GENETIC DETERMINANTS EXCLUDING *BCR::ABL* MUTATIONS OF IMATINIB MESYLATE THERAPY RESPONSE AMONG CHRONIC MYELOID LEUKAEMIA PATIENTS IN MALAYSIA

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by

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LIST OF ABBREVIATIONS

%	Percent
µl	Microliter
ABL1	Abelson Murine Leukaemia Viral Oncogene Homolog 1
ACAs	Additional Chromosome Abnormalities
AE Buffer	Elution Buffer
AHCT	Allogeneic Haematopoietic Cell Transplant
AKT	Ak Strain Transforming
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
AP	Accelerated Phase
AP – 1	Activator protein – 1
AS – PCR	Allele – Specific PCR
ASS1	Arginosuccinate Synthase 1
ATG16L2	Autophagy Related 16 Like 1
ATP	Adenosine Triphosphate
AW1	Washing Buffer 1
AW2	Washing Buffer 2
Bad	BCL 2 Associated Agonist of Cell Death
Bak	Bcl-2 Homologous Antagonist/ Killer
Bax	Bcl-2- Associated X Protein
BCL-2	B Cell Lymphoma 2

BCL-XL	B Cell Lymphoma- Extra Large
BCR	Breakpoint Cluster Region
BCR::ABL1	BCR- Abelson Murine Leukaemia
BECN1	Mammalian Orthologue of The Yeast Apg6/ Vps30
BH3	BCL-2-Homology Domain 3
BIM	BCL2 Like 11
Bim EL	BCL Like 11/ BIM Extra Long
Bim L	BCL Like 11/ BIM Long
Bim S	BCL Like 11/ BIM Short
BM	Bone Marrow
Bmf	Bcl2 Modifying Factor
bp	Base pair
BP	Blast Phase
CCA	Clonal Chromosome Abnormalities
CCA	Conventional Cytogenetic Analysis
CCyR	Complete Cytogenetic Response
CDK2	Cyclin- Dependent Kinase 2
CE	Clonal Evolution
CHR	Complete Haematological Response
CI	Confident Interval
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myeloid Leukaemia
CMR	Complete Molecular Response

CNVs	Copy Number Variations
CO_2	Carbon Dioxide
CP	Chronic Phase
CyR	Cytogenetic Response
DAPI	4’6-Diamidino-2-Phenylindole
ddH_2O	Double Distilled Water
ddPCR	Digital Droplet PCR
DDR	DNA Damage Response
DNA	Deoxyribonucleic Acid
dNTPs	Dinucleotide Triphosphates
dsDNA	Double-Stranded DNA
DPX	Dibutyl Phthalate Polystyrene Xylene
EB	Elution Buffer
EBF2	Early B- Cell Factor 2
ECM	Extra Cellular Matrix
ECs	Endothelial Cells
EDTA	Ethylenediaminetetraacetic Acid
ELN	European LeukaemiaNet
FADD	Fas-Associated Death Domain Protein
FBS	Foetal Bovine Serum
FISH	Fluorescence *In Situ* Hybridization
FOXO3	Forkhead Box-O-3
GNA2	Glucosamine Phosphate N- Acetyl Transferase

GRB2	Growth Factor Receptor- Bound Protein 2
GSK3β	Glycogen Synthases Kinase-3 Beta
GTG	G- Bands by Trypsin Using Giemsa
GUSB	Beta- Glucuronidase
HIF- 1α	Hypoxia Inducible Factor Alpha
hoCT1	Human Organic Cation Transporter Type 1
IL-8	Interleukin 8
IM	Imatinib Mesylate
IS	International Scale
ISCN	International System Human Genomic Nomenclature
JAK	Janus Kinase
kb	Kilo Base Pair
KCl	Potassium Chloride
KD	Kinase Domain
kDa	Kilo Dalton
KDR	Kinase Insert Domain – Containing Receptor
KIT Receptor	Kit Proto- Oncogene Receptor Tyrosine Kinase
KLF 17	Kruppel – Like – Factor 17
L	Litre
MAPK	Mitogen- Activated Protein Kinase
MCL-1	Myeloid Cell Leukaemia 1
MCyR	Major Cytogenetic Response
MDM2	Murine Double Minute 2

MDR1	Multidrug Resistance Mutation
MDS	Myelodysplastic Syndrome
mg	Milligram
$MgCl_2$	Magnesium Chloride
ml	Millilitre
mM	Millimolar
MMR	Major Molecular Response
mRNA	Messenger Ribonucleic Acid
MSD	Minimal Residual Disease
MVD	Micro – Vessel Density
NCBI	National Center for Biotechnology Information
NCCN	National Comprehensive Cancer Network
NEB	New England BioLabs
NF- KB	Nuclear Factor-Kappa B
ng	Nanogram
NHL	Non- Hodgkin Lymphomas
^oC	Degree Celsius
OR	Odds Ratio
OS	Overall Survival
OSCP1	Organic Solute Carrier Partner 1
P13- kinase	Phosphoinositide 3- Kinase
P13K	Phosphoinositide- 3- Kinase- Protein Receptor
PCR	Polymerase Chain Reaction

PCyR	Partial Cytogenetic Response
PdGFR	Platelet- Derived Growth Factor Receptor
PFS	Progression Free Survival
Ph’	Philadelphia Chromosome
Ph-	Philadelphia Chromosome Negative
Ph+	Philadelphia Chromosome Positive
PIGF	Phosphatidylinositol Glycan Anchor Biosynthesis Class F
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RAB36	Ras-Related Protein Rab-36
RAF	Rapidly Accelerated Fibrosarcoma
RAS	Rat Sarcoma
RE	Restriction Enzymes
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
RPMI	Rosewell Park Memorial Institute
rpm	Revolution Per Minute
RT	Room Temperature
RT – PCR	Reverse Transcriptase Polymerase Chain Reaction
SCF	Stem Cell Factor
SH2	Src Homolog 2
SH3	Src Homolog 3
SNPs	Single Nucleotide Polymorphisms
SP – 1	Stimulatory Protein 1

SPSS	Statistical Package for The Social Sciences
SRC	Proto- Oncogene C-Src
SSC Buffer	Saline Sodium Citrate
STAT5	Signal Transducer and Activator of Transcription 5
Ta	Annealing Temperature
TBE Buffer	Tris- Boric Acid- EDTA Buffer
TFAP2A	Transcription Factor AP-2 Alpha
TKD	Tyrosine Kinase Domain
TKI	Tyrosine Kinase Inhibitor
Tm	Melting Temperature
TNF-R	Tumour Necrosis Factor Receptor
TP53	Tumour Protein P53
USM – RU	Universiti Sains Malaysia Research University Grant
UV	Ultra Violet
VEGF	Vascular Endothelial Growth Factor
VEGFA	Vascular Endothelial Growth Factor A
VEGFC	Vascular Endothelial Growth Factor C
VEGFD	Vascular Endothelial Growth Factor D
VEGFR1	Vascular Endothelial Growth Factor Receptor 1
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VRGFB	Vascular Endothelial Growth Factor B
WBC	White Blood Cell
WHO	World Health Organization

LIST OF APPENDICES

PENENTU GENETIK SELAIN DARIPADA MUTASI *BCR:: ABL* BAGI TINDAK BALAS TERAPI IMATINIB MESYLATE DALAM KALANGAN PESAKIT LEUKAEMIA MIELOID KRONIK DI MALAYSIA

