Genetic Alteration and Multidrug Resistant Gene Expression Profile of Acute Leukemia

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

PRADEEP SINGH CHAUHAN

Under the Supervision of

Dr. Sujala Kapur

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN) INDIA

2012

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my deep gratitude to my supervisor, Dr. Sujala Kapur for her generous guidance, kindness, helpful and valuable support in successful completion of my thesis. She has been a tremendously supportive mentor who gave me unlimited support and freedom to develop and apply my intellectual abilities and creativity. I am extremely thankful and lucky for having such a remarkable supervisor.

I would also especially like to express my heartiest appreciation and sincere thanks to Dr. Sunita Saxena, Director of the Institute who let me join this institute and allowed me to pursue this research work under her guidance. Saxena ma'm, thank you for your helpfulness, wise guidance and incessant support throughout the study.

I express my warmest gratitude to Prof. Dr. Bijendra Nath Jain, Vice Chancellor, B.I.T.S Pilani for providing me opportunity to pursue the off campus Ph.D of the Institute. I express my gratitude to Prof. S. K. Verma, Dean, Research and Consultancy Division (RCD), BITS, Pilani for his constant official support and encouragement. In addition, I would like to thank my Doctoral Advisory Committee (DAC) members, Dr Lalita Gupta and Dr. Sandya Mehrotra. I would also like to thank Dr. Hemanth Jadav, Dr. Dinesh Kumar, Ms. Monica Sharma, and Dr. Sharad Shrivastava nucleus members of RCD and all office staff of RCD B.I.T.S, Pilani, for their cooperation and guidance in all official procedures associated with the doctoral program.

I am most grateful to all my collaborators. I wish to thank Dr. Sumita Saluja for his valuable contribution in sample collection. I'd also like to acknowledge all the men who participated in this study, as well as Dr. Vishakha Mittal, Dr. Sumita Chaudhary, Dr Monica Sharma, Dr JM Khunger, Dr DK Gupta, and Dr. S.P Kataria from for their kind help in the selection of patients and the staff at the Department of Hematology, Safdarjung Hospital, for their assistance with the clinical information and the collection of blood samples. I would like to thank all participants in this study who by providing blood samples and personal information, have made it to perform the analysis and conclusion in this work.

This PhD would not be without the financial support from Indian Council of Medical Research (ICMR -No: 3/1/JRF/2005-MPD) and Council of Scientific and Industrial Research (CSIR-F.No.10-2 (5)/2005 (i)-E.U.II), and National Institute of Pathology fellowship (Ref.No. IOP/ RA/ 2011-2012/2347) research grants that were awarded to me.

I wish to express my gratitude to Dr. Saurabh Verma for providing me the support and immense help in the analysis of flow cytometric data. My special thanks to Dr. L.C Singh who helped me many times in my Real-Time PCR experiments and Sequencing. I am also very much thankful to Dr AK Mishra, for his immense help for the statistical analysis of the data. Mishra Sir, thank you for valuable comments and stimulating discussions during the analysis of the manuscript. I enjoyed our discussions and talks very much and I will never forget that you always had a few minutes for me even if you were very busy. I sincerely thank to Dr Usha Agarwal, Dr. Raju and Dr Zeeba for their kind suggestions, encouragement and everyday support.

I am deeply indebted to my senior Dr. Bharat Bhushan, without whom I would have felt very much confused in this new environment. I am extremely grateful to Bharat sir, who has helped me selflessly in so many facets of my work and always had time to listen to my questions and problems. I would also like to thank Dr. Anand Verma, Dr. Indraneel and Dr. Anurupa for their valuable suggestion and inputs.

It is hardly possible to express in words, how much I should thank my good friend and colleagues, Rakhshan Ihsan, Mishi Kaushal and Abhay Kumar Singh (AIIMS) without whom I would not be penning these words. Lucky to have such wonderful and supportive friends. Thank you for all the fun times in the lab and on our conferences trip. Thanks for the time I spent with all of you especially Rakhshan, thanks for all nice walks, chats, tea breaks and for helping me. It has been a joy to face the challenges of both the clinical and scientific work, and to share the various moments of enjoyable free time with you. Thanks to you for making my stay and work so enjoyable.

I would also like to thank my colleagues Dhirender Singh Yadav, Abha Soni, Thoudam Regina Devi for their encouragement and instructive suggestions. It was a pleasure and privilege working with them. I also warmly thank to my colleagues Shanti, Asheema, Nitu, Shobhit, Ashish, Shubham, Angad, Ira, Meena and Shrestha who create a pleasant and congenial working atmosphere that I truly enjoyed.

I also thank to Mr Shivbahadur ji and Mr Surender pal ji for their assistance and help during my research work. I wish to thank Mr PD Sharma (Sharma ji), his assistance in FACS facility very much. I would also like to thank Mr Jagdish Pant (Pant ji) and Mrs Vansamma ji, for their

kind help during these years. I also acknowledge the help provided by lab attendants Mr. Mohan.

I would like to say thank you to those people as well who are not members of the Tumour Biology but supported me during my Ph.D. studies: Vikas sir, Tanvi maam, Banjhit sir, Ashok sir, Harsh sir, Rajneesh sir, and Hemchandra jha ji.

I would also like to thank Mrs Sharda (Administrative Officer) for her help in various matters. I am also thankful to other staff of Office (Ken ji), Store Section (Subash ji), Library (Anita madam) and establishment section (Yogender ji) for their kind help and support.

I wish to thank all the students and staff that have passed through the Institute and friends from the other walks of life- thanks for laughs and keeping me relatively "sane".

I would also like to take this opportunity to give a special thank to my "Hamdard-ANDC College" Friends- Dr. Ritesh Kumar , Dr. Shahnawaz Khan , Dr. Shams Tabrez, Ejaz Ahmed, Shafeeque Azam, Sunil Dhoundiyal, Arjun Singh and Pankaj Sharma.

Finally, to the ones I hold dearest to my heart- my parents for everything, for believing in me when I myself could not. For all their sacrifices. I hope I have made them proud. I would like to thank my brother Kuldeep Singh (Bhaiya) and Mrs Kavita (Bhabhiji), sister Reena and brother in law Mr. Anand Singh (Jijaji) for all their continuous encourage and support throughout the years. I would also like to thank my sweet nephews Shubham (Golu) and Aakash (Chintu) and my little niece Hitesh (Hinnu).

I am enormously appreciative of everyone who has helped me along my journey and supported me in every way possible.

Finally but most importantly, I would like to thank the 'Almighty', for, it is under his grace that we live, learn and flourish.

Finally, I would like to thank myself! Well done!

Pradeep Singh Chauhan

New Delhi, India

Abstract

This dissertation aimed to find out the prevalence and significance of genetic alteration including chromosomal translocation, gene mutation, gene polymorphism and expression of genes involved in drug resistance that may be responsible for poor outcome in patients with acute leukemia (AL). For this, patients diagnosed with acute leukemia were immunophenotyped by flow cytometry to identify and phenotypically characterize the leukemic cells. Immunophenotyping is now part of the standard assessment of AL supplementing morphologic diagnosis and allowing subtyping. In the current study, most of the AL cases in children were of lymphoid type (ALL) with peak incidence at 10-15 years age group whereas in adults they were typically myeloid (AML) in origin with peak incidence in the age group of 31-35 yr which was much lower than the median age of western countries (>60 yrs). Aberrant expression of lymphoid lineage associated antigen (Ly+ AML) was seen more often in cases of children than adults. CD19 was the most common aberrant lymphoid antigen expression in both adult and children followed by CD7 in AML. In B-ALL, aberrant expression of CD33 was seen more often in cases of adults than children.

Clinical and experimental data support the idea that acute leukemic blasts harbor more than one recurring mutations either chromosomal translocation or molecular mutation, suggesting a multi-hit model for leukemogenesis. In this study we investigated the frequency of commonly occurring translocation (BCR/ABL, AML/ETO and PML/RARα) and gene mutation (FLT3 and NPM1) in patient with acute leukemia. The frequency of BCR/ABL, AML/ETO and PML/RARα translocation was 9%, 24% and 4% respectively in AML cases. Further, AML/ETO translocation was significantly associated with the presence of auer rod and lymphadenopathy, low level of hemoglobin and white blood count in the study. The frequency of FLT3 and NPM1 mutations were 25% and 21% respectively in the AML cases. Adult patients showed higher frequency of FLT3 and NPM1 mutation than pediatric patients. FLT3/ITD mutation was associated with higher WBC count while NPM1 mutation with higher platelet counts and absence of hepatosplenomegaly. Higher frequency of FLT3 mutation was found in NPM1 mutation positive cases compared to NPM1 wild cases. Both FLT3 and NPM1 mutations were

observed more frequently in AML/ETO positive cases than PML/RARα translocation cases. These results support the observation that besides chromosomal translocation and gene mutations additional mutations are required for the pathogenesis of acute myeloid leukemia.

An important problem in the treatment of AL is pre-existent or acquired resistance to structurally and functionally unrelated chemotherapeutic compounds. Various cellular mechanisms can give rise to multidrug resistance (MDR). In adult AML patients, MDR1 was the only drug resistance gene that shows significantly higher expression at RNA level by real-time PCR at the time of diagnosis in non-responders compared to responders in adult patient of AML. Expression of anti-apoptotic gene BCL-2 was also higher in non-responders compared to responders in adult. In ALL, expression of MRP1 gene was found to be significantly higher in non-responders compared to responders. In children, drug resistance gene that was significantly different between responders and non-responders patients was BCRP.

The second aspect of this dissertation explores the association of single nucleotide polymorphism in genes involved in the metabolism of xenobiotics/drugs with acute leukemia in a case-control study. The distribution and the potential gene-gene interaction of selected drugs/xenobiotic metabolizing genes were investigated in relation to acute leukemia risk. Patients with acute leukemia and controls matched by age and gender were genotyped for CYP1A1, mEPHX, GSTM1, GSTT1, GSTP1 and SULT1A1 gene polymorphisms. Classification and regression trees (CART) and multifactor dimensionality reduction (MDR) analysis method were used to explore high order gene-gene interactions. GSTM1 null genotype confers reduced risk while CYP1A12A was associated with increased risk of ALL. While exploring non-linear interacting through CART analysis, patients carrying the combination of CYP1A12A, p53 and GSTP1 gene showed highest risk for ALL. Four locus model of GSTP1, p53, EPHX1 exon3 and CYP1A12A was the best model in predicting the ALL risk by MDR analysis. Interaction entropy graph showed that CYP1A12A was the key gene polymorphism in the model associated with the maximum entropy. In AML the highest risk was observed for combination of GSTM1 present, CYP1A12C AA or GG, EPHX1 exon3 TC and EPHX1 exon4 AA or GG genotypes by CART analysis. Four loci model (p53, EPHX1 exon3, CYP1A12A and SULT1A1) was the best model in MDR analysis in predicting risk in AML. In conclusion, the study found complex relationship of gene-gene interaction between xenobiotic gene polymorphism and AL risk.

TABLE OF CONTENTS

List of Tables

List of Figures

Abbreviations

α	=	Alpha
β	=	Beta
γ	=	Gamma
µg	=	Microgram
µl	=	Microlitre
AL	=	Acute Leukemia
ALL	=	Acute Lymphocytic Leukemia
AML	=	Acute Myeloid Leukemia
AR	=	Auer Rod
BAL	=	Biphenotypic acute leukemia
B-ALL	=	B-cell type ALL
Bcl-2	=	B-cell lymphoma protein
BMA	=	Bone Marrow Aspirate
BSA	=	Bovine Serum Albumin
CALLA	=	Common ALL Antigen or CD10
CD	=	Cluster of differentiation antigen
cDNA	=	Complementary Deoxyribonucleic Acid
CL	=	Chronic Leukemia
CLL	=	Chronic Lymphatic Leukemia
CML	=	Chronic Myeloid Leukemia
CNS	=	Central Nervous System
CR	=	Complete Remission or responder
Cyt	=	Cytoplasmic
DEPC	=	Diethyl pyrocarbonate
DHFR	=	Dihydrofolate reductase
DNA	=	Deoxyribonucleic acid
EGIL	=	European Group for the Immunological Characterization of Leukemias
EtBr	=	Ethidium Bromide

FAB	=	French-American-British classification
FCM	=	Flowcytometry
GST-pi	=	Glutathione S-transferase-pi
Hb	=	Hemoglobin
HLA	=	Human leukocyte antigen
HSM	=	Hepatosplenomegaly
ICC	=	Immunocytochemistry
LAP	=	Lymphadenopathy
MDR	=	Multi drug resistance
mg	=	milligram
MLAL	=	Mixed Lineage Acute Leukemia
MPO	=	Myeloperoxidase
mRNA	=	messenger Ribonucleic Acid
NCR	=	No Complete Remission or non responder
NSE	=	Nonspecific Esterase Stain
OD	=	Optical Density
OM	=	Organomegaly
PAS	=	Periodic Acid Schiff
PBMC	=	Peripheral Blood Mononuclear Cells
PBS	=	Phosphate Buffered Saline
PCR	=	Polymerase Chain Reaction
P-gp	=	Poly-glycoprotein
Plt	=	Platelet
RBC	=	Red Blood Cells
REAL	=	Revised European-American Classification of Lymphoid Neoplasms
RNA	=	Ribonucleic acid
RT	=	Room Temperature
RT-PCR	=	Reverse Transcriptase- Polymerase Chain Reaction
SCT	=	Stem Cell Transplantation
T-ALL	=	T-cell type ALL
WBC	=	White Blood Cell
WHO	=	World Health Organization

Chapter 1

Introduction

Chapter 1

Introduction

The acute leukemia (AL) represents a heterogeneous group of neoplasm affecting uncommitted or partially committed hematopoietic stem cells. AL are broadly divided into non lymphocytic (commonly known as myeloid) and lymphoid categories based on cell of origin [Haase *et al.,* 1995]. AL is the most common malignant disease representing 30% of all childhood malignancies [SEER, 2000]. Acute lymphoblastic leukemia (ALL) accounts for 75–80% while acute myeloid leukemia (AML) for 15–20% of all newly diagnosed childhood leukemia cases [Pui *et al.,* 1993]. The incidence of AML progressively increases with age and in adults it accounts for approximately 80% of AL cases [Bhatia and Neglia, 1995]. AML accounts for 25 percent of all leukemias of adults in the Western world, with the highest incidence rates occurring in the United States, Australia and Europe [Deschler and Lubbert, 2006]. The diagnosis and classification of AL is based on standard morphologic criteria best represented by the FAB (French-American-British) criteria that were developed by an international group of investigators in 1976. FAB criteria is based on Romanowsky-stained blast morphology and cytochemical stains [Bennett *et al.,* 1976]. FAB group has divided ALL into three groups from L1 to L3 and AML into eight groups from M0 to M7. Immunophenotyping (i.e. flowcytometric analysis of differentiation-associated molecule expression) analysis plays a central role especially in separating between minimally differentiated AML and ALL [Jaffe *et al.,* 2001]. Immunophenotyping is now part of the standard assessment of AL, supplementing morphologic diagnosis and allowing subtyping.

The genetic alterations associated with the development of AL require collaboration of class I mutation of constitutively activated protein tyrosine kinase such as BCR/ABL and FLT3/ITD mutation with Class II mutation of transcription factors such as AML/ETO, PML/RARα [Gilliland and Griffin 2002]. The class I mutations activate tyrosine kinase signaling pathways and confers a proliferation advantage on hematopoietic cells while class II inhibit differentiation process [Deguchi and Gilliland, 2002]. The class II mutations comprise gene rearrangements affecting transcriptional regulators include

mainly recurrent translocation such as such as t(8;21), inv(16) and t(15; 17), which generate fusion transcripts of AML/ETO, CBFb/MYH11 and PML/RARa [Gilliland *et al.,* 2004; Mrozek *et al.,* 2004]. These chromosomal translocations constitute an important parameter to identify prognostically relevant subgroups in AL. Many of these genes encode for transcription factors which are critical regulators for the differentiation program of hematopoietic stem and progenitor cells. The recurrent translocation t(8;21) juxtaposes the AML1 gene on 21q22 with the ETO gene on 8q22 resulting in the production of a chimeric AML/ETO mRNA and protein. Transcription factor acute myelogenous leukemia (AML1) is a key regulator of hematopoiesisis and the most frequent target of chromosomal translocations in AML [Okuda *et al.,* 1996]. Another recurrent translocation t(15;17) that is PML/RARα inhibits the function of RARα by a dominant negative effect through recruiting the co-repressor complex, in a manner similar to the AML/ETO fusion gene [Frohling *et al.,* 2005]. PML-RARα fusion protein has a negative dominant function in the retinoic pathway, important in myeloid differentiation arrest and apoptosis inhibition [Grignani *et al.,* 1993]. The t(9;22)(q34;q11) translocation which results from a rearrangement between the c-ABL protooncogene (on chromosome 9) and the BCR gene (on chromosome 22) was the first consistent chromosomal abnormality in any malignant disease also known as Philadelphia or Ph chromosome. This translocation generates BCR/ABL fusion protein with an enhanced tyrosine kinase activity. It is involved in the malignant transformation of hematopoietic cells, by enhancing proliferation, reducing apoptosis, and deregulating cell adhesion [Melo and Deininger, 2004].

Proliferation and survival of hematopoietic progenitor cells is regulated by growth factor receptors and their signaling intermediates. The most studied receptors with intrinsic tyrosine kinase activity in progenitor cells include receptors for SCF (stem cell factor), FLT3 and c-KIT respectively. FLT3 (Fms-like tyrosine kinase3, also designated Flk-2 or STK-1) is predominantly, but not exclusively, expressed in hematopoietic tissues. FLT3 may be the single most commonly mutated gene in AML [Gilliland and Griffin 2002]. The FLT3/ITD mutation was characterized first time by Nakao *et al.,* [1996]. The mutation is thought to cause constitutive activation of the receptor although the

mechanism remains unknown. Another most common mutation found in AML is exon 12 of nucleophosmin (NPM1) gene that interferes with cell cycle regulation [Falini *et al.,* 2005]. NPM1 is a nucleus-cytoplasm shuttling protein that is prominently present in Nucleoli [Nishimura *et al.,* 2002]. The most common NPM1 mutation is 4–base pair duplication, 956dupTCTG in exon 12 (called type A), that causes a shift in the reading frame in the C-terminal portion of nucleophosmin. Thiede et al., [2006] provided evidence that NPM1 mutations usually precede the acquisition of FLT3-ITDs, suggesting that NPM1 mutations might constitute a primary event in leukemogenesis. Besides their role as pathogenetic events, these genetic alterations of acute myeloid leukemia (AML) are known to be major determinants of patient response to therapy and outcome [Cheson *et al.,* 2003].

The standard treatment regimen for AL includes chemotherapy with combination of different anti-cancer drugs depending on the leukemia subtype. The standard approaches to the treatment of AML have been predominantly based on cytarabine and anthracyclines. Yet, the outcomes associated with AML continue to be poor, 20% to 40% of patients do not achieve remission with the standard induction chemotherapy, and 50% to 70% of first complete remission patients are expected to relapse within 3 years [Robak and Wierzbowska, 2009]. Failed induction in patients with AL is commonly due to an inherent or acquired drug resistance because of autonomous proliferation or defects in mechanisms that trigger apoptosis. Drug resistance is a major obstacle in the successful treatment and an important cause of death in AL. 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 as multidrug resistance (MDR). Drug resistance in leukemias frequently involves over expression of ABC transport proteins P-glycoprotein (Pgp), or the multidrug resistant related protein (MRP), which provides tumor cells with the capacity of resisting lethal doses by actively pumping drugs out from the inner cell environment. Several molecular biological mechanisms have been identified as being associated with MDR [McKenna *et al.,* 1997]. PGP (P-glycoprotein), product of the multidrug resistance-1 gene (MDR1) is a transmembrane glycoprotein conferring cross-resistance to a variety of mechanistically and structurally unrelated

cytotoxic drugs, such as anthracyclines, taxanes, vinca alkaloids and epipodophyllotoxins [Chauncey 2001]. Another protein, the multidrug resistant related protein (MRP1), is structurally similar to PGP and belongs to the same transmembrane transporter super family also confers in vitro resistance to a wide range of anticancer drugs, such as anthracyclines, vincristine and epipodophyllotoxins [Lockhart et al., 2003]. In addition to these two proteins, a 110 kDa protein has been identified in a PGP-negative MDR lung cancer cell line. This protein was termed the lung resistance protein (LRP) and acts as a major vault protein in humans [Scheper *et al.,* 1993].

