DEVELOPMENT OF DENATURING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (dHPLC) IN DETECTION OF COMMON POLYMORPHISMS IN THE MULTI-DRUG RESISTANCE (MDR1) GENE IN MALAY PATIENTS WITH LEUKEMIA

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DEDICATION

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ABBREVIATIONS

MDR	: Multi-drug resistance
P-gp	: P-glycoprotein
DNA	: Deoxyribonucleic acid
RNA	: Ribonucleic acid
dsDNA	: Double-stranded deoxyribonucleic acid
ssDNA	: Single-stranded DNA
mRNA	: Messenger ribonucleic acid
cDNA	: Complementary deoxyribonucleic acid
PCR	: Polymerase chain reaction
RFLP	: Restriction fragment length polymorphism
SNP	: Single nucleotide polymorphism
AML	: Acute myeloid leukemia
ALL	: Acute lymphoblastic leukemia
CML	: chronic myeloid leukemia
CLL	: Chronic lymphoblastic leukemia
FAB	: French-American-British
WHO	: World Health Organization
dHPLC	: Denaturing high performance liquid chromatography
MDS	: Myelodysplastic syndrome
ABCB1	: ATP-binding cassette sub-family B1
UV	: Ultra violet
ATP	: Adenine Tri-phosphate

ASA : Allele specific amplification

ARMS : Amplification refractory mutation system

SSCP : Single strand conformation polymorphism

DGGE : Denaturing gradient gel electrophoresis

QRT-PCR : Quantitative reverse transcriptase polymerase chain reaction

STR : Short tandem repeat

TEAA : Triethylammonium acetate

FBN1 : Fibrillin 1

CFTR : Cystic fibrosis transmembrane conductance regulator

TSC : Tuberous sclerosis

BRCA : Breast cancer

APC : Adenomatous polyposis

Asn : Asparagine

Asp : Aspartate

Phe : Phenylalanine

Leu : Leucine

Arg : Arginine

Cys : Cystine

Gly : Glycine

Ser : Serine

Ala : Alanine

Thr : Threonine

Ile : Isoleucine

ABSTRACT

Although the use of chemotherapy has evolved remarkably during the past 30 years for treatment in leukemia patients, it remains a difficult disease to treat where the main problem is drug resistance. This is due to over expression of multi-drug resistance gene (MDR1) and it's over expression has been described in many tumors resistant to cytotoxic drugs including leukemias. This gene is polymorphic and studies in different ethnic groups have indicated that the three most frequent single nucleotide polymorphisms (SNPs) are located in exons 12, 21 and 26 of the MDR1 gene. Denaturing high performance liquid chromatography (dHPLC) has been proven to be a feasible method in detecting target DNA for presence of mutations and polymorphisms including in the MDR1 gene. Studies on polymorphisms of the MDR1 gene, however has not been carried out in patients in this institution. Thus, the main objectives of this study were to develop and establish dHPLC technique for screening of these three most frequent polymorphisms located in exons 12, 21 and 26 of the MDR1 gene, to investigate the distribution of these polymorphisms of the MDR1 gene and to compare and combine the results from dHPLC technique with restriction fragment length polymorphism (RFLP) in detection of these polymorphisms of the MDR1 gene in Malay patients with leukemia in this institution. A total of hundred and one DNA samples from Malay patients diagnosed as leukemia were studied and subjected to dHPLC analysis and RFLP analysis. Statistical analysis was done using chi-square (χ^2) and Fisher Exact's test. Out of these, there were 35 (34.7%) patients diagnosed as acute myeloid leukemia (AML), 48 (47.5%) as acute

lymphoblastic leukemia (ALL) and 18 (17.8%) as chronic myeloid leukemia (CML). There were 58 (57.4%) males and 43 (42.6%) females. From the results, if all of the three exons were combined, 25 (25.7%) of the patients showed heterozygous mutation, 7 (6.7%) of them had wild type, while 2 (1.9%) showed homozygous mutation. In all of these patients, there was a significant difference in the distribution of polymorphisms between exons 21 and 26 with a $p<0.05$. PCR-RFLP assays had been performed to confirm the results of dHPLC technique and there were 93.2%, 96.1% and 100% of common polymorphisms detected in exons 12, 21 and 26, respectively. In this study dHPLC was successfully developed for detection of common polymorphisms in the MDR1 gene in Malay patients with leukemia in this institution. The clinical implications of the distribution of these polymorphisms especially in the response of treatment in these patients should be studied for the future.

PEMBANGUNAN TEKNIK KROMATOGRAFI CECAIR DENATURASI BERPRESTASI TINGGI (dHPLC) DALAM MENGESAN POLIMORFISME YANG LAZIM PADA GEN KERINTANGAN PELBAGAI DADAH (MDR1) DI KALANGAN PESAKIT MELAYU YANG MENGHIDAP LEUKIMIA

