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Expression of the multiple drug resistance associated genes: MRP1, LRP and BCRP among leukemia patients in Gaza strip.

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

﴿الَّذِي خَلَقَنِي فَهُوَ يَهْدِينِ * وَالَّذِي هُوَ يُطْعِمُنِي وَيَسْقِينِ * وَإِذَا مَرِضْتُ فَهُوَ يَشْفِينِ﴾

(الشعراء: 78 – 80)

Declaration

I hereby declare that submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university or other degree of the university or other institute, except where due acknowledgment has been made in the text.

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Expression of the multiple drug resistance associated genes: MRP1, LRP and BCRP among leukemia patients in Gaza strip.

ABSTRACT

Hematological neoplasms are usually sensitive to chemotherapy, but with relatively high rate of relapse. Cell resistance to drugs is a major determinant of response to chemotherapy and its detection may be of clinical relevance. The role of expression of transmembrane carriers such as multidrug resistance related Protein 1 (MRP1), breast cancer resistance protein (BCRP) and lung resistance protein (LRP) genes in neoplastic cell survival and risk of relapse for leukemia patients was previously documented. Therefore, the aim of this study was to estimate the level of expression of MRP1, BCRP, and LRP genes in blood cells of leukemia patients in Gaza strip by quantitative real-time RT-PCR technique, and to investigate any correlation between the expression of these genes and other previous and current clinical findings of the patient.

Blood samples were collected from 70 leukemia patients (40 males and 30 females) admitted in the Hematology Departments of Al-Shefa hospital, the European Gaza Hospital and AL-Nasser pediatric hospital in Gaza strip. The specimens were collected during the period between May to November, 2009. Patients' medical data were obtained from their records in the relevant hospitals, and included personal, medical, management and family information (e.g. age, type of disease, severity of case, date of diagnosis of disease, types , protocols of treatments, prognosis, previous tests results and others). A control group of 35 normal healthy individuals was included mainly to correct for any inter-individual expression difference as a result of gender and age variation. This group was also used to compare the levels of gene expression in normal and leukemia patients. The level of expression of MRP1, LRP, and BCRP genes in cells of leukemia patients were quantitated by quantitative Real Time-PCR technique and normalized by the expression level of an endogenous control gene porphobilinogen deaminase (PBGD). The SPSS version 15 was used for statistical analysis.

Five types of leukemia, from different areas of Gaza strip, were included in this study. Thirty cases (42.9%) were acute lymphoblastic leukemia, 5 cases (7.1%) acute myeloblastic leukemia, 12 cases (17.1%) chronic lymphoblastic leukemia, 22 cases (31.4%) chronic myeloblastic leukemia and 1 case (1.4%) small lymphoblastic leukemia.

The mean age of cases was 32.9 ±28.2 years and the mean age of controls was 27.2 ±18.8 years.

MRP1 and LRP but not BCRP mean level of gene expression was significantly higher in leukemia group than normal control group. MRP1 gene expression in ALL patients was lower than all types of leukemia and significantly lower than in AML **(P=0.001)**. LRP gene expression was significantly higher in AML and CML patients than in control group (AML: **P=0.021** and CML: **P=0.001**). LRP gene expression in ALL patients were significantly lower than CML patients (**P=0.024**); and in CML patients higher than CLL patients (P=**0.046**). There was no statistically significant difference between leukemia types in BCRP gene expression levels. MRP1 and LRP mean levels of expression in remission was less than with no remission patients and this decrease of expression was statistically significant (MRP1: **P=0.003** & LRP: **P=0.050**). The mean level of BCRP gene expression in remission patients was also less but with no statistical significance. When comparing the level of MRP1, LRP and BCRP according to management protocols and gender of patient no significant relationship was established.

The outcome of the current study indicates that higher levels of MRP1, LRP and BCRP expression are correlated with chemotherapeutic treatment failure of leukemia patients. Therefore we suggest these factors to be included in the design and application of chemotherapy protocols in Gaza Strip.

Key words: Leukemia, Multidrug resistance, transmembrane protein, Real-Time PCR, Gaza Strip.

التعبير الجيني للجينات المقاومة للعقاقير (MRP1, LRP & BCRP) عند مرضي اللوكيميا في قطاع غزة

ملخص الدراسة:

أورام الدم السرطانية عادة ما تكون حساسة للعلاج الكيميائي في المقام الأول، ولكن معدل الانتكاس و التراجع ما زال مرتفعا في هذه السرطانات.

تحديد العوامل التي من الممكن أن يكون لها دور في الكشف عن استجابة المريض للعلاج هو تحدي مستمر في علم الأورام ، وفي هذا الإطار مقاومة الخلايا السرطانية (Drug resistance) تعتبر سببا رئيسيا من أسباب فشل العلاج الكيميائي والتي تؤدي من ثم إلي الوفاة للمرضي في هذه الأمراض .

خلال العقد الأخير، سعت العديد من الدراسات البحثية في الكثير من بلدان العالم لتحديد النواقل البروتنية علي سطح الخلايا السرطانية الموجودة في غشائها الخلوي مثل جينات MRP1، LRP و BCRP، والتي تبين أن لها دورا مهما في نقل العقاقير الكيميائية من والي الخلايا السرطانية.

في حقيقة الأمر تهدف دراستنا لتحديد وتقدير مستوي التعبير الجيني للجينات مقاومة العقاقير MRP1,LRP & BCRP عند مرضي سرطان الدم في قطاع غزة عن طريق استخدام تقنية Real time PCR والتحقق في أي علاقة بين مستويات التعبير الجيني لهذه الجينات و نسبة الشفاء من المرض و غيرها من المتغيرات السريرة والفيزيائية للمرض.

لتحقيق هذا الغرض أخذت عينات دم من 70 مريض مصاب بسرطان الدم (Leukemia) من أقسام أمراض الدم في مستشفيات الشفاء و النصر للأطفال ومستشفي غزة الأوروبي في الفترة ما بين 1-5-2009 إلي 1-11-2009. و قد كانت 40 حالة من الذكور و 30 حالة من الإناث.

وقد تم جمع البيانات الطبية من سجلات المرضي في المستشفيات المذكورة وشملت المعلومات الشخصية والطبية والعلاجية (مثل: العمر ونوع المرض، شدة الحالة ،تاريخ تشخيص المرض و بروتوكولات العلاج والاختبارات التشخيصية وغيرها).

استخدمت الدراسة تقنية (Real-time PCR) لتحديد المستوي الكمي للتعبير الجيني عن الجينات مقاومة العقاقير (MRP1, LRP & BCRP) وهي تقنية دقيقة وحساسة وفعالة للحصول علي العدد الحقيقي ل RNA المنسوخ من الجين نسبة إلي مستوي التعبير عن جين ضابط (Internal control) وهو جين PBGD. وقد تم تحليل النتائج والمعلومات والبيانات للمرضي باستخدام برنامج التحليل الإحصائي (SPSS) نسخة 15.

شملت الدراسة خمسة أنواع من سرطان الدم وقد كانت ثلاثون حالة (42.9%) ALL، وخمس حالات (7.1%) AML, واثنا عشرة حالة (17.1%) CLL، واثنا وعشرون حالة CML (31.4%) حالة واحدة من (1.4%) SLL.

أخذت مجموعة ضابطة من الأشخاص الأصحاء للمقارنة بينها وبين مجموعة المرضي وقد اخذ بعين الاعتبار المقاربة في الأعمار بين مجموعة مرضي اللوكيميا وبين ا لمجموعة الضابطة وكذلك الجنس.

متوسط العمر للحالات المرضية كان (28.2±32.9) عاما بينما كان متوسط العمر للعينة الضابطة (27.2± 18.2) عاما.

وقد كان متوسط عدد النسخ لجين MRP1 عند مرضي اللوكيميا أعلي منه لدي المجموعة الضابطة وهذا الارتفاع يعتبر ذو دلالة إحصائية (P=0.007). وكذلك الحال أيضا جين LRP (P=0.007) ولكن لم تكن أي دلالة إحصائية في الارتفاع عند جين BCRP.

مستويات التعبير الجيني للجين MRP1 في جميع أنواع اللوكيميا كانت عالية مقارنة بالمجموعة الضابطة ماعدا مرضي (ALL) وكانت جميعها ذات دلالة إحصائية حيث كانت علي النحو التالي:

(ALL: P: 0.365, AML: P: 0.000, CLL: P: 0.037 & CML: P: 0.010)

عند مقارنة أنواع اللوكيميا المختلفة بعضها بعض من حيث مستوي التعبير عن الجينات الثلاثة تبين ما يلي: يختلف مستوي التعبير في الجين MRP1 لدي مرضي ALL اختلاف ذو دلالة إحصائية فقط عنه لدي مرضي AML (P=0.001) حيث كانت الزيادة في التعبير الجيني عند مرضي AML وأيضا بمقارنة أنواع اللوكيميا المختلفة تبين أن مستوي التعبير الجيني عند مرضي AML اعلي منه عند مرضي CLL و مرضي CML وهذا الارتفاع ذو دلالة إحصائية (CLL: P=0.024 & CML: P=0.017).

وقد كان مستوي التعبير عن جين LRP عند مرضي AML و مرضي CML اعلي منه لدي العينة الضابطة (AML: P=0.021 و CML: P=0.001).

أيضا بالمقارنة بين أنواع اللوكيميا المختلفة بعضها بعض تبين أن مستوي التعبير عن جين LRP لدي مرضي CML اعلي منه لدي مرضي ALL و مرضي CLL اختلافا ذو دلالة إحصائية (ALL: P=.024 و CLL: P=0.046). ولم يكن هنالك أي دلالة إحصائية لمستويات التعبير الجيني عن جين BCRP عند مرضي اللوكيميا المختلفة أو المجموعة الضابطة.

عند تقصي نوع وطرق العلاج المتبع مع الحالات المختلفة وجد إن: مرضي اللوكيميا اللذين اخذوا العلاج الكيميائي فقط كان عددهم 65 حالة (92.9%) وجزء بسيط فقط هم خمس حالات بالإضافة للعلاج الكيميائي قاموا بزراعة نخاع العظم . لم توجد أي دلالة إحصائية عند مقارنة مستويات التعبير الجيني للجينات الثلاثة (MRP1, LRP & BCRP) مع أنواع العلاجات.

مستويات التعبير الجيني للجينات MRP1 & LRP عند المرضي الذين تماثلوا للشفاء وأصبحوا في وضع صحي جيد كانت مستويات التعبير الجيني لديهم اقل من الذين لم يتماثلوا للشفاء (LRP: P= 0.050 & MRP1: P=0.003) بينما مستوي التعبير الجيني لجين BCRP لم يعطي أي فرق إحصائي بين المجموعتين. لم يكن هنالك فرق بين مستويات التعبير الجيني للجينات مقاومة العقاقير في الدراسة وبين الجنس (ذكور أو إناث).

أيضا عند مقارنة أنواع اللوكيميا المختلفة وبروتوكولات العلاج المختلفة لهم مع مستويات التعبير الجيني في جينات مقاومة العقاقير بالدراسة لم يكن هنالك فروق إحصائية ذات أهمية بينهم.

أخيرا نجد أن ارتفاع مستوي التعبير الجيني لهذه الجينات يرتبط مع فشل العلاج الكيميائي ارتباط طردي عند مرضي سرطان الدم اللوكيميا. لذلك يفترض أن تكون تقنيات الفحص المخبرية لهذه الجينات واختباراتها ضمن بروتوكولات العلاج والخطط العلاجية للمرضي.

الكلمات المفتاحيه: سرطان الدم، المقاومة للعقاقير، النواقل البروتينية، التقدير الكمي الحقيقي،قطاع غزة.

Dedication

To my beloved ***parents***

To my wife ***Ola***

To my sons

Hala, moaeed & Jana

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ABBREVIATIONS

Abbreviation	Term description
5FU	5-Fluro Uracil
6-MP	6-Mercaptopurine drug
µM	Micro Molar
ABC	ATP-binding Cassette
ABCA	ATP-binding cassette sub-family A
ABCA1,2,3,4,5,6,7,8,9,10,12,13	ATP-binding cassette sub-family A member 1,2,3,4,5,6,7,8,9,10,12,13.
ABCB	ATP-binding cassette sub-family B
ABCB1	ATP-binding cassette sub-family B member 1
ABCB7	ATP-binding cassette sub-family B member 7
ABCC	ATP-binding cassette sub-family C
ABCC 1,2,3,4,5,7,11,12	ATP-binding cassette sub-family C member1,2,3,4,5,11,12
ABCD	ATP-binding cassette sub-family D
ABCD1	ATP-binding cassette sub-family D member 1
ABCE	ATP-binding cassette sub-family E
ABCF	ATP-binding cassette sub-family F
ABCG	ATP-binding cassette sub-family G
ABCG1,2,3,4,5,8	ATP-binding cassette sub-family G member1,2,3,4,5,8
ABCG2	ATP-binding cassette sub-family G member 2
ALD	Adrenoleukodystrophy
ALL	Acute lymphoblastic leukemia
AML	Acute Myeloblastic leukemia
Ara-C	Arabinofuranosylcytosine (cytarabine)
ATP	Adenosine triphosphate
B.M	Bone Marrow
B.M.T	Bone Marrow Transplantation.
BCL-2	B-cells limphoma 2
BCRP	Breast Cancer resistance protein
BCRP-F	BCRP-forward
BCRP-R	BCRP-Reverse
BP	Base pair
CBC	Complete Blood Count
cDNA	Complementary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
CLL	Chronic Lymphoblastic leukemia
CML	Chronic Myeloblastic Leukemia

CR	Complete Remission
CT	Cycle Threshold
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Dinuclotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EPR	Enhanced permeability and retention.
G_1 phase	Growth phase 1
G1-S checkpoint	Growth phase1 --- Synthesis phase checkpoint
G2 – M check point	Growth phase2 --- Mitotic phase checkpoint
G_2 phase	Growth phase 2
GSH	Glutathione
INF-A	Interferon-Alfa
KDa	Kilo Dalton.
LRP	lung resistance protein
LRP-F	LRP- forward
LRP-R	LRP- Reverse
M phase	Mitotic phase
MDR	Multidrug Resistance
MOH	Ministry of health
MRP1	Multidrug resistance associated protein1
MRP1,2,3,4,5,6	Multidrug resistance associated gene type 1,2,3,4,5,6
MRP-F	MRP- Forward
MRP-R	MRP- Reverse
MVP	Major Vault Protein
MXR	Mitoxantrone resistance-associated protein
NBDs	Nucleotide-binding domains
NBFs	Nucleotide-Binding Folds
Ng	Nano gram
PBGD gene	Porphobilinogen deaminase
PBGD-F	PBGD- forward
PBGD-R	PBGD- Reverse
PBP	Permeability binding protein
PGP	Permeability glycoprotein
PML	Plasma Cell Multiple Myeloma
RNA	Ribonucleic acid
RT-PCR	Real Time-Polymerase Chain Reaction
S phase	Synthesis phase
SLL	Small lymphocytic leukemia
TM	Transmembrane protein
TMD	Transmembrane domain
U/L	Unite/Litter
USA	United Stat Of America
WBCs	White Blood Cells

Chapter One

Introduction

1.1 Overview

Cancer is a group of diseases in which the body's cells become abnormal and divide without control. Cancer cells may invade nearby tissues, and they may spread through the blood stream and lymphatic system to other parts of the body.

Cancer is considered one of the most important health problems in both developing and developed countries for its high incidence, cost and associated mortality. Cancer is the third leading cause of death among Palestinians after cardiovascular diseases and cerebrovascular disease, and it is a major cause of morbidity among Palestinian population (1).

Bone marrow cancer is the fifth most common type of cancer in Gaza strip after Trachea, Bronchus & lung cancer, colo-rectal and anus cancer, breast cancer and Brain & other nervous system cancer (2). In children, the most common cases of pediatric cancers are lymphomas (30.7%), the first leading cause of cancer morbidity in male children; and bone marrow (27.8%), the first one in female children under 15 years old (1).

Leukemia is a form of cancer that starts in the blood-forming tissue such as the bone marrow in which the body produces too many white blood cells, and is usually characterized by the presence of the abnormal cells in peripheral blood. In a small number of patients, they are not found (aleukemic leukemia). There are two major kinds of leukemia: chronic and acute (3). Acute leukemia is a rapidly progressing disease involving the proliferation and accumulation of immature red and white blood cells and platelets (i.e., blasts and other very early cells). Accordingly, these cells cannot carry out their normal functions. Acute leukemia has a rapid course lasting for two to four months without treatment, and usually affects younger age groups. It is divided into two categories, depending on the cell type involved. If the disease involves the

lymphocytes it is called acute lymphocytic leukemia (ALL), but if it affects the myelocytes it is known as acute myelogenous leukemia (AML).

Chronic leukemia progresses more slowly and mostly affects older adults. It permits greater numbers of fully developed blood cells to grow, allowing these cells to carry out some of their normal functions. This type of leukemia is also divided in two major types; chronic lymphocytic leukemia (CLL) and chronic myelogenous leukemia (CML) (4).

Although leukemia collectively affects approximately 10 times more adults than children, it is the most common type of cancer among children, with ALL accounting for approximately 78% of all childhood leukemia. The most common type of leukemia in adults is AML, followed by CLL, CML and ALL (4). The only Palestinian comprehensive cancer report was published by the Palestinian Ministry of Health and covered the period 1995-2000 (1). In that period ALL accounted for 36.5% of leukemia cases in Gaza Strip; AML accounted for 20.7%; plasma cell multiple myeloma (PML) accounted for 15.2% of cases; CLL accounted for 12.5% of cases and CML accounted for 15.2% of cases.

All types of leukemia are managed mainly by chemotherapy. In the period 1995-2000 the proportional distribution of marrow cancer therapy in Gaza showed that chemotherapy was applied for 95% of total treated cases and radiation in 23.5%, in addition to other less commonly used types of therapies like biological, surgery, and bone marrow transplantation (1). In Gaza strip hospitals the protocols for chemotherapy treatment applied upon leukemia patients depend on age, weight, WBCs count, stage and type of disease in direction to choose the specific anticancer drug.

According to the chemotherapy protocol for pediatrics non-Hodgkin's lymphoma, the drugs used in treatment of ALL in Oncology Departments of Gaza hospitals are methotreaxate, prednisolone, vincristine, epirubicine, L-asparaginase, cyclophosphoamide, cytosinearabinoside and 6-mercaptopurine which are all administered intravenously. The dose of drug depends on, age, weight, and most importantly the phase of disease.

In other types of leukemia like AML, CML, and CLL which affect adults more than children, the drugs used are cytarabine, daunorubicin, hydroxyurea, busulfan, cytosine arabinoide, Imatinibe mesylate, interferone-alfa and fludarabiune. These drugs are taken by oral and intravenous roots (5).

There are a number of strategies in the administration of chemotherapeutic drugs used today. Chemotherapy may be given with a curative intent or it may aim to prolong life or to palliate symptoms. Combined modality chemotherapy is the use of drugs with other cancer treatments, such as radiation therapy or surgery. Most cancers are now treated in this way. Combination chemotherapy is a similar practice which involves treating a patient with a number of different drugs simultaneously. The drugs differ in their mechanism and side effects. The biggest advantage is minimizing the chances for resistance developing to any one agent.

Chemotherapy of cancer and especially leukemia is frequently associated with dangerous side effects. Furthermore, failure of therapy is one of the major obstacles facing leukemia managements. Treatment failure in acute leukemia may be caused by several different factors, the main determinants of treatment failure in Acute leukemia include: low cellular sensitivity to cytotoxic drugs (cellular drug resistance); increased proliferation potential of leukemia cells between courses of chemotherapy (regrowth resistance) and low systemic exposure of antileukemic drugs(pharmacokinetic resistance) (6).

Drug resistance is setting one of the major obstacles in the success of treatment and is an important cause of death in acute leukemia. Drug resistance, refers to the ability of cancer cell to resist the action of anticancer drugs and inhibit its action. Such resistance may be present before beginning treatment or may develop during chemotherapy. Drug resistance that extends to structurally and functionally unrelated drugs is termed multidrug resistance (MDR) (7). The ATP-binding cassette (ABC) transporter super family contains membrane proteins that translocation a wide variety of substrates across extra- and intracellular membranes including drugs.

ABC genes represent the largest family of transmembrane (TM) proteins. These proteins bind ATP and use the energy to drive the transport of various molecules across all cell membranes (8-10). Proteins are classified as ABC transporters based on the sequence and organization of their ATP-binding domains. Overexpression of certain ABC transporters occurs in cancer cell lines and tumors that are multidrug resistant, and there are a number of important ABC genes which play an important role in MDR of cancer cells, including the multidrug resistance associated protein (MRP1), the lung resistance protein (LRP) and the breast cancer resistance protein (BCRP).

1.2 Objective of the study

Overall objective:

To determine the level of expression of the multiple drug resistance associated genes: MRP1, LRP and BCRP among leukemia patients, in Gaza strip.

Specific objectives:

1. To investigate any correlation between the expression of these genes and clinical variables of the patients like: age, gender, living area and leukemia type.
2. Investigate the correlation between expression of these genes and disease management and remission.

1.3 Significance

To my knowledge this study will be the first to deal with multidrug resistance in cancer and particularly in leukemia patients in Palestine. The establishment of a clinically relevant expression assessment of multidrug resistance genes will aid as a prognostic indicator of chemotherapy responsiveness in human malignancies. It will also aid in defining therapeutic target(s) for reversing multidrug resistance in patients. In addition, it will highlight the need for redefining strategies of induction and consolidation treatment in these diseases.