Despite the identification of these drug resistant proteins, genes involved in apoptosis also play an important role in cellular resistance. Since chemotherapeutic modalities kill leukemic cells primarily through apoptosis therefore altered expressions of p53 and BCL-2 genes in the apoptotic pathway might also contribute to drug resistance [Jia *et al.,* 1999]. Another protein, Survivin, member of inhibitor of apoptosis protein (IAP) family, inhibits apoptosis through a pathway different from the BCL-2 family. Survivin is uniformly expressed in cancer, but not in normal cells [Velculescu *et al.,* 1999]. Survivin is associated with poor response to chemotherapy in solid tumors but its role in AL is still not clear [Adida *et al.,* 1998].

The etiology of AL appears to be multifactorial and the exposure to benzene, ionizing radiation, and cytotoxic therapy has been implicated as the risk factors in the pathogenesis of AL [Nordlinder and Jarvholm 1997]. Although any two unrelated individual share about 99.9% of their DNA sequences, the remaining 0.1% of their DNA sequence is important because it determines individual susceptibility towards environmental exposure that may cause or contribute to the disease. This inter-individual variation is partly due to genetic polymorphisms in genes encoding enzymes involved in xenobiotic and drug metabolism which have the potential to modulate the function of encoded proteins. The metabolism of xenobiotic compounds is divided into two phases. Phase I enzymes, including Cytochrome P450 (CYP) and microsomal Epoxide Hydrolase (mEH), activate several compounds to form genotoxic electrophilic intermediates. Activated metabolites are then, in part, detoxified by phase II enzymes, such as glutathione S-transferase (GST), N-acetyltransferase (NAT) and Sulfotransferase (SULT)

[Garte *et al.,* 2001]. The activity of Phase II enzymes is important since the activated metabolites may react with macromolecules like DNA, RNA and protein forming adducts [McCarver and Hines 2002; Castell *et al.,* 2005]. Further, p53 mutations have also been associated with reduced repair and enhanced cytotoxicity in cell damaged by these adducts [Wani *et al.,* 2000]. A single polymorphism in exon4 Arg72Pro codon has been suggested to modulate *p*53-dependent apoptosis and modify sensitivity to chemotherapeutic agents [Toyama et al., 2007]. The coordinated expression and regulation of phase I and II enzymes determines the outcome of the carcinogen exposure. In addition, interaction of polymorphisms in p53 gene with various phase I and phase II enzymes may also enhance the risk of leukemia.

The 2001 World Health Organization (WHO) classification of myeloid neoplasm is based on genetic criteria [Jaffe et al., 2001]. In the 2008 WHO classification, the category of "AML with recurrent genetic abnormalities," which includes the genetically best defined myeloid neoplasms, underwent major changes [Arber *et al.,* 2008]. This group was characterized by recurrent genetic abnormalities of prognostic significance such as AML with t(8;21)(q22;q22), AML with abnormal eosinophils and inv(16)(p13;q22) or t(16;16)(p13;q11), AML with 11q23 mixed-lineage leukemia abnormalities, and APL with t(15;17)(q22;q11-12). In 2008 WHO classification, provisional categories for inclusion of molecular markers such as mutations of NPM1, FLT3 and CEBPA, in AML have also been incorporated. Among molecular mutations, NPM1 mutation is the most frequent genetic lesions described in adult de novo AML with normal karyotype, compared to FLT3 (20-25%), CEBPA (15%) and MLL-PTD (5-10%). Most of these molecular mutations occur in cytogenetically normal AML and have been confirmed to be an important prognostic factor. Many studies have confirmed that mutation in FLT3 gene confer adverse prognosis in AML [Thiede et al., 2006; Colovic et al., 2007]. In contrast to this AML patients with NPM1 mutations usually show an exquisite response to induction therapy [Falini *et al.*, 2005]. Unless a concomitant FLT3-ITD mutation is present, prognosis is favorable [Schlenk *et al.*, 2008], resembling that of AML with t(8;21) or inv(16). These features appear to be independent of concomitant chromosomal aberrations [Haferlach *et al.*, 2009].

Gap in Existing Research

Genetic changes including chromosomal translocation and gene mutations constitute an important parameter in identification of prognostically relevant subgroups in AL. Recurrent translocation that are commonly found in AL are t(8;21), the t(15;17) and the t(9;22), which typically lead to the formation of fusion genes and their expression as chimeric transcripts that encode proteins with oncogenic potential. Although there are convincing data that these fusion genes alone are not sufficient to cause AL, but do contribute to impaired differentiation of hematopoietic cells. For example, the AML/ETO and PML/RARα fusion proteins are dominant-negative inhibitors of core binding factor and RARα, which are required for normal hematopoietic development. The intensive molecular investigation in recent years has described variety of new molecular mutation which play an important role in molecular diagnostics and estimation of prognosis in acute myeloid leukemia [Buzzai *et al.,* 2008; Schlenk *et al.,* 2009]. The majority of genetic studies in pediatric and adult AML have shown that mutations in the FLT3 and NPM1 gene are common events in AML. Studies particularly in Indian population, taking into account the incidence and prognostic impact of these newly described genetic events in AL patients are warranted. Cellular drug resistance could be a mechanism for the poor clinical outcome of genetic rearrangements like BCR/ABL, FLT3/ITD mutation. Drug efflux by ATP-binding cassette (ABC) genes and activation of anti-apoptotic genes are the two mechanisms primarily involved. Despite extensive investigation into mechanisms of drug resistance in AML, the etiology of therapeutic resistance is unclear. Further clinical studies need to carried out to relate drug resistance gene with genetic alterations as association of genetic alterations (FLT3 mutation and BCR/ABL translocation) with multidrug resistance gene expression is still not clear.

Genetic polymorphisms that affect chemotherapy in AL include variants of Phase 1 metabolizing genes (CYP, EPHX) and Phase 2 metabolism (GST, SULT). These variants might contribute to overall drug efficacy in patients. Drug/Xenobiotic metabolizing genes may influence predisposition or prognosis of AL patients. The

genetic polymorphism underlying the variation in enzyme activity can modify susceptibility to leukemia, probably by influencing the activation and removal of toxicants or drugs. Although single nucleotide polymorphism (SNP) in Phase I and Phase II drug metabolizing genes have been studied extensively with the risk of AL, however, majority of these molecular epidemiological studies consider only the main effects of these SNPs and their observed strength of associations could be challenged by penetrance of the genetic variant. For a complex disease like AL, single gene association studies provide limited information compared to multigenic approaches in predicting risk. Therefore, a high order gene-gene interaction in multigenic approach may help in more precise delineation of the risk groups of the disease.

Thus, the proposed study was undertaken keeping with following aims:

1. Confirmation of diagnosis and typing of acute leukemia by Flow Cytometry immunophenotyping.
2. Detection of commonly occurring genetic alterations (chromosomal translocation and gene mutations) in acute leukemia.
3. To evaluate the expression of Multidrug resistance genes (MDR, MRP, LRP and BCRP) and their correlation with genetic alteration present in acute leukemia.
4. To study the role of Xenobiotic metabolizing (Drug metabolizing) genes and p53 codon 72 polymorphism in acute leukemia.

Chapter 2

Review of Literature

Chapter 2

Review of Literature

Hematopoiesis

Hematopoiesis includes the formation and development of blood cells which is initiated by a small fraction of multipotent hematopoietic stem cells (HSCs). HSCs are located in the bone marrow and they have a capability for self-renewal. HSC can be distinguished by the lack of lineage markers and presence of CD133 (Human cells: $Lin^{-}CD133^{+}$) or c-kit or sca-1 (murine cells; $Lin^{-}Ska^{+}Kit^{+}$) [Wognum *et al.,* 2003]. The self renewal ability of the HSCs maintains the stem cell pool and supplies cells for multilineage hematopoiesis during the entire lifespan of the individual. During the process of hematopoiesis HSCs differentiate into long and short term-HSCs and subsequently into common lymphoid and myeloid progenitors [Akashi *et al.,* 2000]. The common myeloid progenitors differentiates into granulocyte–macrophage progenitors (GMP) which in turn produce monocytes, macrophages and granulocytes, and megakaryocyte-erythroid progenitors (MEP) which differentiate into megakaryocytes, platelets and erythrocytes [Akashi *et al.,* 2000]. Furthermore, the common lymphoid progenitors differentiate into mature T and B cells [Akashi *et al.,* 1999]. Hematopoiesis is highly controlled by intrinsic mechanisms such as the expression of transcription factors or epigenetic modifications, and extrinsic mechanisms like growth factors, cytokines and the microenvironment or stem cell niche [Metcalf, 1998]. The balance between these mechanisms determines whether cells remain quiescent, proliferate, differentiate, self-renew or undergo apoptosis. In normal conditions, the progenitors mature into several types of specialized cells to execute their function in immunity, coagulation or transport of molecules. During life cycle, cell may acquire genetic damage caused by internal or external foreign substances and these genetically damaged immature blasts may fill up the bone marrow and displace normal hematopoietic cells (Figure 2.1).

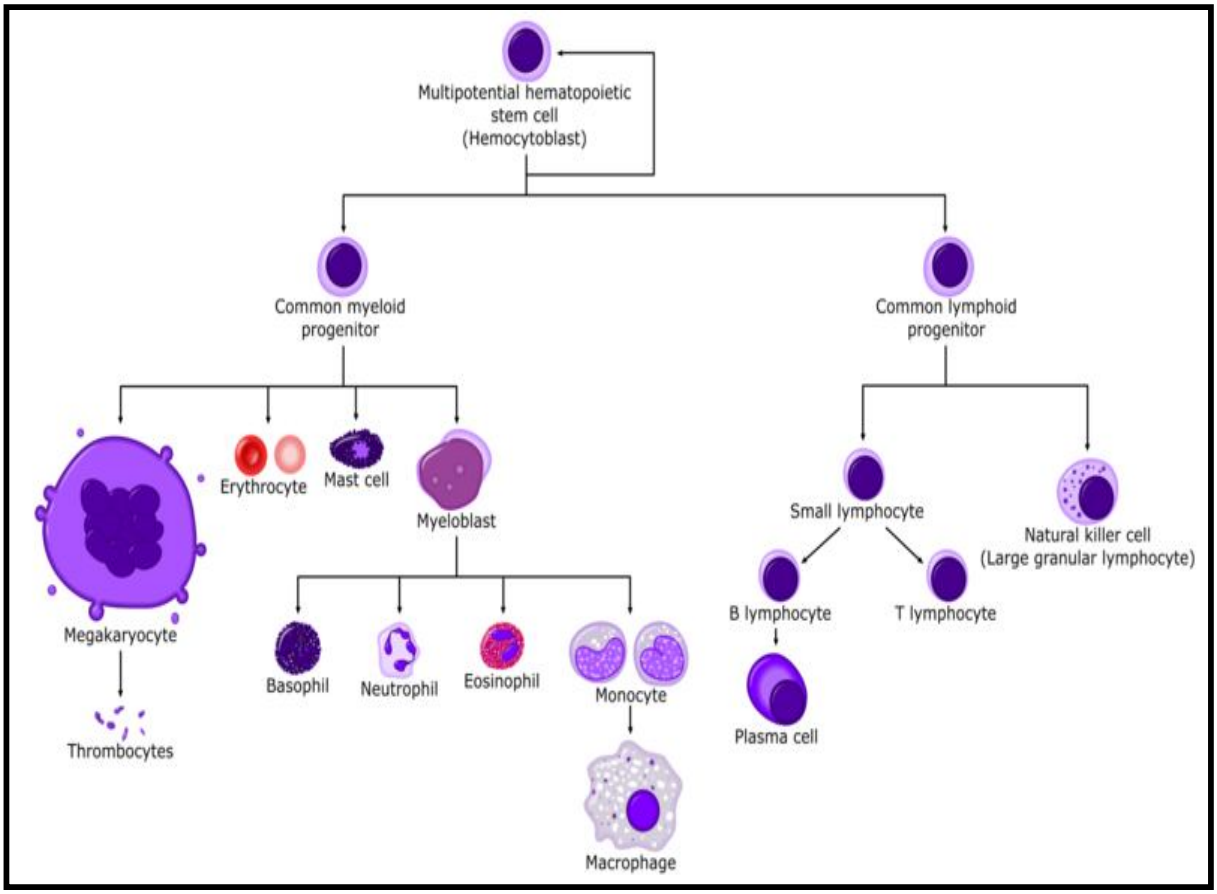


Figure 2.1: Hematopoiesis (Adapted from Adolfsson *et al.,* 2005)

Leukemia

Leukemia is a progressive, neoplastic disease of the hematopoietic system characterized by unregulated proliferation of uncommitted or partially committed stem cells. It includes a heterogeneous group of neoplasms that differ with respect to aggressiveness, cell of origin, clinical features, and response to therapy. Leukemia's are divided into two broad categories that are based on the cell involved (myeloid or lymphoid) and disease aggressiveness (either acute or chronic). Acute leukemia (AL) is malignant disorders of bone marrow hematopoietic precursors. AL is broadly divided into acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) based on cell of origin [Haase *et al.,* 1995]. In most acute leukemias, the malignant transformation is believed to occur in hematopoietic 'sleepers' or inactive multipotent stem or early progenitor cells with the capacity of lineage differentiation (Helleberg *et al.,* 1997). The

hallmark of these diseases is the leukemic blast cells, which ultimately interfere with normal bone marrow elements necessary for survival.

Epidemiology

Worldwide: Leukemias are the 9th most common cancer in men and 10th in women and constitute 3% of the total global cancer burden excluding non-melanoma skin cancer [Ferlay et al. 2008]. The overall age-adjusted incidence rate for leukemia is 5.0 per 100,000 worldwide. The incidence rates were higher in Australia/New Zealand (10.0) followed by North America (9.8) and lowest in Africa (2.7) [Ferlay et al. 2008]. In United States, It is estimated that 44,600 men and women (25,320 men and 19,280 women) were diagnosed with and 21,780 men and women died of leukemia in 2011 [Howlader *et al.,* 2011]. The overall age-adjusted incidence rate for leukemia was 12.5 per 100,000 men and women per year in United States. The age-adjusted incidence rate for ALL and AML was 1.7 and 3.5 per 100,000 men and women per year based on the cases diagnosed in 2004-2008. AML accounts for 25 percent of all leukemias and 80% of acute leukemia in adults in the Western world, with the highest incidence rates occurring in the United States, Australia and Europe than Asia and Latin America [Deschler and Lubbert, 2006; Ries *et al.,* 2006; Yamamoto *et al.,* 2008]. The highest rate of childhood AML is in Asia and the lowest in N.America and India [Miller *et al.,* 2000]. For children, ALL accounts for 73% of all leukemias diagnosed [Xie *et al.,* 2003].

India: In the developing countries, including India, the incidence rate of leukemia are 3 to 4 per 100,000 [Ferlay et al. 2008]. In India, myeloid leukemias were more common than lymphoid leukemias. For myeloid leukemias, Aizawal district showed the highest AAR (5.6) followed by Mizoram state (3.2), Imphal (3.0) and Delhi (2.7) in males. For females, Imphal west district (2.7) registered the highest AAR followed by Thiruvanthapurum (2.3) and Ahmadabad urban (2.1) [ICMR 2010]. In Delhi, leukemia accounts for approximately 5% of all cancers. Male to Female ratio was higher in lymphoid leukemias (2.48) than myeloid leukemias (1.61). In patients, below 14 years of age, lymphoid leukemia is the most common childhood cancer accounting for 42% of all cancers. In adult, myeloid leukemia is more common than lymphoid constituting 63% of all leukemias (ICMR 2010).

Etiology

The exact cause of leukemia is unknown despite many advances in the treatment, but damage to the DNA of hematopoietic stem cells is a key event. Epidemiologic studies of AL have examined possible risk factors, including genetic, infectious and environmental, in an attempt to determine its etiology. The environmental risk factors include ionizing radiation [Infante-Rivard *et al.,* 2000; Mahoney *et al.,* 2004], non-ionizing radiation, chemical, pesticides [Bhatia *et al.,* 1995; Zahm *et al.,* 1999; Glass et al., 2003], alcohol use [Pogoda et al., 2004], cigarette smoking [Pogoda et al., 2002] and illicit drug use. Most environmental risk factors have been found to be weakly and inconsistently associated with either form of AL. Chemotherapeutic drugs (alkylating agents and topoisomerase inhibitors) used to treat cancers such as breast cancer, or lymphomas are also associated with an increased risk of Leukemia [Traweek et al., 1996]. There are a number of well-known risk factors that are established causes while others are presumed causes of leukemia [Zipursky *et al.,* 1992; Deschler *et al.,* 2006; O'Connor *et al.,* 2007].

Diagnosis and classification of acute leukemia (AL)

The standard methods for establishing the diagnosis of acute leukemia are cytomorphology and cytochemistry in combination with multiparameter immunophenotyping. Cytogenetics, fluorescence in situ hybridization, and PCR-based assays add important information regarding biologically defined and prognostically relevant subgroups, and allow a comprehensive diagnosis of well-defined subentities. Therefore, the definitive diagnosis of AL requires demonstration of three diagnostic components:

- The presence of more than 30% (the older The French-American-British (FAB) standard, Bennett *et al.,* 1976 & 1985) or 20% (the newer WHO standard, Harris *et al.,* 1999) leukemic blasts in a bone marrow aspirate.
- The detection of myeloid or lymphoid differentiation marker either by cytochemistry or by analysis of surface antigen markers by flow cytometry.
- Sub-classification according to (i) the FAB that is based on morphology and histochemistry; or a more detailed classification based on morphological characteristics together with clinical history (de novo or secondary leukemia) and genetic analysis in accordance with the new WHO classification.

Morphology

Acute leukemia is broadly divided into acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL). According to the morphological FAB classification, AML can be divided into eight subtypes (M0-M7) and ALL into three categories (L1, L2, and L3) [Bennett *et al.,* 1976 & 1985]. The subtypes differ with respect to the myeloid and lymphoid lineage involved and the degree of leukemic cell differentiation. This FAB system was based solely on morphological and cytochemical findings.

FAB Classification of ALL

The presence and size of nucleoli, nuclear-cytoplasmic ratio, irregularity of nuclear membrane, and size of the leukemic blasts are the criteria used to distinguish between L1 and L2 (Table 2.1). L1 accounts for over 80% of ALL cases in childhood and 30% in adults while L2 is the most frequent ALL found in adults. L3-ALL, also called Burkitt's leukemia, makes up 3% to 5% of ALL cases in children and young adults and has nearly always a mature B-cell immunophenotype. In the contemporary ALL protocols lymphoblast L1/L2 morphology is not used for treatment stratification (Figure 2.2).

Table 2.1: FAB Classification of ALL

Morphological Features	**Subtypes of ALL**		
	L1	**L2**	**L3**
Cell size	Small	Large	Large
Nuclear chromatin	Fine or clumped	Fine	Fine
Nuclear shape	Regular, may have cleft or indentation	Irregular, may have cleft or indentation	Regular, oval to round
Nucleoli	Indistinct or not visible	1 or more/cell; large, prominent	1 or more/cell; large, prominent
Amount of cytoplasm	Scanty	Moderately abundant	Moderately abundant
Cytoplasmic basophilia	Slight	Slight	Prominent
Cytoplasmic vacuoles	Variable	Variable	Prominent
PAS stain	+	±	+

FAB Classification of AML

According to the morphological FAB classification, AML can be divided into eight subtypes (M0-M7) (Figure 2.3 a & b). The subtypes differ with respect to the myeloid lineage involved (e.g., granulocytic, monocytic, erythrocytic, megakaryocytic) and the degree of leukemic cell differentiation as shown in the (Table 2.2).

