INFLUENCE OF *ORM1, PXR, CAR, CYP3A4* AND *CYP3A5* GENE POLYMORPHISMS IN MEDIATING SUSCEPTIBILITY RISK TO CHRONIC MYELOID LEUKAEMIA AND RESPONSE TO IMATINIB MESYLATE THERAPY

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by

NAJLAA BINTI MADDIN

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µL	Microlitre
A	Adenine
ABC	ATP-Binding Cassette Transporter
ABL	Abelson Murine Leukaemia Oncogene
AE	Elution Buffer
AGP	Alpha-1-glycoprotein
AL	Lysis Buffer
ALL	Acute Lymphoid Leukaemia
AML	Acute Myeloid Leukaemia
AP	Accelerated Phase
Arg	Arginine
AS-PCR	Allele Specific - Polymerase Chain Reaction
ATP	Adenosine Triphosphate
AW1	Washing buffer 1
AW2	Washing Buffer 2
BCR	Breakpoint Cluster Region
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BP	Blast Phase
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2

C	Cytosine
CAR	Constitutive Androstane Receptor
CBA	Chromosome banding analysis
CCA	Clonal chromosomal abnormalities
CCyR	Complete Cytogenetic response
CHR	Complete Hematologic response
CHS	Southern Han Chinese
CIs	Confidence Intervals
CLL	Chronic Lymphoid Leukaemia
CML	Chronic Myeloid Leukaemia
CP	Chronic Phase
CYP	Cytochrome P450
CYP1A2	Cytochrome P450 Family 1 Subfamily A Member 2
CYP2C19	Cytochrome P450 Family 2 Subfamily C Member 19
CYP2C9	Cytochrome P450 Family 2 Subfamily C Member 9
CYP2D6	Cytochrome P450 Family 2 Subfamily D Member 6
CYP3A4	Cytochrome P450 Family 3 Subfamily A Member 4
CYP3A5	Cytochrome P450 Family 3 Subfamily A Member 5
CyR	Cytogenetic response
dbSNP	Single Nucleotide Polymorphisms Database
ddH_2O	Deionized Distilled Water
DMEs	Drug Metabolizing Enzymes
DNA	Deoxyribonucleic Acid
dNTPs	Dinucleotide Triphosphate

EB	Elution Buffer
EDTA	Ethylenediaminetetraacetic Acid
ELN	European LeukaemiaNet
FDA	Food and Drug Administration
g	Gram
G	Guanine
GISTs	Gastrointestinal stromal tumours
Gln	Glutamine
HBOC	Hereditary breast/ovarian cancer
HCl	Hydrochloric Acid
HGC	Human Genome Centre
HWE	Hardy Weinberg Equilibrium
I-FISH	Fluorescence in situ hybridization
IM	Imatinib Mesylate
IRIS	International Randomized Study of Interferon and STI571
IS	International Scale
kb	Kilo Base
kDa	Kilodalton
KHV	Kinh in Ho Chi Minh City, Vietnam
L	Litre
Leu	Leucine
mA	Milliamp
MAF	Minor Allele Frequencies
mCyR	Major Cytogenetic response

MDR	Multidrug Resistance
Met	Methionine
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minute
mL	Millilitre
mM	Milimolar
MMR	Major Molecular Response
MMR	Mismatch repair
n	Total Number
NCBI	National Centre for Biotechnology Information
NCCN	National Comprehensive Cancer Network
ng	Nanogram
NHL	Non-Hodgkin Lymphoma
NR1I2	Nuclear Receptor Subfamily 1 Group I Member 2
NR1I3	Nuclear Receptor Subfamily I Group I Member 3
NRs	Nuclear Receptors
OR	Odds Ratio
ORM	Orosomucoid
PB	Phosphate Buffer (Binding Buffer)
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction - Restriction Fragment Length Polymorphism
PCyR	Partial Cytogenetic response
PE	Washing buffer

Ph+	Philadelphia Chromosome Positive
PO_4	Phosphate
Pro	Proline
PXR	Pregnane X Receptor
RCC	Renal Cell Carcinoma
rpm	Revolutions per Minute
RT-Q-PCR	Real-Time Quantitative Polymerase Chain Reaction
s	Seconds
SD	Standard Deviations
SLC	Solute carrier family
SNP	Single Nucleotide Polymorphisms
T	Thymine
TBE	Tris-borate-EDTA Buffer
T_m	Melting Temperature
U	Unit
UV	Ultra Violet
V	Volt
Val	Valine
WHO	World Health Organisation
XME	Xenobiotic Metabolizing Enzymes
YRI	Yoruba in Ibadan, Nigeria

PENGARUH GEN POLIMORFISME *ORM1, PXR, CAR, CYP3A4* DAN *CYP3A5* DALAM MEMPENGARUHI RISIKO MENDAPAT LEUKEMIA MEILOID KRONIK DAN TINDAK BALAS TERHADAP RAWATAN IMATINIB MESYLATE

ABSTRAK

Polimorfisme genetik telah dikenali dengan baik sebagai sumber perbezaan antara individu dalam risiko mendapat penyakit dan tindak balas rawatan. Asosiasi antara varian genetik manusia dan kecenderungan terhadap penyakit serta aktiviti rintangan terhadap pelbagai jenis ubat yang berinteraksi dengan beratus protein seperti reseptor, penggangkut dan enzim metabolisme telah diterangkan. Leukaemia mieloid kronik (CML) merupakan gangguan mieloproliferatif, yang mana molekulnya disasarkan oleh ubat imatinib mesylate (IM), iaitu ubat yang berpiawaian emas. Walaupun keberkesanannya sangat baik, rintangan terhadap IM telah dijumpai dalam sebilangan besar pesakit CML. Pembentukkan rintangan ini boleh disebabkan oleh beberapa faktor. Salah satu faktor yang berpotensi adalah kepelbagaian farmakokinetik yang terhasil daripada polimorfisme genetik dalam gen IM metabolisme. Kajian ini telah dilaksanakan terhadap sejumlah 540 subjek [270 pesakit CML (139 IM rintang dan 131 IM tindak balas baik) dan 250 individu sihat] untuk mengkaji frekuensi genotip dan polimorfisme *ORM1* 520G>A, *PXR* 1792A>G, *CAR* 540C>T, *CYP3A4* 878T>C dan *CYP3A*5 6986A>G terhadap risiko mendapat CML dan tindak balas IM. Penjenisan gen telah dilakukan dengan menggunakan tiga kaedah, iaitu kaedah penjujukan DNA, kaedah Tindak Balas Berantai Polimorfisme-Pemotongan Panjang Cebisan (PCR-RFLP) dan kaedah Alel Khusus-PCR (AS-PCR). Genotip kemudiannya telah dikategorikan kepada