Chapter Two

Literature Review

2.1 Cancer

Cancer is a collective term used for a group of diseases that are characterized by the loss of control of the growth, division, and spread of a group of cells, leading to a primary tumor that invades and destroys adjacent tissues. It may also spread to other regions of the body through a process known as metastasis, which is the cause of 90% of cancer deaths (11). Cancer remains one of the most difficult diseases, and incidence is increasing due to the ageing of population in most countries, but especially in the developed ones.

Cancer is normally caused by abnormalities of the genetic material of the affected cells. Tumorigenesis is a multistep process that involves the accumulation of successive mutations in oncogenes and suppressor genes that deregulates the cell cycle. Tumorigenic events include small-scale changes in DNA sequences, such as point mutations; larger-scale chromosomal aberrations, such as translocations, deletions, and amplifications; and changes that affect the chromatin structure and are associated with dysfunctional epigenetic control, such as aberrant methylation of DNA or acetylation of histones (12). About 2,000–3,000 proteins may have a potential role in the regulation of gene transcription and in the complex signal-transduction cascades that regulate the activity of these regulators.

Cancer is not only a cell disease, but also a tisular disease in which the normal relationships between epithelial cells and their underlying stromal cells are altered (13).

Cancer is presently responsible for about 25% of deaths in developed countries and for 15% of all deaths worldwide. It can therefore be considered as one of the foremost health problems, with about 1.45 million new cancer cases being expected yearly (11). So, cancer is considered one of the most important health

problems in both developing and developed countries for its high incidence, cost and associated mortality.

2.2 Leukemia

The acute leukemia is a heterogeneous group of neoplasm's arising from transformation of uncommitted or partially committed hematopoietic stem cells (14). Acute leukemia represents a group of complex and heterogeneous diseases, which are characterized by accumulation of malfunction and immature leukemia blasts in the peripheral blood and the bone Marrow. Recurring chromosomal abnormalities found in over half of patients are critical for classification of the diseases, risk stratification, and design of treatment regiments (15). Acute leukemia is currently classified pragmatically by a combination of differentiation, pathogenesis, and genetic abnormalities. Lineage assignment (myeloid vs. lymphoid) is based on morphology features, cytochemistry, and immunophenotyping. This classification of each group of leukemia has become essential, as treatment is evolving for specific genetic and pathogenetic groups of disease (16). There are two major kinds of leukemia: chronic and acute. Acute leukemia is a rapidly progressing disease involving the proliferation and accumulation of immature red and white blood cells and platelets (i.e., blasts and other very early cells).

Acute leukemia has a rapid course lasting for two to four months without treatment, and usually affects younger age groups. It is divided into two categories, depending on the cell type involved. If the disease involves the lymphocytes it is called acute lymphocytic leukemia (ALL), but if it affects the myelocytes it is known as acute myelogenous leukemia (AML).

Chronic leukemia progresses more slowly and mostly affects older adults. It permits greater numbers of fully developed blood cells to grow, allowing these cells to carry out some of their normal functions. This type of leukemia is also divided in two major types; chronic lymphocytic leukemia (CLL) and chronic myelogenous leukemia (CML) (4).

2.2.1 Treatment of leukemia

Cancer therapy is based on surgery and radiotherapy, which are, when possible, rather successful regional interventions, and on systemic chemotherapy. Approximately half of cancer patients are not cured by these treatments and may obtain only a prolonged survival or no benefit at all. The aim of most cancer chemotherapeutic drugs currently in clinical use is to kill malignant tumor cells by inhibiting some of the mechanisms implied in cellular division and the antitumor compounds developed through this approach are cytostatic or cytotoxic (11).

Treatment is usually given soon after diagnosis and classification type and stage to all kinds of acute and chronic leukemia's. There are four main types in treatment of leukemia which are chemotherapy, radiotherapy, immunotherapy, bone marrow transplantation.

Treatment depends on prognostic factors, including the total white blood cells counts (WBCs), morphology of cells, cytogentics analysis, type of leukemia, response to treatment, the patient's age, whether leukemia cells are present in the cerebrospinal fluid, and health status of patient.

The chemotherapy treatment is more common in all leukemia patients. Most cytotoxic chemotherapy drugs work by disrupting the ability of cancer cells to grow and multiply. They can be administrated by several routes. When given by mouth, the drugs are rapidly absorbed into the blood stream from the gut and carried throughout the body to reach the cancer cells. Drugs that cannot be given by mouth because they are not well absorbed are injected into subcutaneous fat or muscles or infused directly into a vein; that way they reach cancer cells rapidly and can begin to work without delay (17).

2.2.1.1 Chemotherapy

Chemotherapy refers to treatment of disease by chemicals that kill cells, both good and bad, but specifically cancerous tumors. In popular usage, it refers to

antineoplastic drugs used to treat cancer or the combination of these drugs into cytotoxic standardized treatment regimen.

Most commonly, chemotherapy acts by killing cells that divide rapidly, one of the main properties of cancer cells. This means that it also harms cells that divide rapidly under normal circumstances: cells in the bone marrow, digestive tract and hair follicles (18).

A. Antineoplastic drug

Any of several drugs that control or kill neoplastic (cancer) cells. All have unpleasant side effects that may include nausea and vomiting and hair loss and suppression of bone marrow function (19).

Doxorubicin for example, is antineoplastic drug, (trade name Adriamycin) also known as hydroxydaunorubicin used in cancer chemotherapy (Figure 2.1). It is an anthracycline antibiotic, closely related to the natural product daunomycin, and like all anthracyclines it works by intercalating DNA by intercalation and inhibition of macromolecular biosynthesis (20).

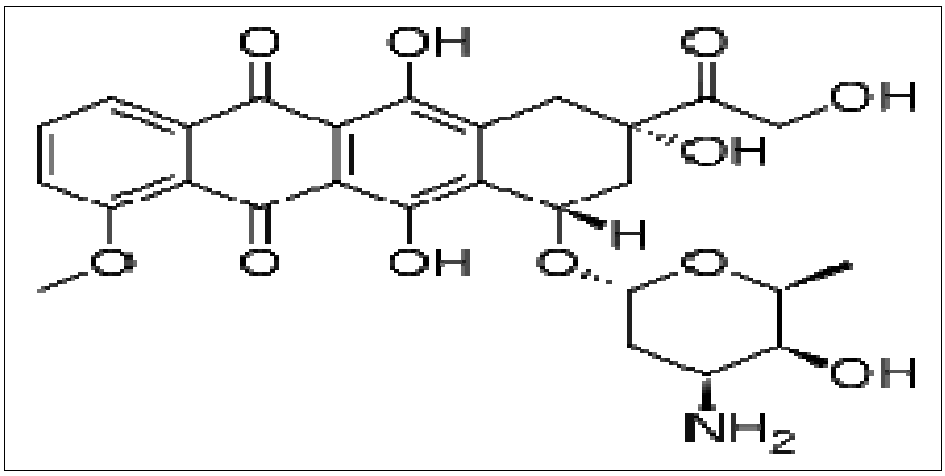

Figure 2.1 Chemical Structure of Doxorubicin drug (20).

It inhibits the progression of the enzyme topoisomerase II, which unwinds DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has

broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication (20).

It is commonly used in the treatment of a wide range of cancers, including hematological malignancies, many types of carcinoma, and soft tissue sarcomas. The drug is administered intravenously, in the form of hydrochloride salt.

All cytotoxic chemotherapy drugs have their own specific modes of action; some kill cancer cells only when they are multiplying, while others kill all cancer cells. They can be used singly or in groups that work together, referred to as combination chemotherapy. Most treatment plans for hematological cancers include combination chemotherapy and, occasionally, radiation therapy, immunotherapy and the newer treatment; monoclonal antibodies. Treatment protocols for hematological malignancies prepared according to many factors that must be take before drug choice for treatment, these factors related to severity of disease, status of patient, body response to treatment, age, weight, change in organ function, increase the risk of toxicity and WBCs count (21, 22).

Important information necessary for the optimal use of these cancer drugs includes: **a.** mechanism of action; **b.** pharmacology, including bioavailability, routes of elimination, and important drug interactions; and **c.** toxicities. Table 2.1 illustrates these parameters (23).

Table 2.1 Classes of chemotherapeutic Agents.

Name (Synonym)	Drug Class	Action	Clearance Route	Major Toxicity
Daunorubicin (Cerubidine)	Antibiotic (anthracycline)	Topoisomerase inhibition, DNA intercalation, free-radical formation	Biliary excretion, hepatic metabolism	Myelosuppression, N&V, cardiomyopathy, vesicant, red urine, mucositis
Busulfan (Myleran)	Alkylating agent	Forms DNA cross-links.	Metabolism	Myelosuppression, hepatotoxicity (veno-occlusive disease), pulmonary fibrosis
Cyclophosphamide (Cytoxan, (Neosar)	Alkylating agent	Cross-links DNA strands	Hepatic metabolism (renal)	Myelosuppression, N&V, cystitis, cardiac (high-dose)
Cytarabine (Cytosar, ara-C, cytosine arabinoside)	Antimetabolite (pyrmidine analog)	Incorporates into DNA; inhibits DNA polymerase	Hepatic metabolism	Myelosuppression, N&V, mucositis, ocular, hepatic
Docetaxel (Taxotere)	Plant alkaloid	Mitotic spindle inhibitor	Hepatic metabolism, biliary excretion	Myelosuppression, hypersensitivity
Chlorambucil (Leukeran)	Alkylating agent	Cross-links DNA	Metabolism	Myelosuppression, pulmonary toxicity, hepatotoxicity

For an antineoplastic drug to be active it must (a) be taken up into a cancer cell and (b) be converted into an active agent. It must then make its way within the cell to its target without being (c) metabolically inactivated, (d) chemically inactivated, or (e) excreted from the cell. Once it interacts with its cellular target or (g) repair the damage to the target (23) Figure 2.2.

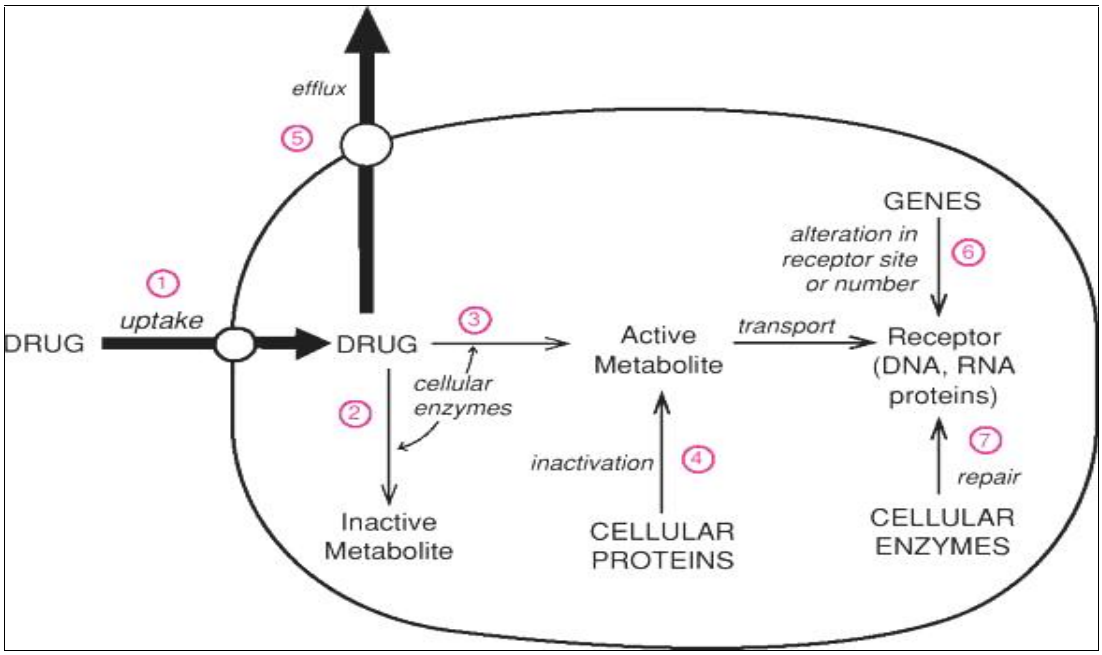

Figure 2.2. Potential pathways for antineoplastic drug disposition in tumor cells. For a drug to be effective, the drug, or its active metabolite, must reach its target site within the cell. Possible steps required for a drug to reach its receptor or to be inactivated include: (1) uptake into a cell through a particular transport protein; (2) enzymatic conversion of the drug to an inactive metabolite; (3) enzymatic conversion of the drug to its active metabolite; (4) binding of an active metabolite by a cellular protein or thiol, thereby inactivating drug; (5) excretion of the drug from the cell via an efflux transport pump; (6) alteration in the genetic makeup of the cell, changing the drug receptor site or number; and (7) changes in the ability of a cell to repair damage of a drug at its receptor (23).

B. Mechanism of antineoplastic drug action

Antineoplastic agents interfere with some essential step required for all cell growth or division. The initial target of antineoplastic drugs varies widely, from direct attack on the DNA molecule to inhibition of the formation of mitotic spindle needed for cell division. All antineoplastic agents cause a disruption in a normal cellular process so significant that it requires the cell to either quickly repair the damage or initiate the process of apoptosis (programmed cell death) so, all of these drugs lead to cell death through initiation of apoptosis.

Apoptosis is the normal physiologic process of cellular suicide, which occurs in all living organisms to eliminate unwanted, functionally abnormal, or harmful cells (24).

C. Cytotoxic drugs initiate apoptosis

An understanding of events occurring in normal cell cycle is important. The cell cycle is composed of four distinct phases during which the cell prepares for and undergoes mitosis. The G1 phase consists of cells that have recently completed division and are committed to continued proliferation. After a variable period of time, these cells begin to synthesize DNA, marking the beginning of the S phase. After DNA synthesis is complete, the end of the S phase is followed by the premitotic rest interval called the G2 phase. Finally, chromosome condensation occurs and the cells divide during the mitotic M phase. Mitosis phase (M-phase), take up only a small part of cell cycle, in most, if not all, cells, the cell cycle is temporarily halted during the G1-S-phase checkpoint and at the G2-M-phase checkpoint. At these times, cells determine whether to continue into S-phase, initiate the process of apoptosis, or undergo DNA repair (25). Passage into a new phase of cell cycle requires activation of a series of enzymes called cyclin-dependent kinases, which activate another enzymes (the cyclins) (26).

If cells are damaged by chemotherapeutic agents and are unable to repair the damage, apoptosis is initiated at the G1-S or G2-M checkpoint, provided that the mechanisms for apoptosis are in place. However, the presence, or absence, of apoptotic proteins is as important as the initial interaction between a cytotoxic drug and its effecter in determining whether tumor cell kill occurs. The antineoplastic agents provide the initial trigger for beginning the pathway to programmed cell death (Figure 2.3)

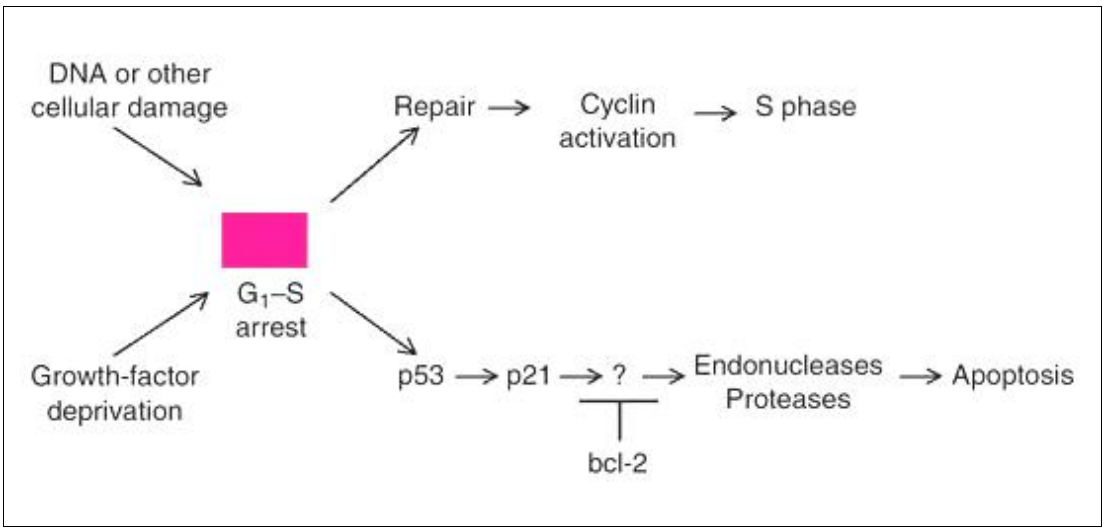

Figure 2.3. Potential pathways involved in cytotoxicity induced by chemotherapy. Chemotherapeutic drugs or growth-factor deprivation damages cells. Cells are arrested at the G_1–S checkpoint. If the damage is sublethal, it may be repaired and the cell proceeds to S phase. If significant DNA damage is present, the process of programmed cell death is initiated. Critical factors, such as p53 and p21 gene products, are required for the cell to undergo apoptosis (23).

D. Factors affecting the activity of drug

Several factors affecting the function of drug within the cell from the drug deposition within the body from the time it is administered until it reaches its target site, these factors are critical to achieving an antitumor response. For a drug to function, it must be taken into the body, and avoid being cleared from the body by metabolism or excretion. It must reach its site of action in active form without being inactivated by protein binding (Figure 2.2).

Pharmacokinetics means how a patient will handle a given dose of drug (27).

The four most important pharmacokinetic parameters are:

a. Bioavailability (drug absorption), drugs are different in bioavailability, which is the percentage of a dose of drug that reaches the plasma compartment, so the drug given intravenously, have 100% bioavailability.

b. Volume of distribution, after a drug reaches the bloodstream, it is distributed into tissues, distribution affected by drug binding to plasma proteins (usually albumin or α-acid glycoprotein).
c. Clearance where a drug can be removed per unit of time.
d. Drug half-life (28-34).

2.2.1.2 Types of chemotherapeutic drugs

Chemotherapeutic agents are classified by mechanism of action and the majority of chemotherapeutic drugs can be divided into: alkylating agents, antimetablolites, plant alkaloids, topoisomerase inhibitors, and antitumor antibiotics. All of these drugs affect cell division or DNA synthesis and cause cancer cell death (18).

A. Alkylating agents

Are so named because of their ability to add alkyl groups to many electronegative groups under intercellular conditions (aqueous solution, 37Cº, pH 7.4). DNA alkylating agents interact with resting and proliferating cells in any phase of the cell cycle, but they are more cytotoxic during the late G1 and S phases. Alkylation prevents DNA replication and RNA transcription from the affected DNA. It also leads to the fragmentation of DNA by hydrolytic reactions and also by the action of repair enzymes when attempting to remove the alkylated bases (35).

They stop tumor growth by cross-linking guanine nucleobases in DNA double-helix directly attacking DNA. This makes the strands unable to uncoil and separate, necessary in DNA replication, and the cells can no longer divide. These drugs acts mainly nonspecifically, some of them require conversion into active substances *in vivo* (e.g. cyclophosphamide). Examples of these agents include: cisplatin, carboplatin, ifosfamide, chlorambucil, busulfan and thiotepa (20).

B. Anti-metabolites

Antimetabolites can be defined as analogs of naturally occurring compounds that interfere with their formation or utilization, thus inhibiting essential metabolic routes. Anti-metabolites as purine or pyrimidine- analogues which are the building blocks of DNA. They prevent these substances from becoming incorporated into DNA during the "S" phase (of the cell cycle), stopping normal development and division. An important example is 5-Fluoro Uracil (5FU), which inhibits thymidylate synthase. Fludarabine inhibits function of multiple DNA polymerases, DNA primase, DNA ligase I and is S phase-specific (since these enzymes are highly active during DNA replication). Methotrexate (being folate antagonist) inhibits dihidrofolate reductase, enzyme essential for purines and pyrimidines synthesis (18).

C. Plant alkaloids

These alkaloids are derived from plants and block cell division by preventing microtubule synthesis and mitotic spindle formation, which is vital for cell division and without them it can not occur. Microtubules are the main target of cytotoxic natural products. Drugs acting on microtubules bind to several sites of tubulin and at different positions of the microtubules but they all suppress microtubule dynamics, thereby blocking mitosis at the metaphase/anaphase transition and inducing cell death.

The main examples are vinca alkaloids such as vincristine, vinblastine and vinorelbine which bind to specific sites on tubulin, inhibiting the assembly of tubulin into microtubules. The new group of taxanes paclitaxel (from *Taxis brevifolia*) with its synthetic derivate (docataxel) inhibits cell division by stimulating tubulin polymerisation, thus enhancing formation and stability of microtubules (18).

D. Topoisomerase inhibitors

Identical loops of DNA having different numbers of twists are topoisomers, that is, molecules with the same formula but different topologies, and their interconversion requires the breaking of DNA strands. DNA topoisomerases are enzymes that regulate the three-dimensional geometry (topology) of DNA, leading to the interconversion of its topological isomers and to its relaxation. This is related to the regulation of DNA supercoiling, which is essential to DNA transcription and replication, when the DNA helix must unwind to permit the proper function of the enzymatic machinery involved in these processes (36).

Topoisomerases are essential enzymes which maintain the topology of DNA. Inhibition of type I or type II topoisomerases interferes with both transcription and replication of DNA by upsetting proper DNA supercoiling. Some type I topoisomerase inhibitors include camptothecins: irinotecan and topotecan. Examples of type II inhibitors include amsacrine, etoposide, etoposide phosphate, and teniposide. The latter are semisynthetic derivatives of epipodophyllotoxins, alkaloids naturally occurring in the root of mayapple (*Podophyllum peltatum*) (18).