Table 2.2: FAB Classification of AML

Subtypes of AML	Morphological Features	AR*	MPO/ SBB
M0 (AML minimally differentiated)	>30% myeloblasts without granules	-	-
M1(AML without maturation)	>30% myeloblasts, with or without granules. <10% show maturation beyond blast stage.	±	+
M2 (AML with maturation)	>30% myeloblasts with granule >10% promyelocytes or mature cells; < 20% monocytic cells	+	+
M3(Acute promyelocytic leukemia)	>30% myeloblasts and promyelocytes with prominent granules	+	+
M4 (Acute myelomonocytic leukemia)	Myeloblasts, monoblasts & promyelocytes >30% marrow cells: moncytic cells >20%	±	+
M5a (Acute monoblastic leukemia without differentiation)	>80% monocytic cells; >80% monoblasts	-	-
M5b (Acute monoblastic leukemia with differentiation)	>80% monocytic cells with monblast, promoncytes (predominant), monocytes,	±	-
M6 (Acute erythroleukemia)	Megaloblastic erythroid precursors >50%; myeloblasts >30%	+	+
M7 (Acute megakaryocytic leukemia)	Megakaryoblasts, lymphoid morphology (L1,L2,M1), cytoplasmic budding	-	-

*AR: Auer Rod , MPO/SBB= Myeloperoxidase/Sudan Black B

Cytochemistry

Cytochemistry in hematology refers to the staining methods used to identify the chemical composition of cells without significantly altering the cell morphology. Most cellular cytochemical markers are organelle-associated enzymes and other proteins. Cytochemical staining reactions are of two types: 1. Enzymatic: e.g. Myeloperoxidase (MPO), 2. Non enzymatic: e.g. Sudan black (Stains lipids) PAS (stains glycogen).

ALL: Lymphoblasts are negative for myeloperoxidase and Sudan Black-B, but may react with periodic acid Schiff (PAS) or non-specific esterase. The PAS positivity is defined by the presence of blocks or coarse granules in 5% or more of the lymphoblasts. The sensitivity of a cytochemical staining combination by PAS positivity and myeloperoxidase (MPO), Sudan Black B and alpha-naphthyl butyrate esterase negativity in defining cases of lymphoblastic leukemia is approximately 50%; however the specificity of this combination of lymphoblastic leukemia is 100%. A positive PAS stain, in combination with negative myeloproxidase, Sudan Black B and alpha-naphthyl butyrate esterase stains, continues to have a diagnostic role in the distinction between lymphoid and myeloid leukemia.

AML: Cytochemistry and light microscopy (primarily Wright-Giemsa or May-Grünwald-Giemsa staining) is the principle method for the diagnosis and FAB-subclassification of AML [Smith *et al.,* 2004]. For diagnosis, all cases of AML, except M0 must stain positive for MPO and Sudan Black. MPO is an enzyme present in the primary and secondary granules of granulocytes and their precursors- metamyelocyte, myelocyte promyelocyte and myeloblast. Monoblasts, monocytes and lymphoblasts are negative. Therefore, MPO positivity is a paramount feature of AML. It is done side by side with a Romanowsky stain. Sudan Black B stains lipid membranes of the granules, which contain enzyme myeloperoxidase. SBB also stains the cells of myeloid series. Other stains that can be briefly mentioned are non-specific esterase (AML-M2, M4 or M5) and chloroacetate esterase (late myeloblast and early promyelocyte stage AML-M3) [Smith *et al.,* 2004].

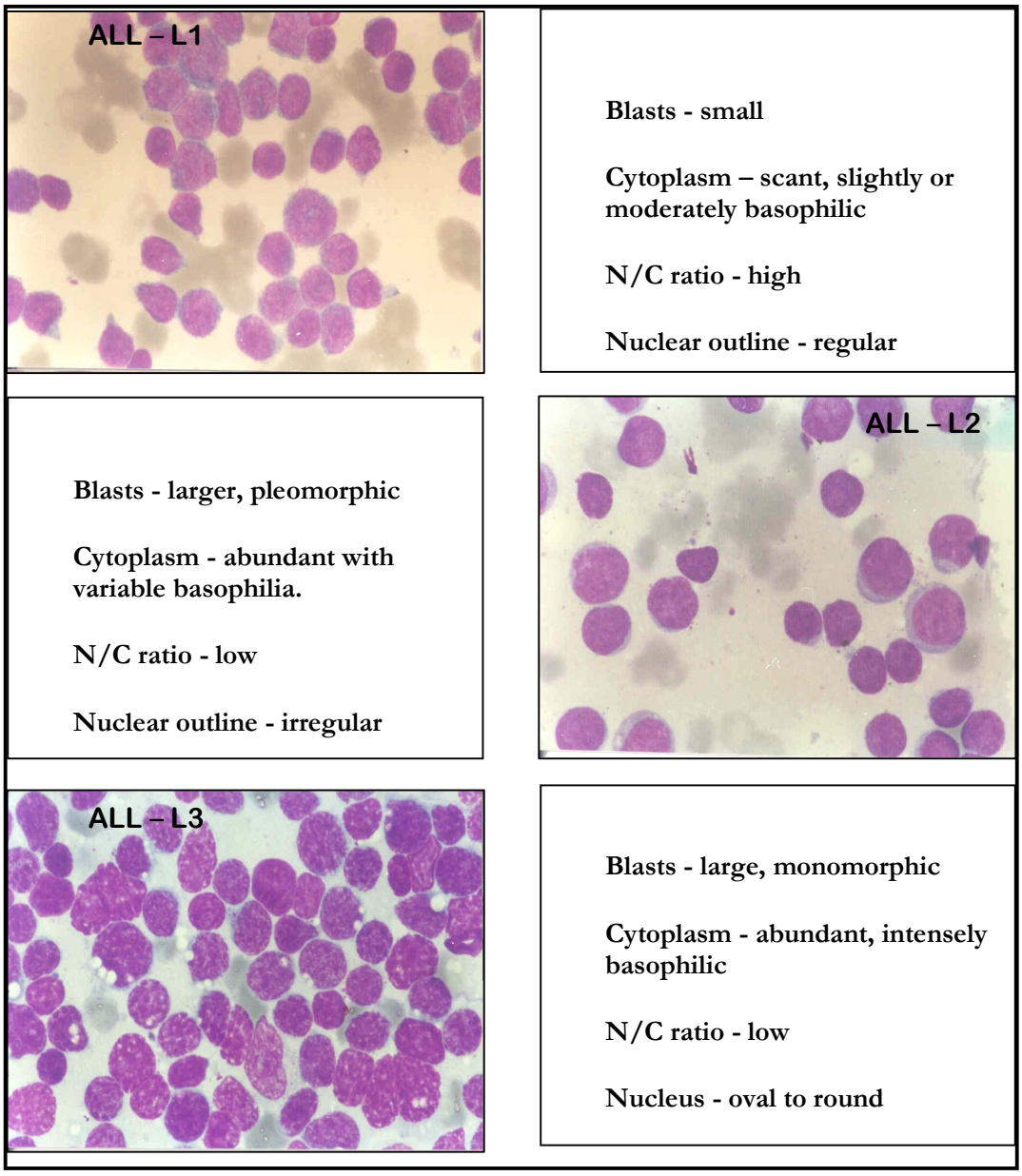


Figure 2.2: FAB subtypes of ALL (L1, L2 and L3)

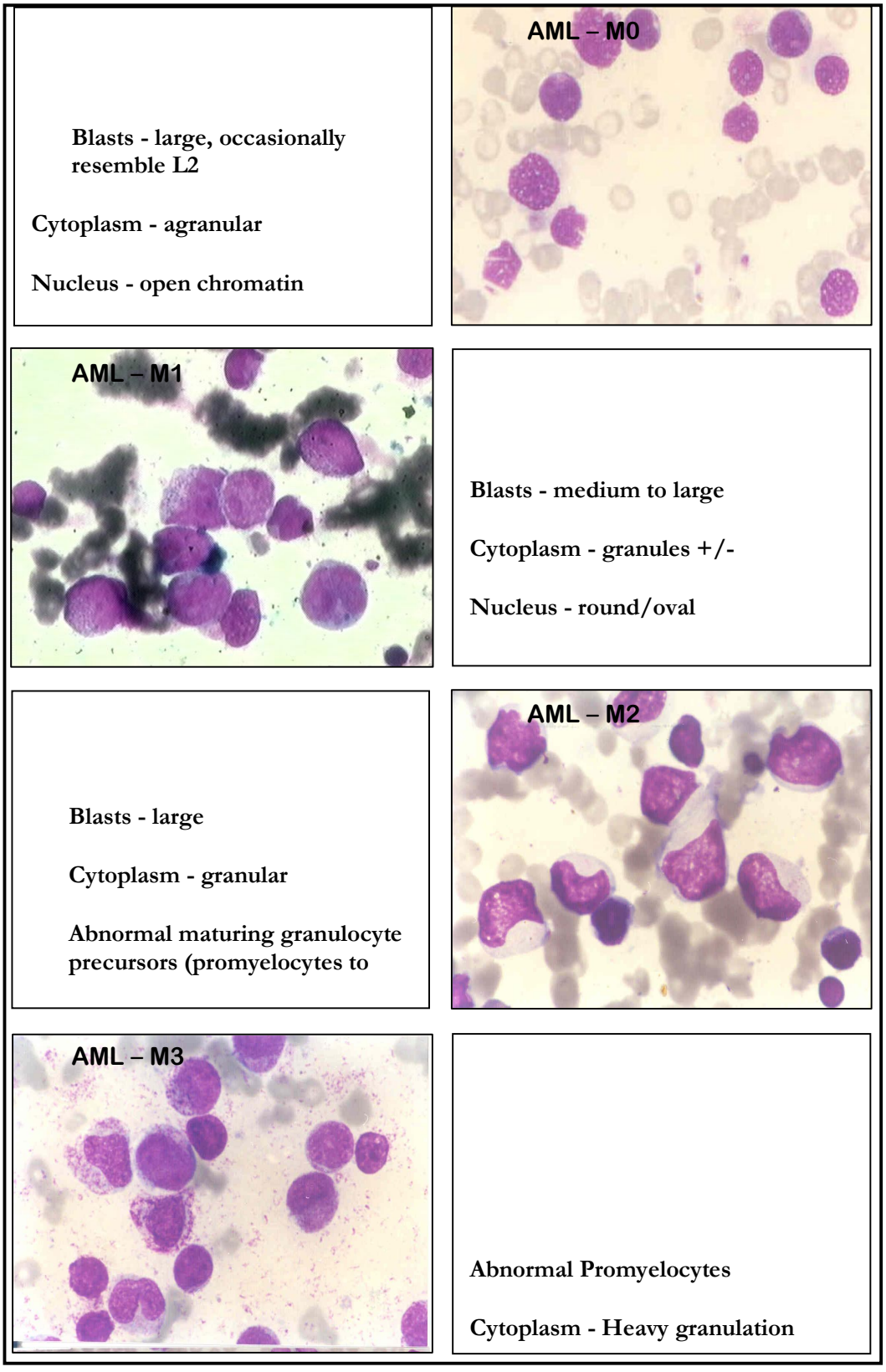


Figure 2.3a: FAB subtypes of AML (M0-M3)

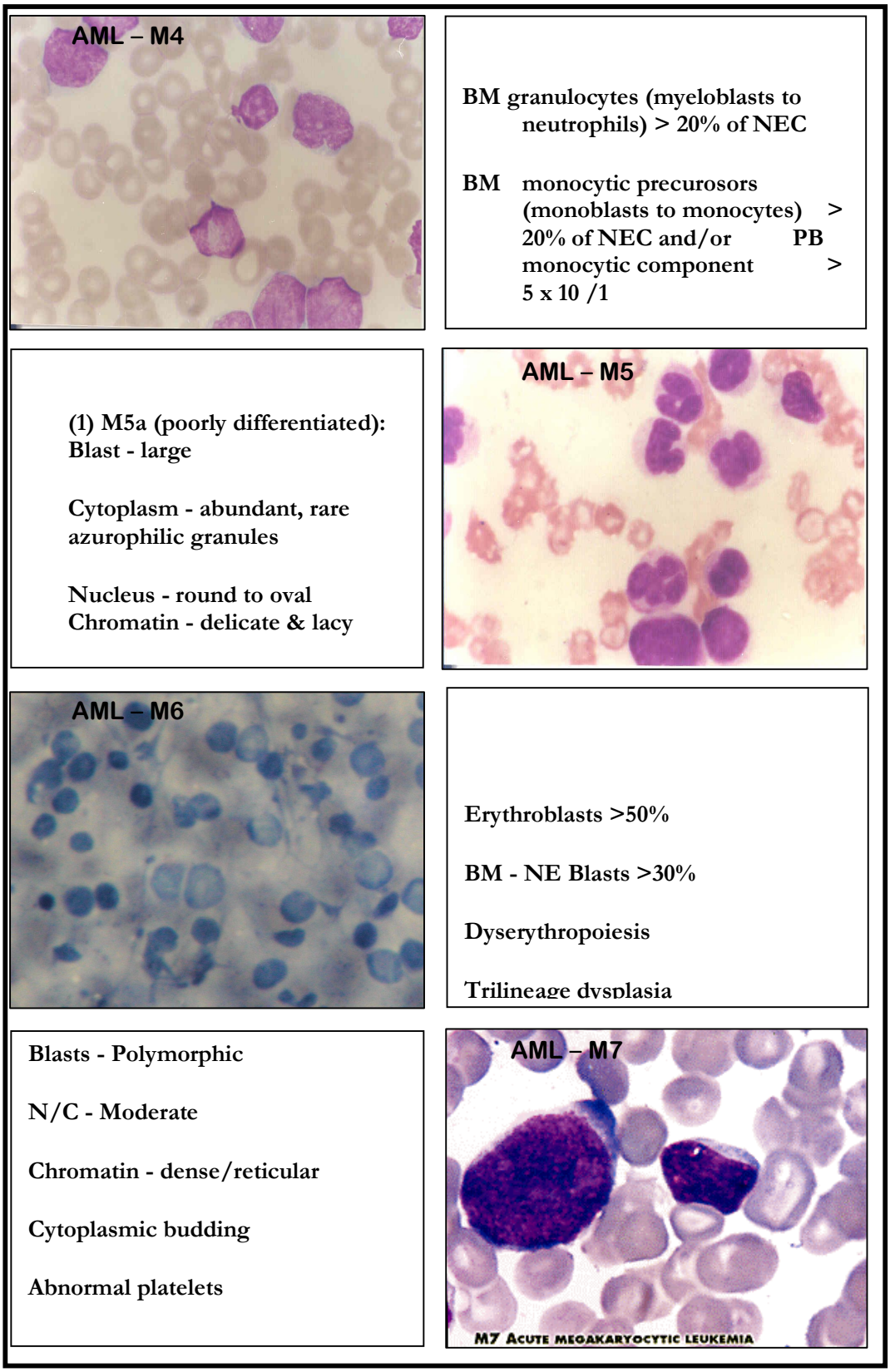


Figure 2.3b: FAB subtypes of AML (M4-M7)

World Health Organization (WHO) Classification of Acute Leukemia (AL)

The discovery of new genetic aberrations, predicting clinical behavior and outcome, necessitated the development of a new classification scheme. The World Health Organization (WHO) classification modifies the FAB classification and takes into account of morphologic, immunophenotypic, genetic and clinical features in an attempt to define entities that are biologically homogenous and that have clinical relevance [Jaffe et al., 2001]. The WHO classification is based on the principles defined in the "Revised European-American Classification of Lymphoid Neoplasms" (REAL) classification originally published by the International Lymphoma Study Group (ILSG) in 1994 [Harris *et al.,* 1994]. Recently, a revised classification has been published to incorporate new scientific and clinical information to refine diagnostic criteria for previously described neoplasms and to introduce newly recognized disease entities [Arber *et al.,* 2008]. The WHO classification is summarized in Table 2.3.

Table 2.3: WHO classification of myeloid neoplasms and acute leukemia

WHO Classification	Description
AML with recurrent genetic abnormalities	♦ AML with t(8;21)(q22;q22); RUNX1-RUNX1T1 ♦ AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 ♦ APL with t(15;17)(q22;q12); PML-RARA ♦ AML with t(9;11)(p22;q23); MLLT3-MLL ♦ AML with t(6;9)(p23;q34); DEK-NUP214 ♦ AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1 ♦ AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1 ♦ Provisional entity: AML with mutated NPM1 ♦ Provisional entity: AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes	♦ Following a myelodysplastic syndrome or myeloproliferative disorder or without antecedent myelodysplastic syndrome
Therapy-related myeloid neoplasms	♦ Alkylating agent-related ♦ Topoisomerase type II inhibitor-related ♦ Other types
AML not otherwise categorized	♦ AML with minimal differentiation ♦ AML without maturation ♦ AML with maturation ♦ Acute myelomonocytic leukemia ♦ Acute monoblastic/monocytic leukemia ♦ Acute erythroid leukemia ♦ Pure erythroid leukemia ♦ Erythroleukemia, erythroid/myeloid ♦ Acute megakaryoblastic leukemia ♦ Acute basophilic leukemia ♦ Acute panmyelosis with myelofibrosis ♦ Myeloid sarcoma

Table 2.3: WHO classification of myeloid neoplasms and acute leukemia (continued)

WHO Classification	Description
Acute leukemias of ambiguous lineage	♦ Acute undifferentiated leukemia ♦ Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); BCR-ABL1 ♦ Mixed phenotype acute leukemia with t(v;11q23); MLL rearranged ♦ Mixed phenotype acute leukemia, B-myeloid, NOS ♦ Mixed phenotype acute leukemia, T-myeloid, NOS ♦ Provisional entity: natural killer (NK) cell lymphoblastic leukemia/lymphoma
B lymphoblastic leukemia/lymphoma	♦ B lymphoblastic leukemia/lymphoma, NOS ♦ B lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities ♦ B lymphoblastic leukemia/lymphoma with t(9;22)(q34;q11.2);BCR-ABL ♦ B lymphoblastic leukemia/lymphoma with t(v;11q23);MLL rearranged ♦ B lymphoblastic leukemia/lymphoma with t(12;21)(p13;q22) TEL-AML1 (ETV6-RUNX1) ♦ B lymphoblastic leukemia/lymphoma with hyperdiploidy ♦ B lymphoblastic leukemia/lymphoma with hypodiploidy ♦ B lymphoblastic leukemia/lymphoma with t(5;14)(q31;q32) IL3-IGH ♦ B lymphoblastic leukemia/lymphoma with (1;19)(q23;p13.3);TCF3-PBX1
T lymphoblastic leukemia/lymphoma	

Immunophenotyping in Acute Leukemia (AL)

Immunophenotyping (i.e. flowcytometric analysis of differentiation-associated molecule expression) analysis plays a central role especially in separating between minimally differentiated AML and ALL [Jaffe *et al.,* 2001]. Immunophenotyping is generally performed by

flow cytometry, but also by immunohistochemistry on slides, and the value of particular markers may differ depending on the technique. The goal of immunophenotyping is the identification and phenotypic characterization of blast cells. Advances in flow cytometry technology and availability of commercially produced monoclonal antibodies directed against hematopoietic and lymphoid cell antigens have opened new horizons for the diagnosis and classification of AL.

Immunophenotyping in ALL

ALL were the first group of hematological malignancies in which immunophenotyping proved to be clinically useful. ALL is initially divided into B and T lineage with the B lineage further subdivided into B cell, pre B cell and early B-precursor types [Pui *et al.,* 1993] (Table 2.4).The immunologic classification of ALL is presented in Figure 2.4.