ABSTRAK

Walaupun keberkesanan penggunaan kemoterapi dalam rawatan terhadap pesakit leukimia adalah jelas terbukti, namun leukimia tetap merupakan penyakit yang sukar dirawat terutamanya dalam masalah kerintangan pesakit terhadap ubat-ubatan (dadah). Ini disebabkan oleh pengekspresan gen kerintangan pelbagai dadah (*multidrug resistance gene*, *MDR1*) yang berlebihan dan ianya telah dilaporkan dalam kebanyakan pesakit tumor yang rintang terhadap dadah sitotoksik termasuk leukimia. Gen ini adalah gen yang polimorfik dan kajian di dalam kumpulan etnik yang berlainan menunjukkan bahawa tiga polimorfisme nukleotida tunggal (SNPs) yang paling kerap berlaku adalah terletak pada ekson-ekson 12, 21 dan 26 pada gen MDR1. Teknik denaturasi cecair berprestasi tinggi (dHPLC) telah dibuktikan sebagai suatu teknik yang berkesan dalam pengesanan mutasi dan polimorfisme, begitu juga dalam gen MDR1. Kajian pengesanan polimorfisme pada gen MDR1 masih belum pernah dijalankan dikalangan pesakit leukimia di institusi ini. Oleh itu, objektif utama kajian ini adalah untuk membangunkan teknik dHPLC bagi menyaring ketiga-tiga ekson tersebut; ekson 12, 21 dan 26 pada gen MDR1, mengkaji taburan polimorfisme gen MDR1 dan seterusnya membanding serta

menggabungkan hasil di antara teknik dHPLC dan *restriction fragment length polymorphism* (RFLP) dalam mengesan polimorfisme ini dikalangan pesakit Melayu yang menghidap leukemia. Sebanyak 101 sampel DNA pesakit-pesakit Melayu yang menghidap leukimia telah digunakan dalam kajian ini. Analisa statistik telah dilakukan menggunakan ujian-ujian *chi-square* (χ^2) dan *Fisher Exact*. Daripada keseluruhan sampel, sebanyak 35 (34.7%) adalah merupakan leukimia myeloid jenis akut (AML), 48 (47.5%) merupakan leukemia lymphoblastic jenis akut (ALL) and 18 (17.8%) merupakan leukemia myeloid jenis kronik (CML). Sebanyak 58 (57.4%) sampel adalah merupakan pesakit lelaki dan 43 (42.6%) sampel adalah perempuan terlibat di dalam kajian ini. Sekiranya hasil daripada ketiga-tiga ekson digabungkan, didapati 25 (25.7%) sampel daripada keseluruhan pesakit menunjukkan mutasi heterozigus, 7 (6.7%) sampel menunjukkan profil jenis liar sementara 2 (1.9%) sampel menunjukkan mutasi homozigus. Di kalangan pesakit-pesakit ini, terdapat perbezaan yang bererti bagi penyebaran polimorfisme di antara ekson 21 dan ekson 26 dengan nilai $p<0.05$. Teknik PCR-RFLP telah dijalankan bagi mengesahkan profil yang telah ditemui oleh teknik dHPLC dan sebanyak 93.2%, 96.1% and 100% polimorfisme yang lazim telah dikenalpasti hadir dalam setiap ekson-ekson 12, 21 dan 26 yang dikaji. Dalam kajian ini, teknik dHPLC telah berjaya dibangunkan untuk mengesan polimorfisme yang kerap berlaku pada gen MDR1 dikalangan pesakit Melayu yang menghidap leukemia di institusi ini. Implikasi klinikal bagi penyebaran polimorfisme ini terutamanya dalam respon pesakit Melayu yang menghidap leukemia terhadap rawatan haruslah dikaji sebagai kajian lanjutan.

CHAPTER I

LITERATURE REVIEW

1.1 Research Background

Leukemia is defined as a neoplasm of the white blood cells (WBC) and its precursor which leads to clonal proliferation and accumulation of WBC in bone marrow. Leukemia is divided into two categories i.e. myeloid and lymphoid each representing the types of cells involved (Denham and Chanarin, 1985). Leukemia can be acute or chronic depending on presentation and progression of the clinical manifestations. The incidence of leukemia varies with morphological type and is also dependent on age, sex, race and locality as will be explained below.

1.1.1 Epidemiology and Etiology of Leukemia

The incidence of leukemia varies with the morphological type. The frequency of any given type of leukemia is also dependent on age, sex, race and locality. In general, acute lymphoblastic leukemia (ALL) is a disease of childhood whereas in acute myelogenous leukemia (AML) there is a higher incidence with increasing of age (Munker *et al.*, 2000).

In Malaysia, there are 26,089 patients diagnosed with cancers according to the first report of the National Cancer Registry (NCR) from 1st January 2002 to 31st December 2002. Out of these, there are 11,815 males and 14,274 females. Unregistered cases are estimated to be 10,656. In terms of risk, 1 in 6 Malaysians is expected to get cancer in his/her lifetime and if taking into account unregistered cases, the risk would be higher i.e.

1 in 4 Malaysians. The crude rate for males is 118.9 per 100,000 population and 148.4 per 100,000 populations in females. Leukemia ranked fourth among cancers in males and fifth among cancers in females (Lim *et al.*, 2002).

Etiology of leukemia remains unknown in the majority of patients. However, several risk factors have been identified. The association of ionizing radiation in leukemia has been firmly established where radiation exposure has been documented by the Atomic Bomb Casualty Commission in Hiroshima and Nagasaki (Rowe and Liesveld, 1995). However, the role of low-level irradiation and of diagnostic x-rays in the etiology of leukemia remains doubtful. Benzene exposure has been strongly associated with development of leukemia. Other chemicals such as phenylbutazone, arsenic and chloramphenicol exposure have also been related to the development of leukemia (Rowe and Liesveld, 1995).