tiga, iaitu homozigot jenis liar, heterozigot dan homozigot varian. Hubungan antara varian alel dengan risiko kerentanan CML dan tindak balas terhadap rawatan IM telah dinilai dengan menggunakan nisbah kemungkinan (OR) dengan 95% selang keyakinan yang dikira menggunakan regresi logistik. Hasil kajian menunjukkan tiada genotip homozigot varian ditemui bagi *CYP3A4* 878T>C dan *ORM1* 520G>A dalam kedua-dua pesakit CML dan individu sihat. Apabila asosiasi antara genotip dengan risiko kerentanan CML dinilai, polimorfisme *PXR* 1792A>G dan *ORM1* 520G>A menunjukkan hubungan yang tidak signifikan terhadap risiko kerentanan CML. Manakala, genotip homozigot varian (TT) *CAR* 540C>T (OR 3.638; 95% CI: 1.779-7.523, p<0.001) dan genotip heterozigot (TC) *CYP3A4* 878T>C (OR 3.387; 95% CI: 1.433-8.007, p=0.005) menunjukkan hubungan yang signifikan terhadap risiko kerentanan CML. Manakala, kedua-dua genotip, heterozigot (AG) dan homozigot varian (GG) *CYP3A5* 6986A>G menunjukkan kesan perlindungan daripada kerentanan terhadap CML (OR 0.171; 95% CI: 0.090-0.324, p<0.001 dan OR 0.257; 95% CI: 0.126-0.525, p<0.001, masing-masing). Seterusnya, apabila asosiasi antara genotip dengan tindak balas IM dalam pesakit CML dinilai, hasilnya menunjukkan bahawa SNP *ORM1* 520G>A dan *CYP3A4* 878T>C tidak signifikan berkaitan dengan tindak balas IM. Namun begitu, kedua-dua genotip, heterozigot dan homozigot varian *PXR* 1792A>G (OR 2.769; 95% CI: 1.290-5.943, p=0.007 dan OR 2.632; 95% CI: 1.030-6.723, p=0.041, masing-masing) dan *CAR* 540C>T (OR 2.700; 95% CI: 1.116-6.536, p=0.028 dan OR 2.923; 95% CI: 1.156-7.393, p=0.023, masing-masing) menunjukkan hubungan yang signifikan dengan rintangan kepada IM dalam pesakit CML. Manakala, pesakit CML dengan genotip heterozigot (AG) dan homozigot varian (GG) *CYP3A5* 6986A>G (OR 0.171; 95% CI: 0.090-0.324, p<0.001 dan OR 0.257; 95% CI: 0.126-0.525,

p<0.001, masing-masing) menunjukkan hubungan yang signifikan dengan tindak balas yang baik terhadap IM dalam kalangan pesakit CML. Akhir sekali, apabila risiko dinilai berdasakan gabungan genotip, beberapa gabungan telah dikaitkan dengan rintangan terhadap IM dan beberapa yang lain telah dikaitkan dengan tindak balas yang baik terhadap IM. Walaupun beberapa genotip berkait dengan risiko kerentanan dan respon terhadap IM dalam kalangan pesakit CML telah dijumpai, kajian lanjut perlu dilakukan pada skala yang lebih besar bagi mengesahkan sama ada polimorfisme ini boleh digunakan sebagai penanda bio prediktif.

INFLUENCE OF *ORM1, PXR, CAR, CYP3A4* AND *CYP3A5* GENE POLYMORPHISMS IN MEDIATING SUSCEPTIBILITU RISK TO CHRONIC MYELOID LEUKAEMIA AND RESPONSE TO IMATINIB MESYLATE THERAPY

ABSTRACT

Genetic polymorphisms are well recognized sources of individual differences in disease risk and treatment response. Associations between human genetic variants and predisposition to diseases and adverse events for different kinds of drug interactions with hundreds of protein like receptors, transporters and metabolizing enzymes have been described. Chronic myeloid leukaemia (CML) is a myeloproliferative disorder for which the molecular targeted drug Imatinib mesylate (IM), is the gold standard drug. Despite its excellent efficacy, resistance to IM emerges in a significant number of CML patients. Development of resistance could be due to several factors. Pharmacokinetic variability as a result of genetic polymorphisms in IM metabolizing genes could be a potential factor. This study was undertaken in a total of 540 subjects [270 CML patients (139 IM resistant and 131 IM good responder) and 250 normal healthy controls] to investigate the genotype frequencies and the impact of *ORM1* 520G>A, *PXR* 1792A>G, *CAR* 540C>T, *CYP3A4* 878T>C and *CYP3A5* 6986A>G polymorphisms towards CML susceptibility risk and IM response. Genotyping was performed by using three methods, DNA sequencing, Polymerase Chain Reaction – Restriction Fragment Length Polymorphisms (PCR-RFLP) and Allele Specific – PCR (AS-PCR) technique. The genotypes were categorized into homozygous wild type, heterozygous and homozygous variant genotype. The association between allelic variants and CML susceptibility risk