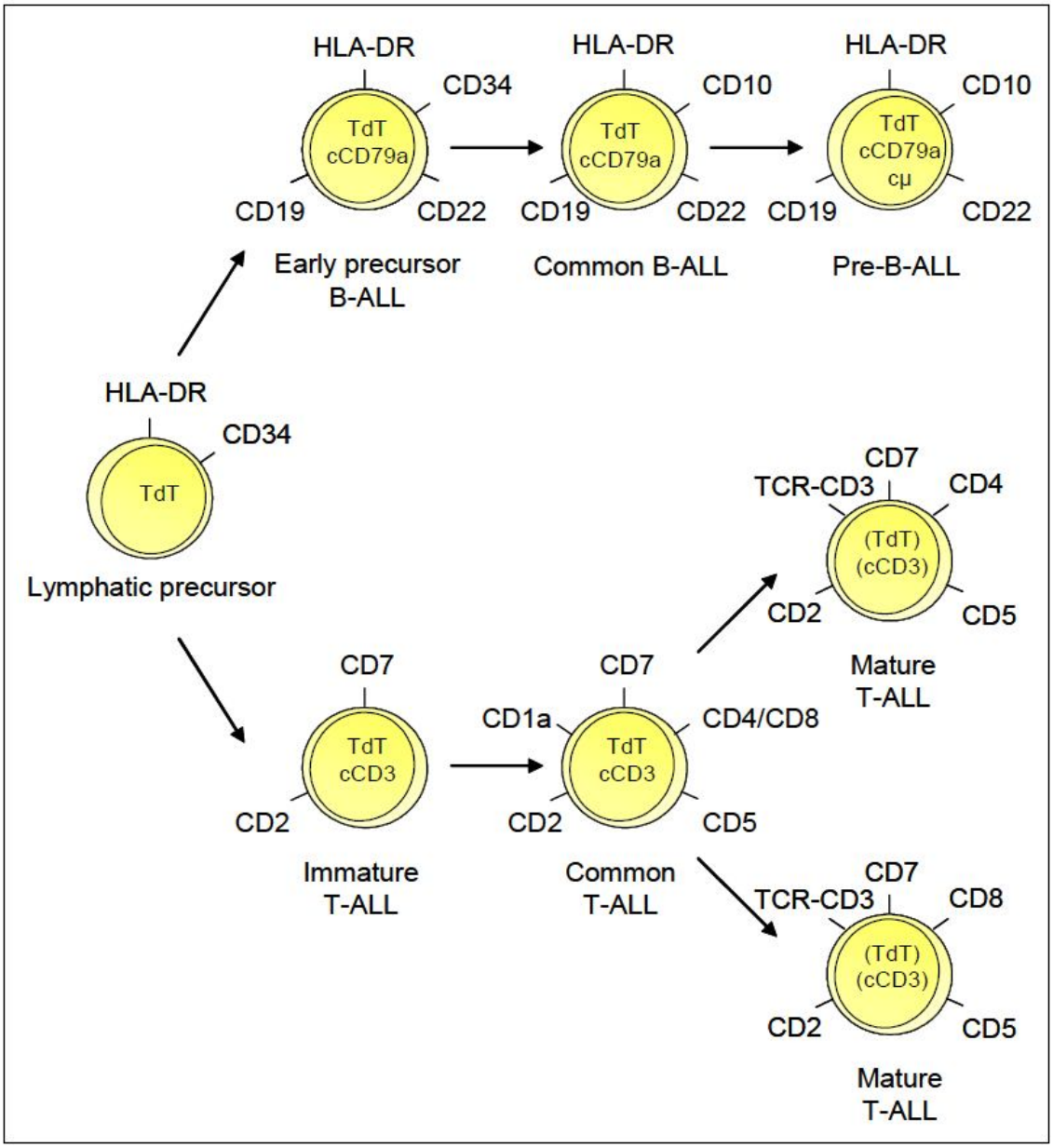


Figure 2.4: Immunophenotype of acute lymphoblastic leukemia

B-ALL: First B-cell associated antigens to be expressed after commitment of an early CD34 positive hematopoietic precursor into the B-lymphoid lineage are CD22, CD10, and CD19 (on the cell membrane), nTdt, and cytoplasmic CD79a (cCD79a). The leukemic cells of early pre-B ALL always express CD19. Almost all cases have cytoplasmic CD22 and CD79a; weak surface CD22 expression is also evident in many cases. Usually rearrangements of the MLL gene

resulting from the t(4;11), t(11;19), or t(9;11) chromosomal translocations are characteristics of this subgroup [Pui *et al.,* 1993]. About 25% of newly diagnosed cases of ALL have a pre-B immunophenotype [Pui *et al.,* 1993]. In Pre-B ALL, cells expresse CD19, CD22 and CD79a and in addition accumulate cytoplasmic immunoglobulin μ heavy chains but no detectable surface immunoglobulins. 20 to 25% cases of Pre B-ALL have either the t(1;19)(q23;p13) or the der(19)t(1;19)(q23;p13) translocation and such cases require more intensive treatment [Pui *et al.,* 1994]. Leukemic cells that express both cytoplasmic and surface immunoglobulin μ heavy chains without k or λ light chains have been designated transitional pre-B ALL [Koehler *et al.,* 1993]. There is no characteristic chromosomal abnormality for this subgroup of ALL. Mature B-cell ALL represents 2 to 5% of all and is equivalent to Burkitts lymphoma in leukemic phase. The phenotype shows B-lineage antigens CD19, CD20, CD22 and CD24 with bright clonal sIg most often IgM. The FAB-L3 subtype commonly belongs to this group. Often these cases represent the leukemic phase of the Burkitt lymphoma. The hallmark of this subset of B-cell ALL is the presence of a reciprocal translocation which include t(8;14)(q24;q32), t(2;8)(p12;q24) and t(8;22)(q24;q11), involve rearrangements of the C-myc gene.

Table 2.4: Immunological Classification of ALL

Category	Cell Markers								
	TdT	**HLADR**	**CD19**	**CD20**	**CD10**	**Cμ***	**sIg#**	**CD7**	**CD2**
Early B-precursor ALL	+	+	+	-	-	-	-	-	-
Common ALL	+	+	+	-	+	-	-	-	-
Pre-B-ALL	+	+	+	+	+	+	-	-	-
B-ALL	-	+	+	+	±	±	+	-	-
Early T-precursor ALL	+	-	-	-	-	-	-	+	-
T-ALL	+	-	-	-	±	-	-	+	+

*Cytoplasmic mu heavy chain # Surface Ig (with kappa or lambda light chain restriction)

T-cell: T-lineage ALL are much less frequent than B-lineage ALL. T-lineage ALL cells have surface CD7 and cytoplasmic CD3 (cCD3) antigens. More than 90% of T lymphoblasts express CD2, CD5, and TdT. T-ALLs also shows expression of CD1, HLA-DR, and TdT. Like precursor B ALL, T-ALL is also divided into four groups [Bene *et al.,* 1995]: pro-T (or TI), pre-T (or TII),

cortical or (TIII), and mature (or TIV) ALL (Table 2.5). Pro-T ALL typically shows coexpression of two early T-cell markers CD7 and cCD3 in the absence of other T-cell-associated antigens. Pre-T ALL shows expression of surface CD2, CD5, and CD8 besides CD7 and cCD3. Precursor T-cell phenotype is found in about 15% to 20% of ALLs and usually has L2 morphology. Some precursor T-cell ALLs also show CD10 expression and tend to exhibit L2 rather than L1 morphology [Consolin *et al.,* 1998]. In cortical-T ALL leukemic cells shows reactivity for CD1a. The T-IV/mature T-ALL phenotype (sCD3+, CD1a-, CD4-, or CD8-) is more often observed among patients presenting with T-lymphoblastic lymphomas than a pure T-ALL. Among all subtype of T-ALL, T-III ALL is the more frequent ones. In both T-III and T-IV T-ALL, surface expression of CD3 may be associated with expression of TCR of either the TCRα/β or TCRγ/δ type.

Table 2.5: Immunological classification of T-Lineage ALL (EGIL classification)

	cCD3	**CD7**	**CD2,CD5,CD8**	**CD1a**	**CD3+/CD1a-**
Pro-T (T-I)	+	+	-	-	-
Pre-T (T-II)	+	+	+	-	-
Cortical-T (T-III)	+	+	+	+	-
Mature-T (T-IV)	+	+	+	-	+

Immunophenotyping in AML

Immunophenotypic studies are not as useful in AML as they are in ALL. The identification of recurrent genetic abnormalities has assumed more priority than immunophenotyping in AML. However, flow cytometric immunophenotypic studies still remain of value in its distinction from ALL, in the diagnosis of minimally differentiated acute myeloid leukemia (AML-MO) where cytochemistry is negative [Bene *et al.,* 2001]. Cases that are devoid of detectable MPO in addition to the absence of lineage restricted lymphoid and megakaryocyte antigen are classified as M0. Immunophenotypic picture of AML-M1 is similar to AML-M0 and blasts are usually CD13+, CD33+ and HLA-DR+ with partial CD15 expression [Venditti *et al.,* 1997]. Leukemic blast cells of AML-M2 commonly express MPO, CD34, CD65, and HLA-DR, but CD13 and CD33 expression is characteristically very weak and sometimes is not detectable [Arber *et al.,*

1997]. About 35 to 45% of childhood M2 AML cases have the t(8;21)(q22;q22) translocation. Expression of CD19, and less often CD56 in cases of M2 is associated with presence of t(8;21) a favorable prognostic marker in adults [Nucifora and Rowley, 1995]. The diagnostic role of immunophenotyping in AML is also widely accepted for M3. Cell of M3 subtype strongly expresses CD13, CD33, MPO and CD65. Contrary to all other subtypes of AML (except possibly M7), HLA-DR and CD34 are usually negative in M3. The t(15;17)(q22-24;q11-21) is the characteristic chromosomal abnormality associated with this subtype. In addition, immunophenotyping is also useful in the identification of megakaryocytic differentiation (AML-M7) with expression of CD41, CD61, and pure erythroid leukemia (AML-M6) with expression of CD235a (glycophorin A) or CD36 in the absence of CD64, myeloperoxidase and other myeloid-associated antigens [Dunphy, 1999].

Biphenotypic Acute Leukemia (BAL): Immunological Characterization of Leukemias (EGIL) has proposed an immunologic classification of AL that includes ALL, myeloid antigen positive ALL, AML, lymphoid antigen positive AML, Biphenotypic AL (BAL), and undifferentiated AL [Bene *et al.,* 1995]. Based on scoring system adopted by EGIL, BAL were distinguished from those with aberrant expression of a marker from another lineage (Ly+AML, My+ALL). This scoring system was based on the number and degree of specificity of the markers expressed by the leukemic cell (Table 2.6). According to this, BAL is defined when the score is greater than two for the myeloid and one of the lymphoid lineages. Table 2.6 shows the markers considered most specific as follows: i) B-lymphoid lineage, CD79a (mb-1) detects a transmembrane protein linked to the immunoglobulin which constitutes part of the receptor for antigen recognition in B lymphocytes, cytoplasmic immunoglobulin and CD22; ii) T-lymphoid lineage, CD3 linked to the T cell receptor complex and expressed in the cytoplasm early in T-cell development; iii) myeloid lineage, myeloperoxidase (MPO) detected by either cytochemical or immunologic methods.

Table 2.6: Scoring system for the definition of Biphenotypic acute leukemia (BAL)

Scoring points	Lineages		
	B-lymphoid	**T-lymphoid**	**Myeloid**
2	CD79a(mb-1), CD22 cyt IgM	CD3, anti-TCRα/β , anti-TCR γ/δ	MPO *
1	CD19, CD10, CD20	CD2, CD5, CD8, CD10	CD117(c-kit),CD13, CD33, CD65s
0.5	TdT, CD24	TdT, CD7	CD14,CD15, CD64

*MPO (myeloperoxidase) demonstrated by cytochemical or immunological methods. Biphenotypic acute leukemia is established when the score from two separate lineages is greater than 2

Prognostic Factors in acute leukemia (AL)

Prognostic features play a critical role in directing therapy for ALL as well as for AML. A variety of clinical and biologic parameters has been examined for potential value in predicting treatment response and survival.

AML: Host factors such as age, gender and race have been associated with the outcome in patients with AML. In general children usually have slightly better outcome than adults**.** Further, in adults, patients < 60 years old have a better prognosis than patients >60 years old [Wheatley *et al.,* 1999]. The adverse clinical outcome of elderly patients may be correlated with a higher rate of unfavourable cytogenetics, a greater frequency of drug resistance phenotypes, and poor treatment tolerance [Grimwade *et al.,* 2009]. Socioeconomic status and gender have recently been identified as important prognostic factors, with a significantly better outcome reported for female [Kristinsson *et al.,* 2009]. A high white blood cell count (WBC) count also predict poor clinical outcome. Patients having hyperleukocytosis show a worse complete response rate and a greater relapse rate [Creutzig *et al.,* 1999; Greenwood *et al.,* 2006]. Based on morphology, both adult and pediatric patients with FAB M3 and M4Eo have a relatively good outcome whereas adults with Ml, M5, M6, and M7 fare worse [Buchner *et al.,* 1996]. Cytogenetic features are the most well-defined prognostic factors. In adult AML patients, monosomy 7, 7q-, t(9;22) and complex karyotypes are accepted as predictors for poor survival while t(8;21) or inv(16) and

t(15;17) are considered as good prognostic chromosomal translocations [Grimwade *et al.,* 1998]. Based on the immunophenotype of myeloid cells, several reports suggested a relationship between some antigens (e.g. CD7, CD13, TdT and CD34) and poor prognosis [Raspadori *et al.,* 1997; Venditti *et al.,* 1998] [Table 2.7].

Table 2.7: Prognostic factors in AML

Prognostic Factors	**Favourable prognosis**	**Unfavourable prognosis**
Age	Children	Elderly
Gender	Female	Male
WBC	Low ($\leq 20\times 10^9$/L)	High ($\geq 100\times 10^9$/L)
Response after Induction	CR	PR/RD
Time to relapse	Long	Short
Performance scale	Good	Poor
FAB Morphology	M3,M4	M0,M5,M6,M7
Karyotype	inv(16), t(16;16), t(15;17), t(8;21)	7q-, 5q-, t(9;22), 11 q23 with MLLrearrangements
Numerical aberrations	-	-5, -7, +8
Immunophenotype	panmyeloid marker	CD34+ve, CD13+ve, CD14+ve, CDllb, CDllc, Biphenotypic (>2 lymphoid markers)

ALL: Various clinically significant features, including initial leukocyte count, age at diagnosis, sex, cytogenetic aberrations, immunophenotype, and response to therapy, have been identified. Age has been recognized as probably the most important prognostic factor in ALL. Apart from the vast differences in prognosis between childhood and adult ALL, age also crucially affects the prognosis among adults. Patients over 60 years have a particularly poor prognosis [Gokbuget and Hoelzer, 2009]. Gender has been reported to be an independent prognostic factor, with male patients having a poorer outcome than female patients [Pui *et al.,* 1999]. The WBC at diagnosis

is also considered to be crucial variable for prognosis of ALL. Higher white blood cell (WBC) count has been correlated with worse outcomes, but the WBC categories for comparison have varied in the different reports. An arbitrary WBC cut-off of $30x10^9$/L for adult and $50x10^9$/L in children has often been used in clinical studies of ALL [Reaman *et al.,* 1999; Hunault *et al.,* 2004; Rowe *et al.*, 2005]. The prognostic significance of L1/L2 morphology is not clear. The early studies of morphology described the L1 subtype as having a better prognosis, unrelated to other risk factors known at that time [Viana *et al.,* 1980]. However, in the contemporary ALL protocols lymphoblast L1/L2 morphology is not used for treatment stratification. Historically, immunophenotyping in ALL has been considered as one of the most important prognostic factors and has a major impact on the therapeutic strategy. B-ALL has been considered to have a more favourable prognosis than T-ALL [Galpin *et al.,* 1997]. However, the pro-B- or pre-pre-B ALL and the immature T-cell precursor are generally associated with a worse prognosis [Gokbuget and Hoelzer, 2009]. In addition, although the presence of myeloid markers such as CD13, CD33, CD14 and CD15, have no particular prognostic significance in most studies [Czuczman *et al.,* 1999; Vitale *et al.,* 2007; Pui *et al.,* 2008] but they can be useful for the detection for minimal residual disease. Cytogenetic in ALL have not only been used for significant prognostic information, but also for the development of targeted drug therapy. The Philadelphia chromosome, comprising the t(9;22)(q34;q11), remains the most frequent and clinically significant abnormality in adult ALL, with an incidence ranging from 15 to 50% in older patients who have B-lineage ALL [Faderl *et al.,* 2003]. Regardless of age, both adult and pediatric patients with Ph+ ALL generally show a higher rate of induction failure and relapse than patients without this cytogenetic abnormality [Larson, 2006]. Other than Philadelphia chromosome, cytogenetic abnormalities comprising translocations t(4;11)(q21;p13.3), t(8;14)(q24.1;q32) or complex karyotype – defined as five or more chromosomal abnormalities excluding patients with an established translocation – as well as those with low hypodiploidy/near triploidy had an inferior outcome. The most common translocation detected in children is the t(12;21) associated with a favorable outcome, although no consensus has been reached of its prognostic value [Baruchel *et al.,* 1997, Seeger *et al.,* 2001] [Table 2.8].

Table 2.8: Prognostic factors in ALL

Prognostic Factors	Favorable prognosis	Unfavorable prognosis
Age	2-10 years	<2 year or > 10 years
Sex	Female	Male
WBC	Less than ≤50× 10^3	High >50× 10^3
Response after Induction	CR	PR/RD
Morphology FAB	L1	L2, L3
Karyotype	t(12;21)	t(9;22), t(4;11), t(1;19)
Ploidy	Hyperploidy	Hypoploidy, Tetraploidy, Pseudoploidy
Immunophenotype	Early Precursor B-ALL (CD10+ve)	T-ALL (CD3+ve) Pre-B ALL

Treatment of Acute Leukemia (AL)

The purpose of treatment is to eradicate all malignant cells. Treatment strategy depends on the type of leukemia but commonly includes one or more chemotherapy, radiation and bone marrow transplants. Patients with disease characterized by favourable clinical and laboratory features respond satisfactorily to standard therapy, whereas those with disease characterized by features portending recurrence require more intensive therapy.

AML: The goal of induction chemotherapy is to significantly decrease the myeloblast count to allow normal hematopoiesis to occur and to provide a complete hematologic remission (CR). The standard induction regimen has remained unchanged for many years and consists of infusional cytarbine for 7 days and an anthracycline (daunorubicin, Idarubicin or mitoxantrone) daily for the first 3 days of therapy ("7+3" regimen). After the completion of induction, a bone marrow aspirate with or without biopsy on day 10-14 of induction is performed to ensure that chemotherapy has induced adequate cytoreduction/marrow aplasia, with <5% marrow blasts signifying successful induction. A complete hematologic remission is defined as an absolute neutrophil count (ANC) >1000 cells/mm^3, platelets >100,000 cells/mm^3, and no evidence of extrameduallary diseases. Following induction, virtually all patients with AML will relapse within several months if not administered consolidation chemotherapy. consolidation therapy in

younger patients is based primarily on cytogenetic profile; favourable risk patients should receive 3-4 cycles of high-dose cytarabine (HiDAC); patients with intermediate risk AML should typically receive (HiDAC) as well as , with HSC (hematopoietic stem cell) transplantation reserved for relapse; patients with high risk cytogenetics, normal karyotype but with poor molecular features (such as FLT3/ITD and absence of NPM1 mutation should be transferred for consideration of HSC transplantation [Table 2.9].

Table 2.9: Treatment Regimens for AML/APL

Induction
AML
"7+3" regimen: cytarbine 100mg/m^2/day IV continous infusion × 7 days, combined with any one:
Idarubicin 12 mg/m^2 IV days 1,2,3; daunorubicin 60-90 mg/m^2 IV days 1,2,3; Mitoxantrone 12 mg/m^2 IV days 1,2,3
APL
AIDA: ATRA 45 mg/m^2 by mouth daily and idarubicin 12 mg/m^2 2,4,6,8 (consider continuous infusion cytarabine in high risk patients)
Consolidation
AML
HiDAC: cytarabine 3000 mg/m^2 IV every 12 hours days 1,3,5
"5+2" regimen: same induction regimen but with 5 days of cytarabine and 2 days of anthracycline
APL
"5+2" regimen or single agent anthracycline for 2-3 cycles with ATRA 45 mg/m^2 by mouth followed by 2 years of ATRA (consider daily 6-mercaptopurine and weekly methotrexate in high-risk patients)
Salvage
Cytarabine 20 mg/m^2 SQ daily for 10 days every 28 days (for elderly, infirm patients)
Gemtuzumab Ozogamicin 9 mg/m^2 IV, repeat in 14 days; premedication with acetaminophen 650-1000 mg by mouth and diphenhydramine 50 mg by mouth

ALL: The primary treatment of ALL involves intensive induction chemotherapy (Methotrexate, cyclophosphamide, cytarabine, dexamethasone or prednisone, vincristine, L-asparaginase, and/or an anthracycline). The aim of induction therapy is to eradicate the initial leukemic cell burden as much as possible and to recover normal hemopoiesis and healthy status. This is followed by intensification or consolidation therapy and prolonged maintenance to eliminate residual leukemia, prevent or eradicate central nervous system leukemia, and guarantee prolongation of

remission. In the current study, a modified BFM 95 protocol in adult and MCP 841 protocol in children was used for the treatment of ALL [Table 2.10].