E. Antitumour antibiotics

Many anticancer drugs in clinical use (e.g. anthracyclines, mitoxantrone, and dactinomycin) interact with DNA through intercalation, which can be defined as the process by which compounds containing planar aromatic or heteroaromatic ring systems are inserted between adjacent base pairs perpendicularly to the axis of the helix and without disturbing the overall stacking pattern due to Watson–Crick hydrogen bonding.

There are many differing antitumour antibiotics, but generally they prevent cell division by several ways: (1) binding to DNA through intercalation between two adjacent nucleotide bases and making it unable to separate, (2) inhibiting ribonucleic acid (RNA), preventing enzyme synthesis and (3) interfering with cell replication. They are products of various strains of the soil fungus *Streptomyces*. Examples are anthracyclines (doxorubicin and daunorubicin

(which also inhibit topoisomerase II), actinomycin, bleomycin, mitomycin and plicamycin. Bleomycin acts in unique way through oxidation of a DNA-bleomycin-Fe (II) complex and forming free radicals, which induce damage and chromosomal aberrations (18).

2.3 Drug resistance

It is obvious that cancer chemotherapy is a very difficult task. One of its main associated problems is the nonspecific toxicity of most anticancer drugs due to their biodistribution throughout the body, which requires the administration of a large total dose to achieve high local concentrations in a tumor. Drug targeting aims at preferred drug accumulation in the target cells independently of the method and route of drug administration (37). One approach that allows improving the selectivity of cytotoxic compounds is the use of prodrugs that are selectively activated in tumor tissues, taking advantage of some unique aspects of tumor physiology, such as selective enzyme expression, hypoxia, and low extra cellular pH. More sophisticated tumor-specific delivery techniques allow the selective activation of prodrugs by exogenous enzymes (gene-directed and antibody-directed enzyme prodrug therapy). Furthermore, the increased permeability of vascular endothelium in tumors (enhanced permeability and retention, EPR effect) permits that nanoparticles loaded with an antitumor drug can extravasate and accumulate inside the interstitial space, where the drug can be released as a result of normal carrier degradation (38).

Another problem in cancer chemotherapy is drug resistance. After the development of a resistance mechanism in response to a single drug, cells can display cross-resistance to other structural and mechanistically unrelated drugs, a phenomenon known as multidrug resistance (MDR) in which ATP-dependent transporters has a significant role (39). Resistance to anticancer drugs may be intrinsic resistance present before treatment or acquired developed during chemotherapy treatment.

During disease progression, leukemia cells acquire a number of genetic alterations, most probably because of increased genomic instability, that may explain the aggressive phenotype, chemotherapeutic drug resistance, and poor

prognosis. Despite the exciting results obtained with drugs like imatinibe mesylate, CML patients eventually show resistance at rate of 80% in blastic phase, 40-50% in acute phase, and 10% in chronic phase post-interferon α failure, at 2 years (40). Identification of the molecular basis of resistance is important, because it could provide insight into disease progression and into the design of novel therapeutic strategies to prevent and overcome treatment resistance.

Drug resistance is setting one of the major obstacles in the successful treatment and is an important cause of death in acute leukemia. Drug resistance, refers to the ability of cancer cell to resist the action of anticancer drug and inhibit its action. Such resistance may be present before beginning treatment or may develop during chemotherapy. Drug resistance that extends to structurally and functionally unrelated drugs is termed multidrug resistance (MDR) (41).

2.3.1 Classification of drug resistance

The efficacy of cytostatic antineoplastic therapy is determined by a sequential cascade of events, including drug delivery, drug-target interaction and the induction of cellular damage. The first part of this cascade corresponds to the pharmacological resistance, and up to now has been the most widely studied mechanism of resistance. Classically, resistance is divided into extrinsic and intrinsic causes (42).

■ **Extrinsic resistance** corresponds to the inability of the drug to reach the tumor cell: this is the case when the bioavailability of the oral form varies greatly from patient to patient, as with 6-mercaptopurine in ALL (43). Defects in tumor vascularisation, frequently observed in solid tumors, are also probably relevant for hematological malignancies (44).

■ **Intrinsic resistance** is directly due to the properties of the tumor cell. This phenomenon can be observed *in vitro*, and can be classified as simple resistance, when the cells are resistant to only one drug, or as multidrug resistance, when a cross-resistance is observed for chemostatic drugs with different biochemical targets. This latter type of resistance is mainly observed in patients, and can be due to several mechanisms. The underlying

pharmacological mechanism corresponds mainly to an active efflux of the drugs out of the tumor cells. Molecular profiles giving rise to broader forms of resistance are now under investigation, and it is believed that a defect in drug-induced apoptosis is at least partly responsible. This could be due to increases in anti-apoptotic signals (survival signals from the micro-environment) and/or increases in anti-apoptotic proteins (ex: bcl-2) or decreases in pro-apoptotic proteins (ex: bax) (Figure 2.4) (42).

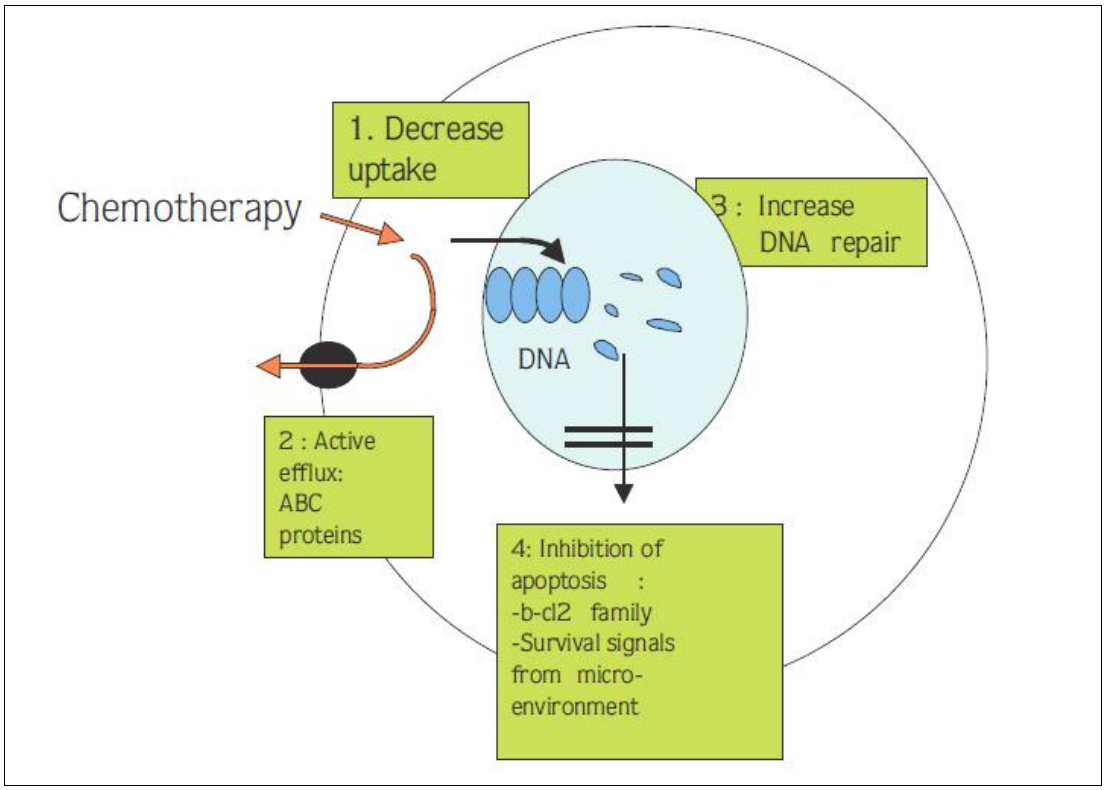

Figure 2.4. Drug resistance in leukemia (42)

2.3.2 Mechanisms of drug resistance

Thirty-one years ago, Goldie and Coldman proposed a mathematical model for drug resistance, assuming that selected subclones of cancer cells eventually became resistant to chemotherapeutic drugs, owing to a high spontaneous mutation rate (45). These cells could escape the effect of cytotoxic drugs, through decreased uptake, increased catabolism, decreased transformation of a prodrug, modification of drug target, increase in DNA repair or resistance to

drug-induced apoptosis. Over the last 25 years, experimental models and clinical research have identified several causes of drug resistance in tumors (46).

2.3.3 Multidrug resistance

It was clinically observed that tumor cells resistant to a class of drugs, are usually also cross-resistant to chemotherapy with a different target. This phenomenon, called MDR, was described *in vitro* by Biedler and Riehm during the 1970s in Chinese hamster ovary cells and is now recognized as a most frequent phenotype developed in cultured tumor cells exposed to anthracyclines or vinca alkaloids (47). This MDR phenotype confers to the cells cross-resistance to a broad range of structurally and functionally unrelated cytotoxic agents, sharing common properties: all are plant or microbial products, known as xenobiotics. The resistant tumor cells maintain lower intracellular drug concentrations than do their sensitive counterparts, and in the large majority of cases express transport proteins of the ABC (ATP-binding cassette) superfamily, responsible for the active efflux of these drugs. More recently, other mechanisms of resistance to a broad spectrum of drugs have been described: increase in DNA repair, and defects of drug-induced apoptosis, either due to strong survival signals delivered to the tumor cells by microenvironment, or because of a defect in the apoptosis pathway (figure 2.5). In man, mutations in genes encoding ABC transporters underlie diverse genetic diseases including cystic fibrosis, Tangier disease, Dubin–Johnson syndrome, sight disorders and adrenoleukodystrophy (48).

2.4 ATP-binding cassette proteins (ABC)

2.4.1 Transport across cell membranes

The cell membrane is not simply a passive barrier, but provides the major interface between the cytoplasm of the cell and the extracellular milieu. Many proteins are ion channels and facilitators –proteins which permit the passive (energy independent) movement of a solute across the membrane down its electrochemical gradient.

ABC (ATP-binding cassette) proteins form one of the largest protein families and members of this family are found in all living organisms from microbes to humans. The wide-spread presence of these proteins with a relatively conserved structure and function suggests a fundamental role. The number of ABC transporters differs widely between species. Eukaryotic cells generally have fewer ABC transporters, presumably because other more sophisticated mechanisms for moving solutes across membranes have evolved.

These transmembrane proteins are specialized in energy dependent cellular transport. The encoding MDR genes are highly conserved between species, from bacteria to man. The role of these proteins is mainly protection against xenobiotics (46).

The minimum structure of the protein is an ABC unit of 200–250 amino acids, consisting of consensus Walker A and B motifs and the ABC signature, located between the two Walker domains, for ATP binding, and six transmembrane domains (TMDs) (such as ABCG2). The more common structure consists of two ABC and 12 transmembrane domains (ABCB1 or P-glycoprotein), and a few members have five more transmembrane domains, with an external N-termination (ABCC1–2–3) (Figure 2.5) (46).

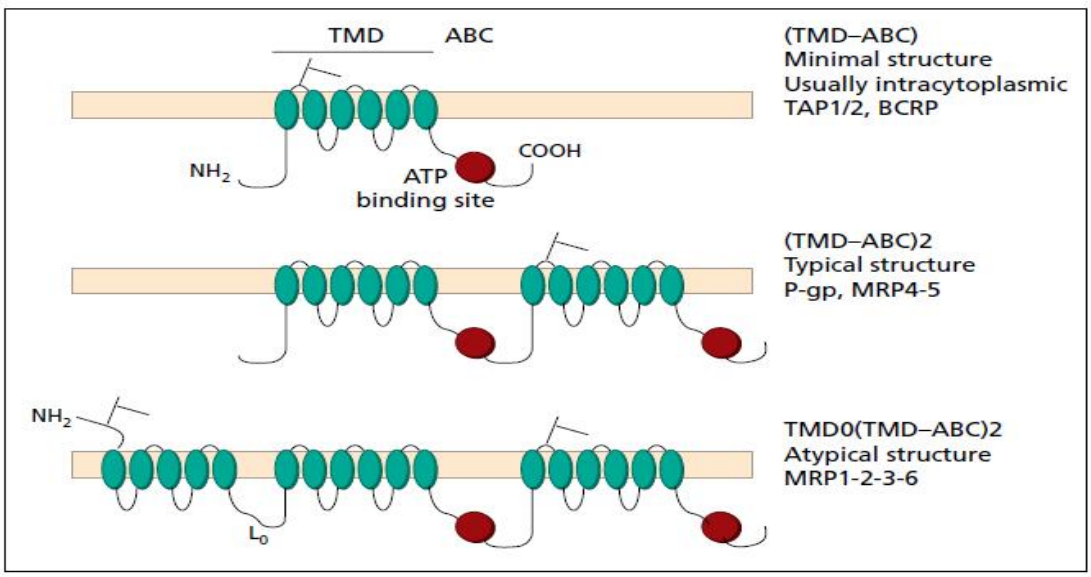

Figure 2.5 Structure of ABC proteins. TMD, transmembrane domain; ABC, ATP-binding cassette; TAP1/2, transporters associated with antigen processing (46).

In the ABC proteins which act as primary active transporters, the transport function depends on the hydrolysis of ATP within the nucleotide-binding domains (NBDs). These cytoplasmic domains are attached to the intracellular regions of the TMDs, and a close interaction provides the functional connection between these two different domains. The nucleotide-binding domains bind cytoplasmic ATP and, in the active transporters, ATP hydrolysis ensures the energy for the uphill transport of a substrate. The specific, close interaction of NBDs with the TMDs provides the transmission gear of the conformational changes caused by substrate binding and the hydrolysis of ATP (49-51).

2.4.2 Mechanism of transport

Transport clearly involves major conformational changes and a conventional enzyme-like mechanism. The transport cycle is initiated by the interaction of substrate with a specific binding site(s) on the TMDs. Substrate binding induces a conformational change in the TMDs, which is transmitted to the NBDs to initiate ATP binding. There is now compelling, but not yet conclusive, evidence that it is ATP binding (rather than hydrolysis) which induces the major conformational changes responsible for altering the affinity and orientation of the substrate-binding site(s) such that substrate is released at the extracellular face of the membrane. Subsequent ATP hydrolysis and ADP/Pi release ‘resets’ the transporter for another cycle. Both NBDs bind and hydrolyze ATP, and there is strong evidence in support of the ‘alternating catalytic cycle’ mechanism. However, it is still unclear whether 1 or 2 ATP molecules are hydrolyzed per molecule of substrate transported; determination of this number, together with determination of the exact number of substrate-binding sites and the nature of the conformational changes involved, is crucial to complete elucidation of the transport cycle (48).

2.4.3 Human ATP binding cassette gene subfamily

The existing eukaryotic genes can be grouped into major subfamilies. A few genes do not fit into these subfamilies, and several of the subfamilies can be further divided into subgroups, (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and

ABCG). The human ABCA subfamily comprises 12 full transporters that are further divided into two subgroups based on phylogenetic analysis and intron structure (52, 53). The first group includes seven genes dispersed on six different chromosomes (ABCA1, ABCA2, ABCA3, ABCA4, ABCA7, ABCA12 and ABCA13), whereas the second group contains five genes (ABCA5, ABCA6, ABCA8, ABCA9 & ABCA10) arranged in a cluster on chromosome 17q24 (54).

The ABCB subfamily is unique in mammals in that it contains both full transporters and half transporters. Four full transporters and seven half transporters have currently been described as members of this subfamily. ABCB1 (MDR/PGY1) is the first human ABC transporter cloned and characterized through its ability to confer a MDR phenotype to cancer cells.

The ABCC subfamily contains 12 full transporters with a diverse functional spectrum that includes ion transport, cell-surface receptor, and toxin secretion activities. The Cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7) protein is a chloride ion channel that plays a role in all exocrine secretions; mutations in CFTR cause cystic fibrosis (55). ABCC8 and ABCC9 proteins bind sulfonylurea and regulate potassium channels involved in modulating insulin secretion. The rest of the subfamily is composed of nine MRP-related genes. Of these, ABCC1, ABCC2, and ABCC3 transport drug conjugates to glutathionine and other organic anions. The ABCC4, ABCC5, ABCC11, and ABCC12 proteins are smaller than the other MRP1-like gene products and lack an N-terminal domain (56) that is not essential for transport function (57).

The ABCD subfamily contains four genes in the human genome and two each in the *Drosophila melanogaster* and yeast genomes (58). All of the genes encode half transporters that are located in the peroxisome, where they function as homo- and/or heterodimers in the regulation of very long chain fatty acid transport.

The ABCE and ABCF subfamilies contain gene products that have ATP-binding domains that are clearly derived from ABC transporters but they have no TM

domain and are not known to be involved in any membrane transport functions (54).

The human ABCG subfamily is composed of six “reverse” half transporters that have an NBF at the N terminus and a TM domain at the C terminus. The mammalian ABCG1 protein is involved in cholesterol transport regulation (59). Other ABCG genes include ABCG2, a drug-resistance gene; ABCG5 and ABCG8, coding for transporters of sterols in the intestine and liver; ABCG3, to date exclusively found in rodents; and the ABCG4 gene that is expressed predominantly in the liver. The functions of the last two genes are unknown.

2.4.4 ATP binding cassette genes and human genetic disease

Many ABC genes were originally discovered during the positional cloning of human genetic disease genes. There are 14 ABC genes have been linked to disorders displaying Mendelian inheritance like cystic fibrosis disease (ABCC7/ CFTR) and adrenoleukodystrophy (ABCD1/ALD) which are lethal diseases (60).

Cells exposed to toxic compounds can develop resistance by a number of mechanisms including decreased uptake, increased detoxification, alteration of target proteins, or increased excretion. Several of these pathways can lead to multidrug resistance (MDR) in which the cell is resistant to several drugs in addition to the initial compound. This is a particular limitation to cancer chemotherapy, and the MDR cell often displays other properties, such as genome instability and loss of checkpoint control, that complicate further therapy. ABC genes play an important role in MDR, and at least six genes are associated with drug transport (61).

Three ABC genes appear to account for nearly all of the MDR tumor cells in human, these are ABCB1 /PGP/MDR1, ABCC1 /MRP1, and ABCG2 /MXR/BCRP (61) (Table 2.2).

Table 2.2 ABC transporters involved in drug resistance (54).

Gene	Substrates
ABCB1	Colchicine, doxorubicin, etoposide, adriamycin, vinblastine, digoxin, saquinivir, paclitaxel
ABCC1	Doxorubicin, daunorubicin, vincristine, VP16, colchicines, VP16, rhodamine
ABCC2	Vinblastine, sulfinpyrazone
ABCC3	Methotrexate, etoposide
ABCC4	Nucleoside monophosphates
ABCC5	Nucleoside monophosphates
ABCG2	Mitoxantrone, topotecan, doxorubicin, daunorubicin, CPT-11, rhodamine

2.4.5 ATP binding cassette transporters in tumor

The multidrug resistance phenotype in tumors is associated with the overexpression of certain ABC transporters, termed MDR proteins. There are two ABC transporters, which have been definitely demonstrated to participate in the multidrug resistance of tumors: the multidrug resistance protein 1 (MRP1/ABCC1) (62-64), and the mitoxantrone resistance protein (MXR/BCRP or ABCG2) (65). Furthermore, other human ABC proteins capable of actively transporting various compounds out of the cells may also be players in selected cases of multidrug resistance. These include the homologues of MRP1, MRP2-MRP5. MRP2 and MRP3 seem to be key players in organic conjugate transport in various tissues, while MRP4 and MRP5, may have special functions as nucleoside transporters (66-70). The three major proteins involved in cancer MRP1 (multidrug resistance protein 1, ABCC1), lung resistance related protein (LRP) and the ABCG2 multidrug transporter (BCRP/ MXR). MDR1 and MRP1 can recognize and transport a large variety of hydrophobic drugs, and MRP1 can also extrude anionic drugs or drug conjugates (71-74).

2.4.6 Multidrug resistance-associated protein (MRP) family (ABCC1–6 genes)

The members of the ABCC family (MRP1–6; 'multidrug resistance-associated protein') have low homology (15%) with MDR1, and mainly act together with glutathione (figure 2.6).

The ABCC1 (MRP1) gene maps to chromosome 16p13.1 and is expressed in tumor cells (75). ABCC1 is adjacent to the ABCC6 gene, and one of these genes undoubtedly arose by gene duplication. It encodes a full transporter that is the principal transporter of glutathione-linked compounds from cells. The ABCC1 gene was identified in the small cell lung carcinoma cell line NCI-H69, a multidrug-resistant cell that does not overexpress ABCB1 (76). The ABCC1 pump confers resistance to doxorubicin, daunorubicin, vincristine, colchicines, and several other compounds, very similar profile to that of ABCB1 (77). However, unlike ABCB1, ABCC1 transports drugs that are conjugated to glutathione by the glutathione reductase pathway (75, 78–81). Anthracyclines and vinca alkaloids, both weak organic bases, are conjugated with GSH, but are transported together with free GSH (Figure 2.6). MRP1 and -2 are particularly involved in the transport of chemotherapeutic agents in human cancer cells.