and response to IM treatment were assessed by means of odds ratio (OR) with 95% confident intervals calculated by logistic regression. Results showed absence of homozygous variant genotype of *CYP3A4* 878T>C and *ORM1* 520G>A in both CML patients and normal healthy controls. When the association of genotypes with CML susceptibility risk was assessed, polymorphisms 520G>A of *ORM1* and 1792A>G of *PXR* showed no significant associations with CML susceptibility risk. Whereas, the homozygous variant (TT) genotypes of *CAR* 540C>T (OR 3.638; 95% CI: 1.779-7.623, $p<0.001$) and the heterozygous (TC) genotype of *CYP3A4* 878T>C (OR 3.387; 95% CI: 1.433-8.007, $p=0.005$) were significantly associated with CML susceptibility risk. In contrast, both heterozygous (AG) and homozygous variant (GG) genotype of *CYP3A5* 6986A>G showed protective effect for susceptibility to CML (OR 0.310; 95% CI: 0.180-0.535, $p<0.001$ and OR 0.140; 95% CI: 0.079-0.246, $p<0.001$, respectively). Next, when the associations of genotypes with IM response in CML patients were evaluated, the SNPs *ORM1* 520G>A and *CYP3A4* 878T>C were not significantly associated with IM response. But, both heterozygous and homozygous variant genotypes of *PXR* 1792A>G (OR 2.769; 95% CI: 1.290-5.943, $p=0.007$ and OR 2.632; 95% CI: 1.030-6.723, $p=0.041$, respectively) and *CAR* 540C>T (OR 2.700; 95% CI: 1.116-6.536, $p=0.028$ and OR 2.923; 95% CI: 1.156-7.393, $p=0.023$, respectively) showed significant association with IM resistance in CML patients. However, CML patients with heterozygous (AG) and homozygous variant (GG) genotypes of *CYP3A5* 6986A>G (OR 0.171; 95% CI: 0.090-0.324, $p<0.001$ and OR 0.257; 95% CI: 0.126-0.525, $p<0.001$, respectively) showed significant association with IM good response to IM. Finally, when the risk was evaluated based on the combination of genotypes, a few combinations were associated with IM good response and a few others with IM resistance. Although a few

genotypes associated with susceptibility risk and IM response in CML patients were observed, further studies are warranted on a larger scale to validate whether these polymorphisms could be used as predictive biomarkers.

CHAPTER 1

INTRODUCTION

1.1 Overview of cancer

Cancer is the leading cause of death worldwide, after heart disease. The incidence of cancer has been reported to be increased from 12.7 million in 2008 to 14.1 million in 2012, and this trend is predictable to continue, with the number of new cases expected to rise a further 75% (Stewart and Wild, 2014). Results from GLOBOCAN show that in 2012 there were an estimated 14.1 million new cases of cancer diagnosed worldwide and 8.2 million estimated deaths from cancer (Organization, 2014). About half of the cancer incidence burden occurs in Asia. According to the Malaysian National Cancer Registry report, a total of 18,219 new cancer cases were diagnosed in 2007 which comprised 8,123 (44.6%) males and 10,096 (55.4%) females. The ten leading cancers among population of Malaysia in 2007 were breast, colorectal, lung, nasopharynx, cervix, lymphoma, leukaemia, ovary, stomach and liver (Zainal Ariffin and Nor Saleha, 2011).

1.1.1 Cancer development

Cancer is the general name for a group of more than 100 diseases, all characterized by uncontrolled division and proliferation of abnormal cells. Mostly, cancer arises when there is a change in or a damage to genes, which is called a gene mutation. Mutation can affect the structure of the gene and stop it from working properly. Basically, gene mutation can occur by chance (sporadic) or be inherited. Mutations are of two types – somatic mutations and hereditary/germline mutations. Somatic mutations result in sporadic cancers. Most of the cancers are sporadic. They occur because of genetic changes in the cells that happen mostly by chance and build up throughout a person's lifetime. They can also be caused by something that damages the cell's DNA, such as carcinogenic agents. Sometimes, cancer causing gene mutation can be inherited from a parent. Inherited mutations are called germline mutations because they are present in parent's reproductive cells, either the father's sperm cell or the mother's egg cell. Germline mutations can be passed from generation to generation. Germline mutations result in hereditary cancer syndromes. Typical examples of germline mutations are *BRCA1/BRCA2* genes resulting in hereditary breast/ovarian cancer (HBOC) syndrome, mismatch repair (MMR) genes in Lynch syndrome etc.

1.1.2 General classification of cancer

Generally, cancers are classified into two ways: by the type of tissue in which the cancer originates (histological type) and the location in the body where the cancer first develops (primary site). From a histological standpoint, cancers are grouped into six major categories, which are carcinoma, sarcoma, myeloma, leukaemia, lymphoma and mixed types.

Carcinoma is a malignant neoplasm of epithelial origin or cancer of the internal or external lining of the body. Carcinomas comprise 80 to 90 percent of all cancer cases. Carcinoma is divided into two major subtypes which are adenocarcinoma and squamous cell carcinoma. Adenocarcinoma develops in organ or gland whereas squamous cell carcinoma originates in the squamous epithelium.

Sarcoma refers to cancer that originates in supportive and connective tissues such as bones, tendons, cartilage, muscle and fat. Most common sarcoma often develops as a painful mass on the bone. Hence, more often than not, this occurs in young adults. Sarcoma tumours usually resemble the tissue in which they arise.

Myeloma is the type of cancer that begins in plasma cells, a type of white blood cell which is made in the bone marrow. Myeloma is also known as multiple myeloma. Normally, plasma cells produce some of the proteins found in the blood. Plasma cells are responsible for producing antibodies (immunoglobulins) which are functioning for maintaining the body's immune system. Mutations in plasma cells result in abnormal

plasma cells which multiply uncontrollably and produce only one type of antibody known as paraprotein which has no useful function.

Leukaemia is a type of cancer that affects the blood and bone marrow. Leukaemia causes overproduction of immature white blood cells. These immature white blood cells do not function normally as they should. Therefore, the patient is often prone to infection. Leukaemia also affects red blood cells and cause poor blood clotting and fatigue due to anaemia.

Lymphoma is a type of cancer that develops in the lymphatic system which affects the immune system. Generally there are two main types of lymphoma which are Hodgkin lymphoma and non-Hodgkin lymphoma (NHL).

Cancer also can be formed in mixed type. The type of components may be within one category or from different categories. Examples of this cancer types are adenosquamous carcinoma, mixed mesodermal tumour, carcinoma and teratocarcinoma.