ABSTRAK

Walaupun imatinib mesylate (IM) merupakan ubat barisan hadapan yang berjaya menangani leukaemia mieloid kronik (CML), sebilangan besar pesakit CML mengalami kerintangan dan memberikan hasil buruk. Objektif kajian ini adalah untuk mengkaji sumbangan polimorfisme terpilih *VEGFA* (+936 C> T dan -634 G> C), *VEGFR2* (1192 C> T, ivs25-29 G> A dan 1416 T> A), *BIM* (penghapusan intron 2 dan c465 C> T), mutasi *TP53* exon 8 dan kelainan kromosom tambahan (ACA) dalam memodulasi tindak balas rawatan IM dan perkembangan penyakit di kalangan 249 pesakit. Untuk kajian ini, pesakit CML terdiri daripada 127 IM tahan dan 122 IM tindak balas baik telah direkrut. Untuk *VEGFA* +936 C> T dan -634 G> C, *VEGFR2* 1192 C> T, ivs25-29 G> A dan 1416 T> A dan *BIM* c465 C> T tindak Balas Berantai Polimerase - Polimorfisme Pemotongan Panjang Cebisan (PCR – RFLP) digunakan dan alel khusus – PCR (AS – PCR) untuk polimorfisme penghapusan *BIM* intron 2, mutasi *TP53* exon 8 disiasat menggunakan penguatan PCR diikuti penjujukan DNA. ACA disiasat menggunakan prosedur sitogenetik standard dan *FISH*. Berkenaan *VEGFA*, kedua-dua SNP +936 C> T dan -634 G> C menunjukkan risiko lebih rendah untuk pengembangan rintangan. Untuk varian homozigot (TT) +936 C>T, menunjukkan OR: 0.11 (95 % CI = 0.02 – 0.56, p = 0.008) dan CC dari -634 G>C menunjukkan OR: 0.17 (95 % CI = 0.07 – 0.41, p = 0.001). Alel C -634 G>C juga dikaitkan dengan risiko lebih rendah untuk pengembangan rintangan IM (OR: 0.49, 95 % CI = 0.34 – 0.71, p = 0.001). Dalam

kes *VEGFR2*, ivs25-29 G>A SNP, hanya varian homozigot (AA) menunjukkan hubungan risiko rendah yang signifikan dengan pengembangan rintangan dengan OR, 0.17 (95 % CI: 0.04 – 0.84, p = 0.029). Untuk SNP 1416 T>A, varian heterozigot (TA) dan varian homozigot (AA) menunjukkan risiko lebih rendah untuk pengembangan ketahanan (OR: 0.25, 95 % CI: 0.11 – 0.59, p = 0.002) untuk varian heterozigot dan OR: 0.27, 95 % CI = 0.12 – 0.62, p = 0.002 untuk varian homozigot masing –masing). Dalam kes *TP53* exon 8 dan penghapusan *BIM* intron 2, semua subjek kajian menunjukkan genotip jenis liar tanpa mutasi pada ekson 8 dan tiada penghapusan yang dikesan dalam intron 2. Untuk *BIM* c465 C> T, varian heterozigot (CT) dan model genetik dominan CT + TT (OR: 2.14, 95 % CI = 1.24 – 3.67, p = 0.006 dan OR: 1.99, 95 % CI = 1.19 – 3.34, p = 0.009) dan alel varian T (OR: 1.57, 95 % CI = 1.03 – 2.39, p = 0.036) menunjukkan risiko lebih tinggi untuk pengembangan ketahanan terhadap IM. ACA dikesan pada 40/ 249 pesakit (16.1 %). 40 pesakit dikategorikan kepada pesakit yang mempunyai Ph + / ACA dan Ph-/ ACA dan dikategorikan kepada empat kumpulan berdasarkan jenis kelainan. Pesakit kumpulan 1 dan 2 menunjukkan prognosis relatif lebih baik sementara pesakit kumpulan 3 dan 4 menunjukkan risiko lebih tinggi untuk perkembangan penyakit. Novel ACA yang melibatkan penyusunan kromosom 11 dan 12 didapati membawa kepada mieloid BP. Stratifikasi berdasarkan ACA individu didapati mempunyai kesan prognostik berbeza dan mungkin merupakan sistem ramalan risiko yang berpotensi untuk memprognosis dan memandu rawatan pesakit CML. Penemuan dari kajian ini menunjukkan hubungan yang jelas faktor genetik inang dan genomik tumour dengan tindak balas rawatan IM dan perkembangan penyakit. Faktor genetik ini boleh menjadi penanda bio yang berpotensi untuk meramalkan tindak balas rawatan IM dan perkembangan penyakit di kalangan pesakit CML Malaysia.

GENETIC DETERMINANTS EXCLUDING *BCR::ABL* MUTATIONS OF IMATINIB MESYLATE THERAPY RESPONSE AMONG CHRONIC MYELOID LEUKAEMIA PATIENTS IN MALAYSIA

ABSTRACT

Despite imatinib mesylate (IM) being the frontline drug for successful treatment of chronic myeloid leukaemia (CML), a significant proportion of CML patients on IM therapy develop resistance and attain poor outcome. The objective of the present study was to investigate the contribution of selected polymorphisms of *VEGFA* (+936 C>T and -634 G>C), *VEGFR2* (1192 C>T, ivs25-29 G>A and 1416 T>A), *BIM* (intron 2 deletion and c465 C>T), *TP53* mutation at exon 8 and additional chromosome abnormalities (ACAs) in modulating IM treatment response and disease progression in 249 Malaysia CML patients undergoing IM treatemnt. For this study, CML patients comprising of 127 IM resistant and 122 IM good response were recriuted. For *VEGFA* +936 C>T and -634 G>C, *VEGFR2* 1192 C>T, ivs25-29 G>A and 1416 T>A and *BIM* c465 C>T Polymerase Chain Reaction- Restriction Enzyme Fragment Length Polymorphims (PCR – RFLP) was employed and allele specific – PCR (AS – PCR) for *BIM* intron 2 deletion polymorphims, *TP53* mutation at exon 8 was investigated using PCR amplification followed by DNA sequencing. ACAs were investigated employing standard cytogenetic procedures and FISH. With regard to *VEGFA,* both the SNPs +936 C>T and -634 G>C showed significant lower risk for the development of resistance. For the homozygous variant (TT) +936 C>T, of showed OR: 0.11 (95 % CI = 0.02 – 0.56, p = 0.008) and CC of the -634 G>C showed OR: 0.17 (95 % CI = 0.07 – 0.41, p = 0.001). The C allele of -634 G>C was also significantly associated with lower risk for development of IM resistance (OR:

0.49, 95 % CI = 0.34 – 0.71, p = 0.001). In the case of *VEGFR2,* ivs25-29 G>A SNP, only homozygous variant (AA) showed significant lower risk association with development of resistance with OR, 0.17 (95 % CI = 0.04 – 0.84, p = 0.029). For SNP of 1416 T>A, the heterozygous variant (TA) and homozygous variant (AA) showed significant lower risk for development of resistance (OR: 0.25, 95 % CI: 0.11 – 0.59, p = 0.002 for heterozygous variant and OR: 0.27, 95 % CI = 0.12 – 0.62, p = 0.002 for homozygous variant respectively). In the case of *TP53* exon 8 and *BIM* intron 2 deletion, all study subjects showed wildtype genotype with no mutation in exon 8 and no deletion detected in intron 2. For *BIM* c465 C>T, the heterozygous variant (CT) and dominant genetic model CT + TT (OR: 2.14, 95 % CI = 1.24 – 3.67, p = 0.006 and OR: 1.99, 95 % CI = 1.19 – 3.34, p = 0.009) and variant allele T (OR: 1.57, 95 % CI = 1.03 – 2.39, p = 0.036) showed higher risk for the development of resistance to IM. ACAs were detected in 40/ 249 patients (16.1 %). For determining the prognosis impact of ACAs, these 40 patients were categorized to those with Ph+/ ACAs and Ph-/ ACAs and further stratified into four groups based on the type of abnormalities. Patients with group 1 and group 2 abnormalities showed comparatively better prognosis while patients with group 3 and 4 abnormalities showed higher risk for disease progression. Novel ACAs consisting of rearrangements involving chromosomes 11 and 12 were found to lead to myeloid BP. Stratification based on individual ACAs found to have differential prognostic impact and might be a potential risk predictive system to prognosticate and guide treatment of CML patients. These findings from the present study demonstrated obvious relationships of host genetic and tumour genomic factors with IM treatment response and disease progression These genetic factors could be potential biomarkers to predict IM treatment response and disease progression in Malaysian CML patients.

CHAPTER 1

INTRODUCTION

1.1 Leukaemia

The cell types for example red blood cells, white blood cells, and platelets in the human blood arise from haematopoietic stem and progenitor cells in the bone marrow. A group of cancer cells that derive from the blood cells of the bone marrow, immune system or lymph system are described as haematological malignancies. Neoplasms with the involvement of the bone marrow and the peripheral blood are classified as leukaemia. Lymphomas are lymphoid tumours that typically show as masses within lymph nodes or other soft tissues.

The cells of origin and typical clinical characteristics are used to define and describe various haematological cancers. Myeloid, myelogenous, or myeloproliferative neoplasms are tumours that contain cells from the myeloid series (granulocytes, red cells, platelets, and their progenitors). The myeloid cancers include acute myeloid leukaemia (AML), and chronic myeloid disorders such as chronic myeloid leukaemia (CML), and the myelodysplastic syndrome (MDS). Tumours comprising of lymphocytes or their progenitors are referred to as lymphoid, lymphocytic, lymphoblastic or, lymphoproliferative neoplasms. The lymphoid cancers include acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), multiple myeloma, and both Hodgkin lymphomas, and non- Hodgkin lymphomas (NHL).

1.2 Overview of Chronic Myeloid Leukaemia (CML)

Despite its relative rarity, chronic myeloid leukaemia (CML) has garnered attention during past two decades. CML is a myeloproliferative disorder of haematopoietic stem cell origin which is characterized by uncontrolled increased proliferation of mature granulocytes (neutrophils, eosinophils and basophils) without the loss of their capacity to differentiate. As a result, increased numbers of granulocytes and their immature precursors, including occasional blast cells are seen in the peripheral blood.

1.2.1 Incidence of CML

CML has an annual incidence of 0.7 – 1.8 per 100,000 population globally and the prevalence is expected to plateau at 35 times its annual incidence by 2050 (Hoglund *et al.*, 2015). This has been attributed to be due to the substantial proportion of survival achieved with the advent of targeted therapy. CML comprises about 15% of all adult leukaemias and approximately 2.5% in children. CML accounts for 15% of approximately 740 new leukaemia cases diagnosed annually in Malaysia (Omar *et al* 2011). The incidence of CML has been reported to be lower in Asian population compared to the Western population (Au *et al.*, 2009). An individual older than 70 years has a risk of more than 20% whereas the risk is less than 5% in children and adolescents. The median age for CML has been reported to be between 45- 55 years old. In Western countries, the median age of CML patients is about 57 years (Hehlmann *et al.*, 2017; Hochhaus *et al.*, 2020; Hoffmann *et al.*, 2015). Meanwhile, in Asia and Africa a younger average median age with <50 years has been reported (Malhotra *et al.*, 2019). CML has been reported to be predominant in males (Krishna

Chandran *et al.*, 2019). However, in Peninsular Malaysia, the male to female ratio has been reported to be 1.1:1 (Azizah *et al.,* 2011; Yusoff *et al.*, 2020).

1.2.2 Pathogenesis of CML

CML arise from balance genetic translocation between chromosome 9 and 22, t(9;22) (q34;q11.2) resulting in shortened of chromosome 22, that is designated as Philadelphia chromosome. **Fig. 1.1** Illustrates the formation of Philadelphia chromosome and **Fig. 1.2** represents a GTG banded male karyotype showing the Ph' chromosome. The molecular sequence of this balanced translocation is the generation of a breakpoint cluster region BCR – Abelson murine leukaemia (ABL1) [*BCR::ABL1*] fusion oncogene. *BCR::ABL1* fusion oncogene codes for a constitutively active tyrosine kinase oncoprotein that promotes growth and replication of haematopoietic cells through down-stream signal transduction pathways such as RAS, RAF, JUN kinase, MYC, and STAT (Carlesso *et al.*, 1996; Sakai *et al.*, 1994). Consequently, this influences leukemogenesis by creating a cytokine- independent cell cycle with aberrant apoptotic signals in response to cytokine withdraw. Although the molecular pathogenesis of CML is well elucidated, the mechanism that leads to the gene translocation is still unknown (Hoffmann *et al.*, 2017).