Table 2.10: Treatment Regimens for ALL

Children (MCP 841 PROTOCOL)		
Cycle	**Chemotherapy**	**Schedule**
Induction 1 (I1)	Prednisone	40 mg/m^2 PO, days 1-28
	Vincristine	1.4 mg/m^2 IV, days 1,8,15,22,29
	L-Asparaginase	6000 U/m^2 IM,ALT×10, 2-20
	Daunorubicin	30 mg/m^2 IV, days 1,8,15
	IT-Methotrexate	12 mg*/m^2 days 1,8,15,22
Induction 2 (I2)	Mercaptopurine	75 mg/m^2 PO, days 1-7 & days 15-21
	Cyclophosphamide	750 mg/m^2 PO, day 1& 15
	Methotrexate	12 mg/m^2 days 1,8,15,22
	Cranial irradiation	180 c*GY*× 9 days (1800c*GY*)
Repeat Induction	Same as I_1,	Same as I_1,
Consolidation	Cyclophosphamide	750 mg/m^2 PO, day 1 & 15
	Vincristine	1.4 mg/m^2 IV, day 1 & 15
	Mercaptopurine	75 mg/m^2 PO, days 1-7 & days 15-21
	Cytarabine	70 mg/m^2 SC, every 12hrs×6 doses, days 1-3 & days 15-17
Maintenance (M, six cycles)	Vincristine	1.4 mg/m^2 IV, day 1
	Prednisolone	40 mg/m^2 PO, day 1-7
	Daunorubicin	30 mg/m^2 IV, day 1
	L-Asparaginase	6000 U/m^2 IM, days 1,3,5,7
	Methotrexate	15 mg/m^2 PO, once a week for 12 weeks begin on day 15
	Mercaptopurine	75 mg/m^2 PO daily, 3 weeks out of every four for a total of 12 weeks begin on day 15

Adult (BFM-95 protocol)		
Cycle	**Chemotherapy**	**Schedule**
Induction	Vincristine	1.4mg/m^2 (2mg) IV on days 8,15,22,29
	Adriamycin/Doxorubicin	45-60/m^2 in 5% dexamethasone infusion
	L-Asparaginase	6000 IU/m^2 (max 10,000 U)
	Prednisolone	60 mg/m^2 daily: days 1-29
Consolidation	Cytosar	75mg/m^2 on days 3-6,10-13,17-20 &24-27
	IT (Cytosar + Dexa + Mtx)	Days 3,10,17,24
	Cyclophosphamide	1000mg/m^2 on days 1,15,29
	Mercaptopurine	60mg/m^2, daily
Maintenance	Prophylactic CRT and CT (6MP, MTX, IT chemo)	-

*1-<2 yrs– 8 mg, 2-<3 yrs – 10mg, ≥3 yrs – 12mg

Genetic Abnormalities in Acute Leukemia (AL)

The genetic alterations associated with acute leukemia can be divided into two functional groups [Gilliland and Griffin 2002] (Figure 2.5). The class I mutations activate tyrosine kinases signaling pathways and confers a proliferation advantage on hematopoietic cells [Frohling *et al.,* 2005]. In contrast, the class II mutations frequently involve transcriptional regulators of normal hematopoietic differentiation, and result in a block of hematopoietic cell maturation and/or aberrant self-renewal capacity [Kelly and Gilliland, 2002]. However, expression of the class II mutations in the hematopoietic system often leads to myelodysplasic changes and acute leukemia after a long latency, suggesting that collaboration of additional genetic alterations might be required. The class II mutations impair differentiation and augment self-renewal properties of hematopoietic progenitors by modulation of chromatin remodeling and recruitment of aberrant co-activator or -repressor complexes, while having modest effects on cell proliferation or survival. This group comprises gene rearrangements mainly recurrent translocation such as such as t(8;21), inv(16) and t(15; 17), which generate fusion transcripts of AML/ETO, CBFb/MYH11 and PML/RARa that affects transcriptional regulators [Vardiman *et al.,* 2002; Gilliland *et al.,* 2004; Mrozek *et al.,* 2004]. Many of these genes encode for transcription factors which are critical regulators for the differentiation program of hematopoietic stem and progenitor cells.

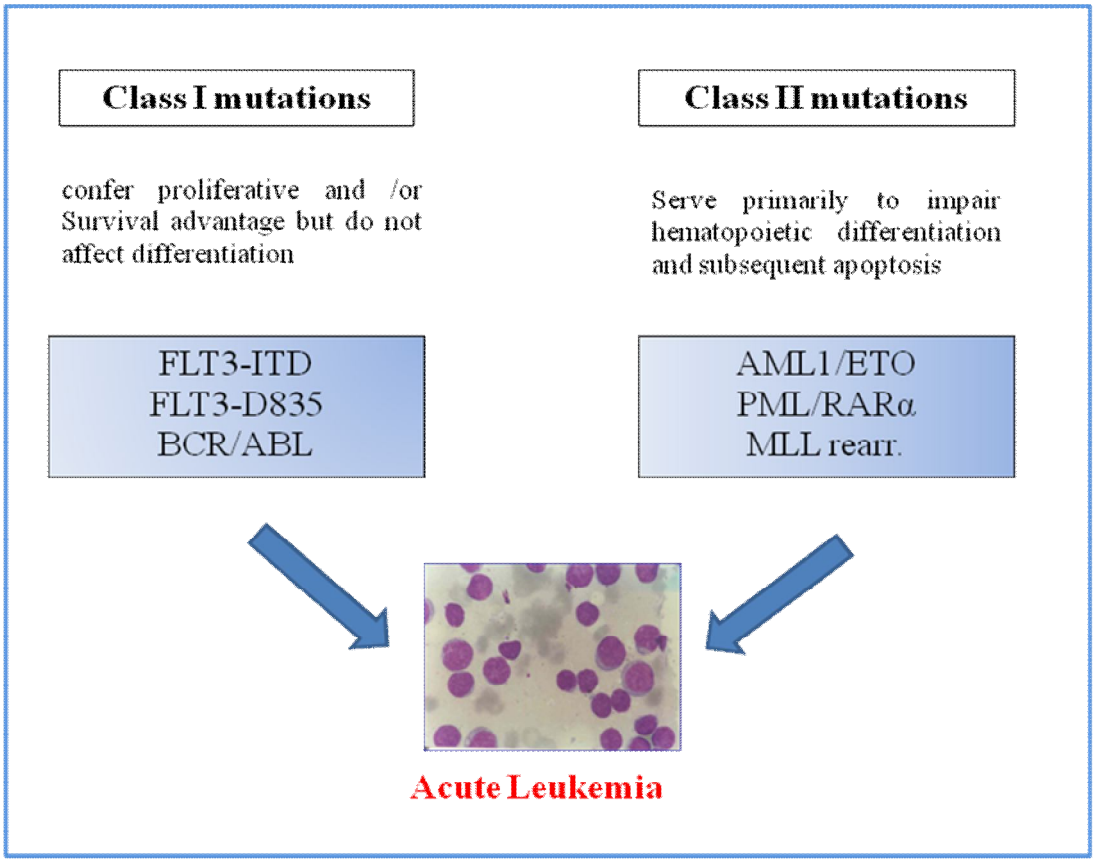


Figure 2.5: A functional collaboration model of genetic alterations leading to acute leukemia: The development of AL is a multistep process that requires at least these two genetic abnormalities for the development of the disease [Adapted from Kelly LM and Gilliland 2001].

Chromosomal Translocation in Acute leukemia (AL)

t(9;22)(q34;q11) (BCR/ABL): The t(9;22)(q34;q11) translocation results from a rearrangement between the c-ABL protooncogene (on chromosome 9) and the BCR gene (on chromosome 22) generating a BCR/ABL fusion protein with an enhanced tyrosine kinase (TK) activity, causes changes of multiple signal transduction pathways and has been demonstrated to be a primary cause of leukemia formation in vivo. It is involved in the malignant transformation of hematopoietic cells, by enhancing proliferation, reducing apoptosis, and deregulating cell adhesion [Melo *et al.,* 2004]. Although BCR-ABL is known to activate some important pathways (such as JAK/STAT, RAS, PI-3 kinase and SCR family), the detailed mechanisms behind BCR-ABL-induced leukemogenesis remain unknown [Hakansson *et al.,* 2008]. The breakpoints in c-

ABL occur in the same region, but BCR can be broken in different clusters, generating distinct proteins. Ph+ ALL childhood patients show a break in the "minor" point cluster region between the BCR exons e1 and e2, forming a fusion protein of 190 kDa (called p190). When the rearrangement of ABL occurs in the "major" breakpoint cluster region of BCR, a fusion protein of 210 Kda (p210) is formed, which is characteristic of chronic myeloid leukemia (CML) and is found in 10% of childhood Ph+ ALL. There is also a rare isoform that forms p230 protein found in uncommon subtype of CML that commits neutrophils [Deininger *et al.,* 2000].

t(8;21)(q22q;22) (AML/ETO) : The core-binding factor (CBF) is a heterodimeric transcription factor composed by the AML1 (also known as RUNX1) and the CBFβ subunits, which are essential for normal hematopoiesis [Speck and Gilliland, 2002]. Thus, disruptions of the AML1/ CBFβ complex might cause differentiation arrest and subsequent leukemic transformation. Indeed, CBF is the target of multiple chromosomal translocations and mutations. Among them, the most frequent and extensively studied is the AML/ETO (RUNX1/ETO) resulting from t(8;21) translocation. Transcription factor, acute myelogenous leukemia (AML1) is a key regulator of hematopoiesisis and the most frequent target of chromosomal translocations in AML [Okuda *et al.,* 1996; Hiebert *et al.,* 1996]. The AML1 gene encodes various isoforms, sharing a DNA binding domain highly homologous to that of the Drosophila runt transcription factor [Bae *et al.,* 1994]. In the t(8;21) chromosome translocation, AML1 recombines with the ETO (eight-twenty-one or MTG8) zinc finger nuclear protein, a putative transcription factor [Nucifora *et al.,* 1995; Hiebert *et al.,* 1996]. The product of AML/ETO plays a critical role in the pathogenesis of AML where behaves as a transcriptional repressor on AML1 target genes and inhibits differentiation of hematopoietic precursors in vivo and results in increased cell proliferation with high self renewal capacity [Westendorf *et al.,* 1998; Downing, 1999].

t(15;17)(q22;q12 ~ 21) (PML/RARα) : The chromosomal rearrangements involving retinoic acid receptor alpha (RARα) on chromosome 15 are exclusively associated with acute promyelocytic leukemia (APL). APL-associated gene rearrangements are characterized by fusing RARα to several different partner genes, of which the most common fusion is PML/RARα resulting from t(15;17). PML/RARα inhibits the function of RARα by a dominant negative effect through recruiting the co-repressor complex, in a manner similar to the AML1/ETO fusions

[Frohling *et al.,* 2005]. RARα belongs to family of nuclear hormone receptor. Nuclear hormone receptor function as ligand-activated-zinc transcription factor. RARα binds to another protein RXR to bind to specific sequence (A/G)G(T/G)TCA in the DNA called as Retinoic response element (RARE). Target genes of RARα are defined by a RARE within their promoter [de the et al., 1990]. In the absence of ligand, RARα/RXR represses gene transcription through methylation and deacetylation by recruiting of corepressors [Jaenisch and Bird, 2003]. Besides repressing transcription of genes, PML/RARα also disturb PML localisaton and its function, impaires p53 stability and its function [Insinga *et al.,* 2004], binds to larger variety of direct repeats other than RARα [Kamashev *et. al.,* 2004], stabilizes the kinase MNK1 at protein level [Worch *et al.,* 2004].

Gene mutation in AML

Disruption of normal hematopoiesis in AML involves mutations and/or epigenetic changes (eg, hypermethylation) of genes operative in the regulation pathways of cell proliferation, maturation, and apoptosis. Many of the genomic alterations driving myeloid leukemogenesis relate to both those detectable microscopically as structural and numerical chromosome aberrations, and those detected as submicroscopic gene mutations and changes in gene expression. Recently, molecular genetic markers such as gene mutations have been identified in AML that allowed further subclassification and prognostic predictions. These recurrent molecular markers mainly increase proliferation and survival potential of hematopoietic stem and progenitor cells, often through constitutive activation of protein tyrosine kinases (PTKs) but normally do not affect cellular differentiation.

FLT3 gene mutation : The most frequent recurrent known genetic markers in AML is mutations within the FLT3 gene which are known as activating mutations that lead to increased cell proliferation. The FLT3 gene, mapped to chromosome band 13q12, member of the class III tyrosine kinase receptor family, is expressed on the surface of hematopoietic progenitor cells. FLT3 and its ligand play an important role in proliferation, survival, and differentiation of multipotent stem cells [Stirewalt *et al.,* 2003]. The most common mutation consists of internal tandem duplication (FLT3-ITD) in the juxtamembrane domain and another FLT3/D835 point mutation in the activation loop of the tyrosine kinase domain (TKD) [Yamamoto *et al.,* 2001;

Kottaridis *et al.,* 2001]. FLT3-ITD results in a constitutively active FLT3 protein that promotes Stat5 phosphorylation. The net consequence of FLT3/Stat5 constitutive activation is uncontrolled hematopoietic cell proliferation [Zheng and Small, 2005]. FLT3-ITDs are located in exons 14 and 15 and vary in insertion site and length of the duplicated segment (from three to 400 nucleotides). FLT3-ITD encodes an abnormal protein that undergoes ligand-independent receptor dimerization, autophosphorylation, and constitutive activation, which turns on downstream signaling pathways involved in cell proliferation, differentiation, and survival. These include the Janus kinase 2 (JAK2) signal transducer and activator of transcription 5 (STAT5) pathway and mitogen activated protein kinase (MAPK) pathway [Grundler *et al.,* 2005; Choudhary *et al.,* 2005]. Several studies have shown that mutations in the FLT3 tyrosine kinase receptor, resulting in its overexpression, are strongly associated with an increased relapse risk and reduced overall survival in patients younger than 60, irrespective of cytogenetics [Kiyoi et al., 1999; Kottaridis *et al.,* 2001; Schnittger *et al.,* 2002].

NPM1 gene mutation : Heterozygous mutations in exon 12 of the nucleophosmin member 1 (NPM1) gene, that interferes with cell cycle regulation and apoptosis have been recently described as one of the most frequent genetic lesions in AML in adult [Suzuki *et al.,* 2005; Verhaak *et al.,* 2005; Chen *et al.,* 2006]. The NPM1 gene is mapped to chromosome 5q35. The most common NPM1 mutation is 4–base pair duplication, 956dupTCTG in exon 12 (called type A), that causes a shift in the reading frame in the C-terminal portion of nucleophosmin. This and several other less-frequent mutations, which are always heterozygous, result in both loss of tryptophan residues 290 and 288 (or 290 only), critical for nucleolar protein localization, and acquisition of an additional nuclear export signal at the C-terminus of nucleophosmin, causing its aberrant cytoplasmic localization [Falini *et al.,* 2006]. Nucleophosmin is a multifunctional protein implicating in many function such as preventing nucleolar protein aggregation [Szebeni and Olson, 1999], intervenes in processing and/or assembly of ribosomes through its nucleo-cytoplasmic shuttling properties and intrinsic RNAse activity [Herrera *et al.,* 1995]. Recent studies in cell lines and knockout mice have shown that NPM1 is involved in the control of genomic stability and contributes to growth-suppressing pathways through its interaction with p53 and Arf tumour-suppressor pathways. Therefore, the loss of NPM1 expression can contribute to tumorigenesis [Grisendi et al, 2006].

Drug Resistance in Acute leukemia (AL)

Cellular multiple drug resistance (MDR) is the phenomenon in which cells have become resistant to the cytotoxic action not only of single drugs but also groups of chemically related and unrelated drugs. The resistance of leukemic cells to these drugs through cellular alterations is considered one of the major causes of failures of chemotherapy. The main mechanisms involved in cellular MDR, however, are cellular alterations, as a consequence of upregulation of specific genes involved in biotransformation processes, cellular efflux, and cell replication [Fojo and Bates, 2003]. In addition anti-apoptosis mechanisms, alteration of tumor suppressor genes, altered immunogenicity and clinical risk factors such as white blood count, age, cytogenetic and other risk factor have been reported to affect drug resistance. These mechanisms which may account for this phenomenon including the impossibility of the drug to reach its intracellular target because of the expression of several efflux pumps on the plasma membrane are listed in the (Figure 2.6).

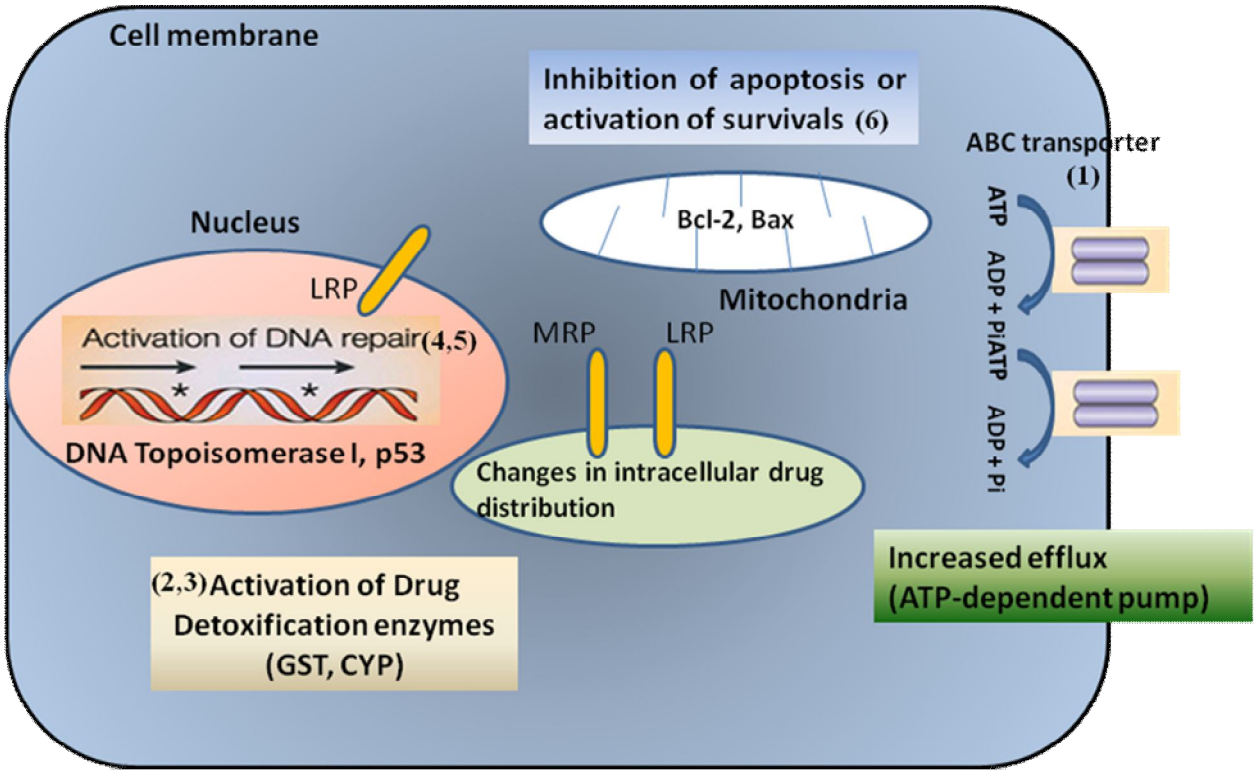


Figure 2.6: Mechanisms of drug resistance. 1. Multidrug resistance proteins. 2. Drug metabolism. 3. Glutathione and its transferases. 4. Molecular target. 5. DNA repair. 6. Apoptosis and its regulation (Adapted from Gottesman *et al.,* 2002).