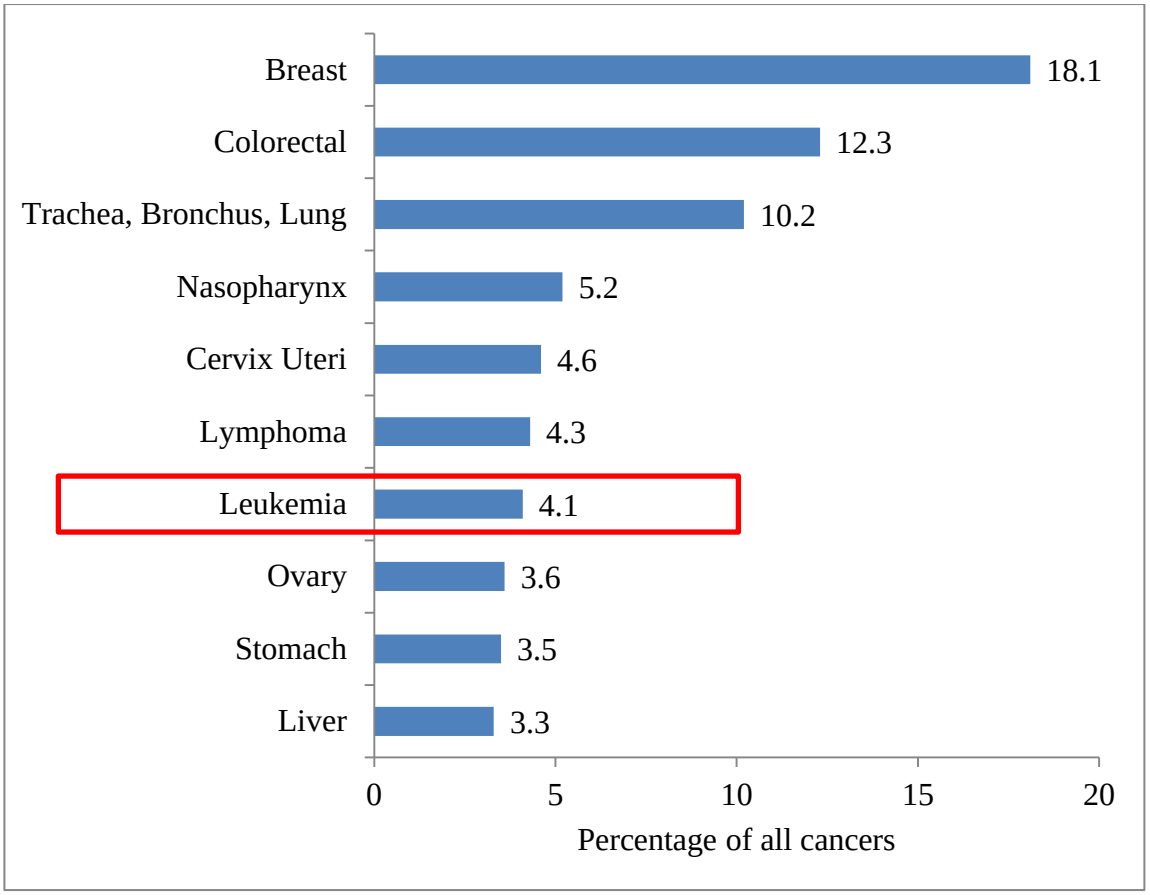

Figure 1.1 Ten most frequent cancers in Malaysia 2007, (adapted from Zainal Ariffin and Nor Saleha, 2011)

1.2 Leukaemia

Leukaemia is a group of neoplastic disorders that arise in haematopoietic stem cells. Leukaemia is characterized by the uncontrolled proliferation and accumulation of immature blood cells in the bone marrow and peripheral blood (Osaro Erhabor and Adias, 2013). Once the disease starts, leukaemia cells begin accumulating in the bone marrow replacing or crowding out normal white and red blood cells and also megakaryocyte. When healthy bone marrow gets replaced by these immature cells, it causes the number of normal red and white blood cells and platelets to be decreased. The most common manifestations of leukaemia include anaemia, neutropenia, thrombocytopenia, weakness and an increase in infections (Osaro Erhabor and Adias, 2013).

In year 2007, leukaemia had been reported to rank as number 7 out of 10 most common cancers in the Malaysian population, with 4.1% (Zainal Ariffin and Nor Saleha, 2011). In Malaysia, there were 741 cases of leukaemia diagnosed in 2007 and registered at NCR, and comprised 419 (56.5%) males and 322 females (43.5%) with the incidence rate as 3.5 per 100 000 and 2.7 per 100 000, respectively. Among male leukaemia patients, Malay male showed higher preponderance (241), followed by Chinese (103) and Indian (29) ethnic groups. Among female leukaemia patients also, Malay female ranked number one which was 184, followed by Chinese (63) and Indian (28) ethnic groups. It seems that Malay ethnic groups are more prone to develop leukaemia, than Chinese and Indian ethnics (Zainal Ariffin and Nor Saleha, 2011).

1.2.1 Classification of Leukaemia

Leukaemia can present in myeloid or lymphoid and acute or chronic forms. If the leukaemia is myeloid in origin, it will involve the myeloid tissue mainly (bone marrow), whereas if the leukaemia started in the lymphoid tissue, it will involve both the lymph nodes and the bone marrow lymphoid tissue. Acute leukaemia can be described as when the malignant cells are immature blasts with a rapid cell proliferation rate. In contrast, leukaemia is described as chronic when the malignant cells are more mature than those of acute leukaemias. Chronic leukaemias have a less devastating clinical course than do acute leukaemias (Al-Tubaikh, 2010).

There are four major types of leukaemia, Acute Lymphoblastic Leukaemia (ALL), Chronic Lymphocytic Leukaemia (CLL), Acute Myeloid Leukaemia (AML) and Chronic Myeloid Leukaemia (CML).

Acute lymphoblastic leukaemia (ALL) also known as acute lymphocytic leukaemia, is a malignant disease of the bone marrow in which early lymphoid precursors proliferate and replace the normal haematopoietic cells of the marrow (Seiter *et al.*, 2014). ALL is characterized by excess lymphoblasts in the bone marrow. It progresses rapidly, replacing healthy cells that produce functional lymphocytes with leukaemia cells that cannot mature properly. ALL is the most common type of leukaemia affecting children.

Chronic lymphocytic leukaemia (CLL) is a lymphoproliferative disorder, composed by monomorphic round B immature lymphocytes involving peripheral blood, bone marrow and lymphoid organs (Swerdllow *et al.*, 2008). Since CLL is a chronic type, it develops slowly. CLL develops when too many abnormal lymphocytes grow, crowding out normal blood cells and making it difficult for the body to fight infection. In CLL, the abnormal lymphocytes take longer time to develop and multiply in the bone marrow. CLL is one of the most common leukaemias among the adults (Scarfò *et al.*, 2016).