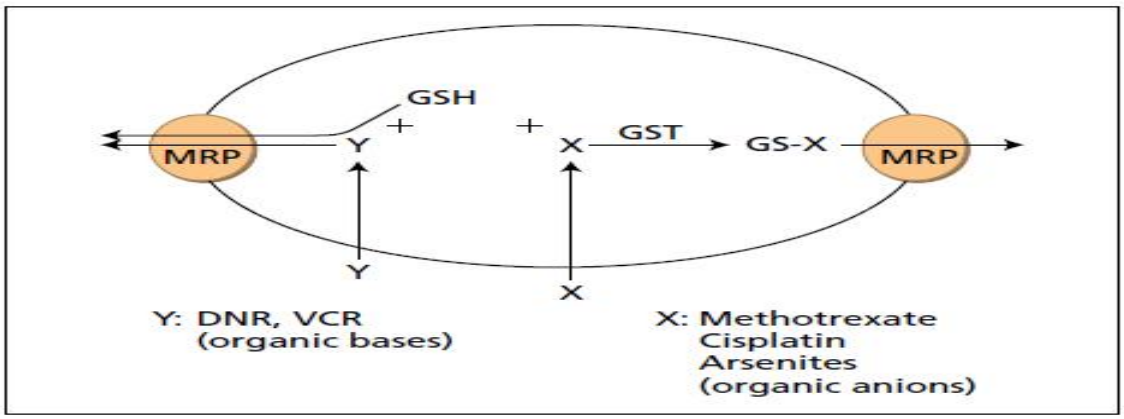

Figure 2.6 MRP1 and -2 mediated drug efflux. Two modalities of drug transport are shown: (I) the GS-X complete is expelled by MRP: organic anions such methotrexate and ciplatine; (ii) Y is contransported by MRP in the presence of GSH: organic bases such as anthracyclines and alkaloids (46).

2.4.6.1 MRP1 and leukemia

The role of other MRPs is still under investigation. Few publications have concerned MRP in ALL, but all showed a measurable level of this protein at diagnosis, comparatively higher than in AML, and some cases showed an increase after treatment. CLL cells express variable levels of MRP at diagnosis and after treatment, but the amount of MRP does not influence the course of the disease (Table 2.3) (46).

2.4.7 Lung resistance protein/major vault protein

A wide variety of P-gp negative multidrug-resistant cancer cell lines expressed the ‘lung resistance-related protein’ (LRP), identified as the major vault protein (MVP). Vaults are not ABC proteins, but cytoplasmic organelles, a small portion of which is localized in the nuclear membrane and nuclear pore complex, and are supposed to mediate the bidirectional transport of a variety of substrates between the nucleus and the cytoplasm. LRP was initially identified in an anthracycline-resistance, non-small cell lung cancer cell line that lacked P-gp overexpression (82). The LRP protein decrease the effectiveness of cytotoxic drugs, either by regulating nucleucytoplasmic transport of cytotoxic drugs away from the nucleus and/ or by involvement in sequestration of cytotoxic drugs in exocytotic vesicles (83) The LRP gene is located on chromosome 16 (16q11.2), close to the MRP1 gene, and encodes a 110-kDa protein (84). LRP has been reported to be involved in resistance to vincristine, doxorubicin and etoposide (85).

In the majority of cases of AML, LRP/MVP is high, and detectable by RT-PCR, and the clinical significance of LRP expression becomes related to leukemia management (Table 2.3). In relapsed childhood ALL, LRP expression was associated with an increased *in vitro* resistance to daunorubicin. In summary, at least four ABC pumps and one non-ABC protein are able to expel several non-related cytotoxic drugs from the leukemia cells, with probable redundancy (Figure 2.7).

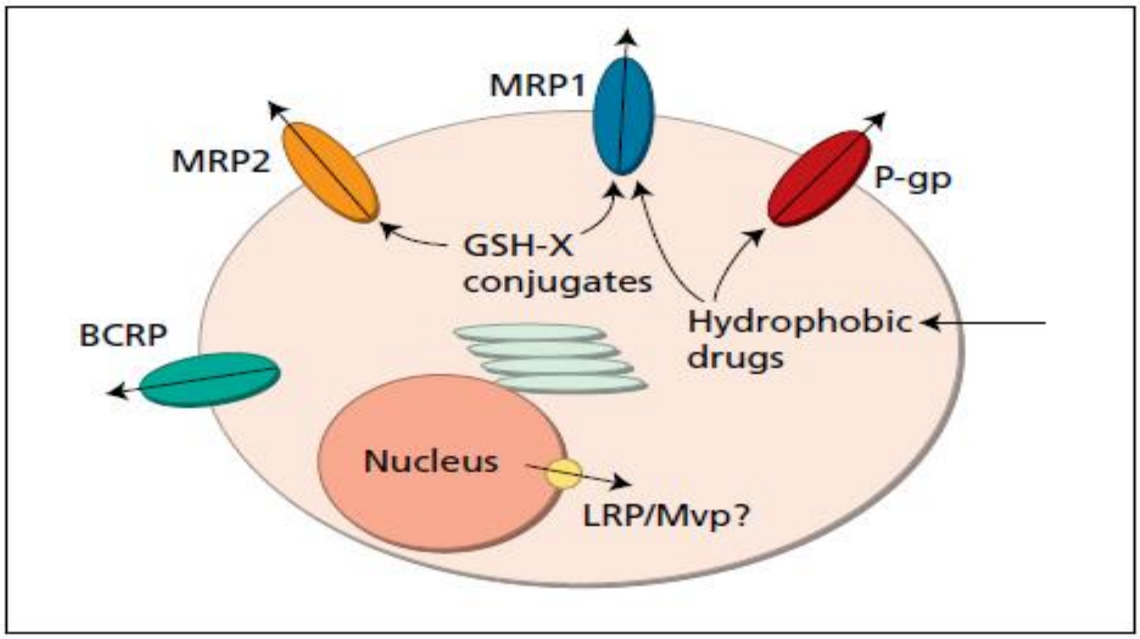

Figure 2.7 Efflux pumps involved in drug efflux in leukemia cells. For LRP/MRP, The demonstration of drug efflux was, until now, not proven by transfection are antisense experiments (46).

2.4.8 ATP-binding cassette sub-family G member 2 gene (ABCG2 gene)

Another member of the ABC superfamily is ABCG2, or breast cancer resistance protein (BCRP). BCRP is a 655- amino acid transmembrane protein coded on chromosome 4q21-22 (86).

It was originally isolated from a breast cancer cell line, MCF-7Adrvp. A 95 KDa protein (P-95) was found to be increased in resistant cell line compared to the parental cell line (87).

BCRP probably function as homodimer or a homotetramer (88). It is suspected that ABCG2 functions as a homodimer because transfection of the gene into cells confers resistance to chemotherapeutic drugs (89). Variations at residue 482 of ABCG2 are found in many resistant cell lines, and the alteration of the wild-type arginine at this position for either threonine or glycine imparts the ability to transport rhodamine and alters the substrate specificity (90).

It is highly expressed by normal stem cells (88), including hematopoietic cells (91), but its physiological role is not fully understood. Its abundant expression in human placenta (92) implies a role in fetal protection and its importance in protection against dietary compounds. In normal hematopiotic stem cells, the expression of BCRP decrease during differentiation, BCRP is also expressed by the so-called "leukemia stem cells" in AML, resulting in lower drug accumulation in these cells compared to more differentiated leukemia cells (93).

Mitoxantrone, like doxorubicin and daunorubicin (94, 95), was initially shown to be a substrate of BCRP. The most important is the ability to extrude anthracyclines and mitoxantrone out side the leukemia cell. The expression and clinical importance of BCRP in leukemia patients have been studied in recent years (Table 2.3).

Table 2.3 Summary of previous studies relating MRP1, LRP and BCRP levels of expression and different leukemia types.

No.	Drug resistance gene	Technique	Correlation	Correlation with treatment	# Ref.
1.	MRP1	RT-PCR	Expression in both ALL & AML.	MRP gene expression was found to be higher in pts. With relapsed de novo AML than pts. Before treated.	96
2.	MRP1 & MDR1	RT-PCR	Expression in de novo AML.	Expression of MRP1 gene tended to be higher in relapsed pts. Than newly diagnosed pts. MDR1 and MRP expression correlated to higher rate of drug resistance.	97
3.	BCRP	RT-PCR	Expression of ALL & AML.	High levels of BCRP gene expression **c**onfer resistance to mitoxantrone, doxorubicin, daunorubicin, and topotecan.	98
4.	MRP1	RT-PCR	Expression in CLL	MRP1 gene expression was significantly high in relation to chemotherapy salvage.	99
5.	MRP1 & LRP	RT-PCR	Expression in ALL & acute non-lymphocytic leukemia group.	Expression of MRP1 & LRP gene associated with decreased in remission rate.	100
6.	LRP & MRP	RT-PCR	Expression in AML	Expression of MRP1 & LRP genes related with relapsed or poor prognosis of chemotherapy treatment.	101
7.	MDR1, MRP1 & LRP	RT-PCR	Expression in ALL	Only increased exp. of LRP was related to worsened event free survival (P=0.05) and related to increased risk of relapsed or death.	102
8.	MRP1 & LRP	RT-PCR	Expression in ALL	Increased of MRP1 & LRP with decreased CR rate.	103
9.	MDR1,MRP1, LRP & BCRP	RT-PCR	Expression in ALL	MDR1 exp. was significantly higher at relapse than at diagnosis.	104
10.	MDR1, MRP1 & LRP	RT-PCR	Expression in acute leukemia	Pts. expressed both MRP1 & LRP m-RNA had poorer outcomes and had worse 2-Yr survival.	105

Chapter Three

Materials and Methods

3.1 Materials

3.1.1 Reagents

Table 3.1 Chemical reagent used in the study.

Used reagents	Manufacturer	Country
TaqMan two-step RT-PCR Master Mix reagents kit	Applied BioSystems	USA
Lytic agent (cell Dyn 1700)	Abbott	USA
Guanidinium thiocynate	Sigma	USA
Phenol	Sigma	USA
Chloroform	Sigma	USA
Isoamyle alcohol: Chloroform (1:49)	Sigma	USA
Isopropanol	Sigma	USA
Sodium acetate	Sigma	USA
Sodium citrate	Sigma	USA
Sarcosyle (10%)	Sigma	USA
Gemsia stain	Sigma	USA
Methanol	Sigma	USA
Frosted slides	Sigma	USA

3.1.2 Chemicals and buffers

Table 3.2 Buffers used in the study.

ID	Composition
Solution D	Consist of 50 ml of guanidinium thiocynate (lysis solution) mix with 0.360 ml of Beta-Mercapto-ethanol; the solution is stable for 3 months.
Sodium citrate (0.5 M & Ph 7.0)	14.7 gm dissolved in 70 ml H2O, pH adjusted 7 by (NaOH), the volume was completed to 100ml.
Sarcosyle (10%)	Weight 10 gm sarcosyle and dissolved in 100 ml H2O (DEPC water).
Sodium acetate (2M & pH 4.0)	16.4 gm dissolved in 100 mlH2O,adjust pH t0 4 by (HCL), complete the volume to 100ml.

3.2 Instruments

Table 3.3 The Instruments used in the study**.**

Used Instruments	Supplier
7500 Real Time PCR	Applied Biosystem, USA
Thermal Cycler	Eppendrof, Germany
Vortex	Hettachi, Germany
Microscope	Olympus, Germany
Micropipette 1--10 µL	Hettachi, Germany
Micropipette1--100 µL	Hettachi, Germany
Micropipette10-1000 µL	Hettachi, Germany
Refrigerated microcentrifuge	Eppendrof, Germany
Refrigerator (- 80 Cº)	Revco, USA
Ice container	Eppendrof, Germany

3.3 Ethical considerations

All parts of the present study were performed in accordance with the Helsinki Declaration of 1975 (106). The approval letter for the present study was

obtained from the Helsinki committee and the Palestinian Ministry of Health (MOH) **(Annex 1, 2)**. In addition, all the subjects involved in the present study gave there oral consent to be involved in the study.

3.4 Target population

This study is a cross sectional study involving a convenient sample within a definite time period. A study sample of 70 leukemia Palestinian patients was collected from both sexes who are admitted to the hematology departments of Al-Shefa hospital, European Gaza Hospital and EL-Nasser pediatric hospital of Gaza strip. A control sample of 35 healthy persons was collected from both sexes and matched age range of patients.

3.5 Settings and place of work

The practical parts of this work were performed in the Molecular Biology Department of the central laboratory, MOH and in the Biology Department laboratory of Islamic University of Gaza.

3.6 Patients data

Patient's medical data were collected from their records in the relevant hospitals. The data included personal, medical, management and family information (e.g. age, type of disease, severity of case, date of diagnosis of disease, treatment and types, protocols of treatments, prognosis, previous tests results and others) **(Annex 3)**.

3.7 Sample collection

Samples were collected from both groups during a six-month period. All patients admitted to Hematology Departments who were newly diagnosis or diagnosed and treated according to leukemia protocols during the period 1/05/2009-1/11/2009 were included. Two peripheral EDTA whole blood samples were collected from each case or control. One subjected to hematological investigation (CBC & blood film), and the second for Molecular investigations (RNA extraction and RT-PCR).

3.7.1 Hematological investigation

a. Complete blood count: The complete blood count (CBC) screening test has many applications, and it can help identify a wide variety of diseases. It is used to measure red blood cell and white blood cell count, total amount of hemoglobin in the blood, hematocrit (the amount of blood composed of red blood cells) and mean corpuscular volume (the size of red blood cells). Results can help detect problems such as dehydration or loss of blood, abnormalities in blood cell production and life span, as well as acute or chronic infection, allergies, and problems with clotting. The test was performed by using blood cell analyzer (cell Dyn 1700, from Abbott Company).

b. Blood Films: A blood film or peripheral blood smear is a slide made from a drop of blood that allows the cells to be examined microscopically. Blood films are usually done to investigate hematological problems (disorders of the blood cells).

A Blood films for each patient was made by placing a drop of blood on one end of a slide, and using a spreader slide to disperse the blood over the slide's length. The aim is to get a region where the cells are spaced far enough apart to be counted and differentiated.

The slide was left to air dry, after which the blood was fixed to the slide by immersing it briefly in methanol. The fixative is essential for good staining and presentation of cellular detail. After fixation, the slide was stained with Geimsa stain to distinguish the cells from each other.

3.7.2 Molecular investigation

a. RNA extraction

Total RNA was extracted from white blood cells (WBCs) using chomczynski protocol (107).

Procedure of extraction

Half ml of EDTA whole blood was placed in 2ml eppendorf tube and 1.5 ml lytic agent reagent was added and mixed by inversion 3 times. The sample was incubated on ice for 10-15 minutes, and vortexed two times during incubation. The sample was centrifuged at 400xg at 4Cº for 10 minutes. The supernatant was discarded completely and then the sediment resuspended in 1ml lytic agent by vortex mixing. It was centrifuged at 400xg at 4Cº for 10 minutes. After formation of pellet the supernatant was removed completely, the WBCs pellet was resuspended with 1ml of solution D (see chemicals & buffers) and mixed by pipetting. 0.1ml of solution (2 M sodium acetate pH 4.o) was added to the mixture, mixed by inversion. 1ml phenol was added to the mixture and mixed by inversion, and then 0.2 ml chloroform: Isoamyle alcohol (49:1) was added and mixed by inversion. The mixture was shacked vigorously for 10 minutes by vortex, and incubated on ice for 15 minutes. The tube was centrifuged at 10000xg for 20 minutes at 4Cº and the upper aqueous phase was transferred to a new 1.5 ml tube containing 1ml Isopropanol and mixed by inversion. The tube was incubated at -70 Cº for 20 minutes, and then centrifuged at 10000xg for 20 minutes at 4 Cº. The supernatant was discarded completely and the pellet was resuspended in 0.3 ml solution D. 0.3 ml of Isopropanol was added to the solution then the mixture was incubated at -70 Cº for 15 minutes. The sample was Centrifuged 10000xg at 4Cº for 10 minutes and the supernatant was completely discarded. The pellet was resuspended in 75% ethanol 0.5 ml and centrifuged at 10000xg at 4Cº for 5 minutes. The supernatant was completely discarded, the tube was air dried for 15 minutes at room temperature and dehydrated with 50 µL of H2O (nuclease free water) at 65 Cº for 10 minutes, the sample was stored at -70 Cº.

b. Quantitative RT-PCR (Reverse transcriptase-polymerase chain reaction)

The cDNA synthesis from total RNA was performed using TaqMan Reverse Transcription Reagents kit (Applied Biosystem, USA). A separate PCR reaction was preformed using previously published specific primers and protocols for each of the genes (MRP1, LRP, and BCRP) with modifications. The applied protocol was performed using the SYBR Green RT-PCR Reagents Kit (Applied

Biosystem, USA) containing: SYPR Green PCR Master Mix and TaqMan Reverse Transcription Reagents.

cDNA Synthesis

Synthesis of cDNA from 500ng total RNA samples was preformed in 10 µL volumes including 1x of the provided buffer, 5.5 mM MgCL2, 500 µM of each dNTPs, 2.5 µM of Random Hexamers, 0.4 u/l RNase Inhibitor and 1.25 u/l MultiScribe Reverse Transcriptase.

The reaction was incubated for 10 minutes at 25 Cº, followed by 48 minutes at 48 Cº and inactivation of the reverse transcriptase for 5 minutes at 95 Cº.

SYBR Green based quantization of targets transcripts

The SYBR Green PCR Master Mix, optimized for real-time PCR analysis, conveniently combines SYBR Green 1 Dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP, Passive Reference dye, and optimized buffer components in easy-to-use premix vials. SYBR Green I dye detects double-stranded DNA, and the Passive Reference dye is required for signal normalization.

The incorporation of SYBR Green I dye into a real-time PCR reaction lets detect any double-stranded DNA generated during PCR. This provides great flexibility because no target specific probes are required, and yet both specific and non-specific products will generate a signal. The use of the hot-start enzyme AmpliTaq Gold DNA Polymerase with SYBR Green I reagent minimizes non-specific product formation. SYBR Green I dyes are ideal for use in target identification (screening assays), or when only a small number of reactions are required for a given assay.

Primers Selections

All primer pairs sequences were obtained from a previously published work in which they were designed by Oligo 6.0 primer analysis software (Table 3.4).

Table 3.4 A list of primers used for real time quantitation.

Primer ID	Sequence (5'----3')	Amplicon size	Reference	position
BCRP-F	5-TGGCTGTCATGGCTTCAGTA-3	205-bp amplicon	AY289766.1	1890-1910
BCRP-R	5-GCCACGTGATTCTTCCACAA-3			2095-2115
LRP-F	5-CAGCTGGCCATCGAGATCA-3	68-bp amplicon	NM_005115.3	2059-2078
LRP-R	5-TCCAGTCTCTGAGCCTCATGC-3			2126-2147
MRP1-F	5-CAATGCTGTGATGGCGATG-3	69-bp amplicon	L05628.1	1633-1652
MRP1-R	5-GATCCGATTGTCTTTGCTCTTCA-3			1702-1725
PBGD-F	5-CTGCACGATCCCGAGACTCT-3	97-bp amplicon	BC000520.2	863-883
PBGD-R	5-GCTGTATGCACGGCTACTGG-3			960-980

Real Time PCR amplification reaction

All reactions were carried out in 20 µL final volumes using the Applied Biosystems 7500 analyzer, USA. Five milliliter of previously prepared cDNA containing about 20ng of RNA, were combined with 15 µL of premixed reaction component, containing: 225 nM of each primer and (1X) of the Sybr green Master Mix. For thermal cycling conditions refer to Table 3.5

Table 3.5 A list of stages and temperature by time of RT-PCR amplification reaction.

Stage	Temperature	Time	# of Cycle
1	50.0 Cº	2:00 min.	1
2	95.0 Cº	10:00 min.	1
3	95.0 Cº	0:15 min.	40
	60.0 Cº	1:00 min.	
4 (Dissociation)	95.0 Cº	0:15 min.	1
	60.0 Cº	1:00 min.	
	95.0 Cº	0:15 min.	
	60.0 Cº	0:15 min.	

Quantitation

The quantitation standards used for the 4 targets (MRP1, LRP, BCRP and internal control Porphobilinogen deaminase (PBGD)) were prepared by qualitatively amplifying fragments of each using conventional PCR and then extraction purification of the amplicon from gel. The concentration of each amplicon was determined by spectrophotometer measurement of its optical absorbance at 260 nm using the NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies Inc., USA).

The number of copies of each amplicon was calculated from the concentration, molecular weight and Avogadro's number using the following equation:

Molecule/ µL= (concentration/ molecular weight) X Avogadro's number.

Serial dilutions of each standard were prepared and used for standard curve construction and quantitation of each target for each patients using standard curve quantitaion method. In order to correct for any errors or deviation in sample preparation or subsequent RT-PCR experiment, the quantitaion value for each target was normalized by division by the quantitaion value of the internal control (PBGD gene), in the so called relative standard curve experiment.

3.8 Biostatistics/ Data analysis

The data was analyzed by the SPSS software (version 15). The one way ANOVA test was used for mean comparisons when indicated with a 95% confidence interval. The figures were prepared and presented by Microsoft office excel 2003 program.

3.9 Limitations of the study:

During the time of experimental work, the researcher had to repeat most of the work as a result of the bombardment of Laboratories building of the Islamic university during the aggressive Israeli intrusion to Gaza. For the same reason, the researcher had to repurchase the reagents again which increased the already high expenses of the study that the researcher personally financed.

Chapter Four

Results

4.1 Study population description

4.1.1 Gender distribution

A total of 105 samples were studied and divided into two groups: The first (case group) included 70 samples (57% were males and 43% females) from previously diagnosed leukemia patients (Figure 4.1). The second group (control) included 35 samples from normal healthy subjects (60% males and 40% females) (Figure 4.2).