Expression of membrane transport associated drug resistance genes

Multidrug resistance 1 gene (MDR1): Several MDR mechanisms have been identified, but the discovery of the membrane transporter P-glycoprotein (MDR1 gene) was a breakthrough in understanding the MDR phenotype of cancer cells [Juliano and Ling, 1976]. MDR1 gene is located on long arm of chromosome 7. Its product P—glycoprotein is a 170 kDa protein, member of the ATP-binding cassette (ABC) superfamily of transport proteins which consists of two structurally homologous domains, each containing six hydrophobic transmembrane segments and a highly conserved ATP binding site [Hyde *et al.,* 1990]. P-gp is expressed in various normal tissues with secretory or barrier functions including lung, placenta, testes, adrenal gland, kidney, liver, pancreas, colon, jejunum and brain [Fojo *et al.,* 1987]. In addition, P-gp is expressed by hematopoietic precursors and lymphocytes [Chaudhary *et al.,* 1992]. The mechanism of action of Pgp is not clearly understood. However, there are three hypothesis proposed for its mechanism are channel formation by its transmembrane segments, "a vacuum cleaner model" and last it might function as a "flippase" transporting drugs from the inner to the outer leaflet of the membrane [Bolhuis *et al.,* 1997]. It has been shown to that P-gp is able to pump out of cell many xenobiotics and confer resistance to a wide variety of anticancer drugs, including anthracyclines, mitoxantrone, taxanes, epipodophyllotoxins and vinca alkaloids by lowering the effective drug intracellular drug concentration. Many of these drugs are used in the treatment of AL and many studies reported that overexpression of Pgp tends to correlate with poor treatment response. Authors have found a significantly higher P-gp expression and/or function in T-ALL compared to B-ALL samples [Kanerva *et al.,* 1998; Plasschaert *et al.,* 2003]. However, P-gp expression did not differ between infant or older ALL patients [Ramakers-van et al., 2004; Stam et al., 2004].

Multidrug resistance related protein (MRPl): For several years, MDR1 was the only ABC transport protein associated with drug resistance. However, reports describing drug-resistant cell lines without MDR1-overexpression indicated the presence of other MDR-conferring proteins. These observations led to the discovery of MRPI in cell lines that showed a typical MDR phenotype without elevated P-gp expression [Cole *et al.,* 1992]. The MRP1 gene, mapped on chromosome 16 (16p13.1), encodes a 190 kDa N-glycosylated hydrophobic anion pump

localized on both the plasma and intracytoplasmic membranes, including the endoplasmic reticulum and Golgi apparatus [Lautier *et al.,* 1996]. MRP1 has a broad substrate specificity including glutathione S-conjugates, glucuronide conjugates, sulphate conjugates, the estrogen glucuronide E217βG, sulphated bile acids, anticancer drugs, heavy metals, organic anions and lipid analogues [Jedlitschky *et al.,* 1996; Rigato *et al.,* 2004]. Glutathione (GSH) plays an important role in the MRP1 transport process. However, the exact mechanism by which GSH participates in the MRP1-mediated efflux is unknown. MRP1 expression has been detected in most of the tissues in the human body, especially in lung, testes, kidney, skeletal muscle, epithelial and hematopoietic cells [Flens *et al.,* 1996]. However, the role of MRP1 in inducing the multidrug resistance phenotype in leukemia is still controversial. Fujimaki et al., [2003] found that increased MRD1 expression not MRP1 expression at the time of diagnosis correlated with clinical resistance in leukemia patients. Similarly, Mahjoubi et al., [2008] finding suggest that high MRP1 expression was associated with the clinical outcome in AML but not in ALL patients.

Breast cancer resistance protein (BCRP): Chen et al., 1990 detected a 95 kDa ABC transporter in the human breast cancer cell line MCF-7/AdrVp resistant to doxorubicin. The transporter is known as ABCG2, placental transporter or mitoxantrone resistance protein. BCRP is encoded by the ABCG2 gene which was mapped on chromosome 4 (4q22). In contrast to the MDRI and MRPI gene ABCG2 gene encodes a protein which is a half-transporter molecule requiring dimerizalion in order to function [Ewart and Howells, 1998]. In vitro, high BCRP expression causes resistance to anthracyclines (e.g. doxorubicin and daunorubicin), topo-isomerase I inhibitors (e.g. topotecan), topo-isomerase II inhibitors (e.g. bisantrene, etoposide and mitoxantrone), cell-cycle inhibitors (e.g. flavopiridol) and antifolates (e.g. methotrexate) [Robey *et al.,* 2001; Volk *et al.,* 2002]. BCRP overexpression has been described in resistant ovary, breast, colon and gastric cancer [Maliepaard *et al.,* 2001]. The significance of BCRP for clinical outcome in AL is presently unclear. Some studies but not others have found worse outcome to chemotherapy [Benderra et al., 2004; Damiani et al., 2006]. Further, one study also suggested that BCRP has a limited function in the drug efflux related resistance in AML in comparison to MDR1 and MRP1 gene [van der Pol et al., 2003].

Lung resistance protein (LRP): The lung resistance protein (LRP) was initially identified in an anthracycline-resistant, non small cell lung cancer cell line which lacked P-gp overexpression [Scheper *et al.,* 1993]. The LRP gene is located on chromosome 16 (16p11.2), close to the MRP1 gene, encodes a 110 kDa protein [Slovak *et al.,* 1995]. LRP is not considered an ABC transporter as it lacks characteristic ATP-binding sites however is a constituent of ribonucleoprotein particle: the vault complexes which are evolutionary conserved organelles present in eukaryotic cells [Chugani *et al.,* 1993]. LRP overexpression has been observed in epithelia of the bronchus and digestive tract as well as in keratinocytes, adrenal cortex and macrophages. These results suggest that vaults play a role in detoxification processes [Izquierdo *et al.,* 1996]. The functional role of vaults in MDR is still unclear however; transfection studies in cell lines have suggested that vaults may contribute to resistance to anthracyclins and platinum derivates [Raaijmakers *et al.,* 1998; Kitazono *et al.,* 1999]. Although clinical data on LRP expression in AL is limited, it has been reported that LRP expression may be associated with poor response to chemotherapy in AML than ALL [Michieli *et al.,* 1999; Zhang *et al.,* 2005].

Glutathione S-Transferases (GST) and Multidrug Resistance

The glutathione S-transferases (GSTs) comprise a family of dimeric phase II detoxification enzymes that catalyze the conjugation of glutathione to a wide variety of endogenous and exogenous electrophilic compounds. An important member of this family especially of the π class (GSTP1 gene) is responsible for clinical multidrug resistance [Stavrovskaya *et al.,* 2000]. The overexpression in tumours of GSTs, especially of GSTP1-1 is, considered as a possible mechanism of tumour cell drug resistance [Cote *et al.,* 2005; Zhou *et al.,* 2005]. In addition to its role of a detoxification enzyme GSTP1 also acts as inhibitor of the MAP kinase pathway [Townsend and Tew, 2003]. Specifically, GSTP1 was shown to be an endogenous inhibitor of c-Jun N-terminal kinase 1 (JNK1), a kinase involved in stress response, apoptosis, and cellular proliferation [Adler *et al.,* 1999]. Inhibition of the JNK signaling pathway leads to a decrease in cisplatin induced apoptosis, while overexpression of c-jun increases the sensitivity of cells toward cisplatin [Potapova *et al.,* 1997]. The contribution of any GST to drug resistance is likely to vary with cell type and drug, as well as with the expression profile of other enzymes and transporters. So inhibitors of GST catalytic activity are considered as a potential therapeutic tool.

Drug resistance through expression of apoptosis related gene

Anticancer drugs such as cisplatin, citarabine, etoposide and doxorubicin act in part by inducing apoptosis in tumor cells [Ormerod *et al.,* 1996]. Therefore, alterations of the mechanisms leading to the activation of apoptosis often result in enhanced resistance to these drugs. Drug resistance associated with altered apoptotic response includes drug resistance with altered p53 expression and function [Amson et al., 1996] and resistance associated with altered expression of BCL-2 and the BCL-2 family of proteins [Jia *et al.,* 1999; Kaufmann *et al.,* 1998]. Bcl-2 overexpression was initially described in follicular lymphomas as a consequence of a t(14;18) translocation, and as a poor prognostic marker in acute myelogenous leukemia (AML) and non-Hodgkin's lymphomas. Further overexpression of Bcl-2 prevents apoptosis induced by most chemotherapeutic drugs, including alkylating agents, and topoisomerase inhibitors [Kamesaki *et al.,* 1993]. Some authors revealed that loss of the p53 tumor suppressor gene can also result in a MDR phenotype in cells that normally require p53 to undergo apoptosis following appropriate stress such as DNA damage [Lowe *et al.,* 1994].

Survivin (BIRC5), another regulator of apoptosis is structurally unique inhibitor of apoptosis (IAP) family which block apoptosis induced by variety of non-related apoptosis triggers including antitumor agents [Eckelman *et al.,* 2006; Li *et al.,* 2008]. Survivin is a dual function protein. Besides inhibiting the apoptosis, it is also implicated in both cell survival and regulation of mitosis by stabilizing microtubules [Pennati *et al.,* 2007]. Survivin is preferentially and highly expressed in cancer cells, with little expression in most normal non-dividing adult tissues except CD34+ hematopoietic stem cells, placenta, basal cells of the colonic epithelium, and thymus [Ambrosini *et al.,* 1997]. Moreover, Survivin is identified as the top fourth transcript uniformly expressed in cancer, but not in normal cells [Velculescu *et al.,* 1999]. Over-expression of survivin has been demonstrated to induce drug-resistance to various chemo-therapeutic agents such as cisplatin (DNA damaging agent) and paclitaxel (microtubule stabilizer) in many cancers [Takai *et al.,* 2002; Ferrandina *et al.,* 2005]. Despite profound research, the clinical relevance of cellular resistance, mediated by MDR related transport proteins, remains unclear.

Table 2.11: Genes involved in different mechanism of drug resistance

Mechanism	Protein/Gene	Drugs
Transmembrane Transport	P-glycoprotein (MDR1), MRP, BCRP	Taxanes, etoposide, doxorubicin, vinca alkaloids, Methotrexate, topotecan, mitoxantrone, doxorubicin
Intracellular entrapment	MRP/LRP	Methotrexate, etoposide, doxorubicin, vinca alkaloids
Drug Efflux	MRP	Anthracyclines, vinca alkaloids, epipodophyllotoxins, methotrexate,
Drug Detoxification	GSTs	Chloroethylnitrosoureas, cisplatin, thiotepa, anthracyclines, phosphanides, acrolein, melphalan, cyclophosphamide
Altered nuclear target	DNA Topoisomerase	Chloroethylnitrosoureas, epipodophyllotoxins, anthracyclines
Apoptotic response	Bcl-2 ;p53;Survivin	Taxanes and other cytotoxic Agents such as cisplatin, etoposide

Xenobiotic metabolizing (Drug metabolizing) gene polymorphism in acute leukemia (AL)

Acute leukemia (AL) is a heterogenous disease resulting from the number of predisposing factors and exposures. In all likelihood, the cause or causes of the acute leukemia is multifactorial, including genetic, immune, infectious, and environmental factors. Though the clinical, biological and immunophenotypical aspects of AL have been fairly well explored, little is known about the leukemogenesis, particularly with respect to the genetic susceptibility and the environment factors. Carcinogens present in these environmental factors damage DNA after metabolic activation by xenobiotic enzymes (Figure 2.7). Such interactions at the level of hematopoietic precursors cells predispose the individual to develop AL. The carcinogenic effect of xenobiotics is influenced by a series of genes codifying enzymes involved in oxidation/activation (phase I) and conjugation/ detoxification (phase II) of these compounds. However, these enzyme genes exhibit polymorphisms leading to variability to metabolize environmental carcinogens [Taningher *et al.,* 1999].

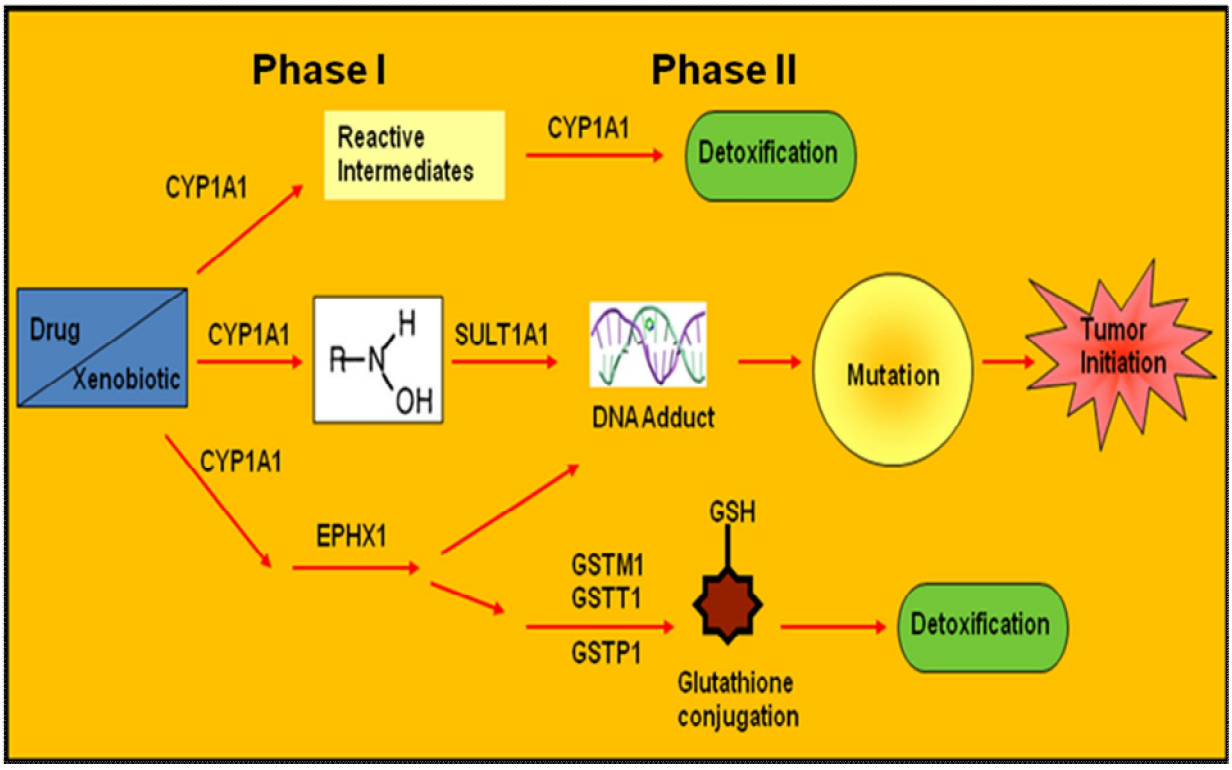


Figure 2.7: Overview of Xenobiotic metabolizing (Drug metabolizing) genes in cancer

Cytochrome P450 (CYP) isoenzymes are one major kind of phase I enzymes that catalyzes oxidative reactions and activates xenobiotics to carcinogenic reactive metabolites. The CYP1A1 gene which encodes for aryl hydrocarbon hydroxylase (AHH) is predominantly extrahepatic, microsomal enzyme involved in the bioactivation of PAHs including benzo(a)pyrene. Polymorphisms in CYP1A1 (chr15q24.1) are the most frequently studied. Two functional polymorphisms are known in the CYP1A1 gene; one is 3698T>C substitution (CYP1A1*2A, rs 4646903) creating an MspI restriction site in the 3'-flanking region, and the other is 2454A>G substitution (CYP1A1*2C, rs 1048943) resulting in an amino acid change in exon 7 (Ile462Val) [Bartsch *et al.,* 2000]. The CYP1A1*2A and CYP1A1*2C alleles are putatively linked to higher inducibility of the enzyme, probably with increased risk of certain types of cancer including leukemia [Sinnett et., al 2000; Agundez, 2004].

Microsomal epoxide hydrolase (EPHX1) another important Phase I biotransformation enzyme is involved in the first-pass metabolism of highly reactive epoxide intermediates and oxygen radicals. EPHX1 plays a dual role in carcinogenesis depending on the exposure to type of environmental substrates. Besides providing protection against the toxicity of reactive epoxides intermediate, EPHX1 along with CYP enzymes plays a key role in the metabolic activaton of procarcinogens such as benzo(a)pyrene (BP) present in tobacco smoke leading to highly reactive carcinogenic diolepoxides [Miyata *et al.,* 1999]. Literature about the role of EPHX1 polymorphism in the development of AL is very scanty. One study reported a protective role of EPHX1 in leukemogenesis of childhood ALL while another study considered it as a risk factor in adult AML with t(8:21) translocation [Lebailly *et al.,* 2002; Silveira *et al.,* 2009]. These phase 1 metabolic enzymes transfer electrons onto substrate toxicants to create highly reactive intermediates, which are then available for detoxification by a variety of phase 2 enzymes including glutathione S-transferases and sulfotransferases.

Glutathione S-transerases (GSTs) are phase II biotransformation enzyme that convert the activated metabolites of procarcinogens of phase I reactions into non-reactive and water-soluble compounds. The GSTs catalyze the transfer of glutathione to reactive electrophiles, a function that serves to protect cellular macromolecules from interacting with electrophiles that contain electrophilic heteroatoms (-O, -N, and -S) and in turn protects the cellular environment from damage. The co-substrate in the reaction is the tripeptide glutathione, which is synthesized from γ-glutamic acid, cysteine, and glycine. Glutathione exists in the cell as oxidized (GSSG) or reduced (GSH), and the ratio of GSH: GSSG is critical in maintaining a cellular environment in the reduced state. The addition of GSH to the xenobiotic compound gives it a molecular 'flag' which allows the xenobiotic-conjugate to be removed from the cell during phase III of drug metabolism, a process which requires the participation of drug transporters such as multi-drug resistance associated protein [Hayes and Mclellan, 1999]. Once transported out of the cell, the peptide portion of the GSH-conjugate is subjected to peptidase attack, by γ-glutamlytransferase and either aminopeptidase M or cysteinylglycine dipeptidase to yield a cysteinyl conjugate which is in turn N-acetylated to form a mecapturic acid. It is the mercapturic acid that is typically the final metabolite that is eliminated from the body in urine.

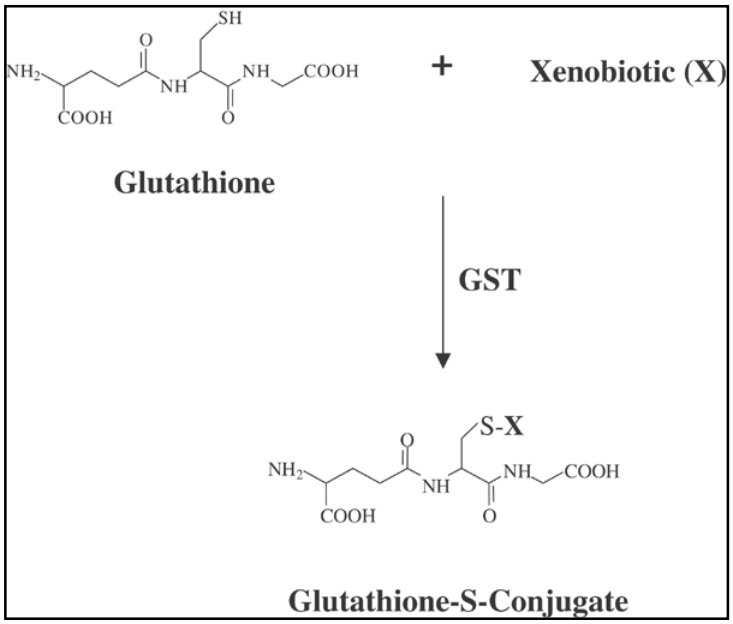


Figure 2.8: Formation of a glutathione-S conjugate - Glutathione conjugation to generic xenobiotic (X) vis GST enzyme (Adapted from Townsend and Tew, 2003)

GSTs have evolved with GSH, and are abundant throughout most life forms. GSTs are divided in to two distinct super-family members: the membrane bound microsomal and cytosolic family members. Cytosolic GSTs are subject to significant genetic polymorphisms in human populations. They are divided into six classes [alpha (GSTA2, mu (GSTM1), pi (GSTP1), theta (GSTT1), zeta (GSTZ1) and omega (GSTO1)], which share ~30% sequence identity. The GSTM1 and GSTT1 both exhibit deletion polymorphism that results in lack of enzyme activity. GSTP1, located on chromosome 11 (11q13), encodes the major enzyme involved in the inactivation of tobacco-related procarcinogens. The GSTP1 Ile105Val polymorphism is associated with reduced catalytic activity which may result in an increased susceptibility to cancer [Ali-Osman *et al.,* 1997]. Individuals with the GSTM1 null genotype and GSTP1 Val/Val allelic variant have significantly higher levels of hydrophobic DNA adducts [Ryberg *et al.,* 2002]. These GST variants have been investigated as a risk factor for predisposition to many

cancers but there are very few studies reported on the association of GST polymorphism with AL risk.