Acute Myeloid Leukaemia (AML) is a clonal haematopoietic disorder resulting from genetic alterations in normal haematopoietic stem cells. These alterations disrupt normal differentiation, leading to excessive proliferation of abnormal immature leukemic cells known as blasts (Kumar, 2011). AML is the most common type of acute leukaemia affecting adults. AML is characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal cells (Chaudhary and Chaudhary, 2011). Because AML is an acute type of leukaemia, it can spread quickly to the blood and other parts of the body, such as lymph nodes, liver, spleen, brain and spinal cord, and testicles if not quickly treated (Society, 2014). It also can be fatal.

The fourth type of common leukaemia is chronic myeloid leukaemia (CML). This study is focused on Chronic Myeloid Leukaemia.

1.3 Chronic Myeloid Leukaemia (CML)

Chronic Myeloid Leukaemia (CML) is a haematopoietic stem cell disorder characterized by excessive proliferation of leucocytes of myeloid series in the bone marrow and circulating blood (Hoyle *et al.*, 2011). CML normally comprises 3 clinically recognized phases. Approximately 90% of patients are diagnosed during the typically indolent chronic phase (CP), which is followed by an accelerated phase (AP) and a terminal blastic phase (BP). In CML, the bone marrow produces too many white cells, called granulocytes. These cells, gradually crowd the bone marrow, interfering with normal blood cell production. They also spill out of the bone marrow and circulate around the body in the bloodstream. Over time, a shortage of red cells and platelets can cause anaemia, bleeding and/or bruising. Most cases of CML occur in adults and comprises 15%-20% of all adult leukaemias, with a median age of diagnosis of 50 years (Rumjanek *et al.*, 2013). Rarely CML occurs in children too. Among childhood leukaemias, CML is a rare entity with an annual incidence of one case per million children (Nikumbh *et al.*, 2012).

CML is characterized by the Philadelphia (Ph) chromosome, created by a reciprocal translocation between chromosomes 9 and 22 t(9:22)(q34;q11 (Al-Achkar *et al.*, 2013). This translocation initially occurs in a cell in the bone marrow. This abnormal cell divides further and produces a clone of abnormal cells which results in development of CML. Figure 1.2 shows the formation of Philadelphia chromosome. Philadelphia translocation t(9;22)(q34;q11) is detected in 95% of chronic myeloid leukaemia (CML) cases (Zheng *et al.*, 2009). This reciprocal t(9:22) translocation, transfers the Abelson

(ABL) proto-oncogene on chromosome 9 to the breakpoint cluster region (BCR) of chromosome 22, resulting in the formation of a BCR/ABL fusion gene (Al-Achkar *et al.*, 2013), which function as an oncogene. BCR/ABL fusion gene encodes for a 210 kDa protein with increased tyrosine kinase activity.

ABL and BCR are normal cellular genes on chromosomes 9 and 22, respectively. The ABL gene encodes a tyrosine kinase enzyme whose activity is tightly regulated (controlled). In the formation of the Ph chromosome, two fusion genes are generated: BCR/ABL on the Ph chromosome 22 and ABL-BCR on the chromosome 9 participating in the translocation. The BCR/ABL fusion gene encodes a 210 kDa protein with deregulated (uncontrolled) tyrosine kinase activity. The presence of this protein in the CML cells is strong evidence of its pathogenic (disease-causing) role.

A tyrosine kinase plays an important role in communicating signals within a cell (signal transduction) and regulating cellular activity, such as cell proliferation, differentiation, migration, metabolism and programmed cell death. The protein kinase can be mutated and causing transformation of its functions due to mutations, overexpression and autocrine paracrine stimulation leading to malignancy (Paul and Mukhopadhyay, 2004). Activation of BCR/ABL gene promotes phosphorylation of a chimeric protein, which is responsible for the proliferation of a clone of malignant myeloid cells in the bone marrow due to excessive tyrosine kinase activity. This activation is responsible for CML pathogenesis (Danier *et al.*, 2011).