Environment has also been recognized as one of the factors contributing to the development of leukemia. These include maternal irradiation, *in utero* irradiation, early childhood viral diseases and maternal history of fetal wasting. These factors are often difficult to measure and in many instances it is likely that the disease progression are by multiple factors rather than a single factor (Rowe and Liesveld, 1995). It is also observed that frequency of leukemia is increased in the identical twins of leukemia patient, also in those with congenital disorders such as Down syndrome, Bloom syndrome, Fanconi syndrome, ataxia-telangiectasia, Kleinfelter syndrome and congenital aneuploidy. Most of these congenital disorders are associated with non-random chromosomal aberrations

where they might play a role in leukemeogenesis. Relatives of patients with cystic fibrosis also may have an increased risk of leukemia (Rowe and Liesveld, 1995).

1.1.1.1 Acute leukemia

Acute leukemia is a heterogeneous group of malignant disorders characterized by uncontrolled clonal proliferation and accumulation of blast cells in the bone marrow, blood and other tissues. Acute leukemia is usually rapidly fatal if untreated while chronic leukemia is associated with slower progression (Pallister, 1999). Acute leukemia affects all age groups from neonates to old age (Provan and Henson, 1998). The accumulation of cells in the bone marrow replaces haemopoietic precursor cells and results in bone marrow failure. At presentation at least 30% and usually more than 80% of marrow cells are blasts.

The disease is divided into two main subgroups, acute myeloid (myeloblastic) leukemia (AML) and acute lymphoblastic leukemia (ALL), and further subdivided into various subcategories. The French-American-British (FAB) scheme divides AML and ALL into various subtypes. There are additional unusual types [see also World Health Organisation (WHO)] classification in later part of this dissertation. ALL is the most common malignancy of childhood where it accounts for 85% of childhood leukemia. The FAB system classifies ALL (**Table 1.1**) into three subtypes L_1, L_2 and L_3.

ALL is commonest in the age group of 2-10 years with a peak of incidence at 3-4 years. The incidence then decreases with increasing age, though there is a secondary rise after 40 years. ALL affecting B lymphocyte precursors with CD10+ subtype is the most

common subtype in children and has an equal sex incidence. On the other hand, there is a male preponderance for ALL of T lymphocyte precursor subtype (Provan and Henson, 1998).

Hematological investigations may reveal a normochromic, normocytic anemia with thrombocytopenia in most cases of acute leukemia. The total white cell count may be decreased, normal or increased up to $200x10^9$/l or more. Blood film examination typically shows variable number of blast cells. The bone marrow is hypercellular with more than 30% of leukaemic blasts (Munker *et al.*, 2000).

The signs and symptoms of acute leukemia result from the infiltration of the malignant blasts into bone marrow and other organs. Signs of anemia are pale skin and mucous membranes and easy fatigability. Thrombocytopenia may be a feature as manifested by gum bleeding, petechiae, retinal bleeding and easy bruising. Affected children may also have bone and joint pains and sometimes a painful enlargement of the spleen.

Table 1.1: Table shows the French –American British (FAB) classification of acute lymphoblastic leukemia (ALL) into three subtypes. Adapted from (Munker *et al.*, 2000)

FAB type	Morphological features
L1	Small homogeneous with scanty cytoplasm, moderate basophilia, inconspicuous nuclei.
L2	Larger, heterogeneous cells, variable cytoplasm, basophilia, prominent nucleoli.
L3	Larger, homogeneous cells with dark basophilic cytoplasm, prominent vacuoles, prominent nucleoli.

On the other hand, AML is the commonest leukemia (80%) of adulthood where the median age at presentation is 60 years old. The incidence of AML is about two to four new cases per 100,000 people. AML is rare in children and young adults (Munker *et al.*, 2000) and forms only a minor fraction of the leukemias in childhood. The sex preponderance for AML is the same for both males and females (Provan and Henson, 1998).

However, this is not the case in older patients where males are more susceptible. This is most likely due to the fact that myelodysplastic syndromes (MDS) are more common in men, and advanced MDS frequently evolves into AML rather than ALL (Provan and Henson, 1998). AML is an aggressive hematologic neoplasm that requires urgent treatment. The myeloid stem cell is transformed into blasts or myeloblasts which then proliferate in the blood and bone marrow and suppresses normal hematopoiesis. Myeloblasts proliferate under the influence of myeloid growth factors, some of which are produced by the leukemic cells themselves. Consistent with the clonal transformation of myeloid stem cells, myeloblasts express cytogenetic abnormalities in 50-70% of cases. Cytogenetic studies performed on bone marrow also provide important prognostic information.

According to the FAB classification there are three groups of myeloid malignancies whilst WHO classification system groups diseases that show similar characteristics of both myeloproliferative and myelodysplastic conditions into a new separate group i.e. myeloproliferative/myelodysplastic diseases. This difference in classification is compared in **Table 1.2**.

The FAB classification, divides AML into eight sub-types (**Table 1.3**). The FAB subtypes are based on characteristic patterns of cytochemical stains and immunophenotype while WHO classification is based on chromosomal aberration involved. **Table 1.4** compares the different sub-types of AML classification by FAB and WHO.