Sulfotransferases (SULTs), the enzymes of phase II metabolism catalyzes the sulfation of a variety of phenolic and estrogenic compounds including endogenous and environmental estrogens. Cytosolic SULT isoforms include two major subgroups, phenol SULT1 and hydroxysteroid SULT2. The most commonly expressed isoform of the phenol SULT1 family is SULT1A1. SULT1A1 is one of the most important members in phenol SULT1 family due to its extensive tissue distribution and abundance [Glatt *et al.,* 2000]. A polymorphism in the SULT1A1 gene (Arg213His) has been identified which has functional consequences for the translated protein in the variant allele (His213, SULT1A1*2) [Nowell *et al.,* 2000].

The equilibrium between phase I and II enzymes determines the outcome of the carcinogen exposure. Genetic polymorphism in these genes might be a determinant of the inter-individual variation in the metabolism of xenobiotic compounds [Perera, 1996]. Altered metabolism of carcinogens has been associated with increased risk of acute leukemia [D Alo *et al.,* 2004; Majumdar *et al.,* 2004] and several solid tumors such as lung, breast, colon and bladder cancer [Talseth *et al.,* 2006; Zienolddiny *et al.,* 2008]. Further, p53 mutations have been associated with reduced repair and enhanced cytotoxicity in cell damaged by benzo(a)pyrene diol epoxide-DNA adducts [Wani *et al.,* 2000]. Numerous polymorphism in the p53 gene have been reported both in coding and non coding regions [Pietsch *et al.,* 2006]. However the most common is codon 72 polymorphism which is a single base substitution of cytosine for guanine, leading to arginine (A72) replaced by proline (P72) [Pietsch *et al.,* 2006]. P53 codon 72 polymorphism has been reported to be associated with the risk of several cancers [Wu *et al.,* 2002; Tandle *et al.,* 2001; Rogounovitch *et al.,* 2006; Mitra *et al.,* 2005]. Interaction of polymorphisms in p53 gene with various phase I and phase II enzymes has been associated with increased risk of solid tumors only [Miller *et al.,* 2002; Quinones *et al.,* 2006]. However, the role of phase I and phase II enzymes along with p53 codon 72 polymorphism still need to be explored in the acute leukemia.

Chapter 3

Aims and Objectives

Chapter 3

Aims and Objectives

Acute leukemia (AL) is a heterogeneous group of disease that has different clinical and prognostic characteristics. Considerable advances have been made over the last three decades in the diagnosis, treatment and survival of the patients with acute leukemia. However, the management of acute leukemia is still a challenge due to heterogeneity at molecular level. The aim of the current study was to find out the clinical relevance of genetic alteration such as chromosomal translocation, gene mutation and genes involved in the multidrug resistance phenomenon that may be responsible for poor outcome in patients with AL. The study also evaluated the significance of polymorphism in genes involved in Phase I and Phase II metabolism of xenobiotic (drug) compounds. For this, samples with confirmed diagnosis of acute leukemia using flow cytometer were analyzed for the aberrant expression of CD markers, fusion genes (BCR/ABL, AML/ETO and PML/RARα), gene mutation (FLT3 and NPM1), genetic polymorphism in xenobiotic enzymes (GSTM1, GSTT1, GSTP1, CYP1A1, EPHX1, and SULT1A1) and the expression of drug resistance genes (MDR1, MRP1, LRP, BCRP) by Real time PCR.

The specific objectives were as follows:

1. Confirmation of diagnosis and typing of acute leukemia using Flow Cytometer

FAB diagnosed samples of acute leukemia were confirmed with a panel of fluorochrome conjugated antibodies for immunophenotyping using FACS Calibur Flow Cytometer (Beckton Dickinson). Heparinised whole blood samples/bone marrow were processed by the direct immunofluoroscence method. Flow cytomeric analysis was performed on using Cell Quest™ software. Aberrant expression of cross lineage antigen marker was also evaluated in samples of patients with acute leukemia.

2. **Detection of commonly occurring genetic alteration in acute leukemia**

Genetic rearrangement like BCR/ABL, AML1/ETO and PML/RARα fusion genes were detected by reverse-transcription polymerase chain reaction (RT-PCR) in samples of acute leukemia patients. Polymerase Chain reaction (PCR) and DNA sequencing was used to detect mutation in molecular markers such as FLT3 and NPM1 mutation in AML patients. The association between these genetic markers with clinical, hematological features and immunological marker in patients with acute leukemia was also determined.

3. **Study of multidrug resistance genes in acute leukemia**

To understand the mechanism of multidrug resistance in acute leukemia, expression of genes involved in drugs efflux (ATP dependent binding pump MDR1, MRP1, LRP, BCRP), drug metabolism (GSTP1, DHFR) and apoptosis (p53, BCL-2, Survivin) were studied by Real-Time PCR. Correlation of expression of Multidrug resistance genes with genetic rearrangements was also assessed.

4. **Role of Xenobiotic metabolizing (Drug metabolizing) genes and p53 codon 72 polymorphism in acute leukemia**

In this objective, a multigenic approach was applied to identify the association of eight polymorphisms in six xenobiotic/drug metabolizing genes (GSTM1, GSTT1, GSTP1, CYP1A1, EPHX1, and SULT1A1) and p53 codon 72 gene polymorphism with the risk of AL. Classification and regression trees (CART) and multifactor dimensionality reduction (MDR) analysis method were used to explore high order gene-gene interactions.

The present study will contribute in accurate diagnosis, stratification of risk on the basis of molecular and genetic marker and understanding the mechanisms responsible for drug resistance and the role of genetic polymorphism in susceptibility to acute leukemia

Chapter 4
Confirmation of diagnosis and typing of acute leukemia using Flow Cytometer

Chapter 4

Confirmation of diagnosis and typing of acute leukemia using Flow Cytometer

Introduction

Acute leukemia (AL) is a progressive, neoplastic disease of the hematopoietic system charaterized by unregulated proliferation of uncommitted or partially committed stem cells. It includes a heterogeneous group of neoplasms that differ with respect to aggressiveness, cell of origin, clinical features, and response to therapy. Immunophenotyping or surface membrane phenotype analysis is an increasingly important tool for identifying immunological abnormalities, diagnosing lymphocyte neoplasms, and monitoring changes in immune status in conjunction with various therapeutic manipulations. Flow cytometric characterization of leukemia and lymphomas using monoclonal antibodies against lineage and differentiation markers has helped in more precise and objective classification of these malignancies. Acute lymphoblastic leukemias (ALL) were the first group of hematological malignancies in which immunophenotyping proved to be clinically useful. The FAB classification, which utilizes the morphological and cytochemical criteria, identifies lymphoblastic and myeloblastic leukemia but has some limitations. First, the reproducibility of the morphologic separation of the different acute myeloid leukemia (AML) subtypes in various studies ranges from 60 to 90%. Second, absence of cytogenetics and molecular genetics features in FAB classifications has been shown to have significant clinical impact in AL. Third, FAB criteria for separating MDS from AML are not practical and easily reproducible. Fourth, FAB classification does not directly address the cases of Biphenotypic acute leukemia (BAL).

Advances in flow cytometry technology and availability of commercially produced monoclonal antibodies directed against hematopoietic and lymphoid cell antigens have opened new horizons for the diagnosis and classification of acute leukemia. Antibodies are assigned cluster designations (CD) by International Workshops on Leukocyte Differentiation Antigens. Using these antibodies, the first immunologic classifications of AL separated lymphoblastic from

myeloblastic lineages and recognized B- and T-lineage ALL subtypes. Further, expression patterns of CD34 and HLA-DR were used in distinguishing acute promyelocytic leukemia (APL/AML M3) from AML M1/M2 [Stelzer *et al.,* 2000]. The European Group for the Immunological Characterization of Leukemias (EGIL) has proposed an immunologic classification of AL that includes ALL, myeloid antigen positive ALL, AML, lymphoid antigen positive AML, BAL and undifferentiated AL [Bene *et al.,* 1995]. Based on scoring system adopted by EGIL, BAL was distinguished from those with aberrant expression of a marker from another lineage (Ly+AML, My+ALL). This scoring system was based on the number and degree of specificity of the markers expressed by the leukemic cell.

Immunophenotyping of blasts has become indispensable in the diagnosis of ALL [Khalidi *et al.,* 1999]. Specific phenotypic characterization of ALL blast cells is usually performed using lineage associated markers such as CD19 (B-cells) and CD7 (T-cells). ALL is initially divided into B and T lineage with the B lineage further subdivided into B cell, pre B cell and early B-precursor types [Pui *et al.,* 1993]. The correlation of prognosis and choice of therapy with FAB classification alone is much less clear especially for L1 and L2, as these include many different immunological subtypes of ALL. Thus, therapy and clinical outcome are better correlated with immunophenotype than morphology alone [Copelan *et al.,* 1995]. Expression of CALLA (or CD10) by T-ALL is associated with an improved clinical outcome. Mature B-ALL patients may require more aggressive therapy compared to T-ALL which is less common but has a more favourable prognosis with new treatment regimens particularly in adults [Boucheix *et al.,* 1994]. The diagnostic role of immunophenotyping in AML is not as successful as in ALL due to lack of monoclonal antibodies to lineage specific antigens. Immunophenotyping is mostly useful for diagnosing AML subtypes, such as the M0, M6 and M7 and especially M3 [Rothe and Schmitz 1996; Dunphy, 1999].

Aberrant phenotype is a well known phenomenon in which lymphoid-associated markers or myeloid associated markers are co-expressed in myeloblasts and lymphoblasts respectively. Occurrence of aberrant phenotype has been reported in both ALL and AML with varying frequencies. Immunophenotyping has also highlighted ‘‘aberrant’ antigen expression in acute

leukemia [Khalidi *et al.,* 1998]. One of the T-cell lineage markers, CD7, has been detected on the leukemic cells in a minority of AML cases. In a recent study, expression of aberrant myeloid antigen (CD13 and/or CD33) has been reported in 35% cases of ALL [Vitale *et al.,* 2007]. Several reasons have been suggested for the conflicting reports in the occurrence of aberrant phenotype. These include, use of different reagents against the CD surface antigens, different cut-off levels, analysis of fresh or frozen cell material, and most importantly the difference between phenotypic characteristics of blast cells of children and adult patient.

Prognostic value of aberrant phenotype and its association with adverse clinical, hematological, and other biological prognostic factors in adult and childhood AL is still controversial. In some studies, the aberrant phenotypes were found to be of prognostic significance while other studies reported no difference between aberrant and normal phenotype. In the literature, Ly+AML phenotypes have been shown to be associated with both poor as well as favorable prognosis [Casasnovas *et al.,* 2003; El-Sissy *et al.,* 2006]. Similarly, in ALL, opinions are conflicting in the literature about the clinical outcome of My+ALL phenotype in both children and adultspoor prognosis has been reported in adults others disagree with this [Wiersma *et al.,* 1991; Pui *et al.,* 1993; Suggs *et al.,* 2007; Vitale *et al.,* 2007]. The significance of aberrant expression of antigen is not clearly established. Biologically, it could represent aberrant lymphoid antigen expression on myeloid blasts or may be a marker of a developmental stage in myelopoiesis.

With this background, the specific objectives of this study were

- To determine the frequency and significance of CD markers in FAB diagnosed samples of acute leukemia by flow cytometry and their correlation with age, sex and clinico-hematological features.
- To determine the frequency of aberrant expression of lymphoid antigen markers in AML and myeloid antigen markers in ALL.
- To correlate the expression of aberrant phenotypes with known clinical and hematological features of acute leukemia.

Experimental methods

Materials

Dulbecco's Phosphate buffered Saline, Heparin, Sodium azide (Himedia, India), Histopaque, Tris base, EDTA, Nacl Paraformaldehyde and other chemicals from Sigma, USA. Flow cytometry antibodies and BD cytofix/cytoperm kit were purchased from BD Pharmingen, USA.

Selection criteria of patients

Patients with a confirmed diagnosis of acute leukemia admitted to the Division of Hematology, Safdarjang Hospital, New Delhi for induction chemotherapy from the year 2006-2010 were included in the study. The diagnosis of acute leukemia was made on routinely stained bone marrow aspiration/biopsies and peripheral blood smears and evaluated according to the French-American-British (FAB) criteria. Cytochemical stains were used to differentiate AML from ALL and to sub-classify them into FAB sub types. Written informed consent, according to the protocol approved by the Ethical Committee of Safdarjang Hospital was obtained from all patients.

Inclusion criteria

- Patients diagnosed with acute leukemia at Safdarjung Hospital, New Delhi.
- All patients treated with the standard '3+7' induction chemotherapy or ATRA (all-trans-retinoic acid) therapy in AML and standard vincristine, adriamycin/daunorubicin, L-asparaginase, prednisone and methotrexate (BFM95 in adult and MCP841 in children) in ALL were included in the study.

Exclusion criteria

- Patients unwilling to give consent.
- Patients who were too ill to participate in the study were excluded.
- Patients who had taken any form of treatment earlier (Secondary cases) were also excluded from the study.
- Patients with a documented myelodysplastic syndrome, or blastic phase of CML were also excluded.

Patients' details

Relevant clinical details and investigations were obtained for all patients included in the study at the time of diagnosis. The name, age, sex and address of each patient, along with the history and presenting complaints of fever, weight loss, petechiae, gum bleeding, bleeding from other sites, and duration of symptoms was recorded. Findings of physical examination including hepatosplenomegaly (HSM), lymphadenopathy (LAP) were noted. Hematological parameters like Hemoglobin%, WBC count, platelet count and blast% at diagnosis and clinical response after induction chemotherapy on BMA were also recorded.

Collection of blood samples/bone marrow aspirates

Peripheral blood samples (4-5 ml) were obtained from all patients in heparinised vials (10-50 U/ml of blood) and transported to the laboratory on ice. In addition, bone marrow aspirates were collected in heparin containing phosphate buffered saline (PBS) when possible. All samples were processed on the same day. One ml of whole blood/bone marrow sample was used for Immunophenotyping studies by flow cytometry and remaining 3-4 ml of blood/bone marrow was used for separation of PBMC as described below for genetic studies and gene expression analysis.

Separation of peripheral blood mononuclear cells

Leukemic peripheral blood mononuclear cells (PBMC) were isolated by a standard density gradient/centrifugation method using Histopaque-1077 solution (Sigma). Blood/bone marrow was diluted with PBS (1:1) and mixed well by inversion. To a 15 ml conical centrifuge tube, 3 ml of histopaque was added. Carefully 8 ml of diluted blood was layered onto the histopaque. Tube was then centrifuged at 400g at room temperature for 15 minutes. After centrifugation opaque interface containing the mononuclear cells was aspirated and transferred into a 1.5 ml tubes. PBS was then added and mixed by inversion. Tubes were then centrifuged at 250g for 10 minutes. Supernatant was removed from the tube and pellet was resuspended in PBS and mixed by gentle aspiration with the pipette. Tubes were again centrifuged at 250g for 10 minutes. This step was repeated again. Final pellet was resuspended in 1 ml of PBS. From this, 10 µl of cell suspension was taken out for the purpose of cell counting using hemocytometer chamber. Finally concentration of cell suspension was adjusted to $2x10^7$ cells/ml of PBS. Cells were stored in RNA later solution and kept at -70^0C for genetic studies and Real Time-PCR experiments.

Flow cytometric immunophenotyping of acute leukemia

FCM immunophenotyping was used for identification and phenotypic characterization of blast cells using a panel of fluorochrome conjugated antibodies [Fluorescein isothiocyanate (FITC) & Phycoerythrin (PE)]. All antibodies were purchased from Becton Dickinson (BD) Biosciences (USA). The following panel of fluorochrome conjugated antibodies was used (Table 4.1).

Table 4.1: Panel of antibodies used for the diagnosis of acute leukemia

Non-lineage	B-Lymphoid	T-lymphoid	Myeloid
FITC labeled CD34 and TdT	FITC labeled CD19, PE labeled CD10	FITC labeled CD3, CD5 and CD7	FITC labeled CD14, CD 33 , cyt MPO PE labeled CD13

- FITC labeled CD 34 and PE labeled CD45 for selection of blast cells.
- FITC and PE conjugated isotypes were used as control.
- Unstained cells were used for instrument setting.

Procedure for staining cell surface antigen

Heparinised whole blood samples were processed by the direct immunofluoroscence method using PharM Lyse solution according to manufacturer's protocol (Becton Dickinson Biosciences, USA).

STEP1. Antibody Incubation- To 1.5 ml tube, whole blood (≤ 50 µl containing $1x10^6$cells) was incubated at 4°C for 30 minutes with 10 µl of monoclonal antibodies in the dark. (Separate tubes were taken for each antibody)

STEP2. Lysis of RBCs-After incubation, one ml of 1x PharM lyse solution was added to each tube. Tubes were vortexed, incubated for 10 minutes and centrifuged at 2000 rpm for 6 minutes to get the cell pellet. Supernatant was removed carefully from each tube without disturbing the pellet.

STEP3. Washing of cell pellet- One ml of PBS (pH 7.4) was added to the cell pellet. Tubes were vortexed to resuspend the pellet and then centrifuged at 2000 rpm for 6 minutes. Color of

the pellet was observed. If, the color of the pellet was reddish (indicates incomplete lysis of RBCs), Step 2 was repeated. If, the color of the pellet was whitish, step 3 was repeated again for proper washing of cells. Cell pellet washing was done twice.

STEP4. Fixation of cells - Carefully supernatant was removed from all the tubes and cell pellet was resuspended in 0.5 ml of 1% paraformaldehyde solution for fixation of cells.

For staining Intracellular cytoplasmic antigen

For myeloperoxidase (MPO) antigen and TdT (intracellular), BD cytofix/cytoperm kit was used.

FACS Analysis

Set up of the instrument for acquisition of cells (Data acquisition and storage)

- Using Cell Quest software, following dot plots were created: 1) FSC vs. SSC and 2) FL2 vs. FL1.
- From the Acquire menu, connect to cytometer and parameter description and from the Cytometer menu, detectors/amps, threshold and compensation were selected.
- Using detectors/amps (FSC and SSC gain), majority of the cells were positioned near the midpoint of the FSC axis. Cell debris was excluded from being counted using threshold option.
- Using detectors/amps (FL1 and FL2 PMT voltage), FL1 and FL2 voltage was adjusted so that the majority of unstained cells appear in the lower-left corner of the FL1 vs. FL2 dot plot or show between 10^0 and 10^1 fluorescence units (U) on the dot plot.
- After optimum settings, 10,000 cells/events were acquired from the tube.
- Using these settings, 10,000 cells from the Isotype control (FITC and PE) tubes were acquired. Fine adjustment was made to Compensation and other settings if necessary.
- After final adjustment to compensation and detector settings, a total of 10,000 cells from each samples were acquired.

Data analysis

Cell Quest^{TM} software was used to analyze the data. Results were obtained by gating the blast cells using side scatter (SSC) versus forward light scatter (FSC) parameters and examining the associated FITC and PE fluorescence on FL1 and FL2 detectors, respectively (Fig. 4.1). In ALL, aberrant expression of CD13 and CD33 while in AML, aberrant expression of CD3, CD5, CD7, CD10 and CD19 was studied. The percentage of population showing high intensity of CD45 (representing the contamination of normal cells in the gated area) was subtracted from the total positivity of particular aberrant marker in order to calculate the exact percentage of an aberrant marker. Antigen expression was considered to be positive when the percentage of positive blast cells was equal or greater than 20%. Similarly, aberrant phenotypes were defined when at least 20% of the blast cells expressed the particular aberrant marker.