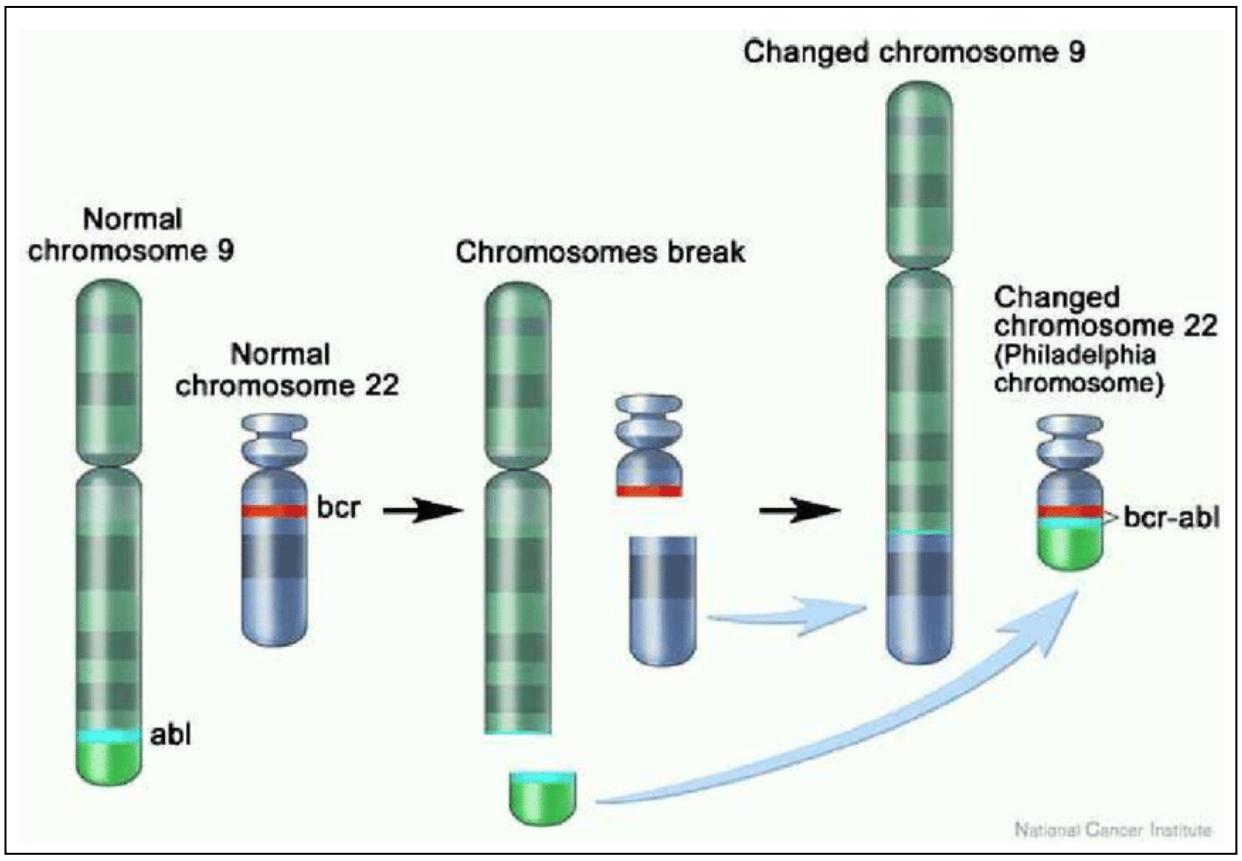

Figure 1.1: Illustration of Philadelphia chromosome from translocation t(9;22)(q34;q11.2) and formation BCR::ABL1 fusion gene (Adapted from National Cancer Institute website www.cancer.gov/Templates/db alpha.aspx?CdrID=44179)

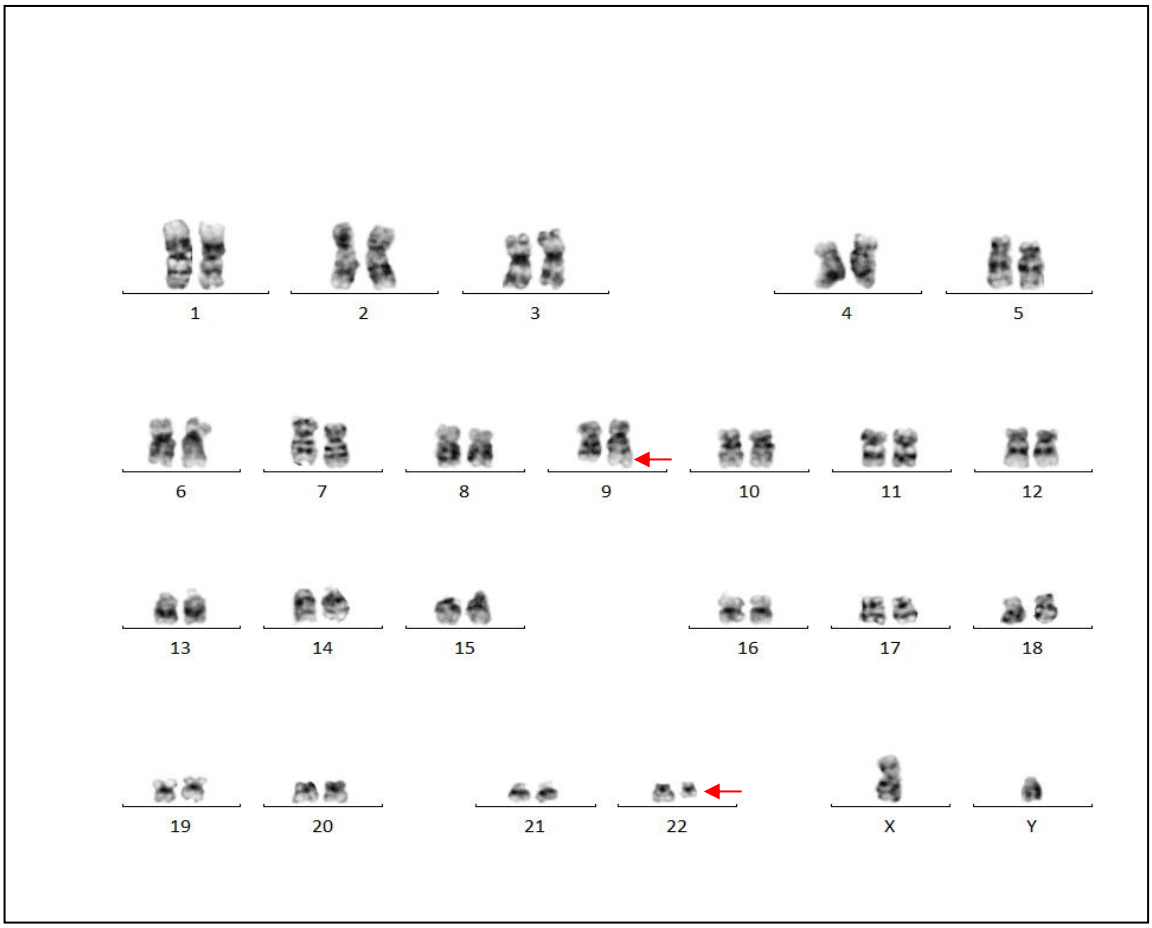

Figure 1.2: GTG banded of 46,XY,t(9;22) (q34;q11.2)

1.2.3 Variant Ph chromosome

The typical t(9;22) is seen in 95 % of CML patients and the remaining 5 % have variant translocations. Variant translocations can be simple involving chromosome 9, 22 and one other chromosome or can be complex involving more chromosomes in addition to chromosome 9 and 22. Studies had showed CML patients with Ph more frequently have a small deletion potion on derivative chromosome 9. This small deletion can be only detected using FISH or molecular method (El-Zimaity *et al.*, 2004; Herens *et al.*, 2000; Marzocchi *et al.*, 2011; Sinclair *et al.*, 2000). Various chromosomes can be involved in a variant translocation with a pattern cluster seen. The breakpoint cluster to chromosome band 1p36, 3p21, 5q13, 6p21, 9q22, 11q13, 12p13, 17p13, 17q21, 17q25, 19q13, 21q22, 22q12 and 22q13 (Johansson *et al.*, 2002a). **Fig. 1.3** and **Fig. 1.4** show two karyotypes showing variant Philadelphia chromosome.