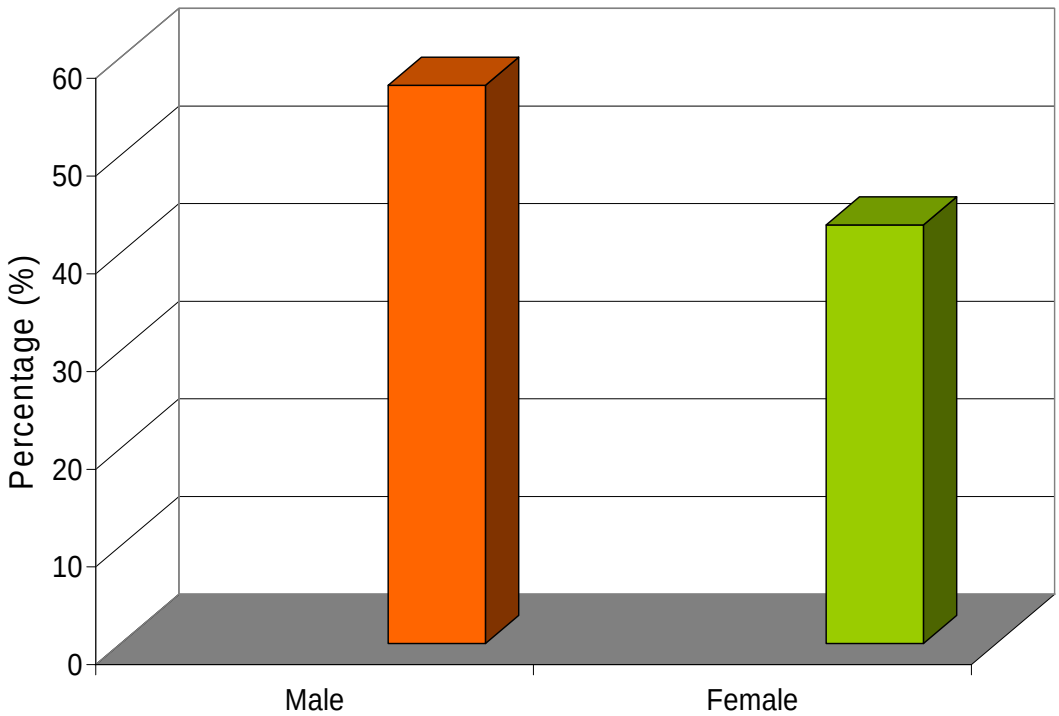

Figure 4.1 Distribution of cases by gender.

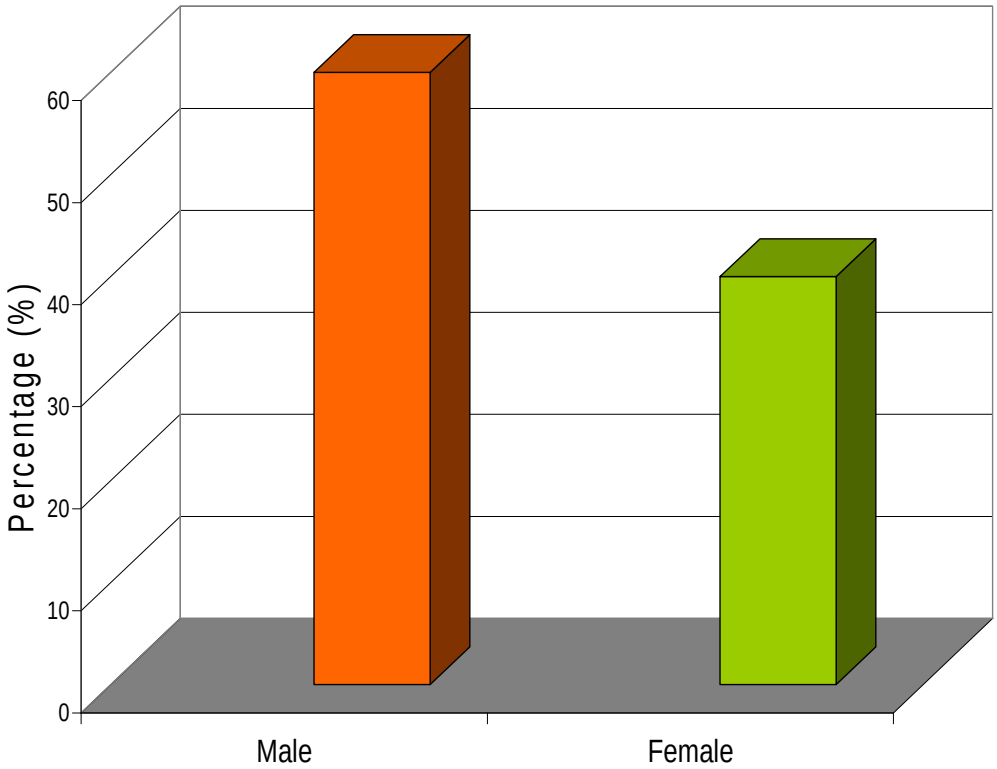

Figure 4.2 Distribution of controls by gender.

4.1.2 Living area

Leukemia patients were distributed allover Gaza strip as follows: 11.4% from North Gaza, 41.4% from Gaza City, 4.2% from Middle Area, 32.9% from Khan Younis City and 10% from Rafah City (Figure 4.3).

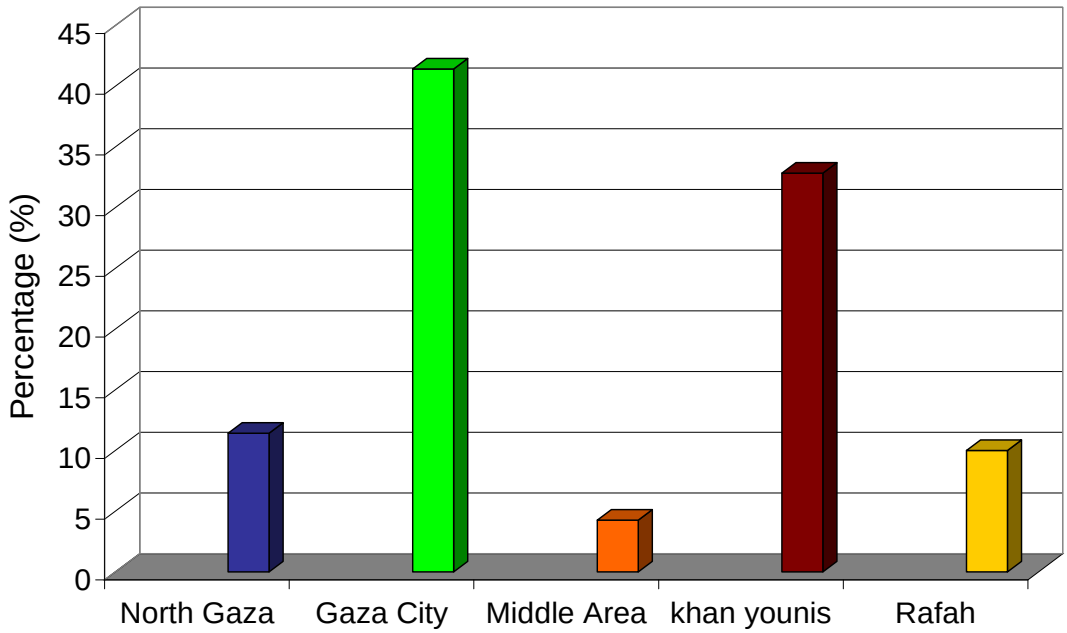

Figure 4.3 Distribution of cases by place of living.

4.1.3 Age distribution

The mean age of cases was 32.9 ±28.2 years and the mean age of controls was 27.2 ±18.8 years.

The leukemia patients were divided into age groups according to age range of incidence of each type of leukemia as follows: 33 patients (47.1%) were < 15 years old, 2 patients (2.9%) were 16-35 years old, 5 patients (7.1%) were 36-45 years old and 30 patients (42.9%) were > 45 years old (Figure 4.4).

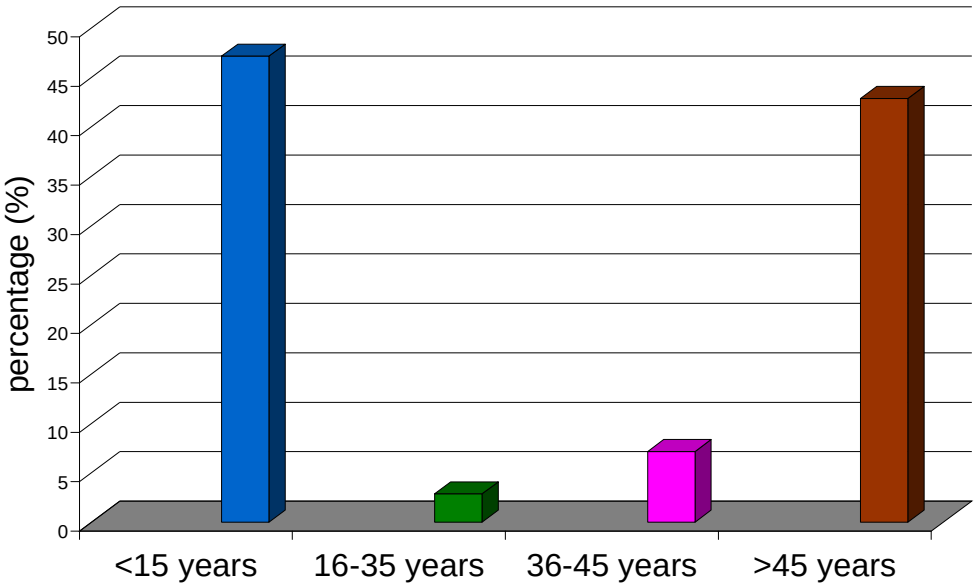

Figure 4.4 Distribution of cases by age groups.

4.1.4 Case distribution according to place of sample collection

The patient samples were collected from leukemia patients admitted to Hematology Departments of the three hospitals in Gaza Strip which were EL Shefa Hospital (13 cases, 18.6%), European Gaza Hospital (28 cases, 40%) and EL Nasser pediatric Hospital (29 cases, 41.4%) (Figure 4.5).

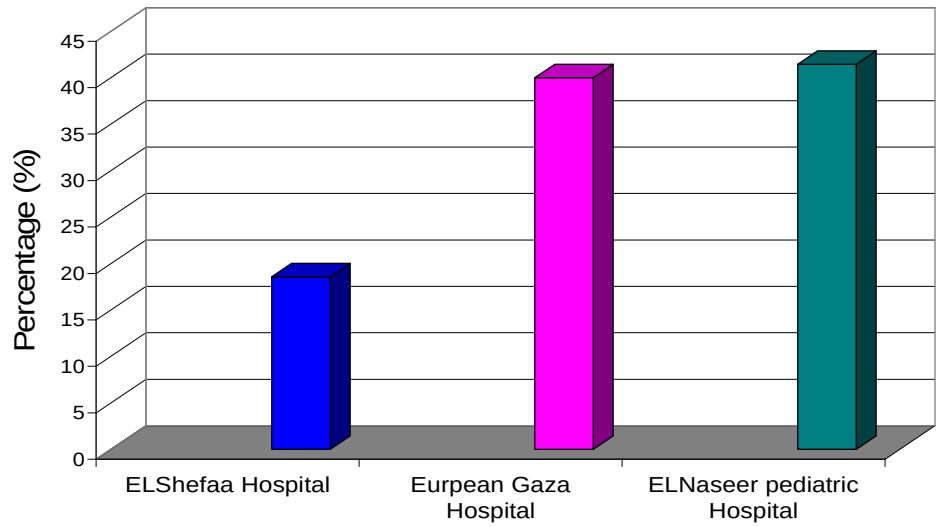

Figure 4.5 Distribution of leukemia samples by place of collection.

4.1.5 Distribution of cases by type of leukemia.

Five types of leukemia were included in this study. Thirty cases (42.9%) were acute lymphoblastic leukemia, 5 cases (7.1%) were acute myeloblastic leukemia, 12 cases (17.1%) were chronic lymphoblastic leukemia, 22 cases (31.4%) were chronic myeloblastic leukemia and 1 case (1.4%) was small lymphoblastic leukemia (Figure 4.6)

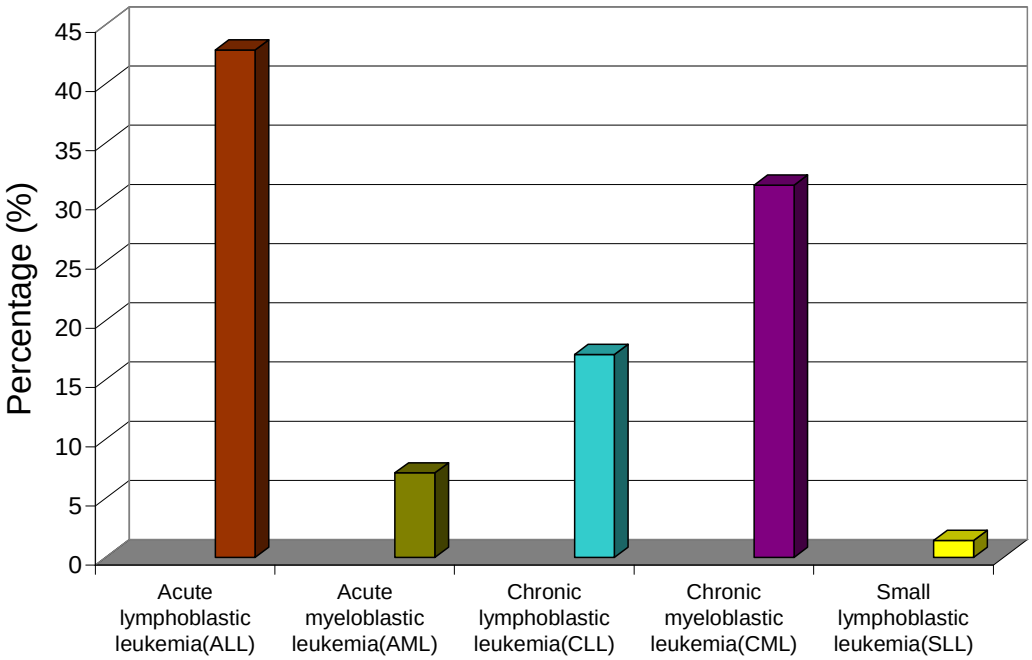

Figure 4.6 Distribution of cases by type of leukemia.

The diagnosis of each type of leukemia was confirmed by blood film at time of sample collection (Figure 4.7) and other hematological investigation (CBC).

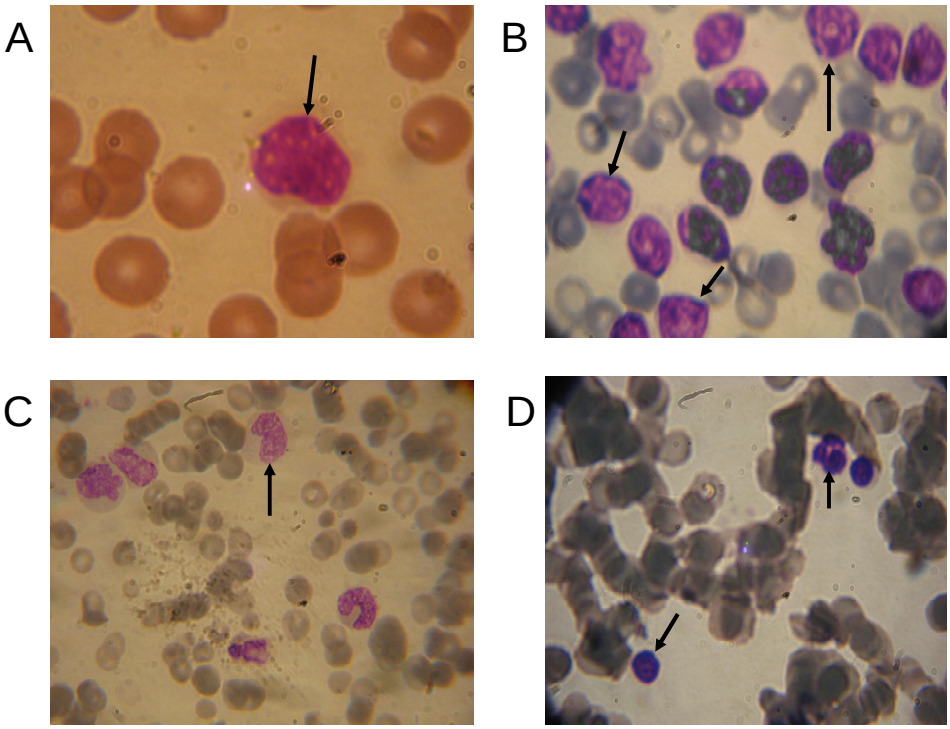

Figure 4.7 A representative microphotograph of a blood film showing blast cells:

(A) A myeloblast cell in AML case, (B) Blast cells in CLL case, (C) A metamyelocyte cell in CML case and (D) Lymphocyte cells in ALL case. The blast cells in all types are pointed by arrows.

4.1.6 Types of cases management

Out of the 70 patients, 65 cases (92.9%) received chemotherapy for leukemia management, whereas 5 cases (7.1%) received chemotherapy and bone marrow transplantation in "Israel" and Egypt (Figure 4.8).

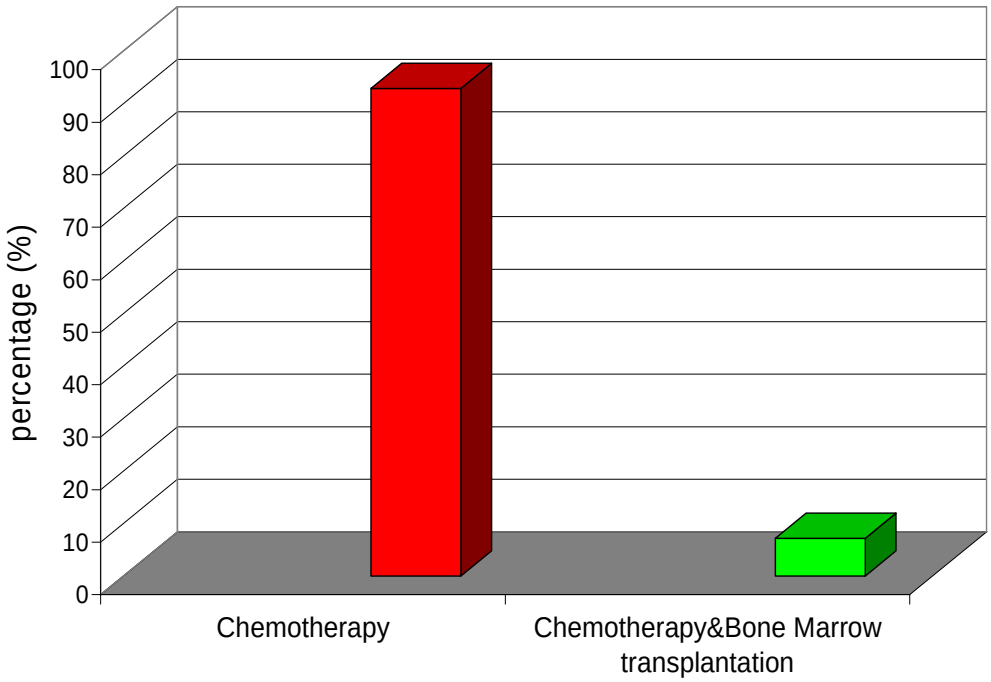

Figure 4.8 Distribution of cases by type of managements.

Out of the 70 patients, the 30 case (42.9%) affected with Acute Lymphoblastic Leukemia (ALL) were treated according to Standard Berlin-Frankfurt-Munster Study Group protocol (BFM-ALL) **(Annex 4)**. All ALL patients received Prednisone (60 mg/M²), Vincristine (2 mg/M²), L-asparaginase (5,000 unites/ M²), 6-mercaptopurine (6-MP) (50 mg/ M² /day/2years), and Methotrexate (20 mg/ M² /weekly/2 years).

The Five cases (7.1%) of acute myeloblastic leukemia (AML) were treated according to a protocol that contains two phases of treatments: Induction phase and Consolidation phase. Also relapsed and refractory disease was treated according to high-dose cytarabine (Ara-C) /Mithoxantrone (HAM) protocol **(Annex 5)**. The drugs used in AML treatment were Cytosar (100 mg/m²), daunorubcine (45 mg/m²), Fludarabine (30 mg/m²) and Mitoxantrone (12 mg/m²).

The Twelve (17.1%) Chronic Lymphoblastic Leukemia (CLL) cases received Fludarabine (25 mg/m²), Cyclophosphomide (40mg/m²), vincristine (1.4mg/m²), Prednisone (80mg/m²) and Chrorambucil (0.1mg/kg/day). Every patient

received different types of these drugs according to situation case and according to protocol **(Annex 6).**

The 22 cases (31.4%) of Chronic myeloblastic leukemia (CML) received the following drugs as a CML regimen protocol **(Annex 7)**: Interferon-Alfa 2a (IFN) (5 million unite/m²/day), Hydroxyurea (500mg/day), Cytosine arabinoside (Cytosar) (500mg/day), Imatinib mesylate (Gleevec) (300mg/day) and Busulfan (4 mg/m²/day).

The Small lymphocytic leukemia (SLL) received the drugs Fludarabine (25mg/m²) 3 days, and Cyclophosphoamide (40 mg/m²) 3 days. Combination between the two drugs and was repeated every 28 days and prednisone (80 mg/m²) **(Annex 8)**.

4.2 Quantitation of drug resistance.

4.2.1 Validation of Syber-green quantitation experiments.

Standard curves were constructed from mean cycle threshold (CT) values obtained from at least 4 separated experiments. The regression and slopes of standard curves were within accepted limits (Figure 4.9 A, B, C and D).