Table 1.2 Classification of myeloid malignancies based on French-American-British (FAB) and World Health Organization (WHO) (http://xenia.sote.hu/depts/pathophysiology/hematology/e/who-classification.html)

FAB	WHO
Chronic myeloproliferative diseases	Chronic myeloproliferative diseases
	Myelodysplastic/myeloproliferative diseases
Myelodysplastic syndromes	Myelodysplastic syndromes
Acute myeloid leukemia	Acute myeloid leukemia

Table 1.3: French-American-British (FAB) classification of acute myeloid leukemia (AML) into eight subtypes. Adapted from (Munker *et al.*, 2000).

FAB types	Morphological features
M0	Undifferentiated -lymphoid markers negative, reactive with myeloid markers (CD33, CD13), ultrastructural peroxidase positive
M1	Myeloid, no maturation -poorly differentiated blasts with rare azurophilic granules
M2	Myeloid with maturation -myeloblasts, promyelocytes, occasionally eosinophils and basophils, often Auer rods.
M3	Promyelocytic -Two variants: hypergranular (90%) and atypical microgranular (<10%)
M4	Myelomonocytic -leukemic myeloblasts and monoblasts, variant: M4Eo with abnormal eosinophils.
M5	Monoblastic -leukemia monoblasts
M6	Erythroleukemia -more than 50% abnormal erythropoietic cells, >30% myeloblasts of all nonerythroid cells
M7	Megakaryoblastic -usually associated with myelofibrosis, platelet markers present

Table 1.4: Shows difference in classification systems of acute myeloid leukemia (AML) by French American British (FAB) compared with World Health Organization (WHO) and classification systems.
(http://xenia.sote.hu/depts/pathophysiology/hematology/e/who-classification.html

AML classification	
FAB	**WHO**
M0: minimally differentiated	**AML with recurrent cytogenetic translocations** -AML with t(8;21)(q22;q22) AML1/CBFalpha/ETO -Acute promyelocytic leukemia: AML with t(15;17)(q22;q12) and variants PML/RARalpha -AML with abnormal bone marrow eosinophils inv(16)(p13;q22) vagy t(16;16)(p13;q22) CBFbeta/MYH1 -AML with 11q23 MLL abnormalities
M1: myeloblastic leukemia without maturation M2: myeloblastic leukemia with maturation M3: hypergranular promyelocytic leukemia M4: myelomonocytic leukemia	
M4Eo: variant, increase in marrow eosinophils M5: monocytic leukemia M6: erythroleukemia (DiGuglielmo's disease)	**AML with multilineage dysplasia** With prior MDS Without prior MDS

Table 1.4, continued

AML Classification	
FAB	**WHO**
M7: megakaryoblastic leukemia	**AML with myelodysplastic syndrome, therapy related**
	Alkylating agent related
	Epipodophyllotoxin related
	Other types
	AML not otherwise categorized
	AML minimally differentiated
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monocytic leukemia
	Acute erythroid leukemia
	Acute megakaryocytic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis

1.1.1.2 Chronic Leukemia

Chronic leukemia has a slower progression in terms of clinical manifestation as compared to acute leukemia. There are two types of chronic leukemia, chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL). CML is a clonal disorder of the pluripotent stem cell and is classified as one of the myeloproliferative disorders. The disease accounts for around 15% of all leukemias and may occur at any age.

This disease occurs in both sexes with a male: female ratio of 1.4:1 and most frequently between the ages of 40 and 60 years. However, it may occur in children and neonates and the very old (Pallister, 1999, Provan and Henson, 1998). The diagnosis of CML is rarely difficult and is assisted by the characteristic presence of the Philadelphia (Ph) chromosome. This results from the reciprocal translocation of chromosomes 9 and 22 as a result of which part of the Abelson proto-oncogene (ABL) on chromosome 9 is moved to the BCR gene on chromosome 22 and part of BCR gene on chromosome 22 moves to ABL gene on chromosome 9. The abnormal chromosome 22 is the Ph chromosome or t (9; 22) (q34; q11).

On the other hand, CLL has a peak incidence between 60 and 80 years of age and rarely before the age of 40. The male to female ratio is 2:1 (Pallister, 1999, Provan and Henson, 1998). It is characterized by a chronic persistent lymphocytosis and subtypes can be distinguished by morphology, immuno phenotype and cytogenetics. DNA studies may be useful in showing a monoclonal rearrangement of either immunoglobulin or T-cell receptor genes.

1.2 Chemotherapy Resistance

The use of chemotherapy in the treatment of leukemia has evolved remarkably during the past 30 years, particularly in childhood ALL. However, leukemia remains a difficult disease to treat despite recent progress in chemotherapy. The biggest problem limiting the effective use of cytotoxic chemotherapy is drug resistance. Tumors differ in their initial chemo sensitivity where some tumors, are very sensitive, resulting in a relatively high percentage of responses. Others, such as small-cell lung cancer, are initially sensitive but then recur; whereas in others, such as pancreatic cancer, cytotoxic drugs have little activity. These differences reflect differences in the biology of the tumor, relating to factors such as the growth fraction, the activity of specific drug resistance mechanisms, and changes in proteins mediating apoptosis.