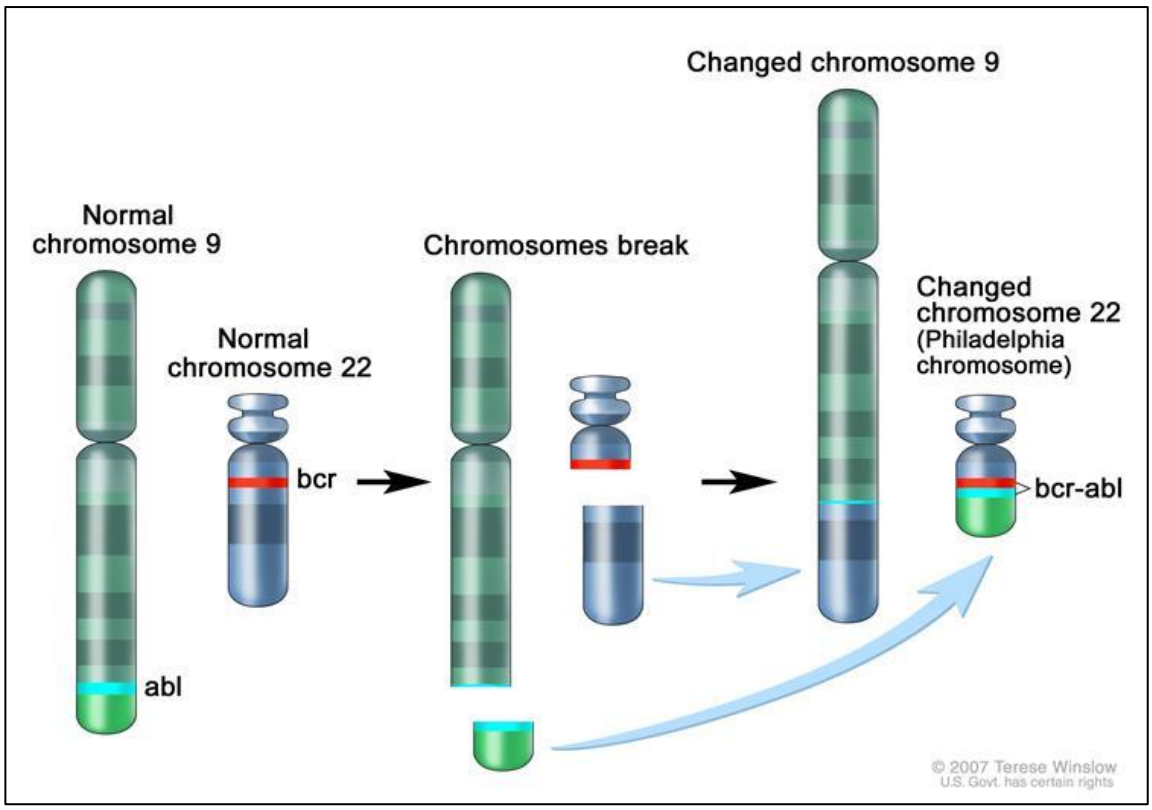

Figure 1.2 The formation of Philadelphia chromosomes as a result of reciprocal translocation between chromosome 9 and chromosome 22 (Adapted from website National Institute of Health, National Cancer Institutes)

1.3.1 Staging of Chronic Myeloid Leukaemia

Chronic myeloid leukaemias have three clinical phases, chronic phase (CP), accelerated phase (AP) and blast phase (BP). The CP, AP and BP are defined according to European LeukaemiaNet (ELN) (Baccarani *et al.*, 2009; Baccarani *et al.*, 2013; Baccarani *et al.*, 2006) and by the World Health Organisation (WHO) (Swerdllow *et al.*, 2008) recommendations. Approximately 90% of patients with CML are in the chronic phase at the time of diagnosis. Patients in CP usually are asymptomatic or show only mild symptoms. Without adequate and effective treatment, patients in this phase can potentially progress to an accelerated phase.

According to European LeukaemiaNet (ELN), CML patients are considered to be in the accelerated phase when they have following criteria: blasts in blood or marrow contains 15-29%, or blasts plus promelocytes in blood or bone marrow >30%, with blasts <30%, basophils in blood ≥20%, persistent thrombocytopenia (<100 x 10^9/L) unrelated to therapy and having clonal chromosome abnormalities in Ph+ cells (CCA/Ph+), major route, on treatment. According to World Health Organisation (WHO), accelerated phase of CML is defined as presence of 10-19% blasts in blood or bone marrow, ≥20% basophils in blood, persistent thrombocytopenia (<100 x 10^9/L) unrelated to therapy, CCA/Ph+ on treatment, thrombocytosis (>1000 x 10^9/L) unresponsive to therapy, and increasing spleen size and increasing white blood cell count unresponsive to therapy. The accelerated phase is often a sign that the disease is progressing and transforming to blast crisis and that the current treatment is ineffective.

Blast phase is the final phase in the progression of CML. Blast phase is characterized by the presence of rapid disease progression and shorter cell survival. According to ELN, a CML patient is said to be in the blast phase if he/she present the following criteria during his/her active treatment: ≥30% blast in the blood or bone marrow, and extramedullary blast proliferation, apart from spleen. Based on WHO, a CML patient is defined to be in the blast phase when he/she has the following criteria: presence of ≥20% blasts in blood or bone marrow, presence of large foci or clusters of blasts in the bone marrow biopsy and extramedullary blast proliferation, apart from spleen.

1.4 Problem statement

The most exciting breakthrough in the development of CML has been the development of Imatinib mesylate (IM) as an orally bioavailable therapeutic agent. IM, also known as Glivec/Gleevec, is a molecularly targeted drug developed against BCR/ABL fusion product and has been approved and successfully introduced in the treatment of CML. Gleevec was approved by the Food and Drug Administration (FDA), United States of America since 2001 (Paula, 2012).

Despite the clinical success, with IM demonstrating long-term survival for the majority of patients, a significant proportion of patients (30-40%) show suboptimal response to IM or develop resistance to IM. Development of resistance to IM could be due to a heterogeneous array of mechanisms. There are two broad mechanisms that are associated with development of resistance to IM in CML patients. These two

mechanisms are 1) those involving BCR/ABL dependent pathway and 2) those involving BCR/ABL independent pathways. BCR-ABL dependent mechanism generally includes point mutations within the BCR/ABL kinase domain that interfere with imatinib binding and also the over-expression or amplification of the BCR/ABL gene. An earlier study from HGC had shown that IM resistance through BCR/ABL dependent mechanism (BCR/ABL mutation and BCR/ABL amplification) occur in only approximately 30% of CML patients showing resistance to IM. This showed that the mechanism of IM resistance among the rest of CML patients might be due to BCR/ABL independent mechanisms. Among BCR/ABL independent mechanisms, a number of factors may influence the plasma and tissue levels of IM and under certain circumstances, may contribute to pharmacologic resistance.

The mechanisms that may affect IM sensitivity include drug dependent mechanisms due to modification of drug disposition resulting in altered expression of the proteins. There are three main systems that are involved in the intracellular drug levels, (1) the drug influx system of the solute carrier family (SLC), (2) the drug efflux system of ATP binding cassette transporters (ABC transporter, also called MDR multidrug resistance transporter) and (3) the xenobiotic (drug) metabolizing enzymes (XME). Drug uptake and efflux transporters are likely to be involved in IM absorption, distribution and excretion, thereby influencing pharmacokinetics. The efficacy and toxicity of IM seem to depend on both IM pharmacokinetics, influenced by several enzymes and transporters, and IM pharmacodynamics, influenced by the mutational studies of the target. Recently, attention is focused on inter-patient pharmacokinetic

variability. Pharmacokinetic differences between patients might be due to the patient's genetics.