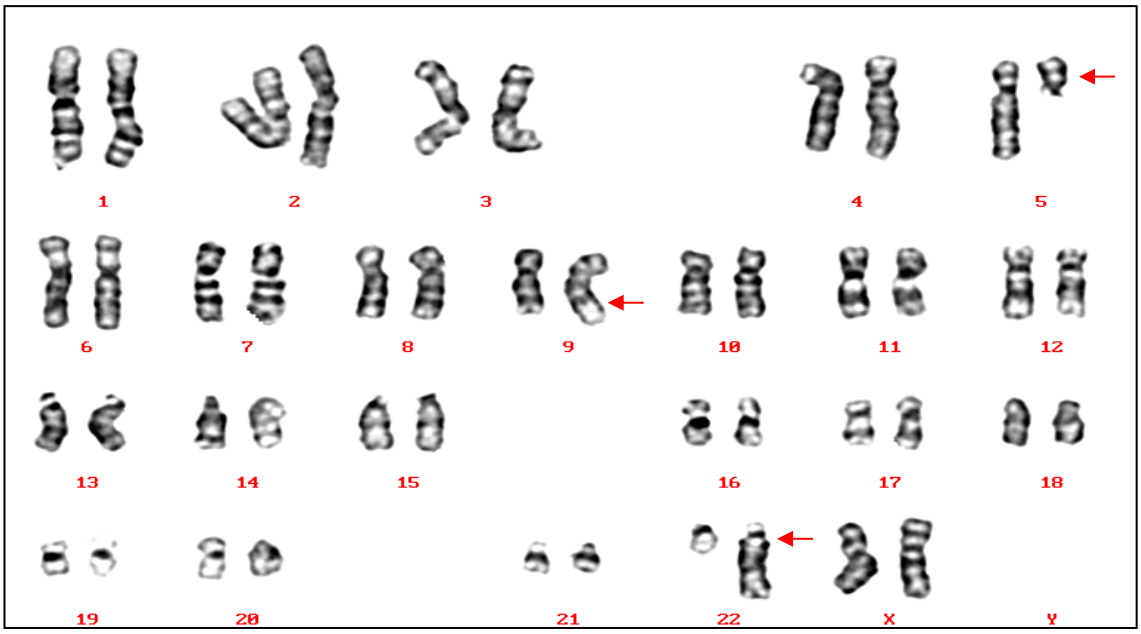

Figure 1.3: Karyotype for 46,XX,der(22) t(5;9;22) (q11;q34;q11)
(aberrations shown as red arrows)

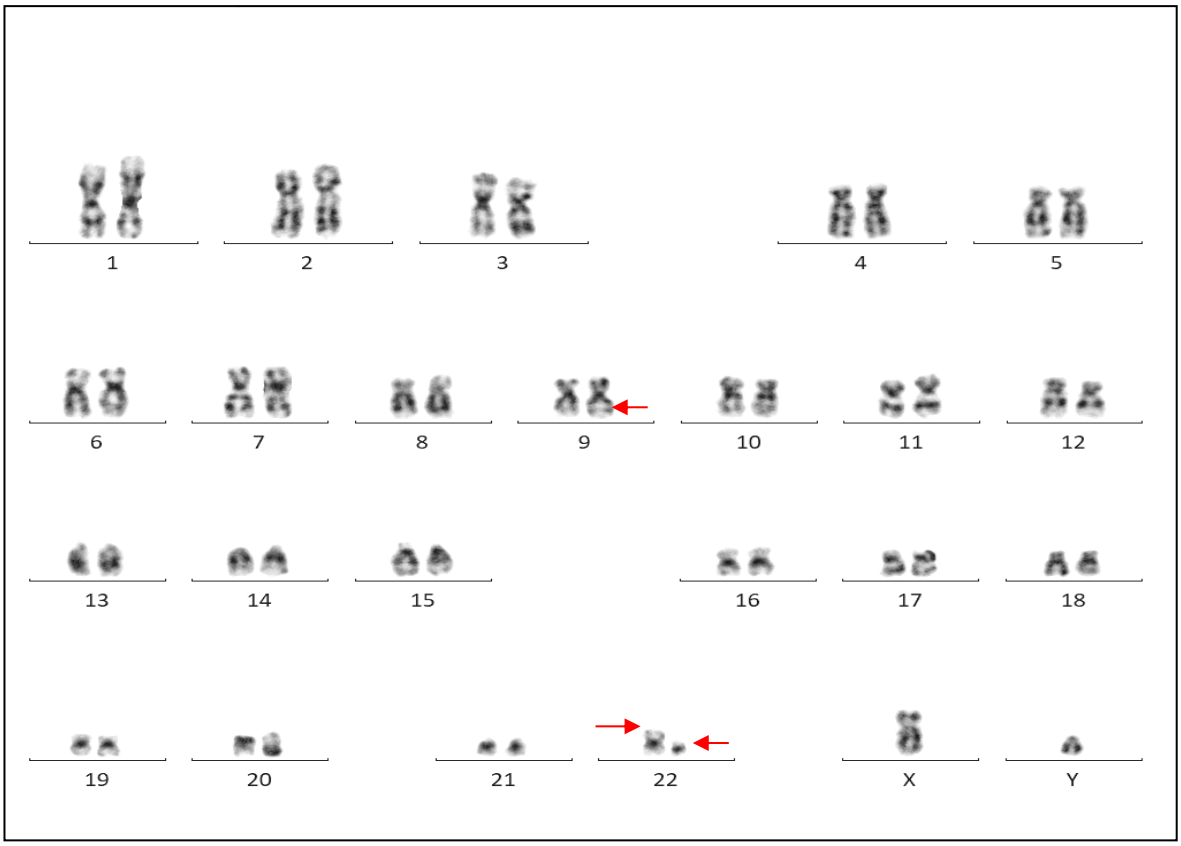

Figure 1.4: Karyotype for 46,XY,t(9;22;22) (q34;q10;q11) (aberrations shown as red arrows)

1.2.4 Diagnosis of CML

CML is a disease that typically evolves in three distinct clinical phases (triphasic disease). Most patients present in an indolent chronic phase (CP) course in which the symptoms can be easily controlled with therapy for more than five years. But without effective medical intervention, the disease can progress through a period of increasing instability known as accelerated phase (AP) and/or to terminal transformation to an acute leukaemic-like illness which is known as the blast phase (BP). Blast phase is characterized by rapid expansion of a population of myeloid or lymphoid blasts of at least 30 % in the peripheral blood or bone marrow and a median survival time measured in few months (Kantarjian *et al.*, 1988).

Patients are typically diagnosed in CP based on a characteristic blood count and differential (left – shifted leukocytosis). Patients presenting in chronic phase show one or more of the frequent symptoms and signs such as fatigue, night sweats, malaise and weight loss, left upper quadrant pain, discomfort, satiety and splenomegaly (the most common sign). Less frequent symptoms include priapism, retinal haemorrhages, thrombosis/bleeding or both, bone pain, hepatomegaly and lymphadenopathy (Apperley, 2015).

For confirmatory diagnosis of CML, standard laboratory tests include bone aspiration or biopsy for haemato – pathological examination, bone marrow aspiration for cytogenetic detection of the Ph' chromosome translocation or fluorescence in situ hybridization (FISH) for the detection of *BCR::ABL* fusion signal or reverse transcriptase polymerase chain reaction (RT- PCR) for detection of BCR::ABL fusion transcripts. In laboratories where facilities are available, quantitative real time

polymerase chain reaction (qRT-PCR) is also carried out at the time of diagnosis to quantitate the base line *BCR::ABL* transcripts. These tests are carried out not only at diagnosis, but also repeated periodically thereafter for monitoring haematological, cytogenetic, and molecular responses to treatment.

1.2.5 Treatment

Elucidation of the molecular pathogenesis of CML led to the targeted approach in the development of small molecule tyrosine kinase inhibitors (TKIs) that could exploit the presence of the aberrantly expressed BCR::ABL1 protein in CML cells. Thus, the introduction of targeted drugs dramatically altered the natural history of the disease, improved 10 – year overall survival (OS) from 20 % to 80 – 90 % (Deininger *et al.*, 2009; Jemal *et al.*, 2010). The first TKIs, Imatinib Mesylate (IM) is a molecular targeted drug used in positive CP CML patients. The development of IM resistant in CML patients, leads to development of second and third generation of TKIs.