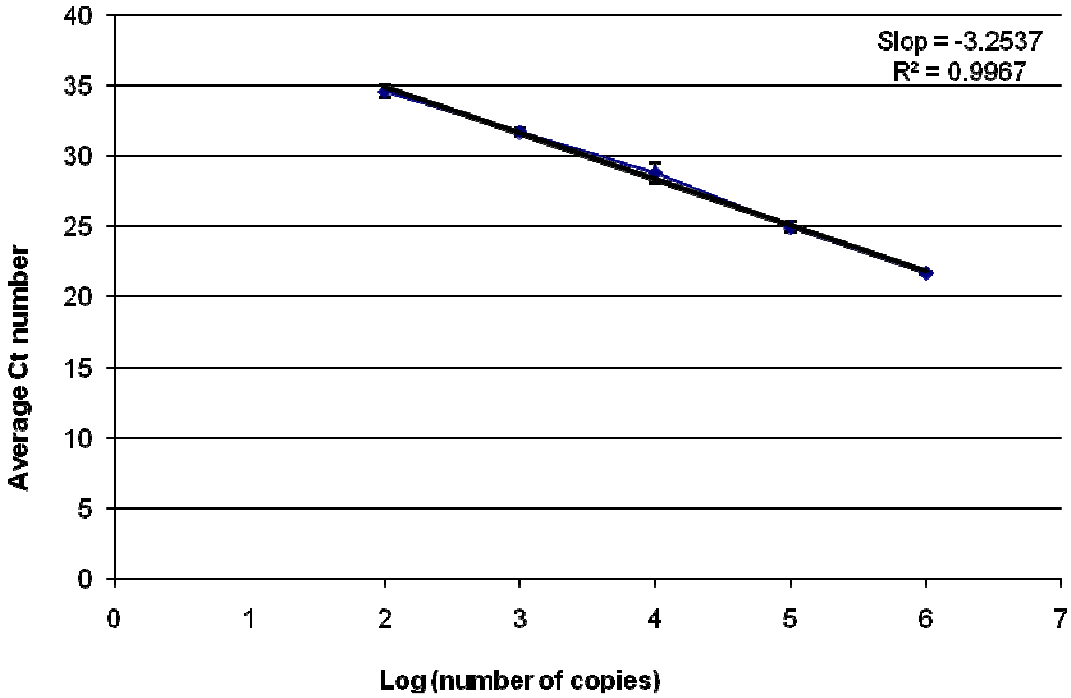

Figure 4.9-A the standard curve of PBGD gene.

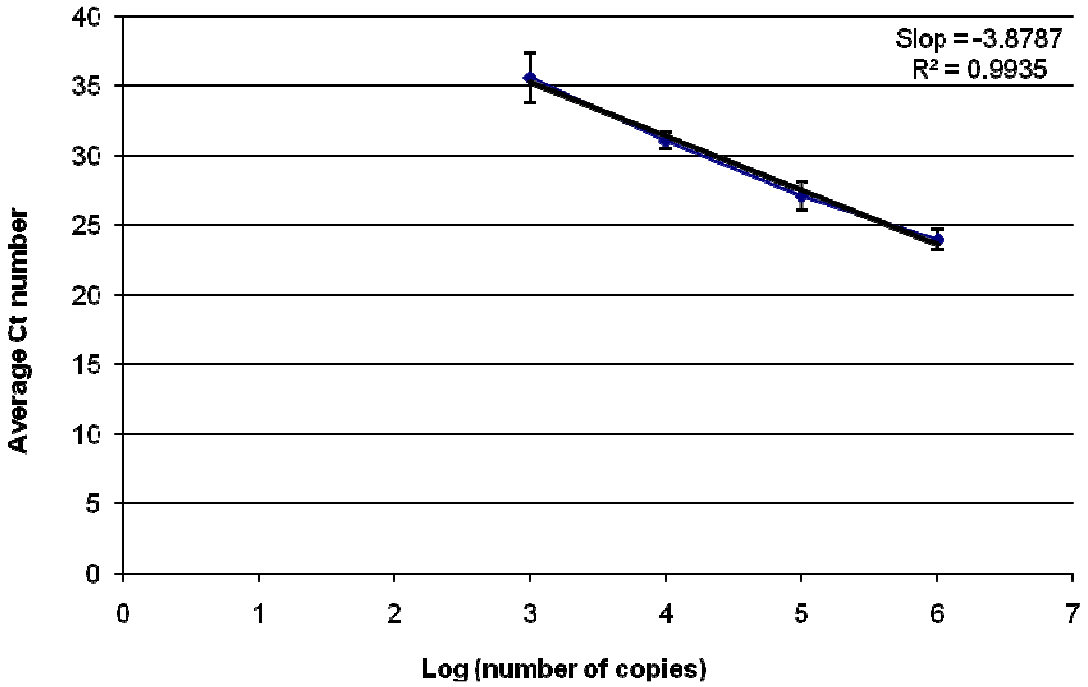

Figure 4.9-B The standard curve of MRP1 gene.

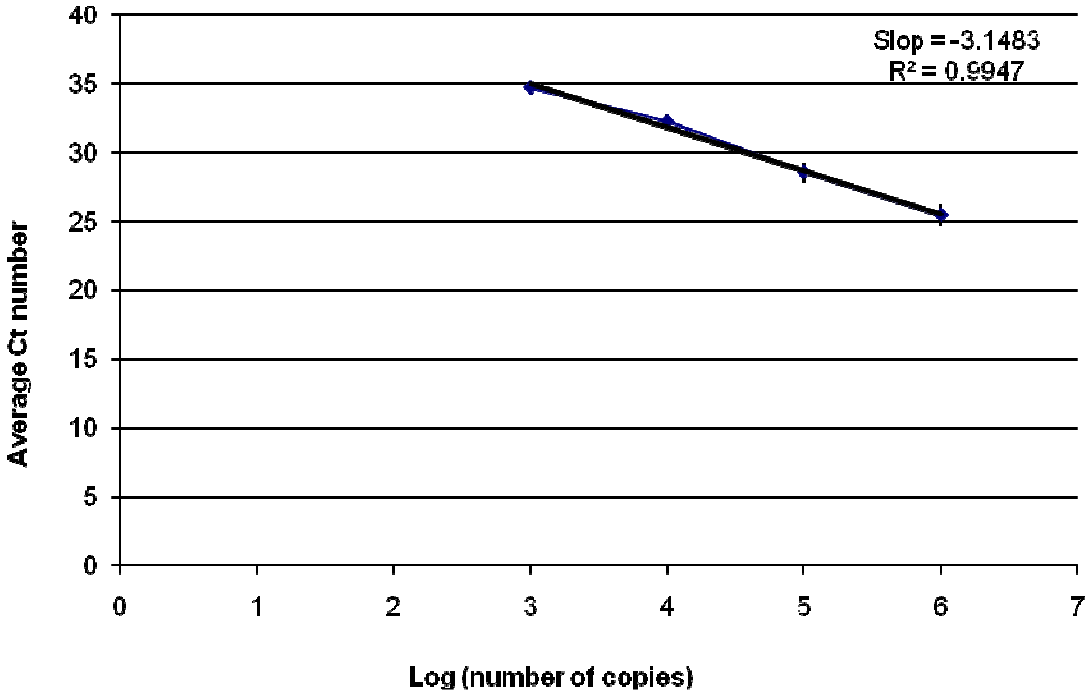

Figure 4.9-C The standard curve of LRP gene.

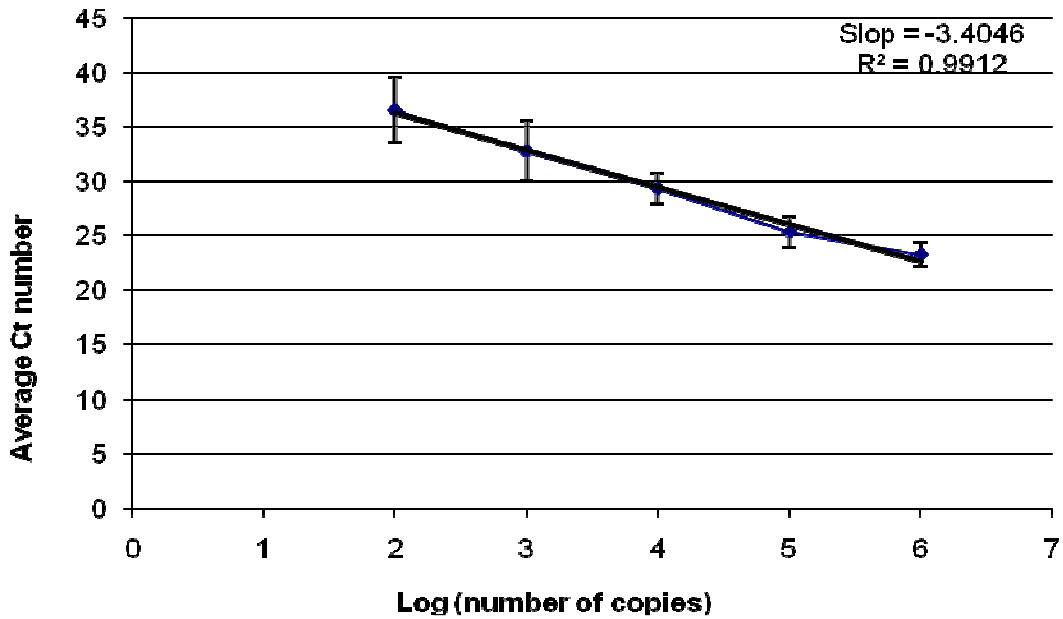

Figure 4.9-D The standard curve of BCRP gene.

As with all Syber green quantitation experiments the stringency of reactions must be increased in order to reduce non specific amplification that may represent a bias in quantitation values. Therefore, the primers concentration and templates amount were optimized and melting curves for the product of each target were evaluated (Figure 4.10— 4.13). No noticeable non specific amplification was present in the final reactions.

However, this high stringency requirement resulted in reduced sensitivities of the test. The detection limit of MRP1 and LRP PCR was 10^3 copies and for BCRP 10^2 copies.

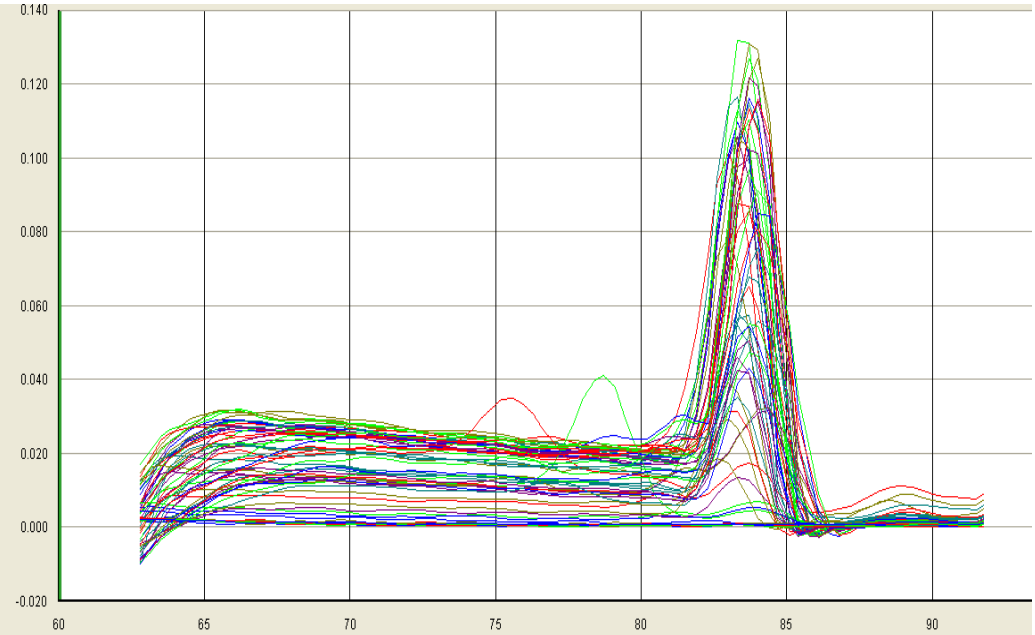

Figure 4.10 Dissociation curves of PBGD gene in case samples:

The x-axis represents Temperature Cº and y-axis fluorescence value.

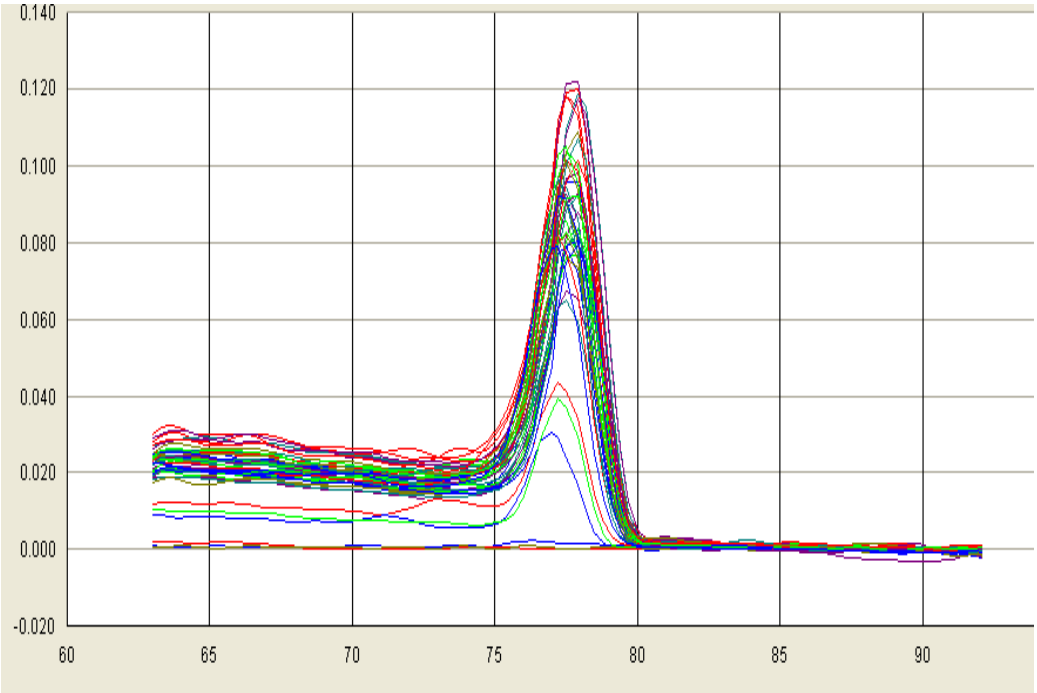

Figure 4.11 Dissociation curves of MRP1 gene in case samples:

The x-axis Temperature C° and y-axis fluorescence value.

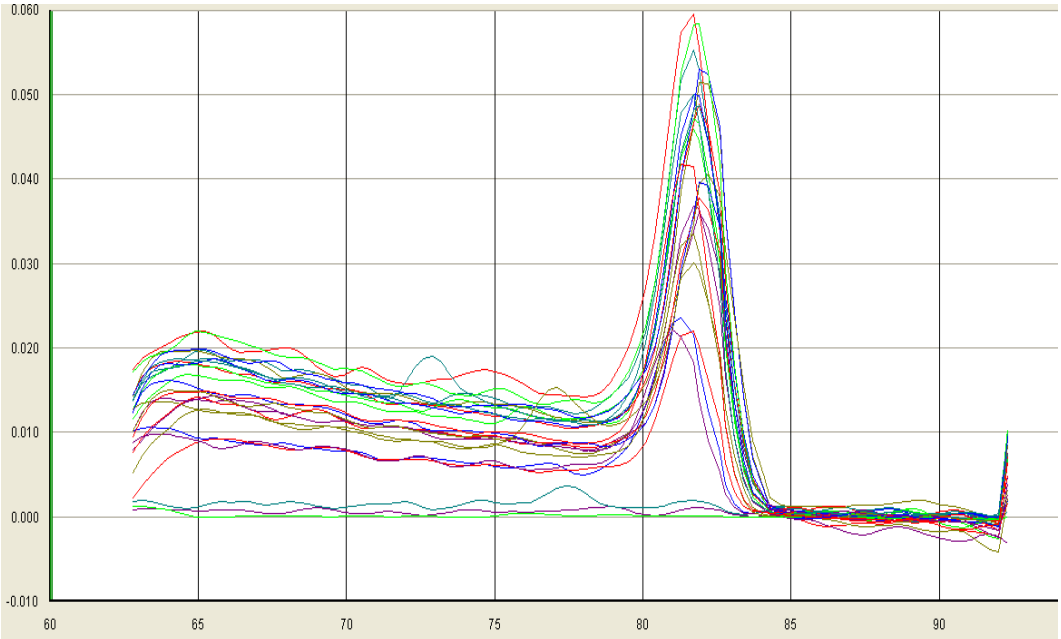

Figure 4.12 Dissociation curves of LRP gene in case samples:

The x-axis Temperature C° and y-axis fluorescence value.

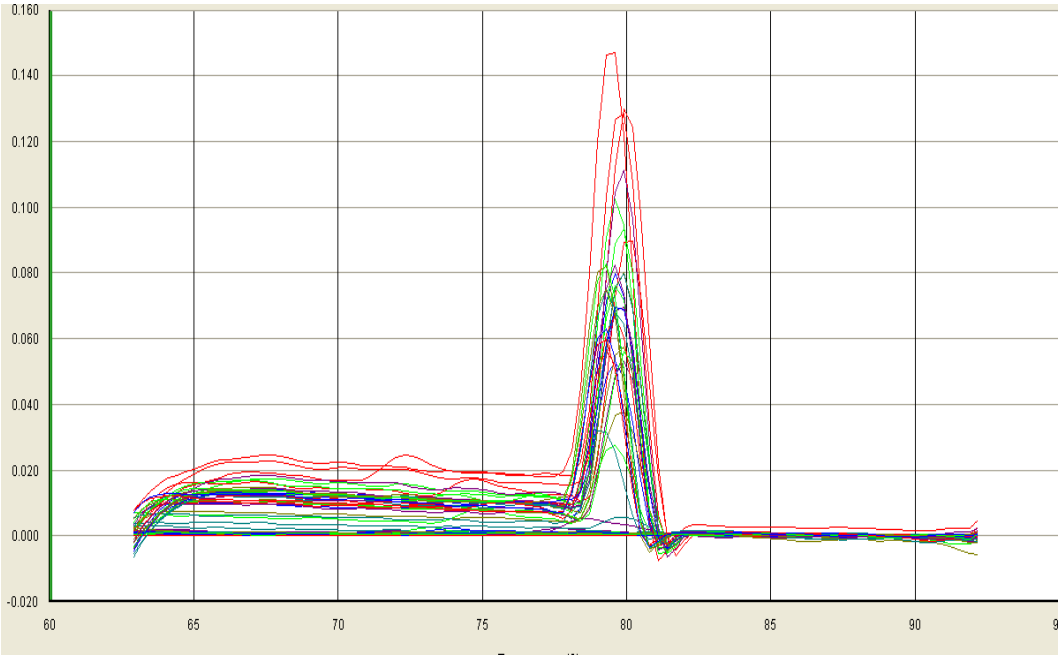

Figure 4.13 Dissociation **curves of BCRP gene in case samples:**

The x--axis Temperature Cº and y-axis fluorescence value.

The samples were found negative for MRP1 gene in eight cases (11.4%) of leukemia, and no negative samples in controls, for LRP gene 12 (17.1%) samples were negative in the cases and seven samples in controls, and 31(44.9%) samples were negative in the cases and 15 samples in controls for BCRP gene expression.

4.2.2 The levels of MRP1, LRP and BCRP drug resistance genes expression

As previously indicated all gene expression values are represented as normalized copy number of gene RNA, calculated as number of copies of target gene RNA divided by number of copies of endogenous control gene RNA. The mean normalized copy number of MRP1 gene was significantly higher in cases compared to control (P value =0.007) (Table 4.1). Also, the mean normalized copy number of LRP gene was significantly higher in cases than in controls (P value= 0.007). In BCRP gene, the mean of normalized number of copies in cases was higher than in controls, but the change was not significant (P value=0.358).

Table 4.1 The mean number of copies to genes (MRP1, LRP & BCRP) in cases and controls.

Gene		Number of cases	Mean	Std.Error	Sig.(*p value)
MRP1gene	Case	61	5.07	1.09	
	Control	35	1.12	0.25	**0.007**
	Total	96	3.63	0.07	
LRP gene	Case	57	12.5	2.88	
	Control	28	1.15	0.29	**0.007**
	Total	85	8.76	2.02	
BCRP gene	Case	38	0.13	0.04	
	Control	20	0.08	0.04	0.358
	Total	58	0.18	0.03	

*P-value calculated by One-Way ANOVA analysis SPSS.

4.2.2.1 The levels of MRP1, LRP and BCRP genes expression by gender

The gene expression levels of MRP1, LRP and BCRP genes in case group was generally higher in females compared to males. However this difference was not statistically significant (Table 4.2).

Table 4.2 The gene expression of MRP1, LRP & BCRP genes in male and female cases.

Gene		Number of cases	Mean	Std.Error	Sig.(*p value)
MRP1 gene	M	36	4.90	1.43	
	F	25	5.32	1.70	0.850
	Total	61	5.07	1.08	
LRP gene	M	32	11.93	3.26	
	F	25	13.21	5.16	0.829
	Total	57	12.49	2.88	
BCRP gene	M	22	0.09	0.03	
	F	16	0.20	0.08	0.147
	Total	38	0.14	0.04	

*P-value calculated by One-Way ANOVA analysis SPSS.

4.2.2.2 The levels of MRP1, LRP and BCRP genes expression by age grouping.

As mentioned previously, leukemia patients in the present study divided into 4 categories according to age, when compared the levels of MDR genes expression between these groups. The age group < 15 years was lower than age group of patients 36-45 years, in both MRP1 and LRP genes respectively, (p= 0.038, p=0.045) (Table 4.3).

Table 4.3 The gene expression of MRP1, LRP & BCRP gene by age grouping.

