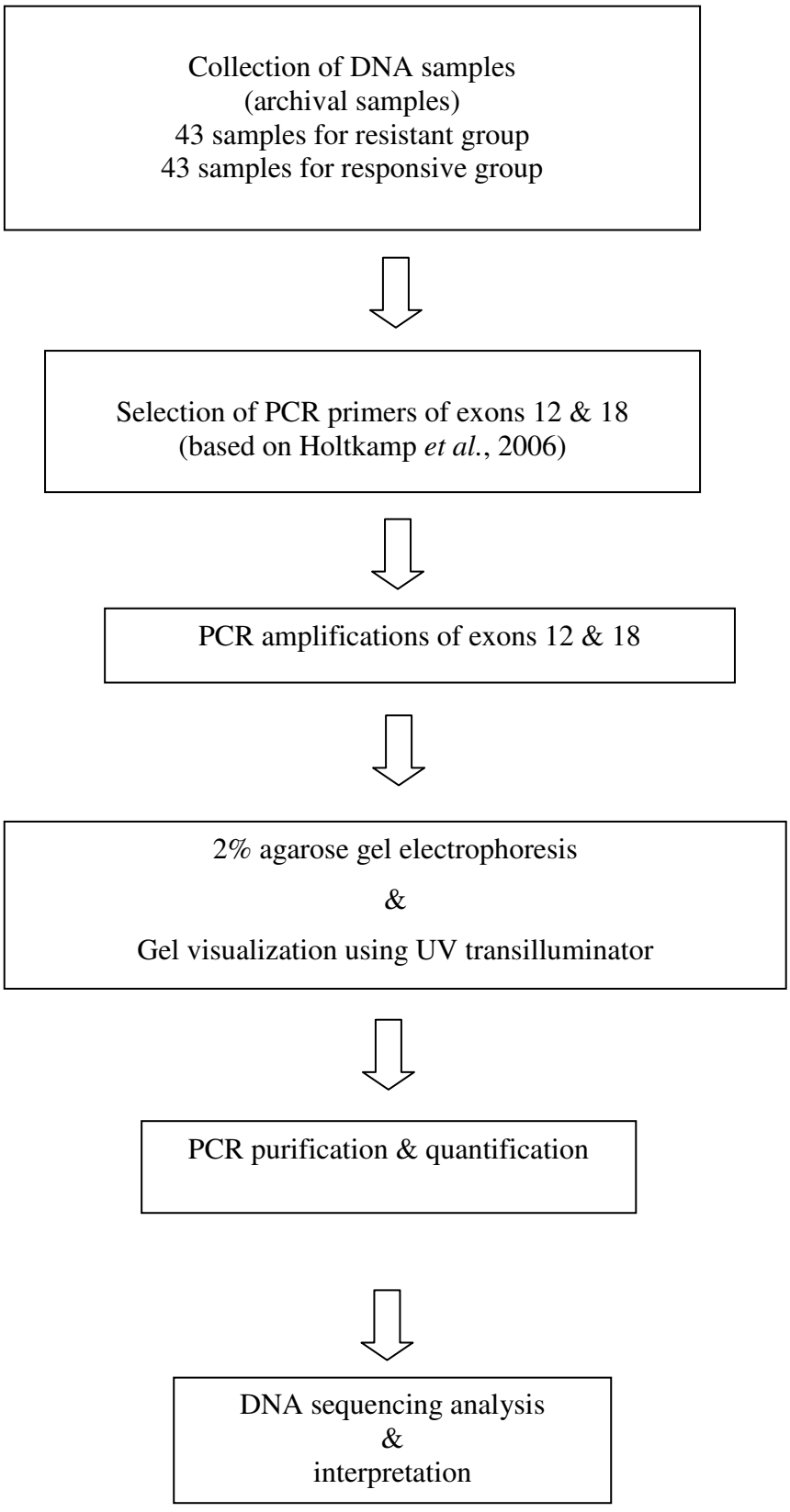

Figure 2.1 Flowchart of the study

2.4 Inclusion criteria

IM-resistant CML patient included in this study was those;

i. Confirmed clinically and hematologically to have CML with presence of Ph chromosome.
ii. Undergone IM treatment (400 mg, 600 mg or 800mg dose) for at least 12 months
iii. Experienced signs of primary or secondary resistance to the treatment.
iv. Showed suboptimal response and failure to IM treatment at cytogenetic, hematological and molecular level (based on European LeukemiaNet 2013 (ELN) guidelines) (Baccarani *et al.*, 2009).

IM-responsive CML patient included in this study was those;

i. Confirmed clinically and hematologically to have CML with presence of Ph chromosome.
ii. Undergone IM treatment (400 mg, 600 mg or 800mg dose) for at least 12 months
iii. Showed optimal response to IM treatment at cytogenetic, hematological and molecular level (based on ELN 2013 guidelines) (Baccarani *et al.,* 2009).

2.5 Exclusion criteria

i. CML patients with absence of Ph chromosome confirmed by molecular test.
ii. CML patients who were treated with IM but less than 12 months.
iii. CML patients who are not treated with IM.

2.6 Methodology

2.6.1 Genomic DNA extraction

Genomic DNA (gDNA) was extracted from the patient's peripheral blood which was stored in EDTA tube using a commercial GeneAll Exgene Blood SV mini kit (GeneAll Biotechnology, Korea) according to manufacturer's instruction. Included in this kit are GeneAll Proteinase K, Buffer CL, Buffer BL, Buffer BW, Buffer TW and Buffer AE. The protocol begins with 20 µl of Proteinase K solution was pipetted into the bottom of a 1.5 ml tube. This followed by 200 µl of blood from each sample was transferred to the tube and then 200 µl of Buffer BL was pipetted into the same tube. The tube was vortexed for 15 seconds to mix it. The mixture was incubated at 56^0C for 10 minutes using Thermomixer (Eppendorf, Germany) before being centrifuged briefly to remove any drops from internal lid wall.

Next, 200 µl of absolute ethanol was added to the sample to precipitate the DNA. The tube was placed on pulse-vortex for 15 seconds to mix the samples thoroughly, and then centrifuged briefly for about 10 seconds. The mixture was transferred to the SV column carefully, followed by centrifugation at 8000 rpm for 1 minute to resuspend the cell pellet. The collection tube was then discarded and replaced with the new one. Next step was the addition of 600 µl of Buffer BW into each SV column and then was centrifuged for 1 minute at 8000 rpm. Once again, the collection tube was discarded and replaced with new collection tube. A volume of 700 µl of Buffer TW was added and subjected to 1 minute centrifugation at 6000 rpm. The pass-through was discarded and the SV column was reinserted back into the collection tube. Centrifugation was then done at full speed for 1 minute to remove residual