For newly diagnosed CML patients, Imatinib mesylate (IM) is recommended as a reasonable first line option. The standard dosage of imatinib is 400 mg daily and 600 mg daily may lead to a higher molecular response. The recommended dose of imatinib is 400 – 800 mg daily. However, the selection needs to depend on the risk score, (Sokal, and Hasford), physician's experience, toxicity profile, patients's age, tolerance, and adherence to therapy as well as co – morbidities (Sundar and Radich, 2016). However, to ensure that the CML patients treated with IM meet the treatment milestones, both NCCN and ELN guidelines emphasize adequate monitoring and measurement of residual disease through the sensitive qRT – PCR method, even for

those who achieve CCyR (Baccarani *et al.*, 2013; Castagnetti *et al.*, 2015; Cortes *et al.*, 2016; Deininger, 2015; O'Brien *et al.*, 2013).

For CML patients who fail treatment goals as a result of primary resistance, intolerance to IM, haematologic disease recurrence or emergent *BCR::ABL1* kinase domain mutations, substitution with another TKI is the best option, as IM dose escalation may not be sufficient to control the disease (Jabbour *et al.*, 2010; Jabbour *et al.*, 2014; Pallera *et al.*, 2016).

Second generation TKIs represented by Nilotinib, Dasatinib, and Bosatinib, and third generation inhibitor represented by Ponatinib were developed to overcome IM resistance. Nilotinib binds to the inactive conformation of ABL1 kinase domain through lipophilic and weak Van der Wall interactions and blocks its catalytic activity (Manley *et al.*, 2006). Nilotinib is 10 – 30 fold more potent than IM in inhibiting *BCR::ABL1* activity and proliferation of BCR::ABL+ cells and is effective against 32/33 *BCR::ABL1* mutations at physiologically relevant concentrations (Kantarjian *et al.*, 2007). Dasatinib exclusively targets the active conformation of ABL kinase, but with less stringent – conformation requirement and reduced selectivity (Eck and Manley, 2009). This binding characteristics makes Dasatinib – 325 fold more potent against wildtype *BCR::ABL1* than IM and effective against majority of *BCR::ABL1* mutations except for T315I (Olivieri and Manzione, 2007).

Imatinib 400 mg once daily, dasatinib 100 mg once daily, or nilotinib 300 mg twice daily has been prescribed by the (Baccarani *et al.*, 2015) ELN (2015) and European Society of Medical Oncology ESMO (2017) (Hochhaus *et al.*, 2017) as the first line treatment of CP – CML patients. Bosatinib has also been recommended by the

current updated NCCN guidelines (2018) (Radich *et al.*, 2018) as an option for first line treatment along with the aforementioned TKIs. The third generation TKI, Ponatinib possess a linear structure, which possibly helps it avoid steric clashes with hydrophobic gatekeeper residues and make it a potent inhibitor of the known *BCR::ABL1* mutations, particularly T315I (O'Hare *et al.*, 2009).

Although IM, dasatinib and nilotinib are all recommended for first line use, IM remains the most popular first line therapy for CML because of its efficacy in majority of the patients and no/or less severe toxic effects. CML patients who show sub-optimal response to IM evaluated carefully and one of the TKIs such as dasatinib, nilotinib, bosatinib, or ponatinib is appropriately selected and prescribed as alternative treatment options. For treatment selection, factors such as the patient's disease, state, prior therapy, type of mutation, co – morbidities, treatment toxicity, and therapy goals need to be considered (Kantarjian *et al.*, 2010; Radich *et al.*, 2012). In order to provide the optimal treatment, regular monitoring of *BCR::ABL1* level, effective management of toxicities, and patients education on adherence to TKIs therapy are highly essential.

Before the advent of TKI therapy, allogeneic haematopoietic cell transplant (AHCT) was the treatment of choice for CML. However, AHCT is now generally reserved for CML patients who show resistance to multiple or unable to tolerate TKIs, those who have the T315I *BCR::ABL1* mutation, and those who are not suitable for prolonged ponatinib therapy (Sundar and Radich, 2016).

1.3 Rationale of the study

1.3.1 Development of resistance of Imatinib Mesylate (IM)

Despite increased efficacy and better clinical outcomes, a significant proportion of CML patients on IM develop drug resistance. Clinical response to IM has been documented to be determined by somatic mutations as well as by germline genetic variations (Kim *et al.*, 2017). Resistance to IM could be due to either BCR::ABL1 dependent pathways or BCR::ABL1 independent pathways. An earlier study from USM (Elias *et al.*, 2014) reported that BCR::ABL1 mechanism involving mutations and amplifications of *BCR::ABL1* tyrosine kinase domain account for only 24.6 % of CML patients in showing IM resistance. This implied that the mechanisms of resistance to IM in the remaining 75.4 % of CML patients with IM resistance could be due to the involvement of BCR::ABL1 independent and other pathways. With the completion of the human genome sequencing, it has become clear that the genetic makeup of an individual can affect response to drugs, either optimum response or development of resistance. This has generated great interest in examining the germline genetic profiles of CML patients who are undergoing IM treatment. Genetic factors that can contribute to a patient's response to drugs include single nucleotide polymorphisms (SNPs), haplotypes or other heritable mutations, copy number variations (CNVs), and chromosome alterations (Nath *et al.*, 2017). Hence examination and identification of genetic factors that influence IM response in CML patients is important to provide prognostic information to predict treatment outcomes and improve the efficacy of treatment for individual patients.

1.3.2 Present study

Although IM has been shown to elicit high response rates among CML patients, the extent and duration of these responses are heterogeneous between patients. A significant proportion of CML patients on IM therapy have been found to develop resistance due to factors other than *BCR::ABL* mutations and amplifications. This suggests the existence of *BCR::ABL* independent genetic factors as modifiers that affect CML patients' response to IM. Development of *BCR::ABL1* independent mechanism of resistance in CML patients undergoing IM treatment cannot be explained in a simple and singular way. It appears to be more complex than speculated. This study was designed to elucidate the *BCR::ABL* independent mechanisms of resistance to IM in *BCR::ABL* non-mutated CML patients.

Based on the complexity of IM resistance, multiple approaches have been examined by different group of researchers, as candidate mechanisms. Previous researchers from CML research group in USM had already investigated the contribution of several SNPs involved absorption, metabolism, cellular influx and efflux of IM, in modulating resistance to IM (Au *et al.*, 2014; Maddin *et al.*, 2016; Makhtar *et al.*, 2017). Therefore, this study aimed to investigate few other genetic factors including multiple candidate SNPs of genes involved in angiogenesis and apoptosis pathways, *TP53* mutation status and emergence of additional chromosome abnormalities (ACAs) for their association with response and resistance in CML patients undergoing IM treatment.