Gene	(I) Age Group	(J) Age Group	Mean Difference (I-J)	Std. Error	Sig.(*P value)
MRP1 gene	<15 years	36-45 years	-8.52	4.02	**0.038**
		>45 years	-2.54	2.21	0.256
	36-45 years	>45 years	5.98	4.02	0.142
LRP gene	<15 years	36-45 years	-23.36	11.41	**0.045**
		>45 years	-7.39	5.84	0.211
	36-45 years	>45 years	15.97	11.38	0.166
BCRP gene	<15 years	36-45 years	0.02	0.18	0.931
		>45 years	-0.04	0.08	0.620
	36-45 years	>45 years	-0.06	0.18	0.758

*P-value calculated by One-Way ANOVA analysis SPSS.

4.2.3 MRP1 gene expression in different types of leukemia

The mean MRP1 gene expression was significantly higher in all types of leukemia (except for ALL) than in control group (ALL: P=0.365, AML: P=0.000, CLL: P=0.037 and CML: P=0.010) (Table 4.4).

The levels of MRP1 gene expression in different types of leukemia varied (Table 4.4). By comparing these types of leukemia, It was found that the mean difference of expression levels of ALL patients was significantly less than AML patients (P=0.001). The mean difference between ALL patients, CLL patients and CML patients was not significant. The mean difference in expression levels between AML patients higher than CLL patients and CML patients, and the increased statistically significant (CLL: p= 0.024 and CML: p = 0.017).

In addition, the mean difference between CLL patients and CML patients was not significant.

Table 4.4 The mean difference of MRP1 gene expression between leukemia types & control.

(I) leukemia type	(J) leukemia type	Mean Difference (I-J)	Std. Error	Sig.(*P value)
	AML	-11.92	3.49	**0.001**
ALL	CLL	-3.24	2.34	0.170
	CML	-3.24	1.93	0.097
AML	CLL	8.68	3.80	**0.024**
	CML	8.68	3.56	**0.017**
CLL	CML	-0.01	2.44	0.998
Control	ALL	-1.53	1.68	0.365
	AML	-13.45	3.43	**0.000**
	CLL	-4.77	2.25	**0.037**
	CML	-4.77	1.82	**0.010**

*P-value calculated by One-Way ANOVA analysis SPSS.
- The mean difference is significant at the 0.05 level.

4.2.4 LRP gene expression in different types of leukemia

The mean LRP gene expression was significantly higher in AML patients and CML patients than in control group (AML: P=0.021 and CML: P=0.001).

It was also higher in ALL and CLL patients than in control group but not statistically significant (Table 4.5).

By comparing the LRP gene expression levels in leukemia types, It was found, that the mean difference of expression levels of ALL patients was less than CML patients and this decrease is statistically significant (P=0.024). The mean difference between ALL patients, AML and CLL patients in expression levels was not statistically significant. Also the mean difference between AML patients and CLL and CML patients was not statistically significant. The levels of LRP gene expression in CLL patients was less than in CML patients and this decrease was statistically significant (P=0.046) (Table 4.5).

Table 4.5 The mean difference of LRP gene expression between leukemia types and control.

(I) leukemia type	(J) leukemia type	Mean Difference (I-J)	Std. Error	Sig.(*P value)
ALL	AML	-15.34	9.38	0.106
	CLL	1.46	6.54	0.823
	CML	-12.26	5.33	**0.024**
AML	CLL	16.81	10.28	0.106
	CML	3.08	9.56	0.748
CLL	CML	-13.72	6.79	**0.046**
(control) Normal	ALL	-6.44	4.83	0.186
	AML	-21.78	9.28	**0.021**
	CLL	-4.98	6.40	0.439
	CML	-18.70	5.16	**0.001**

*P-value calculated by One-Way ANOVA analysis SPSS.
-The mean difference is significant at the 0.05 level.

4.2.5 BCRP gene expression in different types of leukemia

The mean expression levels of BCRP gene was not statistically significant in all types of leukemia compared with control group (ALL: P=0.528, AML: P=0.953, CLL: P=0.557 and CML: P=0.088) (Table 4.6).

The mean difference of BCRP gene levels between all types of leukemia was also not statistically significant (Table 4.6).

Table 4.6 The mean difference of BCRP gene expression between leukemia types and control.

(I) leukemia type	(J) leukemia type	Mean Difference (I-J)	Std. Error	Sig.(*P value)
ALL	AML	0.03	0.16	0.829
	CLL	0.10	0.10	0.312
	CML	-0.09	0.08	0.256
AML	CLL	0.07	0.17	0.697
	CML	-0.13	0.16	0.443
CLL	CML	-0.19	0.11	0.074
Control (Normal)	ALL	-0.04	0.07	0.528
	AML	-0.01	0.16	0.953
	CLL	0.06	0.10	0.557
	CML	-0.14	0.08	0.088

*P value calculated by One-Way ANOVA analysis SPSS.

-The mean difference is significant at the .05 level.

Expression of each of the MDR genes in the study was independent of the WBCs count and blast cells count at sample of collections and living area.

4.2.6 Leukemia managements and levels of gene expression

When comparing the level of expression of the three genes (MRP1, LRP& BCRP) according to management protocols (chemotherapy & Bone Marrow transplantation), no significant relationship was established (Table 4.7).

Table 4.7 The relation between gene expression levels of MRP1, LRP& BCRP genes and leukemia managements.

Gene	Leukemia managements	N	Mean	Std. Error	Sig.(*P value)
MRP1 gene	Chemotherapy	56	5.11	1.15	0.896
	Chemotherapy & Bone marrow transplantation	5	4.59	3.52	
	Total	61	5.07	1.08	
LRP gene	Chemotherapy	52	13.28	3.13	0.384
	Chemotherapy & Bone marrow transplantation	5	4.32	2.11	
	Total	57	12.49	2.88	
BCRP gene	Chemotherapy	35	0.14	0.04	0.719
	Chemotherapy & Bone marrow transplantation	3	0.09	0.05	
	Total	38	0.14	0.04	

*P-value calculated by One-Way ANOVA analysis SPSS.
-The mean difference is significant at the 0.05 level.

4.2.7 Remission and gene expression

All patients received chemotherapy protocols for their leukemia managements, and only 5 patients underwent bone marrow transplantation (B.M.T) in addition to chemotherapy protocol. The number of cases that responded and showed good prognosis (remission) were 47 cases (67.1%), and 22 cases (31.4%) had bad prognosis with no remission. Also one case (1.4%) died. The mean expression levels of MRP1 gene and LRP gene in remission patients was less than no-remission patients and this decrease of expression was statistically significant (MRP1: P=0.003 & LRP: P=0.050). The mean level of BCRP gene expression in remission patients was less than no-remission patients, but without statistical significance (Table 4.8).

Table 4.8 The mean levels of gene expression of MRP1, LRP & BCRP genes in remission (Yes or No).

Gene	Remission	N	Mean	Std. Error	Sig.(*p value)
	Yes	40	2.80	0.67	
MRP gene	No	21	9.39	2.68	**0.003**
	Total	61	5.07	1.08	
	Yes	36	8.27	2.99	
LRP gene	No	21	19.74	5.67	**0.050**
	Total	57	12.49	2.88	
	Yes	26	0.10	0.03	
BCRP gene	No	12	0.21	0.09	0.191
	Total	38	0.14	0.04	

*P-value calculated by One-Way ANOVA analysis SPSS.

4.2.7.1 Remission and gender

The total number of leukemia cases was 70 cases, among which 40 cases were male and 30 were females. The remission cases in male were 28 (70%) while 12 cases (30%) had no remission. The female cases 19 cases (63.3%) were in remission while the other 11 cases (36.7%) not remission (Figure 4.14). The highest number of remission was in ALL patients (27 cases, 90%) and the lowest number in CML patients (10 cases, 45%) (Table 4.9).

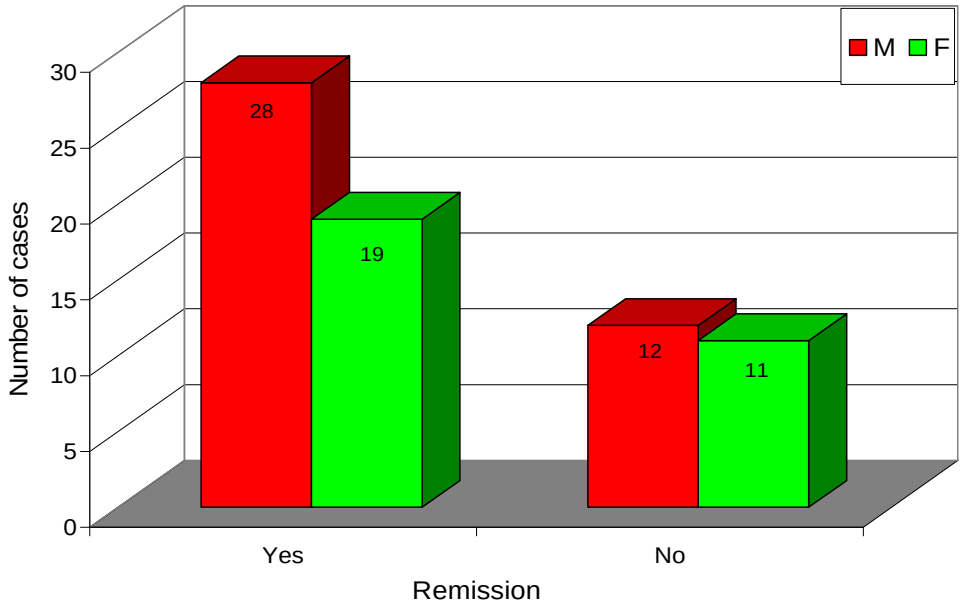

Figure 4.14 The relation between gender and remission in cases.

Table 4.9 The remission among leukemia types.

	Remission		
Leukemia type	**Yes**	**No**	**Total**
Acute lymphoblastic leukemia (ALL)	27	3	30
Acute myeloblastic leukemia (AML)	3	2	5
Chronic lymphoblastic leukemia (CLL)	6	6	12
Chronic myeloblastic leukemia (CML)	10	12	22
Small lymphoblastic leukemia (SLL)	1	0	1
Total	47	23	70

4.2.8 Drugs and levels of (MRP1, LRP & BCRP) gene expression

All patients received chemotherapy protocols according to their type of leukemia. Many factors are considered in determining type and dosage of chemotherapeutic drug. Five families of chemotherapeutic drugs (explained in chapter 2) are alkylating agents, anti-metabolites, plant alkaloids,

topoisomerase inhibitors and anti-tumor antibiotics. All drugs administrated to patients in this study belong to these families **(Annex 9-12)**. When the mean expression of MRP1, LRP and BCRP genes were compared between different types of chemotherapy drugs, no significant difference was found.

4.3 Study Case

For one of the patients included in this study and suffering from ALL, the number of WBCs at diagnosing was 93.9x10³ cell/ µL and 80% blast cells was found in peripheral blood. The patient was managed by chemotherapy for 36 months; he initially was treated according to Standard Berlin-Frankfurt-Munster Study Group protocol (BFM-ALL). The treatment protocol included three phases:

(1) Induction phase:

a. Induction phase I: He received in induction phase 1 (Vincristine 2 mg, Prednisone 60 mg/M² & L-asporaginase 5X10³unites/M²).

b. Induction phase II: Also received (Cyclophosphamide 650mg/M², Cytarabine 75 mg/ M² & 6-MP 60 mg/ M²).

(2) Consolidation phase:

a. Consolidation I: also received in consolidation phase I (Vincristine 2mg, Doxorubicin 25 mg/M² & Dexamethasone 10 mg/M²).

B. consolidation phase II: (Cyclophosphomide 650 mg/M², Cytarabine 75 mg/M² & 6-Thioguanine 60 mg/M²).

(3) Maintenance phase: He received 6-MP 60 mg/M² & Methotrexate 20 mg/M². He was complete the three phases. The blast cells at time of sample collection were 82% after 36 months of chemotherapy, at this time he become in relapsed case and received Etopsoide 100mg/M², Cyclophosphamide 750mg/ M², Aracytin 100mg/M², and high dose Methotrexate 6-gm/M². The levels of MRP1, LRP and BCRP gene expression were extremely high compared to other patients (MRP1 : 2774 normalized copy numbers compared to case mean (4.5), LRP: 1525 compared to case mean (10.3) and BCRP: 1.5 compared to case mean (0.07). The patient suffered from a relapse and unfortunately passed away

Chapter Five

Discussion

Although the antineoplastic drugs currently available are usually effective for the treatment of various tumors, they may prove to be relatively ineffective in the treatment of some primary or recurrent neoplasias. The identification of factors that might effectively predict response of the patient to treatment is a constant challenge in oncology. Cell resistance to drugs is a determinant of the response to chemotherapy, and its detection via RT-PCR may be of clinical importance.

During the last decade, several studies have defined a role for expression of transmembrane carriers such as multidrug resistance related Protein (MRP1), breast cancer resistance protein (BCRP) and lung resistance protein (LRP) genes in neoplastic cell survival and risk of relapse for leukemia patients (97, 99-103 & 108-110).

The application of relatively simple techniques such as RT-PCR may be comparable to more sophisticated techniques such as biological methods in identifying the role of upregulation of these genes in resistance to chemotherapy. Therefore in this study we determined the level of expression of MRP1, BCRP, and LRP genes in cells of leukemia patients in Gaza strip by quantitative RT-PCR technique and its clinical significance as prognostic factors for the treatment outcome.

In the present study, the case group was 70 samples (57% were males and 43% females), the control group was 35 samples (60% males & 40% females). The main goal of control group inclusion was to correct for any expression difference between individuals as a result of gender and age difference. This group was also used to compare results from cases whenever possible, particularly because of the almost matched gender and age distribution.

The study included living areas allover Gaza strip. The case group included five types of leukemia (42.9% ALL, 31.4% CML, 17.1% CLL, 7.1% AML and 1.4% SLL).

The diagnosis was confirmed by blood film and other hematological investigation such as CBC.

As shown in our results, there is significantly higher level of expression of MRP1 and LRP genes among leukemia patients than normal control individuals. This result is in agreement with previously published studies (99-101 & 103, 104). However, our results of BCRP gene expression don't agree similarly. This may be due to low sensitivity of our BCRP RT-PCR experiments as evident from the relatively high number of negative cases and controls for the gene.

As might be expected, these genes don't show any gender related difference in respect of their expression (Table 4.2). The leukemia patients were divided into age groups according to previously described range of age onset of each type of leukemia. When the age groups of our study cases were compared, it was found that patients who are < 15 year's old show significantly lower MRP1 and LRP genes expression than other groups (Table 4.3). This may be due to the fact that the majority of this age group is comprised from ALL patients who show a lower level of MRP1 expression (Table 4.4) than other types of leukemia (such as AML: P=0.001). Moreover, ALL patients usually show higher rates of remission (about 75%) than other types (110).

Also ALL patients' level of MRP1 gene expression was not significantly different from the normal control group, although it is slightly higher (Table 4.4). Other studies showed higher levels of MRP1 gene expression in ALL patients compared to normal (103, 104).

When the level of expression of MRP1 was compared between the different leukemia types, the only statistically significant relation was established between AML, with higher expression and ALL with lower expression (Table 4.4). This difference is in accordance with the results of other studies. For example, MRP1 gene expression was found to be higher in AML compared to ALL in Iranian patients (101). In the same study also the increased expression

in AML was correlated with poor outcome, in contrast to ALL. Other studies positively correlated the increased expression of MRP1 gene in increased relapse (99, 103 and 108).

Likewise, when LRP gene expression was compared between the different types of leukemia as well as to the control group, significant correlations were found. First, both CML and AML showed significantly higher LRP level of expression than normal (Table 4.5), a result that is in consistence with other previous studies (95, 96, 99).

It is well documented that expression of multiple drug resistance genes, particularly MRP1 and LRP, are involved in decreased remission rate (99, 100, 101, 103 and 108). In this regard our results support such conclusion. We found a significantly decreased remission rate with expression of MRP1 and LRP genes in all types of leukemia.

On the other hand, we found no significant difference in MRP1 and LRP gene expression in relation to the type of chemotherapeutic drugs used. This may be due to the fact that all patients suffering from the same type of leukemia were systematically managed using the same chemotherapeutic protocol, including the calculated dose and type of drug (see Annex 9-12). Only 5 patients were managed by BMT who also received the same chemotherapeutic agents as others at one stage of their disease progression.

All in all, our results show that no matter, what the drug resistance gene is over-expressed, the leukemia patient will suffer from an increased risk of relapse. This is evident by the fact that different leukemia types overexpress the MRP1 and LRP genes to different extents. And at the same time they will relapse if they overexpress any of them.

Chapter Six

Conclusions and Recommendations

6.1 Conclusions

- The overexpression of MRP1 and/ or LRP genes, among others genes like MDR1 gene, may represent a hallmark of different leukemia types, particularly if managed by chemotherapy.
- No matter what drug resistance associated gene is overexpressed, the leukemia neoplasm will have an increased risk of relapse and finishing of therapy as a result.
- Extremely high levels of a particular drug resistance associated gene alone or in combination with others, may be lethal due to non-responsiveness to treatment and the resulting toxicity associated with the demand of proportionally increase drug doses.
- If to be used for prediction of treatment outcome, one has to expand the number of genes tested to cover all possibly overexpressed ones. Accordingly, a more comprehensive technique for testing, such as microarray technique, should be applied.
- The proposed prognostic role of MRP1 and LRP in predicting chemotherapy outcome may be more profound in AML, CML and CLL patients but not ALL.

6.2 Recommendations

- The decisive role of MDR genes in response of patient to chemotherapy should be taken in consideration when planning for management protocols.
- Profiling of level of expression of such genes in different leukemia types should be done, in order to establish diagnostic guidelines and protocols.
- More studies should be conducted in which other MDR targets are analyzed in leukemia as well as other malignancies.

Chapter Seven

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APPENDIX

Annex 1:

Palestinian National Authority
Ministry of Health
Helsinki Committee

السلطة الوطنية الفلسطينية
وزارة الصحة
لجنة هلسنكي

Date: 1/6/2008

التاريخ: 1/6/2008

Name: Hany El Sadoni

الاسم: هاني السعدوني

I would like to inform you that the committee has discussed your application about:

نفيدكم علماً بأن اللجنة قد ناقشت مقترح دراستكم حول:-

Expression of the multiple drug resistance associated genes: MDR-1, MRP, LRP and BCRP among leukemia patients of Gaza Strip.

In its meeting on June 2008
and decided the Following:-
To approve the above mention research study.

و ذلك في جلستها المنعقدة لشهر يونيو ٢٠٠٨
و قد قررت ما يلي:-
الموافقة على البحث المذكور عاليه.

Consent form

Signature
توقيع

Member
عضو

Member
عضو

Chairperson

Conditions:-
- Valid for 2 years from the date of approval to start.
- It is necessary to notify the committee in any change in the admitted study protocol.
- The committee appreciate receiving one copy of your final research when it is completed.

Gaza Etwam – Telefax 972-7-2878166

Annex 2:

الجـــــــــامعة الإسلامية – غزة كلية العلوم
The Islamic University of Gaza
مدير برنامج ماجستير العلوم الحياتية

التاريخ/ ٢٠٠٨/٥/٢٥م

الأخ الدكتور/ محمد الكاشف مدير عام المستشفيات حفظه الله ،،،

السلام عليكم ورحمة الله وبركاته .

الموضوع / تسهيل مهمة باحث

نود أن نعلم سيادتكم بان الطالب/ ***هاني حسني السعدوني*** طالب في ماجستير العلوم الحياتية – تخصص ***تحاليل طبية*** في الجامعة الإسلامية يقوم بإجراء دراسة بعنوان:

التعبير الجيني للجينات مقاومة العقاقير بين مرضى سرطان الدم (اللوكيميا) في قطاع غزة.

لذا نرجو من سيادتكم تسهيل مهمة الطالب في الحصول علي المعلومات والعينات اللازمة لبحثه.

ولكم منا جزيل الشكر والتقدير ،،،

مدير برنامج ماجستير العلوم الحياتية
د. عبود ياسر القيشاوي

الجامعة الإسلامية-غزة – الرمال ص.ب:108 فلسطين
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الجـــــــــامعة الإسلامية – غزة كلية العلوم
The Islamic University of Gaza
مدير برنامج ماجستير العلوم الحياتية

التاريخ/ 2009/5/20م...

الأخ الدكتور/ ناصر أبو شعبان مدير عام تنمية القدرات البشرية حفظها الله ،،،

السلام عليكم ورحمة الله وبركاته ،،،

الموضوع / تسهيل مهمة باحث

نود أن نعلم سيادتكم بأن الطالب: ***هاني حسني السعدوني*** طالب في برنـــامج ماجســـتير العلـــوم الحياتية في الجامعة الإسلامية يقوم بإجراء دراسته للماجستير والتي بعنوان:

"التعبير الجيني في جينات مقاومة العقاقير بين مرضى سرطان الدم " اللوكيميا " في قطاع غزة"

حيث ان الطالب المذكور سابقا قام بجمع عينات من مستشفي ***غزة الاوروبي*** – قسم ***امراض الدم*** وقام بحفظها في مختبرات الجامعة وقد تعرضت هذه العينات للدمار نتيجة لتعـــرض مختبـــرات الجامعة للتدمير من قبل الاحتلال في العدوان الاخير علي غزة!

لذا نرجو من سيادتكم تسهيل مهمة الطالب في الحصول علي المعلومات والعينات اللازمة لبحثه من جديد.

ولكم منا جزيل الشكر والتقدير ،،،

مدير برنامج ماجستير العلوم الحياتية
د. عبود باسر الفيشاوي

الجامعة الإسلامية-غزة- الرمل من.ب:108 فلسطين
Islamic University P. Box 108 Al Rimal Gaza Palestine
Tel:(970/8)2860700 Fax:(970/8)2860700 2863552 e-mail:public@mail.iugaza.edu Web Site:www.iugaza.edu

Annex 3

Patient's data

Personal information

□ Name:……. □ Age:…… □ Weigh:……..