A tumor is commonly referred to as resistant if the activity of standard chemotherapy in that patient is less than that to be expected in a typical population. Resistance can be intrinsic, such that the tumor is resistant to a particular drug or drugs at presentation, or it can be acquired, such that resistance develops during treatment. The cellular mechanisms mediating drug sensitivity and resistance include target-related changes, changes in drug efflux mechanism, drug deactivation, changes in DNA repair and changes in apoptosis. In drug efflux process, a number of mechanisms for effluxing xenobiotics exist, their normal function being to protect the cell from potentially damaging compounds. Not surprisingly, many cytotoxic drugs are effluxed by this mechanism, resulting in decreased intracellular drug concentrations.

The first of these pumps to be described was P-glycoprotein (P-gp), a member of the adenosine triphosphate (ATP)-binding cassette (ABC) super family of transmembrane transport proteins. Increased expression of this protein and activity of the pump have been described in tumors that are resistant to specific cytotoxic drugs. Moreover, the selection of drug-resistant cells by prolonged exposure to specific cytotoxic drugs that are P-gp substitutes results in the accumulation of cells with increased P-gp activity that are also resistant to a number of other structurally, unrelated compounds that are P-gp substrates. This phenomenon has been termed multi-drug resistance (MDR) because resistance to one P-gp-effluxed drug is associated with resistance to multiple other agents.

P-gp is commonly detected in the leukemic cells of patients with AML in who increased P-gp, or MDR1 expression, is correlated with increase risk for treatment failure. The influence of P-gp and MDR1 on the outcome of patients with ALL is less clear. P-gp consists of two homologous halves of six hundred and ten amino acids joined by a flexible linker consisting of sixty amino acids (Shen *et al.*, 1986). It is an ATP-dependent efflux pump that confers protection to the body from environmental toxins. It transports a huge variety of structurally diverse compounds and it was first identified by because it was over expressed in human tumor cells.

Studies have helped to explain the mechanism of action of human P-gp which indicated that mutations in membrane-spanning domains or nucleotide binding domains of P-gp are involved. According to (Fojo *et al.*, 1985), drug resistance is due to decrease

accumulation of these drugs because of an energy-dependent increase in drug efflux which results from increased expression of a membrane glycoprotein.

Recent studies indicate that over expression of P-gp plays a more general anti-apoptotic role that extends beyond resistance to chemotherapy. The cells over express P-gp are resistant to a wide range of apoptotic inducers. These include serum starvation, fas ligand ligation, UV-irradiation and also exposure to tumor necrosis factor (TNF) as well as complement-mediated cytotoxic action (Scotto *et al.*, 2003).

P-gp is embedded in lipid membranes, facilitate the import of nutrients into cells or releasing the toxic products into the surrounding medium (Higgins, 1992). It is expressed in very high levels of the secretory cell type such as adrenal gland and kidney. An intermediate level has also been reported in the lung, liver, lower jejunum, colon and rectum while low levels are reported in many other tissues where the normal function is thought to involve the excretion of toxic metabolites (Schinkel, 1997, Fojo *et al.*, 1986).

This protein is also highly expressed in hematopoietic stem cells, where it is thought to protect these cells from toxins. It also has been shown to play a role in the migration of dendritic cells (Schinkel *et al.*, 1997). P-gp binds to ATP (**Figure 1.1**) and use energy to drive the transport of various molecules across all cell membranes (Higgins, 1992, Dean *et al.*, 2001). This phosphorylation process will cause conformational changes that are transmitted to the membrane-spanning domains where finally it transports substrates from one side of the membrane to the other.

In general, P-gp transports a wide range of substances including ions, sugars, amino acids, glycans, peptides, proteins, phospholipids, toxins and drugs. In eukaryotes, most P-gp move compounds from the cytoplasm to the outside of the cell or into an intracellular compartment such as endoplasmic reticulum (ER), mitochondria and peroxisome (Dean *et al.*, 2001).

The P-gp consists of two structurally homologous halves, each with six transmembrane domains, one ATP binding site and highly conserved 'Walker A' and 'Walker B' motif (**Figure 1.2**). These two halves are probably derived from internal gene duplication. It was also reported that phosphorylation of P-gp might be essential for drug transport (Hrycyna *et al.,* 1996). However, mutation in the major phosphorylation sites within P-gp does not affect its transport function. The glycosylated at extracellular sites of P-gp are not involved in routing and stability of the protein but it also serve as an antigens for monoclonal antibodies recognizing P-gp (Sonneveld, 2000, Schinkel *et al.*, 1993). P-gp not only removing an anticancer drug from the inside to the outside of the cell membrane using ATP energy, but also prevents the influx of drugs through the cell membrane (Juliano and Ling, 1976).

The classical phenomenon of MDR is seen where levels of P-gp expression was found in a large range of cell-lines that were selected through exposure to different cytotoxic agents (Kartner *et al.*, 1983). There are two different genes encoding for P-gp in humans, MDR1 gene and MDR3 gene, both located on chromosome 7. Despite their homology, only MDR1 gene is a drug efflux protein which is related to MDR (Hrycyna *et al.*, 1998). Several studies suggested that phosphorylation of P-gp might be essential for drug transport (Germann *et al.*, 1996). The glycosylated sites of P-gp at the extracellular site

are probably involved in routing and stability of the protein as well as serve also as an antigen for monoclonal antibodies in recognizing P-gp (Schinkel *et al.*, 1993).