1.4 Genetic variations in vascular endothelial growth factors (*VEGF*) and *BIM* (BCL2L11) in association with IM response and disease progression

Bone marrow microenvironment has been implicated to play a role in haematological malignancies. An important process that aids the growth of leukaemic cells is angiogenesis. Angiogenesis which deals with development of new blood vessels from a pre – existing vascular network is a tightly regulated process (Medinger *et al.*, 2010). The reports (Medinger *et al.*, 2010) that leukaemia, similar to solid tumours, also have high bone marrow micro – vessel density (MVD), suggested that angiogenesis plays an important role in the progression of leukaemia. Vascular Endothelial Growth Factor (VEGF) has been identified as the specific growth factor for angiogenesis (Song *et al.*, 2012). It has been demonstrated by earlier researchers that development of haematopoietic stem cells and remodelling of both the extra cellular matrix and the regeneration of inflammatory cytokines are regulated by VEGF (Cursiefen *et al.*, 2004; Kato *et al.*, 1995). Bone marrow vascularisation has been reported to be correlated with VEGF production in CML patients (Lundberg *et al.*, 2000). Several leukaemic cell strains and primary cells have been shown to synthesize and secrete VEGF which in turn affect and modulate the malignant biological behaviour of leukaemic cells by two positive – feedback systems such as paracrine and autocrine loops (Kay *et al.*, 2002).

In Ph+ CML cells, BCR::ABL1 oncoprotein has been reported to directly induce VEGF expression (Mayerhofer *et al.*, 2002). VEGF secreted by leukaemic cells stimulate endothelial cells to produce growth factors by interacting with relevant

receptors on the endothelial cell surface. These growth factors had been reported to act on leukaemic cells which results in an increase in their proliferative activity and drug resistance (Rafii *et al.*, 2002a). Treatment with IM has been reported to reverse bone marrow angiogenesis and thereby decrease the levels of VEGF in CML patients (Yıldırım *et al.*, 2016). However, genetic variability in the host possibly may influence the production and function of VEGF.

As angiogenesis is largely a host- mediated event rather than a process mediated by somatic mutations in cancer, germline SNPs in angiogenesis pathway could be logical candidates to study. Host's genetic variations in *VEGF*, such as single nucleotide polymorphisms (SNPs) in VEGF pathway especially in *VEGF* and its receptor *VEGFR2* may be responsible for inter- individual differences in VEGF expression and circulating plasma concentrations. SNPs in *VEGFA* and *VEGFR2* might influence angiogenesis dependent biological pathways, influence sensitivity to various therapies, disease progression and outcome. Not many studies have been carried out on the impact of *VEGF* SNPs in modulating IM treatment response and disease progression in CML patients and nil in Malaysian CML patients. Therefore, genetic variations in *VEGFA* and *VEGFR2* were chosen as logical candidates to study and the contribution of SNPs in *VEGFA* and *VEGFR2* in modulating IM treatment response and disease progression in Malaysian CML patients was designed as one of the objectives of this study.

The anti-leukaemia mechanism of IM that selectively inhibit the proliferation of CML cells has been shown to be by inducing apoptosis (Deininger *et al.*, 1997). Early researches have demonstrated that up – regulation of a pro – apoptotic protein BIM, [a member of the B – cell CLL/ Lymphoma 2 (BCL2) family of proteins] is

required for TKIs to induce apoptosis in kinase driven cancers including CML (Belloc *et al.*, 2007; Kuribara *et al.*, 2004). *BIM* has emerged as a mediator of apoptosis signal pathways triggered by IM. It has been reported that IM activates several pro- apoptotic BH3 protein that are important to IM induced apoptosis of *BCR::ABL1* in CML patients (Kuroda *et al.*, 2006). BH3 domain plays an important role in cell apoptosis which is one of the pivotal pathways for cancer cell death induced by IM. Therefore, genetic alterations in *BIM* sequence could affect BH3 protein production and apoptosis and thereby lead to imatinib resistance. Suppression of BIM expressions a result of *BIM* deletion polymorphism has been reported to confer resistance in in – vitro (Cragg *et al.*, 2007; Gong *et al.*, 2007; Kuroda *et al.*, 2006). One group of researchers have a discovered a common intron 2 deletion polymorphism of *BIM* among East Asians, compared with other ethnic groups (Ng *et al.*, 2012). These researchers demonstrate that *BIM* deletion polymorphism has profound effect on TKIs sensitivity and that one copy of the deleted allele was sufficient to render cells intrinsically resistant to TKIs. They reported that East Asian CML patients harbouring *BIM* deletion polymorphism experienced significantly inferior response to TKIs than did CML patients without the polymorphism (Ng *et al.*, 2012). But in the study by Augis *et al* (2013) in the French population, deletion polymorphism of *BIM* in the coding sequence was not detected. Instead, they detected a single nucleotide polymorphism c465 C>T in the *BIM* in the French population of patients with CML treated with IM.

It seems that these two variants in *BIM* might be private to different ethnic populations. However, no reports are available on genetic variation in *BIM* in Malaysian CML patients. So, it was of interest to investigate the deletion 2

polymorphism and SNP c465 C>T in CML patients undergoing IM treatment in Malaysian and to elucidate their contribution in modulating IM resistant and disease progression.

1.5 *TP53* mutation at exon 8 in association with IM response and disease progression

The *TP53* is a multifunctional tumour suppressor gene which is frequently adjusted in several types of cancers (Meplan *et al.*, 2000; Olivier *et al.*, 2010). Normally, the *TP53* combines with an array of proteins occupied in biological pathways and provides a potent barrier to tumourigenesis. *TP53* does this by triggering cell cycle checkpoint, cellular senescence or apoptosis in response to DNA damage and aberrant proliferation signals.

For their optimal anti – tumour effects, conventional chemotherapy agents also exploit the same signalling networks influenced by *TP53*. Mutations in *TP53* usually results in either complete or partial loss of function of *TP53* protein, depending on the site of mutation (Rossi and Gaidano, 2012). Accordingly, mutations in *TP53*, acquired as a by-product of tumour evolution, has been implicated to promote drug resistance. In murine cells *TP53* mutations have been shown to promote genomic instability by producing drastic changes in cell physiology (Brusa *et al.*, 2003). However, it is not clear whether *TP53* might influence the response of human CML cells to IM. Few earlier studies reported the existence of substantial cross – talk between the BCR::ABL and TP53 – signaling networks (Brusa *et al.*, 2005; Goldberg *et al.*, 2004; Trotta *et al.*, 2003) and also the accompaniment of *TP53* mutations during CML disease progression (Nakai and Misawa, 1995). Wendel *et al*

(2006) reported that inactivation of *TP53* can hinder the sensitivity to IM in vitro and in vivo without preventing *BCR::ABL* kinase inhibition. Another study (Mir *et al.*, 2013) found higher frequencies of *TP53* exon 8 codon 282 mutations in Indian CML patients displaying a poor or minimal haematological response is equivalent to patients who showed a major haematological response. This study suggests that mutation in *TP53* exon 8 contributes to resistance to TKI therapy and promotes the disease progression. However, no reports are available indicating the association of *TP53* mutations with IM resistance and disease progression in Malaysian CML patients undergoing IM treatment. Hence, the contribution of mutations in exon 8 codon 282 of *TP53* as a genetic determinant modulating response to IM treatment in CML patients was also selected for investigation. Exon 8 codon 282 of *TP53* was selected because it is an evolutionarily preserved region of *TP53* and the most common mutational hotspot of *TP53.* This codon 282 of exon 8 DNA binding domain is also considered to carry some genetic inherent instability or chemical predilection to single nucleotide substitutions. The objective was to investigate whether the acquisition of *TP53* mutations during IM therapy was correlated with the development of resistance and treatment failure in CML patients.