Genetic polymorphisms in key genes encoding transporters, metabolizing and binding enzymes of IM may influence the intracellular IM delivery and therefore, on the effectiveness of the drugs. Genetic polymorphisms of candidate genes could affect expression of corresponding proteins and thus may bring about differences in response to IM. Given that genetic differences between individuals or population can impact the efficacy of drugs, **defining pharmacogenetic differences among patients is regarded as an important aspect which needs to be addressed in understanding the development of resistance to IM in CML patients.**

What are the factor(s) that cause the development of CML? It is still largely unknown. A few risk factors have been identified such as exposure to radiation or radiation therapy. Previous chemotherapy for other types of cancer has also been associated with development of CML. Long term exposure to high levels of certain environmental carcinogens (chemicals such as benzene 1-3 butadiene, dioxin, metals) are the other risk factors for CML development. Benzene which is used in massive amounts in large number of industries such as agricultural industry, petroleum industry, is a known carcinogen. However, not all individuals exposed to these risk factors do develop CML and individuals who are never exposed to these risk factors also develop CML. So also, a few earlier studies (Biernaux *et al.*, 1995; Bose *et al.*, 1998) had demonstrated the presence of very low levels of BCR/ABL fusion gene in the blood of healthy people, but who never developed CML. All the above facts reiterates that, in

addition to Ph chromosome translocation, other predisposing risk factors are also necessary for development of CML. **This indicates the importance of undertaking studies to identify host genetic susceptibility factors for the development of CML**.

1.5 Significance of the present study

Resistance to IM could be directly or indirectly caused by pharmacokinetic factors such as an increase in cellular efflux, a decrease in cellular influx of IM, intrinsic variations in the metabolism of IM, incomplete adherence of IM etc. Pharmacogenetics is concerned with understanding individual genetic variability and how it affects response to treatment. It seems clear that the effect of IM depends on several genes. An approach involving multiple candidate genes is clearly needed to elucidate the real impact of candidate gene polymorphisms on IM response.

The xenobiotic metabolizing and transporter enzymes play important roles in the process of drug disposition and detoxification of numerous foreign and endogenous chemicals. Polymorphisms in genes coding for metabolizing enzymes and drug transporters can affect drug efficacy and toxicity. Thus, such genetic variations can influence pharmacokinetic and pharmacodynamic parameters of anticancer drugs (Bosch, 2008). Other than that, interindividual genetic variations also cause different individuals to respond differently to the same dosage of a drug (Bosch, 2008; Dasgupta and Wahed, 2014).

The detoxification processes occurring in both tumour and healthy cells include oxidation/reduction reactions by phase I enzymes such as cytochrome P450 (CYP)-related enzymes. Enhanced activity of phase I enzymes may result in the inactivation of drug and enhanced chemoresistance. On the other hand, several anticancer agents are administered as inactive pro-drugs and hence require metabolic activation by phase I proteins. Accordingly, reduced sensitivity to these drugs may be due to the reduced expression or function of the enzymes. Altogether, alterations in drug metabolism that are due either to an enhanced production of inactive metabolites or a reduced activation of pro-drugs may decrease the intracellular concentrations of the active agent, reducing the efficacy of chemotherapy (Marin *et al.* 2010). Changes in the expression or function of these enzymes that are due to genetic variations especially single nucleotide polymorphisms, in these genes involved in drug biotransformation in cancer cells, may dramatically alter the final success of pharmacological treatment (Yu *et al.* 2012).

CYP belongs to a large group of more than 60 enzymes that are responsible for the activation or inactivation of most clinically important drugs including cancer chemotherapeutic drugs such as IM. CYP enzymes can play important roles in reducing the intracellular concentration of drugs and in affecting cancer drug resistance. The expression of drug metabolizing enzymes can therefore either potentiate or reduce the toxicity of chemicals and drugs. Variations in both the activation and inactivation pathways are important variables that can lead to drug resistance. The CYP genes are highly polymorphic and certain genetic variants have been demonstrated to be clinically relevant. IM is a potent mechanism-based inhibitor of CYP3A4 and *CYP3A5* enzymes. The SNPs *CYP3A4*18* and *CYP3A5*3* exhibit inter individual differences in their

mRNA expression levels. So it was aimed to investigate the genotype frequencies of SNPs *CYP3A4*18* and *CYP3A5*3* in CML patients undergoing IM therapy and determine their association with IM response.

Most of the early works had focused on drug metabolizing enzyme genes because of their direct involvement in conversion of drugs and their removal from the body. The expression of many XMEs and transporters is up-regulated by a group of ligand-activated transcription factors namely nuclear receptors (NRs) in order to accommodate chemical challenges. The synergy and co-operation of the three systems is the result of co-ordinated regulation of the expression of these systems by nuclear receptors. The mammalian NR superfamily of transcription factors such as Pregnane X receptor (*PXR*, *NR1I2*) and Constitutive Androstane receptor (CAR, *NR1I3*) are involved in xenobiotic regulation of gene expression at the transcriptional level (Tolson and Wang, 2010). Mutations in either one of the NR will affect the functions of NR as well as cause failure or reduce efficacy in the XME and transporters. However, in order to gain a better understanding, variations in the nuclear receptors that affect the regulation of DMEs are also necessary. Nuclear receptors (a superfamily of transcription factors) especially Pregnane X Receptor (*PXR*) and Constitutive Androstane Receptor (*CAR*) play major role in detoxification process by controlling a network of signaling pathways that regulate the expression of specific batteries of genes involved in the detoxification machinery (Germain *et al.*, 2006). Because they interfere with other signaling pathways, chronic activation of these NRs could alter disease promotion. Some aspects of drug metabolism and transport are regulated by *PXR* and *CAR* genes (Evans, 2005).

According to their role as master xenobiotic responsive receptors, *CAR* and *PXR* might contribute to the well known intra and inter-subject variability in anticancer drug response. Genetic factors affecting *CAR* or *PXR* (expression or activation levels) may affect the cytotoxic threshold of tumour cells to chemotherapy which can consequently mask or attenuate pharmacogenetic associations. Recently, *PXR* pathway has also been implicated in drug resistance via the upregulation of drug metabolizing genes such as *CYP3A4* and *CYP3A5* (Basseville *et al.*, 2011). Genetic variation in NRs will affect the functions of NRs as well as cause failure or reduced efficacy in regulating the XMEs and transporters which in turn affect treatment response. So this study aimed to investigate the frequencies of SNPs 1792A>G of *PXR* and SNP 540C>T of *CAR* in CML patients on IM therapy and determine the association with IM response.