Several studies have reported the association between genotype variations with the reduction of P-gp expression levels. A single nucleotide change in the coding region of MDR1 gene has been found to be associated with the expression level of P-gp. These single nucleotide changes are also known as single nucleotide polymorphisms (SNPs).

MDR represents a form of pleotropic drug resistance that has an adverse prognostic value in AML, refractory multiple myeloma and non-Hodgkin's lymphoma. It may affect the outcome of current chemotherapy protocols (Sonneveld, 2000). Numerous studies have investigated the expression of the MDR1 gene products in leukemia, lymphoma and myeloma (Hegewisch-Becker and Hossfeld, 1996). Studies in myelogenous leukemia and myeloma have so far provided the best evidence for a significant correlation between P-gp expression and response to chemotherapy (Sonneveld, 2000).

1.2.1 MDR1 Gene also known as Drug Transporter Gene

Drug transporter gene is the gene that allows drugs to enter cells and in some cases, acts to keep them out from membrane cell. Transporter genes may account for discrepancies in the way drugs such as antidepressants, anticonvulsants, and chemotherapy agents work in different people. The drug-resistant phenotype is due to a membrane alteration which reduces the rate of drug permeation (Juliano and Ling, 1976). Surface labeling studies reveal that in Chinese hamster, the ovary cell membranes possess a carbohydrate-containing drug-resistant component. This component is shown to be a cell surface

glycoprotein and has a weight of 170 000 Dalton (170 kD) in size. Since this glycoprotein is unique to mutant cells displaying altered drug permeability, it has been designated as the P-gp. It has been reported to be an important factor in understanding a natural resistance of tumors to cytotoxic drugs (Juliano and Ling, 1976).

1.2.2 Mechanisms of Multi-drug Resistance

There are two mechanisms of resistance to anticancer drugs, firstly, those that damage delivery of anticancer drugs to tumor cells and secondly, those that arise in the cancer cell it self due to genetic and epigenetic alterations which can affect drug sensitivity (Jain, 2001). Cancer cells can become resistant to a single anticancer drug or a class of drugs with a similar mechanism of action (Gottesman *et al.*, 2002). It arises either by altering the drug's cellular target or by increasing repair of drug-induced damage, commonly by DNA. After selection for resistance to a single drug, cells might also show cross-resistance to other structurally and mechanistically unrelated drugs.

Cancer cells are genetically heterogeneous because of the mutated phenotype. Tumor cells that are exposed to chemotherapeutic agents will be selected for their ability to survive and grow in the presence of cytotoxic drugs. Thus, in any population of cancer cells that is exposed to chemotherapy, more than one mechanism of multi drug resistance can be present. This phenomenon has been called as multi factorial MDR (Gottesman et al., 2002).

MDR can also result from activation of coordinately regulated detoxifying systems, such as DNA repair and the cytochrome P450 mixed function *oxidases*. It can be induced after

exposure to any drug (Schuetz *et al.*, 1996). Recent evidence indicates that certain orphan nuclear receptors might be involved in mediating global response to environmental stress (Synold *et al.*, 2001). MDR can also result from defective apoptotic pathways which might occur as a result of malignant transformation as in those cancers with mutated or non-functional p53 gene (Lowe *et al.*, 1993). Alternatively, cells might acquire changes in apoptotic pathways during exposure to chemotherapy, such as changes in cell-cycle machinery, leading to an activated checkpoints which prevents initiation of apoptosis (Lowe *et al.*, 1993).

1.2.2.1 ABCB1 Gene Family

Classical MDR results from expression of ATP-dependent efflux pumps with broad drug specificity (Gottesman *et al.*, 2002). These pumps belong to a family of ATP-binding cassette (ABC) transporters which share sequence and structural homology. So far, fourty-eight human ABC genes have been identified and divided into seven distinct subfamilies (ABCA, ABCB, ABCC, ABCD,… and ABCG) on the basis of their sequence homology and domain organization (Dean *et al.*, 2001). Drugs that are affected by classical MDR include vinca alkaloids (vinblastine, vincristine), the anthracyclines (doxorubicin, daunorubicin), the RNA transcription inhibitor actinomycin-D and the microtubule-stabilizing drug paclitaxel (Ambudkar *et al.*, 1999).

The ABCB1 gene maps to chromosome 7q21.1 and is the best characterized ABC drug pump (**Figure 1.3**). Formerly known as MDR1 gene or P-gp, ABCB1 gene was the first human ABC transporter cloned and characterized through its ability to confer a MDR phenotype to cancer cells that had developed resistance to chemotherapy drugs (Roninson

et al., 1986, Juliano and Ling, 1976). The gene is thought to play an important role in removing toxic metabolites from cells. It also expressed in cells at the blood–brain barrier and may play a role in transporting compounds into the brain that cannot be delivered by diffusion (Hoffmeyer *et al.*, 2000).

P-gp belongs to the ABC transporter subfamily B1 and is encoded by the ABCB1 gene (Ueda *et al.*, 1987). ABCB1 gene is found to be over expressed in all MDR human cell lines. It consists of 28 exons and 26 introns and encodes a transporter P-gp.