1.6 Additional chromosome abnormalities (ACAs) in association with IM response and disease progression

Additional chromosome abnormalities (ACAs) are chromosomal abnormalities observed in addition to the Ph chromosome in CML patients. Earlier, the emergence of ACAs, which is also called clonal cytogenetic abnormalities (CCA) or clonal evolution was thought to reflect genetic instability of the leukaemic cells and as a sign of disease progression (Marktel *et al.*, 2003b). ACAs emerge in Ph positive

CML patients who show no/ minimal/ partial cytogenetic response and also in CML patients who have become negative for Ph' chromosome as a result of complete cytogenetic response (CCyR). In small percentage Ph positive CML patients, ACAs can be seen at the time of diagnosis.

ACAs have been stated to reflect intrinsic aggressiveness of the disease, less amenable to subsequent alternative treatment and thereby negatively affect overall survival (Schoch *et al.*, 2003). ELN guidelines (Baccarani *et al.*, 2009) considers the presence of ACAs at diagnosis as a warning signal which affect the patient's response to TKIs therapy and sign of imminent disease progression. In CML patients undergoing IM treatment, the appearance of ACAs has been associated with reduced response to IM, increase in relapse of the disease, clonal evolution, and disease progression (Melo and Barnes, 2007). The appearance of ACAs during IM treatment in CML patients has been implicated to play an important role in IM resistance (Perrotti *et al.*, 2010) and considered as a sign of treatment failure. Based on the frequency and types, ACAs have been stratified differently by different groups of researchers. However, not many reports and no consensus are available on the differential prognostic impact of ACAs on CML disease progression. Therefore, another aspect of this study was to investigate the ACAs observed at the time of diagnosis, during course of treatment, and to determine the differential prognostic impact of ACAs, especially on treatment response and disease progression in Malaysian CML patients undergoing IM treatment.

1.7 Hypothesis

1. Single nucleotide polymorphisms in *VEGFR2* (rs1531289, rs1870377 and rs2305948), *VEGFA* (rs2010963 and rs3025039) and *BIM* (intron 2 deletion polymorphism and SNP rs724710) have potential impact on clinical response (either good response or resistance) and disease progression in CML patients on IM treatment.
2. *TP53* mutation at exon 8 and additional chromosome abnormalities (ACA) contribute to IM response and disease progression in CML patients on IM treatment.

1.8 Objectives of the study

Broad objective:

The broad objective of this study is to investigate the contribution of selected polymorphisms of *VEGFR2, VEGFA, BIM*, *TP53* exon 8 mutations and additional chromosome abnormalities (ACAs) in modulating IM treatment response and disease progression in chronic myeloid leukaemia patients.

The specific objectives of this study are:

1. To investigate the genotype, allele and haplotype frequencies of three polymorphisms of *VEGFR2* (rs1531289, rs1870377 and rs2305948), two polymorphisms of *VEGFA* (rs2010963 and rs3025039) among Malaysian chronic myeloid leukaemia (CML) patients undergoing Imatinib mesylate

(IM) treatment and determine their association with IM treatment response and disease progression.

2. To investigate the frequency of intron 2 deletion polymorphism and the genotype and allele frequencies of polymorphism c465 C>T (rs724710) of *BIM*, among Malaysian chronic myeloid leukaemia (CML) patients undergoing Imatinib mesylate (IM) treatment and determine their association with IM treatment response and disease progression.
3. To investigate the frequencies and types of mutations in exon 8 of *TP53* in Malaysian CML patients undergoing IM treatment and determine its association with IM response and disease progression.
4. To determine the types and proportion of additional chromosomal abnormalities (ACAs) at the time of diagnosis and also during the course of IM therapy and determine their impact on treatment response and disease progression in CML patients .
5. To correlate the clinical variables, polymorphisms of *VEGFR2, VEGFA, BIM, TP53* mutation at exon 8 status and ACAs with the prognosis of these CML patients and evaluate their contributory role as predictive biomarkers of IM treatment response and disease progression in Malaysian CML patients.

CHAPTER 2

LITERATURE REVIEW

2.1 Chronic Myeloid Leukaemia (CML)

2.1.1 CML phases

The World Health Organization (WHO) and European LeukaemiaNet (ELN) have classified CML into 3 phases – chronic phase (CP), accelerated phase (AP) and blast phase (BP). The presence of <10 % blasts in the white blood cell and platelet count left shift and normally ($\geq$ 1000 x 10^9/L) with basophilia, and peripheral blood shows leukocytosis (12- 1000 x 10^9/L) suggestive of CP of CML with minor symptoms of weight loss, night sweat, anaemia and fatigue, (Cortes and Kantarjian, 2012). 50% of the CML patients had palpable splenomegaly due to the infiltration of the red pulp cords by the mature and immature granulocytes. According to WHO classification, the blast cell count for AP is 15 – 19 % and > 20 % for the blast phase. Meanwhile, according to ELN guidelines (2015) (Baccarani *et al.*, 2015) the AP is defined as when there is 15 – 29 % of blast cells present or 30 – 49 % blast cells present with promyelocytes in the peripheral blood or the bone marrow aspirate, or by the platelet count <100 x 10^9/ L with no related to the treatment and >100 x 10^9 /L with not responding to treatment or emergence of clonal chromosome abnormalities in Ph+ cells (Ph+/ACAs). Also, the progressive splenomegaly with increased WBC>10x 10^9/L occurs due to unresponsive to the treatment.

The blast phase is defined by the presence of blast cells percentage ≥ 30 % in the bone marrow or peripheral blood or involvement of blast cells in non-hematopoietic tissues excluding the liver and spleen. According to ELN (2020) guidelines, resistance to two TKIs treatments, detection of *BCR::ABL1* kinase domain (KD) mutation or the emergence of additional chromosome abnormalities in the Ph+ cells (Ph+/ ACAs) during the treatment regime indicate disease progression (Baccarani *et al.*, 2013; Hochhaus *et al.*, 2020; Vardiman *et al.*, 2008).

2.2 Molecular consequences of *BCR::ABL1* fusion

The BCR::ABL1 fusion gene formed as a result of Ph translocation is an oncogene which encodes a constitutively activated tyrosine kinase protein with a molecular weight of p190, p210 or p230 kDa depending on the breakpoint located within BCR gene that is fused together with the *ABL1* on exon a2 to form subtypes of e1a2, e19a2 and e14a2 respectively (Vigil *et al.*, 2011). The BCR::ABL fusion protein is mostly located in the cytoplasm meanwhile the c – ABL kinase is situated inside the nucleus. This chimeric activity of BCR::ABL protein express the molecular activity of CML pathogenesis (Constance *et al.*, 2012; Druker, 2008). The oncogenic tyrosine kinase protein is responsible for the leukaemic phenotype, through the constitutive activation of multiple signaling pathways (Quintás-Cardama and Cortes, 2009).

The genomic DNA breakpoint in *BCR* occurs inside the intronic sequences spanning 5.8 kb between the exon 11 and 16. The genomic breakpoint in *ABL* is generally more variable, spanning up to 180 kb from 5' to the first alternative first exon to the start of exon 2 (Laurent *et al.*, 2001). After splicing, these highly variable intronic sequences are missing from *BCR::ABL* mRNA, and the most of majority CML