□ Height:…… □ Place:……. □ Occupation:…….

Medical information (This data will be completed from the patient medical record)

1- Type of leukemia:
□ Acute lymphoblastic leukemia (ALL)

□ Acute myeloblastic leukemia (AML)

□ Chronic lymphoblastic leukemia (CLL)

□ Chronic myeloblastic leukemia (CML)

□ Acute Non-lymphoblastic leukemia (ANLL)

□ Promyelocytic leukemia(PL)

2- Date at diagnosis: ..... .

3- Hematological investigation at diagnosis date:
□ Leukocyte count ……..

□ Platelet count……..

□ Type of blast cell: Lymphoblast cell……

Myeloblast cell……

4- Other investigation if applicable:
□ Imunophenotypic classification.

□ FAB classification.

□ Blood Film / Bone Marrow Aspiration / Biopsy.

Shown……..

□ Karyotyping, PCR, FISH.

Shown……..

Management information

1- Type of management:

□ Chemotherapy (Name and dosage of drug)

……

……....

□ Radiation

□ Bone Marrow Transplantation.

□ Other.

□ None.

2- Duration of treatment.

3- Date of last treatment.

4- Is the patient currently in remission?
□ Yes □ No

If yes, how long?

…… .

5- Are you on any medication?
□ Yes □ No

6- Failure of treatment:
□ Yes □ No

If yes, at time of relapse

□ WBCs count……..

□ Blast cells count……

□ Ratio of blast cells in Bone Marrow…….

Family information

1- Is there a family history of cancer, in first degree relation?
□ Yes □ No

If yes, the degree of relation:

□ Mother □ Father □ other relatives.

2- Treatment:
□ Yes □ No

- Current situation

- Treatment succeed

- Failure

- Death.

Annex 4: BFM-ALL protocol

Acute Lymphocytic Leukemia

Hoelzer Regimen (BFM)

INDUCTION—PHASE I				
	Vincristine	2 mg	IV	days 1,8,15,22
	Daunorubicin	25 mg/M²	IV	days 1,8,15,22
	Prednisone	60 mg/M²	PO	days 1-28
	L-asparaginase	5,000 units/M²	IV	days 1-14
INDUCTION—PHASE II				
	Cyclophosphamide	650 mg/M²	IV	days 29,43,57
	–maximum dose 1000 mg			
	Ara-C	75 mg/M²	IV	days 31-34,38-41, 45-48, 52-55
	6-Mercaptopurine	60 mg/M²	PO	days 29-57
CNS PROPHYLAXIS—weeks 5 through 8				
	Methotrexate	10 mg/M²	IT	days 31,38,45,52
	–maximum dose is 15 mg			
	Cranial radiotherapy	1800-2400 cGy		given with phase II induction
CONSOLIDATION—PHASE I–begins week 20				
	Vincristine	2 mg	IV	days 1,8,15,22
	Doxorubicin	25 mg/M²	IV	days 1,8,15,22
	Dexamethasone	10 mg/M²	PO	days 1-28
CONSOLIDATION – PHASE II				
	Cyclophosphamide	650 mg/M²	IV	day 29
	– maximum dose is 1000 mg			
	Ara-C	75 mg/M²	IV	days 31-34,38-41
	6-Thioguanine	60 mg/M²	PO	days 29-42
MAINTENANCE				
	6-Mercaptopurine	60 mg/M²	PO	daily weeks 10-18,29-130
	Methotrexate	20 mg/M²	PO/IV	weekly weeks 10-18,29-130
	REF: Hoelzer et al. Blood 1988; 71:123-131			
	PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and 12 hours after: daunorubicin, doxorubicin, and cyclophosphamide 2. Compazine 10 mg PO/IV 30 minutes before: cytarabine and L-asparaginase			

Anthracyclines—monitor cumulative dose for possible cardiac toxicity; vesicant–avoid extravasation

Methotrexate—use 75% dose for CrCl < 50; 50% dose if CrCl < 25; do not give if patient has an effusion ("reservoir effect")

Vincristine—vesicant–avoid extravasation; cumulative neurotoxicity—may produce severe constipation; maximum 2 mg per administration

6-Mercaptopurine—reduce dose by 75% when used in conjunction with allopurinol

L-asparaginase—be prepared to treat anaphylaxis at each administration; giving with or immediately before Vincristine may increase Vincristine toxicity

Annex 5:

Acute Myelogenous Leukemia

INDUCTION CHEMOTHERAPY

Agent	Dosage
7+3 cytarabine (ara-c)/ daunorubicin	Ara-C 100 mg/M²/d CIV days 1-7 Daunorubicin 45 mg/M² IV days 1-3 REF: Yates et al. Blood 1982; 60:454-462 PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and Q12 hours during chemotherapy on days 1-7 2. Dexamethasone 20 mg IV 30 minutes before chemotherapy on days 1-3 Daunorubicin—monitor cumulative dose for possible cardiac toxicity; vesicant—avoid extravasation CONSOLIDATION—repeat the above drugs for 5 and 2 days respectively
7+3+7 cytarabine (ara-c)/ daunorubicin/ etoposide (VP-16)	Ara-C 100 mg/M²/d CIV days 1-7 Daunorubicin 50 mg/M² IV days 1-3 VP-16 75 mg/M² IV (over 1 h) days 1-7 REF: Bishop et al. Blood 1990; 75:27-32 PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and Q12 hours during chemotherapy on days 1-7 2. Dexamethasone 20 mg IV 30 minutes before chemotherapy on days 1-3 Daunorubicin—monitor cumulative dose for possible cardiac toxicity; vesicant—avoid extravasation CONSOLIDATION—repeat the cytarabine for 5 days and the daunorubicin for 2 days (and optional 5 days of etoposide)
Idarubicin/ cytarabine (ara-c)	Ara-C 100 mg/M²/d CIV days 1-7 Idarubicin 13 mg/M² IV days 1-3 REF: Wiernick et al. Blood 1992; 79:313-319 PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and Q12 hours during chemotherapy on days 1-7 2. Dexamethasone 20 mg IV 30 minutes before chemotherapy on days 1-3

Agent	Dosage
	Idarubicin—monitor cumulative dose for possible cardiac toxicity; vesicant—avoid extravasation CONSOLIDATION—repeat the above drugs for 5 and 2 days respectively
Mitoxantrone/ cytarabine (ara-c)	Ara-C 100 mg/M²/d CIV days 1-7 Mitoxantrone 12 mg/M² IV days 1-3 REF: Arlin et al. Leukemia 1990; 4:177-183 PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and Q12 hours during chemotherapy on days 1-5 2. Dexamethasone 20 mg IV 30 minutes before chemotherapy on days 1 and 2 Mitoxantrone—watch cumulative dose—do not exceed 140 mg/M²; possible cardiac toxicity CONSOLIDATION—repeat the above drugs for 5 and 2 days respectively
TAD 9 daunorubicin/ cytarabine (ara-c)/ 6-thioguanine (6-TG)	Ara-C 100 mg/M²/d CIV days 1-2 –followed by Ara-C 100 mg/M² IV (over 30 min) Q12H days 3-8 Daunorubicin 60 mg/M² IV days 3-5 6-TG 100 mg/M² PO Q12H days 3-9 REF: Buchner et al. J Clin Oncol 1985; 3:1583-1589 –there are several variations of the DAT/TAD regimen PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and Q12 hours during chemotherapy on days 1-8 2. Dexamethasone 20 mg IV 30 minutes before chemotherapy on days 1-5 Daunorubicin—monitor cumulative dose for possible cardiac toxicity; vesicant—avoid extravasation
CONSOLIDATION CHEMOTHERAPY	
HiDAC high-dose cytarabine (ara-c)	–has been used as consolidation chemotherapy or for recurrent disease Ara-C 3000 mg/M² IV (over 3 h) Q12H days 1,3,5 –note that this is given with an anthracycline, as in the above regimens REF: Mayer et al. NEJM 1994; 331:896-903

Agent	Dosage
	–there are several variations of the HiDAC regimen PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and 12 hours after chemotherapy on days 1, 3, and 5 2. Dexamethasone 20 mg IV 30 minutes before chemotherapy on days 1, 3, and 5 3. Dexamethasone eye drops 2 drops each eye Q3H during and for 48-72 hours after completion of cytarabine Repeat every 28 days (as consolidation) for 2 or 3 courses Ara-C—high doses can cause CNS toxicity (cerebellar dysfunction); neurotoxicity increases as infusion time increases

RELAPSED/REFRACTORY DISEASE

HAM high-dose cytarabine (ara-c)/ mitoxantrone	Ara-C 3000 mg/M^2 IVQ12H(over 3 h) days 1-4 Mitoxantrone 10 mg/M^2 IV(over 30 min) days 2-5 or 6 REF: Hiddemann et al. Blood 1987; 69:744-749 PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and Q12 hours during chemotherapy on days 1-5 2. Dexamethasone 20 mg IV 30 minutes before chemotherapy on days 1-4 3. Dexamethasone eye drops 2 drops each eye Q3H during and for 48-72 hours after completion of cytarabine Ara-C—high doses can cause CNS toxicity (cerebellar dysfunction); neurotoxicity increases as infusion time increases Mitoxantrone–watch cumulative dose–do not exceed 140 mg/M^2; possible cardiac toxicity
High-dose cytarabine (ara-c)/ fludarabine	Fludarabine 30 mg/M^2 IV(over 30 min) days 2-6 –followed 31/2 hours later by Ara-C 1000 mg/M^2 IV(over 2 h) days 1-6 REF: Estey et al. Leuk Lymphoma 1993; 9:343-350 PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and Q12 hours during chemotherapy on days 1-5 2. Dexamethasone 20 mg IV 30 minutes before chemotherapy on days 1-5 3. Dexamethasone eye drops 2 drops each eye Q3H during and for 48-72 hours after completion of cytarabine Ara-C—high doses can cause CNS toxicity (cerebellar dysfunction); neurotoxicity increases as infusion time increases

Agent	Dosage			
Mitoxantrone/ etoposide (VP-16)	INDUCTION			
	VP-16	100 mg/M^2	IV	days 1-5
	Mitoxantrone	10 mg/M^2	IV	days 1-5
	CONSOLIDATION			
	VP-16	75 mg/M^2	IV	days 1-5
	Mitoxantrone	8 mg/M^2	IV	days 1-5
	Ara-C	75 mg/M^2	IV Q12H	days 1-5
	REF: Ho et al. J Clin Oncol 1988; 6:213-217			
	PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and Q12 hours during chemotherapy on days 1-5 2. Dexamethasone 20 mg IV 30 minutes before chemotherapy on days 1-5			
	Mitoxantrone—watch cumulative dose—do not exceed 140 mg/M^2; possible cardiac toxicity			
Gemtuzumab zogamicin (Mylotarg)	–also called CMA-676			
	Mylotarg	9 mg/M2	IV	days 1,15
	REF: Sievers et al. Blood 1999; 94 (Suppl 1):abstract 3079			
	PREMEDICATIONS 1. Benadryl 25-50 mg PO/IV 30 minutes before 2. Tylenol 650 mg PO 30 minutes before			
	Day 15 dose is given regardless of blood counts			

Annex 6:

Chronic Lymphocytic Leukemia

Agent	Dosage			
COP cyclophosphamide/ vincristine/ prednisone	Cyclophosphamide	400 mg/M²	PO	days 1-5
	Vincristine	1.4 mg/M²	IV	day 1
	Prednisone	80 mg	PO	days 1-5
	REF: Raphael et al. J Clin Oncol 1991; 9:770-776			
	PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and 12 hours after chemotherapy on days 1-5			
	Repeat every 21 days			
	Vincristine—vesicant–avoid extravasation; cumulative neurotoxicity; may produce severe constipation; maximum 2 mg per administration			
FCR - cyclophosphamide/ fludarabine/ rituximab	Cyclophosphamide	250 mg/M²	IV	days 1-3
	Fludarabine	25 mg/M²	IV	days 1-3
	Rituximab	375 mg/M²	IV	day 1
	–for cycles 2-6, dose is increased to 500 mg/M² –infusion is started at 50 mg/hr (25 mg/hr in patients with circulating tumor cells) and slowly increased to a maximum of 400 mg/hr (300 mg/hr during initial infusion)			
	REF: Keating et al. Proc Am Soc Clin Oncol 2000; abstract 2214			
	PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and 12 hours after chemotherapy 2. Tylenol 650 mg PO 30 minutes before rituximab 3. Benadryl 25 mg PO/IV 30 minutes before rituximab			
	Trimethoprim-sulfamethoxazole DS BID for 2 days each week for patients who require any corticosteroids			
	Repeat every 28 days			
Chlorambucil daily	Chlorambucil	0.1 mg/kg	PO	QD
	REF: Dighiero et al. NEJM 1998; 338:1506-1514			
	Given daily			
	–adjust dose based on CBC			
Chlorambucil pulse	Chlorambucil	0.3 mg/kg	PO	days 1-5
	Prednisone	40 mg/M²	PO	days 1-5
	REF: Dighiero et al. NEJM 1998; 338:1506-1514			

	Agent	Dosage		
	OR			
	Chlorambucil	30 mg/M²	PO	day 1
	Prednisone	100 mg/M²	PO	days 1-5
	REF: Raphael et al. J Clin Oncol 1991; 9:770-776			
	Repeat every 28 days			
	–adjust dose based on CBC			
Cyclophos-phamide—oral	Cyclophosphamide	1-2 mg/kg	PO	daily
	REF: Huguley et al. Cancer Treat Rev 1977; 4:261-273			
	–there are multiple variations of this regimen			
	Cyclophosphamide—precautions against hemorrhagic cystitis			
Cyclophos-phamide–IV	Cyclophosphamide	20 mg/kg	IV	day 1
	REF: Huguley et al. Cancer Treat Rev 1977; 4:261-273			
	–there are multiple variations of this regimen			
	PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and 12 hours after chemotherapy 2. Dexamethasone 20 mg IV 30 minutes before chemo-therapy			
	Repeat every 14-21 days			
	Cyclophosphamide—precautions against hemorrhagic cystitis			
Fludarabine	Consider prophylactic use of trimethoprin-sulfamethoxazole			
	Fludarabine	25 mg/M²	IV	days 1-5
	REF: Keating et al. J Clin Oncol 1991; 9:44-49			
	Repeat every 28 days			

Annex 7:

Chronic Myelogenous Leukemia

Agent	Dosage
Interferon-alfa 2a (IFN)/ cytarabine (Ara-C)	IFN 5 X 10^6 units/M^2 SQ daily Ara-C 10 mg SQ daily REF: Kantarjian et al. J Clin Oncol 1999; 17:284-292 PREMEDICATIONS 1. Tylenol 650 mg PO before IFN 2. Compazine 10 mg PO before prn
Busulfan	Busulfan 4-8 mg PO daily REF: Bolin et al. Cancer 1982; 50:1683-1686 Hold for WBC count < 20,000; resume for WBC > 50,000
Hydroxyurea	Hydroxyurea 500-2000 mg PO daily REF: Bolin et al. Cancer 1982; 50:1683-1686
Interferon-alfa 2a (IFN)	IFN 5 X 10^6 units/M^2 SQ daily REF: Alimena et al. Blood 1988; 72:642-647 PREMEDICATIONS 1. Tylenol 650 mg PO before IFN prn Interferon—adjust dose as tolerated to maintain WBC count 3000-5000
Thiotepa	–this agent can be used for persistent thrombocythemia in CML patients who have adequate WBC count Thiotepa 75 mg/M^2 IV day 1 REF: Rodriquez-Monge et al. Cancer 1997; 80:396-400 PREMEDICATIONS 1. Compazine 10 mg PO/IV 30 minutes before chemotherapy Repeat every 14-21 days

Annex 8: Chemotherapy protocol for SLL.

Small Lymphocytic Leukemia (SLL)

FCR - cyclophos-phamide/ fludarabine/ rituximab	Cyclophosphamide	250 mg/M²	IV	days 1-3
	Fludarabine	25 mg/M²	IV	days 1-3
	Rituximab	375 mg/M²	IV	day 1
	–for cycles 2-6, dose is increased to 500 mg/M²			
	–infusion is started at 50 mg/hr (25 mg/hr in patients with circulating tumor cells) and slowly increased to a maximum of 400 mg/hr (300 mg/hr during initial infusion)			
	REF: Keating et al. Proc Am Soc Clin Oncol 2000; abstract 2214			
	PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and 12 hours after chemotherapy 2. Tylenol 650 mg PO 30 minutes before rituximab 3. Benadryl 25 mg PO/IV 30 minutes before rituximab			
	Trimethoprim-sulfamethoxazole DS BID for 2 days each week for patients who require any corticosteroids			
	Repeat every 28 days			
Chlorambucil daily	Chlorambucil	0.1 mg/kg	PO	QD
	REF: Dighiero et al. NEJM 1998; 338:1506-1514			
	Given daily			
	–adjust dose based on CBC			
Chlorambucil pulse	Chlorambucil	0.3 mg/kg	PO	days 1-5
	Prednisone	40 mg/M²	PO	days 1-5
	REF: Dighiero et al. NEJM 1998; 338:1506-1514			

	Agent	Dosage		
	OR			
	Chlorambucil	30 mg/M²	PO	day 1
	Prednisone	100 mg/M²	PO	days 1-5
	REF: Raphael et al. J Clin Oncol 1991; 9:770-776			
	Repeat every 28 days			
	–adjust dose based on CBC			
Cyclophosphamide—oral	Cyclophosphamide	1-2 mg/kg	PO	daily
	REF: Huguley et al. Cancer Treat Rev 1977; 4:261-273			
	–there are multiple variations of this regimen			
	Cyclophosphamide—precautions against hemorrhagic cystitis			
Cyclophosphamide–IV	Cyclophosphamide	20 mg/kg	IV	day 1
	REF: Huguley et al. Cancer Treat Rev 1977; 4:261-273			
	–there are multiple variations of this regimen			
	PREMEDICATIONS 1. Kytril 1 mg PO/IV 30 minutes before and 12 hours after chemotherapy 2. Dexamethasone 20 mg IV 30 minutes before chemotherapy Repeat every 14-21 days Cyclophosphamide—precautions against hemorrhagic cystitis			
Fludarabine	Consider prophylactic use of trimethoprin-sulfamethoxazole			
	Fludarabine	25 mg/M²	IV	days 1-5
	REF: Keating et al. J Clin Oncol 1991; 9:44-49			
	Repeat every 28 days			

Annex 9: Data of ALL patients. (Management types & chemotherapy drugs).

Type of leukemia	Patient #	Type of management	Chemotherapy Drugs				
			Drug I	Drug II	Drug III	Drug IV	Drug V
ALL	3	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	4	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	6	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	7	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	8	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	9	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	15	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	20	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	21	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	22	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	23	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	24	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	25	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	26	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	27	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	43	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	45	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	46	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	47	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	49	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	59	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	60	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	61	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	62	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	65	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	66	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	67	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	68	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	69	Chemotherapy & B.M.T	L-asparaginase	vincrstine	prednisone	6-MP	MTX
	70	chemotherapy	L-asparaginase	vincrstine	prednisone	6-MP	MTX

Annex 10: Data of AML patients.

Type of leukemia	Patient #	Type of managements	Chemotherapy drugs				
			Drug I	**Drug II**	**Drug III**	**Drug IV**	**Drug V**
AML	2	chemotherapy	daunorubicin	cytarabin	Cytarabine (high dose)	MTX	-------
	11	Chemotherapy & B.M.T	cytarabin	daunorubicin	-------	-------	-------
	13	chemotherapy	cytarabin	daunorubicin	Fludarabine	Cytarabine (high dose)	-------
	51	chemotherapy	cytarabin	daunorubicin	MTX	------	------
	64	Chemotherapy& B.M.T	cytarabin	daunorubicin	MTX		

Annex 11: Data of CLL patients.

Type of leukemia	Patient #	Type of management	Chemotherapy drugs			
			Drug I	Drug II	Drug III	Drug IV
CLL	1	chemotherapy	cyclophosphamide	vincristine	Prednisone	Chlorambucil
	10	chemotherapy	cyclophosphamide	vincristine	Prednisone	Chlorambucil
	28	chemotherapy	Fludarabine	cyclophosphamide	vincristine	prednisone
	30	chemotherapy	cyclophosphamide	vincristine	prednisone	chlorambucil
	31	chemotherapy	cyclophosphamide	fludarabine	rituximab	chlorambucil
	35	chemotherapy	cyclophosphamide	vincristine	prednisone	chlorambucil
	41	chemotherapy	cyclophosphamide	Fludarabine	Rituximab	-------
	48	chemotherapy	cyclophosphamide	fludarabine	rituximab	chlorambucil
	52	chemotherapy	cyclophosphamide	vincristine	Prednisone	Chlorambucil
	53	chemotherapy	cyclophosphamide	vincristine	prednisone	-------
	56	chemotherapy	cyclophosphamide	Fludarabine	Rituximab	-------
	57	chemotherapy	cyclophosphamide	vincristine	prednisone	-------
SLL	18	chemotherapy	cyclophosphamide	Fludarabine	prednisone	-------

Annex 12: Data of CML patients.

Type of leukemia	Patient #	Type of management	Chemotherapy drugs				
			Drug I	Drug II	Drug III	Drug IV	Drug V
CML	5	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	12	Chemotherapy & B.M.T	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	14	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	16	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	17	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	19	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	29	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	32	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	33	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	34	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	36	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	37	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	38	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	39	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	40	Chemotherapy & B.M.T	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	42	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	44	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	50	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	54	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	55	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	58	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a
	63	chemotherapy	Hydroxyurea	Busulfan	Cytarabine	Gleevec	Interferon-a