Orosomucoid (*ORM*), also known as α-1 Acid Glycoprotein (*AGP*), is a circulating serum protein that has been proposed as a factor that reduces drug efficacy through nonspecific binding to the drug. Following absorption, IM bind to plasma protein such as albumin and α-1 acid glycoprotein (*AGP*) and distribute from the intravascular spaces to their targets in their extra vascular space. Only unbound (free) drugs are pharmacologically active. So, plasma AGP concentration has a marked influence on total IM concentration. Previous mouse models had implicated a possible role for IM sequestration in the plasma by the serum α-1 acid glycoprotein, as a mechanism of therapy resistance. Individual differences in *AGP* binding may explain some of the inter-patient variability in the observed total plasma exposure to IM. Elevated levels of *AGP* had been observed in patients with CML and with further increased levels in patients with disease progression (Gambacorti-Passerini *et al.*, 2003).

It is presumed that modulation of IM binding to α-1 acid glycoprotein (*AGP*) in plasma may contribute to IM response (Rochal *et al.* 2008). Difference in IM binding to AGP protein could be due to single nucleotide polymorphisms in the *AGP* gene. So, it was planned to analyse the impact of SNP of *AGP*, namely 520G>A on IM response.

Although BCR/ABL fusion gene formed as a result of Philadelphia chromosome translocation t(9;22)(q34;q11) is essential for the development of CML, other putative inherited factors also are associated with the risk of development of CML. Inherited genetic susceptibility factors accounting for CML include genes involved in the activation and/or detoxification of xenobiotics including carcinogens and repair of DNA damage (Irigaray *et al.*, 2007). Individual variations in metabolism of carcinogens account for the differences in susceptibility to cancer and could be an attributable risk factor. It is possible that CML develops as a result of exposure to risk factors in genetically susceptible individuals. How to identify individuals who have higher susceptibility risk for CML? Being metabolizing genes and their transcription regulators, do SNPs in these genes have any role in modulating susceptibility risk of Malaysian CML patients? No information is available. This was another research question addressed in this study. So this study aimed to address this issue also.

The frequencies of the above mentioned SNPs vary across normal populations worldwide due to genetic heterogeneity. What are the frequencies of the SNPs 520G>A of *ORM1*, 878T>C of *CYP3A4*, 6986A>G of *CYP3A5*, 1792A>G of *PXR* and 540C>T of *CAR* in Malaysian population? No data is available. Genetic characterization of these SNPs in Malaysian population needs to be built up. So this study aimed to investigate

the genotype frequencies of variants of all the above SNPs in normal healthy controls belonging to three predominant ethnic groups (Malay, Chinese, and Indians) in Malaysian population.

1.6 Hypothesis

Single nucleotide polymorphisms in *ORM1* (520G>A), *PXR* (1792 A>G), *CAR* (540C>T), *CYP3A4*18* (878T>C) and *CYP3A5*3* (6986 A>G) genes have potential influence on susceptibility risk and response to IM, either good response or resistance, in Chronic Myeloid Leukaemia patients.

1.7 Objectives of the study

Broad objective:

The main objective of this study was to investigate the genotype frequencies of selected genetic polymorphisms of *ORM1*, *PXR* (*NR1I2*), *CAR* (*NR1I3*), *CYP3A4* and *CYP3A5* genes in normal controls and chronic myeloid leukaemia patients in Malaysian population and to elucidate the association of the variant genotypes with CML susceptibility risk and with clinical response to Imatinib Mesylate (IM) in Chronic Myeloid Leukaemia patients.

The specific objectives of this study are:

i. To investigate the genotype frequencies of SNP 520G>A of *ORM1*, 1792A>G of *PXR*, 540C>T of *CAR*, 878T>C of *CYP3A4* and 6986 A>G of *CYP3A5* genes in normal healthy controls belonging to 3 ethnic groups (Malays, Chinese and Indian) in Malaysian population.

ii. To investigate the genotype frequencies of SNP 520G>A of *ORM1*, 1792A>G of *PXR*, 540C>T of *CAR*, 878T>C of *CYP3A4* and 6986 A>G of *CYP3A5* in CML patients undergoing IM therapy (both IM good response and IM resistant CML patients).

iii. To determine the association of the above SNPs in modulating CML susceptibility risk.

iv. To determine the association of any of these gene polymorphisms, either in single or in combination, with good response as well as resistance to IM in CML patients.

v. To assess whether genotyping for these gene polymorphisms could be utilized as a potential pharmacogenetic tool in the future for early identification of good or poor responders to IM among CML patients.

CHAPTER 2

LITREATURE REVIEW

2.1 Imatinib Mesylate

Imatinib mesylate (IM) is a first generation tyrosine kinase inhibitor that was approved for the frontline therapy in chronic myeloid leukaemia (CML) patients by the Food and Drug Administration (FDA) in 2001. Imatinib mesylate is also known as Glivec or Gleevec. It is the first molecularly targeted drug developed for treatment of CML. IM is also used in treatment of gastrointestinal stromal tumours (GISTs). In CML, IM is specifically designed to inhibit the breakpoint cluster region (BCR)-Abelson (ABL) fusion protein. BCR/ABL fusion protein results from the chromosomal abnormality known as Philadelphia chromosome translocation t(9;22)(q34;q11). Imatinib has become first-line treatment in newly diagnosed patients with Philadelphia chromosome positive (Ph+) chronic phase CML, Ph+ accelerated phase or blast-crisis CML, and in patients with Ph+ chronic phase who have failed to respond to interferon-α therapy. Imatinib is well absorbed after oral administration with a biovailability exceeding 90% (Petain *et*

al., 2008). Since its approval in 2001, for frontline management of CML, IM has proven to be very effective in achieving high remission rates and improving prognosis.

Imatinib inhibits the BCR/ABL tyrosine kinase by competitive binding at the adenosine triphosphate (ATP)-binding pocket of the ABL kinase domain which prevents a change in conformation of the protein that would otherwise convert the molecule to its active form, and IM binding thereby leads to the apoptosis of target cells (Dulucq and Krajinovic, 2010). Figure 2.1 shows the mechanism of action of imatinib. In the absence of imatnib, ATP binds to BCR/ABL tyrosine kinase and transfer phosphate (PO_4) from ATP to tyrosine residues on various substrates. This causes excess proliferation of myeloid cells, which is characteristic of CML. In the presence of imatinib, imatinib blocks the binding of ATP to the BCR/ABL tyrosine kinase, inhibiting the activity. In the absence of tyrosine kinase activity, substrates required for BCR/ABL function cannot be phosphorylated, and subsequent cellular events are abrogated (O'Dwyer *et al.*, 2003).