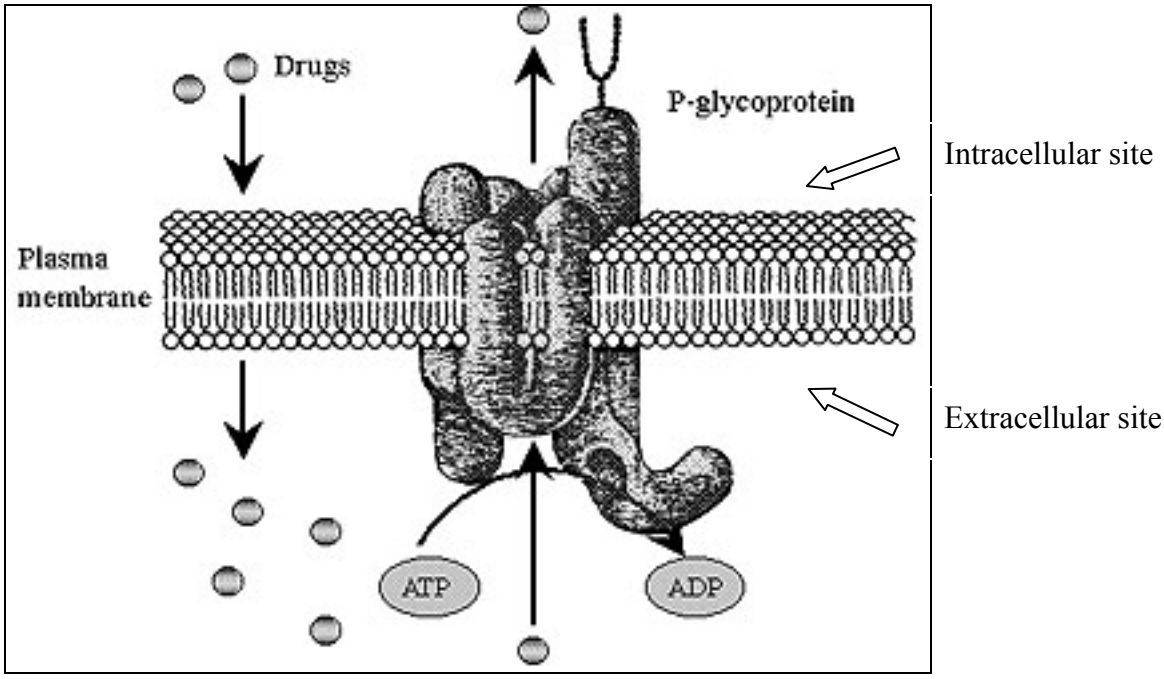

Figure 1.1: The figure shows the process of phosphorylation of P-gp. This process is essential for transport activity of the drugs into the membrane cell. This figure cited from http://images.search.yahoo.com/search/images?p=+p-glycoprotein&ei=UTF 8&fl=0&imgsz=all&fr=FP-tab-img-t&b=41.

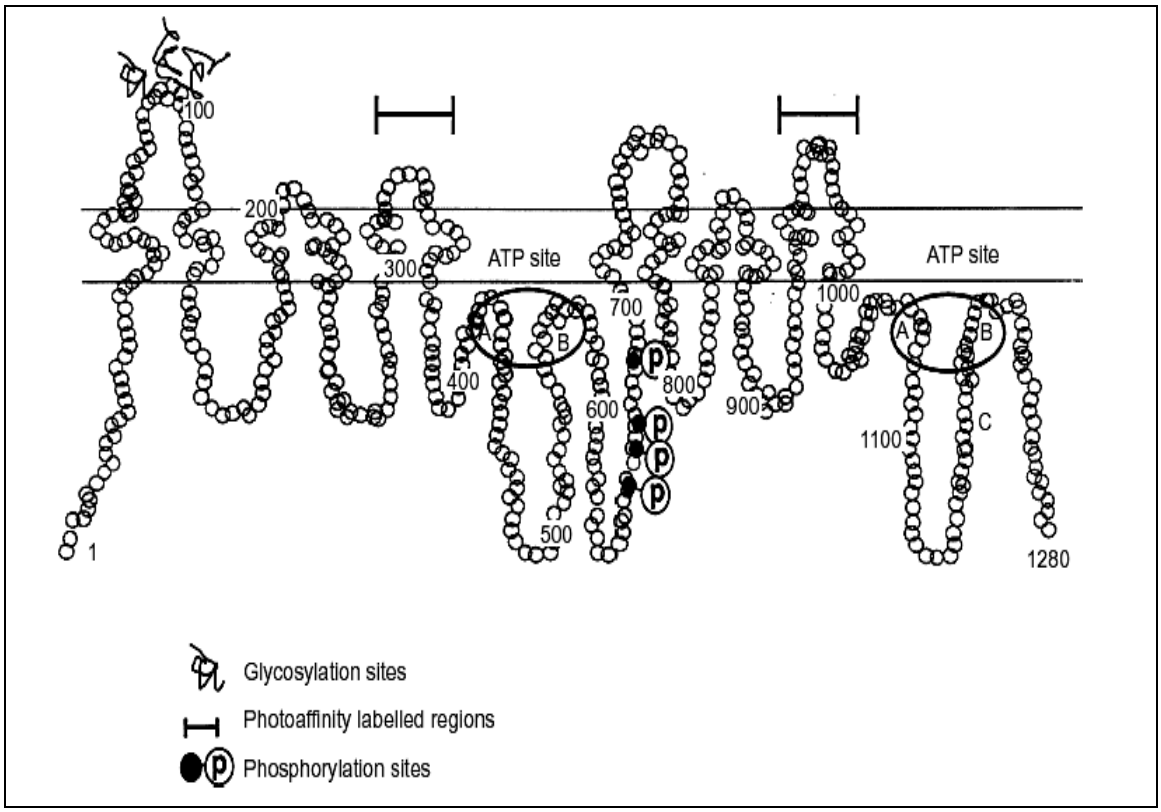

Figure 1.2: Schematic representation of P-gp. It contains two ATP-binding sites, phosphorylation sites and glycosylation sites as well as comprises of 1280 amino acid sequences (Sonneveld, 2000).

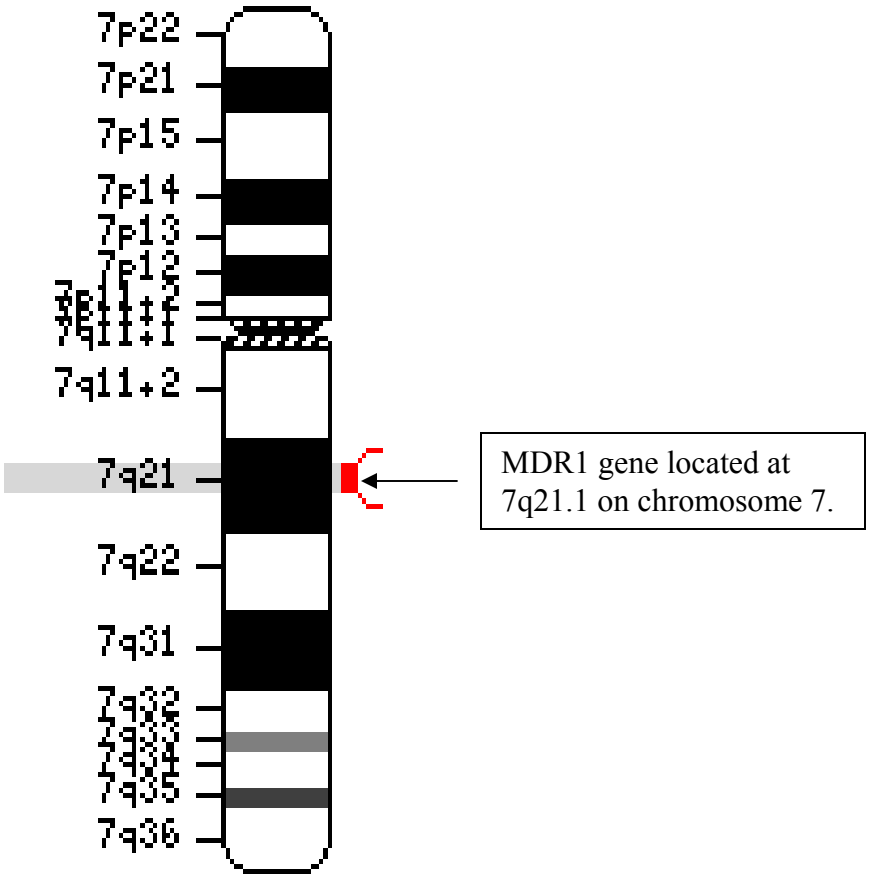

Figure 1.3: The MDR1 gene is located at the q-arm of chromosome 7 at position 21.1. The ideogram shows the location of MDR1 gene on the chromosome. (http://www.ncbi.nlm.nih.gov/mapview/maps.) The OMIM reference code: # 171650.

1.2.3 Expression of P-gp in Normal Cells.

P-gp is differentially expressed in normal tissues as a consequence of differentiation triggers and response to environmental challenges. High levels of P-gp expression are found in the kidney and adrenal glands. Expression of P-gp is also high in the colon and in the endothelial cells of the blood-brain and blood-testes barrier, where it plays a role in the pharmacokinetic and bio-distribution of xenobiotics. Furthermore, environmental factors such as heat shock, cytokines, hormones, differentiation agents, chemotherapeutics, dietary products, UV and X-irradiation, receptor agonists, oncogenes and tumor suppressor genes can influence the levels of specific P-gp isoforms in different systems. Therefore, P-gp expression is controlled by the highly complex interplay of a variety of factors that are involved in multiple regulatory pathways (Schwab *et al.*, 2003).

P-gp is involved in limiting absorption of xenobiotics from the gut lumen, in protection of sensitive tissues (brain, fetus, testes) and in biliary and urinary excretion of its substrates (Schwab *et al.*, 2003). P-pg has an important role in regulating central nervous system permeability. The brain is protected against blood-borne toxins by the blood-brain barrier (BBB) and the blood-cerebrospinal-fluid (CSF) barrier. P-gp is located on the luminal surface, preventing the penetration of cytotoxins across the endothelium (Rao *et al.*, 1999).

P-gp plays an important role to protect the testicular tissues and transports toxins into the capillary lumen in the testes (Cordon-Cardo *et al.*, 1990). In the placenta, P-gp is localized on the apical syncytiotrophoblast and involved in developing of fetus by protecting from toxic cationic xenobiotic. In the liver, gastrointestinal tract and kidney, P-gp is responsible to excrete toxin thus protecting the entire organisms (Schinkel, 1997).