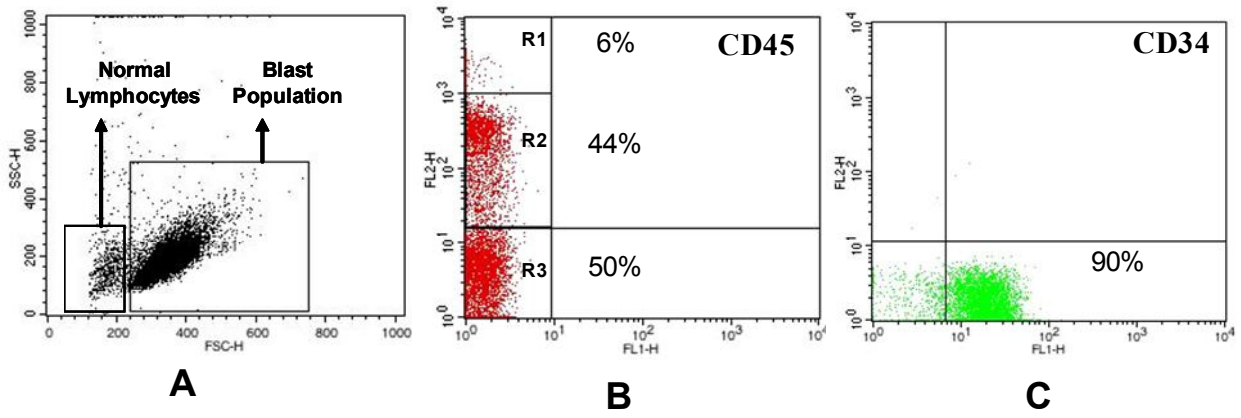


Figure 4.1 : Analysis for aberrant antigen expression (single color immunophenotyping) : A. a showing selection of blast population (gating), B. showing three cluster or region of different intensity of CD45 from the gated blast population (R1 high intensity, R2 moderate intensity, R3 negative/nill). Both R2 and R3 regions represent blast population as these cells have nill or moderate expression of CD45 while R1 represents contamination of normal cell population in the gated area of blast population as these cells have high intensity of CD45. Since normal cells have high expression of CD45 and appear in R1 region thus in order to calculate the actual percentage of particular aberrant marker, % of R1 region (CD45 positive population of high intensity) was subtracted from the total positivity of that particular aberrant marker. C. showing positivity for CD 34 marker, an immature cell marker, this also helps in the selection of blast cell population.

Treatment Protocol and Response to Induction Chemotherapy

AML patients received the standard '3+7' induction chemotherapy. The standard induction chemotherapy regimen in ALL patients was MCP841 in children and modified BFM 95 regimen in adults. The details of the treatment protocol have been described previously in chapter 2. In this study clinical response of the patients was evaluated after completion of induction chemotherapy only. Complete response (CR) to induction chemotherapy was defined clinically by disappearance of abnormal physical findings attributable to the leukemia and return of the patient to good physical health and hematologically by the return of the peripheral blood to normal values with respect to hemoglobin, total and differential white blood cell count and platelets and less than 5 per cent morphologically normal blast cells in a marrow preparation of normal cellularity. Patients who did not meet the criteria of CR after induction therapy were considered non complete response (NR).

Statistical analysis

Analyses were performed using SPSS 19.0 (SPSS inc.) statistical analysis software. Chi-square/Fisher's exact test was used to see the association of aberrant phenotype with adverse prognostic factors and clinical outcome. Two sided p-value = ≤ 0.05 was considered as statistically significant.

Result

Flow cytometric immunophenotyping of Acute Leukemia (AL)

In the current study, 289 FAB-diagnosed acute leukemia samples were analysed for the confirmation of diagnosis and typing of AL by flow cytometric (FCM) immunophenotyping. Of the 289 FAB-diagnosed AL samples, 137 (47.4%) were diagnosed as AML, 145 (50.1%) were diagnosed as ALL, 3 (0.4%) case were of MLAL and 4 (1.4%) were diagnosed as BAL (Table 4.2).

By FCM immunophenotyping 133 (46.0%) cases were diagnosed as AML, 112 (38.7%) as B-ALL, 33 (11.4%) as T-ALL and 7 (2.4%) were diagnosed as mixed lineage acute leukemia (MLAL) and 4 (1.4%) were diagnosed as BAL (Table 4.2 and Figure 4.2). FCM immunophenotypic analysis revealed discrepant results in 9 FAB-diagnosed cases. Of these 4 cases of FAB-diagnosed AML; 2 were diagnosed as B-ALL, 1 as T-ALL, and 1 as MLAL. 6 cases of FAB-ALL were diagnosed as of MLAL type. In addition FCM was useful in diagnosing 3 L0/M0 cases as ALL cases; 2 as B-ALL and 1 as T-ALL (Table 4.3).

Table 4.2: Diagnosis of AL

Types of AL	Diagnosis of AL		
	FAB (n=289)	FCM (289)	
AML (%)	137(47.4%)		133(46.0%)
ALL (%)	145(50.1%)	**B-ALL**	112(38.7%)
		T-ALL	33(11.4%)
BAL (%)	4(1.4%)		4(1.4%)
MLAL (%)	3(1.0%)		7(2.4%)

Note: Table shows the distribution of AL patient diagnosed by FAB and FCM immunophenotyping
AL: Acute leukemia; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia; BAL: Biphenotypic acute leukemia; MLAL: Mixed lineage acute leukemia. Number in parenthesis represents percentage

Figure 4.2: Pie chart showing distribution of AL cases by FCM Immunophenotyping

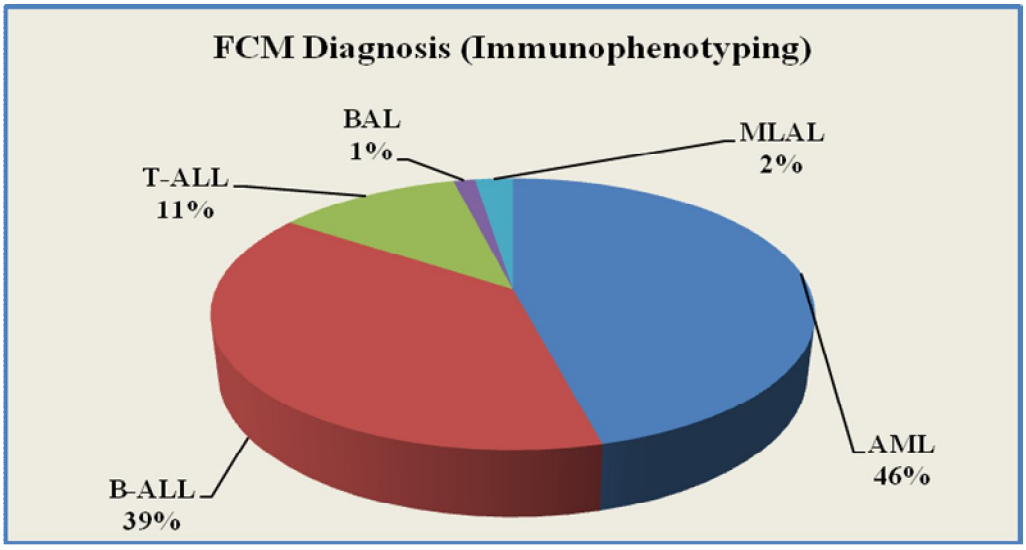


Table 4.3: Discrepant cases of AL

S. No.	Age	Sex	FAB Diagnosis	FCM Diagnosis
1	8	F	L1	MLAL
2	10	F	L2	MLAL
3	9	M	L2	MLAL
4	24	M	L2	MLAL
5	15	M	M5a	B
6	30	M	M1	B
7	22	F	M5b	MLAL
8	50	M	AML	T
9	24	F	L2/M0	T
10	18	M	L2/M0	T
11	32	M	L2	MLAL
12	25	F	L2/M0	B

Note. Table shows the discrepant cases of AL diagnosed differently by FAB and FCM immunophenotyping

Distribution of AL patients according to FAB subtypes

AML: Of 133 FCM diagnosed AML cases, M2 (51/133, 38%) was found to be more common FAB subtype followed by M0/M1 (22/133, 16%). The frequency of M3 was the least one among FAB subtype (8/133, 6%). The same pattern was seen in adults. M2 (36/97, 37%) was the most common FAB subtype followed by M0/M1 (14/97, 14%), M4 (15/97, 15%). In children also M2 was the most common followed by M0/M1>M4>M5>M3. Distribution of FAB types according to gender (male and female) is shown in the table 4.4.

B-ALL: Of 145 FCM diagnosed ALL cases, 128 were of B-ALL and 37 were of T-ALL. According to FAB morphology, L2 was found to be the most common subtype (55/112, 49%) followed by L1 subtype (43/112, 38.3%) in B-ALL. Similarly, L1 was more common in children than adults in B-ALL (39 vs 23 in children: 20 vs 16 in adults) (Table 4.5).

T-ALL: In T-ALL also, L1 was found more often than L1 (18/33 vs. 12/33). In children, L1 (8/17, 47%) and L2 (7/17, 41%) were equally common, however, in adults L1 (10/16, 62.5%) was twice more common than L2 (4/12, 31.2%) (Table 4.5). Distribution of FAB types according to gender (male and female) is shown in the same table.

Table 4.4: Distribution of AML cases according to FAB subtypes

Age group	FAB Subtypes in AML (n=133)													
	M0/M1(21)		M2(51)		M3(8)		M4(16)		M5(18)		M6(0)		AML(19)	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Children (36)	6	1	11	4	2	0	1	0	5	0	0	0	4	2
Adults (97)	11	3	24	12	4	2	10	5	6	7	0	0	7	6

M= Male, F= Female

Table 4.5: FAB distribution of B-ALL and T-ALL cases

Age group		FAB subtypes in **ALL** (n=145)					
		L1 (n=73)		**L2** (n=55)		**ALL** (17)	
		M(52)	F(21)	M(41)	F(14)	M(12)	F(5)
B-ALL (112)	**Children (69)**	28	11	16	7	6	1
	Adults (43)	11	5	15	5	3	4
T-ALL (33)	**Children (17)**	5	3	6	1	2	-
	Adults (16)	8	2	4	1	1	-

M= Male, F= Female

Distribution of AL patients according to Age and Gender

AML: Of the 133 AML patients, 97 patients (73%) were adults while 36 patients (27%) were children (Figure 4.3). The median age of the children and adults were 9.5 and 35 years (Range=1-15 yr and 16-85 yr) respectively. The peak incidence of AML in adults was seen in the age group of 31-35 yr while in children incidence was more common in the age group of 6-10 yr (Figure 4.4).

B-ALL: Of the 112 B-ALL patients, 69 patients (62%) were children while 43 patients (38%) were adults (Figure 4.3). The median age of children and adults were 7 and 24 years (Range=1-15 yr and 16-67 yr) respectively. The peak incidence of B-ALL was seen in 11-15 age group (Figure 4.4).

T-ALL: Of the 33 T-ALL patients, 17 patients (52%) were children while 16 patients (48%) were adults (Figure 4.3). The median age of the children and adults were 9 and 21 years (Range=2-15 yr and 16-50 yr) respectively. The incidence of T-ALL in children was found to be high in the age group of 6-10 yr while in adult peak incidence was seen in the age group of 16-20 yr (Figure 4.4).

Figure 4.3: Distribution of acute leukemia cases in children and adults

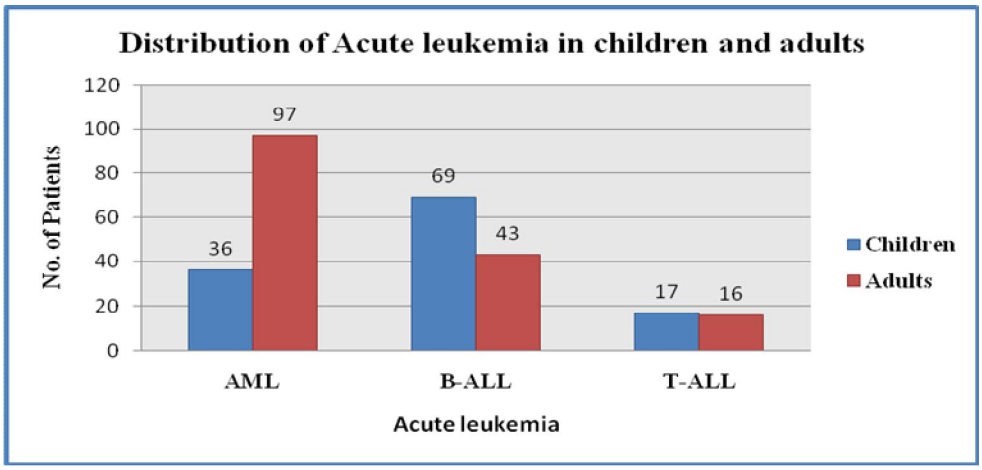
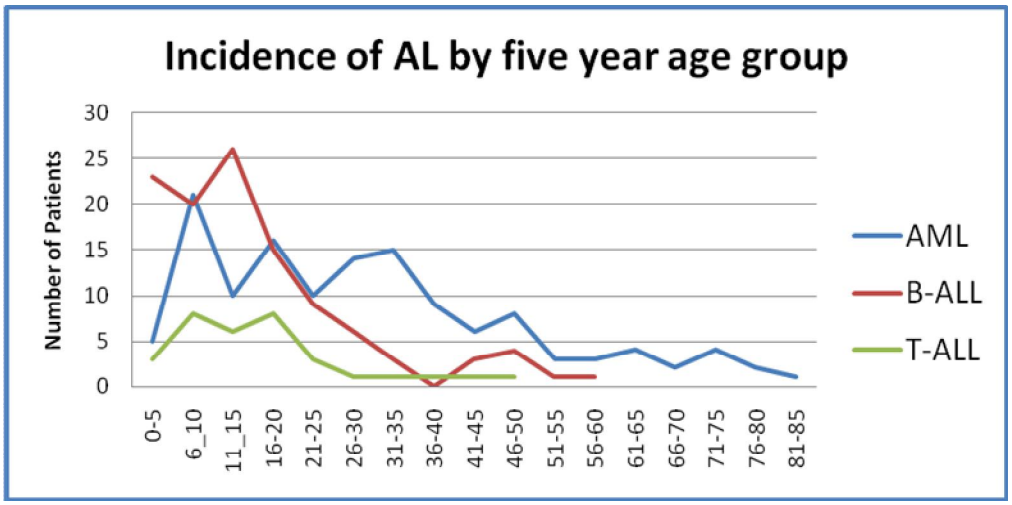


Figure 4.4: Age wise distribution of patients with AML, B-ALL and T-ALL



Clinical, Hematological and Cytochemical findings in AML patients

In AML, the frequency of male was higher in both children and adults than female [64%, (62/97) in adults and 77%, (28/36) in children]. Male to female ratio was 1.8 in adults and 3.5 in children. MPO positivity was seen more in adults than children though it was not statistically significant (93% vs 79%, p=0.06). In AML, hepatosplenomegaly and lymphadenopathy was present in higher number of adults than children but only LAP was statistically significant (80%

vs. 56% respectively (p=0.007). The median level of Hb was also found to be significantly higher in adults compared children (6.5 vs 5.2, p=0.0007). However, no significant difference was found in the Platelet and WBC count between children and adults (Table 4.6).

Table 4.6: Clinical and hematological characteristics of 133 AML patients

		AML (133)	
Clinical and laboratory findings	**Adult (97)**	**Children (36)**	**p-value**
Gender (M/F)	62/35	28/8	0.14
MPO stain (Cytochemical)%(n)	93.3(70/75)	78.6 (22/28)	0.06
Auer rod present %(n)	47.4 (46/97)	50.0 (18/36)	0.84
Hepatosplenomegaly (HSM) %(n)	50.5(49/97)	33.3 (12/36)	0.08
Lymphadenopathy (LAP)%(n)	80.4(78/97)	55.6 (20/36)	**0.007***
Hb (g/dl) (median, range)	6.5(2-13.8)	5.2 (3.3-10.8)	**0.006***
WBC ($\times 10^9$/l) (median, range)	28(1-428)	34.9 (4.8-256)	0.45
Plt ($\times 10^9$/l) (median, range)	20 (1-343)	29 (2-130)	0.55
Peripheral Blasts (median, range)	69 (10-98)	70 (17-96)	0.82

Note. Table shows the clinical and haematological features of patients with AML in adult and children. *Bold values represent significant p value <0.05 calculated by $\chi 2$ test. HSM and LAP were significantly higher in adult AML compared to Chidhood AML.

Clinical, Hematological and Cytochemical findings in ALL patients

B-ALL: Of the 112 B-ALL patients, males were over-represented in B-ALL cases (72% in children and 67% in adults). In childhood and adult B-ALL, male to female ratio was 2.6:1 and 2:1 respectively (Table 4.7). HSM was present in 82.6% and 79.1% cases of children and adult B-ALL while LAP was seen in equal percentages of cases in children and adults respectively. There was no significant difference in the median level of Hb, Plt and WBC count of the children and adults B-ALL (Table 4.7).

T-ALL: Of the 33 T-ALL patients, 79% (26/33) were males while 21% (13/33) were females. The male to female ratio was 3.7:1. In childhood and adult B-ALL, male to female ratio was 3.2:1 and 4.3:1 respectively (Table 4.7). In T-ALL cases, HSM was present in 64.7% and 87.5%

cases of children and adult T-ALL while LAP was seen in 64.7% and 50% cases of adults and children respectively (Table 4.7). The children and adults patients showed no significant difference in their median level of Hb, Plt and WBC count. PAS positivity was seen in higher number of cases of children than adults (75% vs 58.3%) (Table 4.7).

Table 4.7: Clinical and hematological characteristics of 145 ALL patients

	B-ALL (112)			T-ALL (33)		
Clinical and laboratory findings	**Children (69)**	**Adult (43)**	**P**	**Children (17)**	**Adult (16)**	**P**
Gender (M/F)	50/19	29/14	0.67	13/4	13/3	1.0
PAS positivity %(n)	65.6(40/61)	59.4(19/32)	0.65	75.0(9/13)	58.3(7/12)	0.66
HSM%(n)	82.6(57/69)	79.1(34/43)	0.63	64.7(11/17)	87.5(14/16)	0.22
LAP%(n)	49.3(34/69)	48.8(21/43)	1.0	64.7(11/17)	50.0(8/16)	0.49
Hb (median, range)	6.4(2.8-1.9)	5.8(2-12.4)	0.60	6.7(3.2-2.8)	7.4(3-112)	0.90
WBC(median, range)	22 (3-160)	20(1-115)	0.31	56(1.4-395)	68(2.4-240)	0.82
Plt (median, range)	40.6(1.7-320)	33.4(0.9-234)	0.32	25(1.6-173)	20(1-160)	0.46
Blast (median, range)	80(12-96)	80(10-96)	0.53	76(10-99)	73(25-90)	0.66

Note. Table shows the clinical and hematological features of B-ALL and T-ALL patients in adult and children. P- p value calculated by $\chi 2$ test. Hb in g/dl while WBC and Plt in ($\times 10^9$/l).

Analysis of lineage specific antigen markers expressed in AL patients

AML: One hundred thirty three (133) patients with AML (36 children and 97 adults) were evaluated for the expression of myeloid lineage specific marker and CD34 (Table 4.8). Myeloid lineage marker, CD33 was detected in 83.3% and 89.7% cases, CD13 in 85.7% and 87.7% while MPO was detected in 46.2 % and 22.4% cases of children and adults respectively. There was marginal significant difference in the expression of stem cell marker, CD34 between children and adult. The CD34 marker was detected in higher number of children cases than adults (72.2% vs 53.5%, p=0.07) A representative case of AML diagnosed on FCM immunophenotyping is shown in Figure 4.5.

In B-ALL: One hundred and twelve (112) patients with B-ALL (43 children and 69 adults) were analysed (Table 4.8). The expression of B-cell lineage marker i.e. CD19 was detected in all

samples. The CALLA antigen i.e. CD10, was found in 94% and 91% cases of children and adults respectively. CD34 marker was found more often in adults (51%) as compared to children (39%). However there was no significant difference in the expression of any of the above markers between children and adults in B-ALL (Table 4.8). A representative case of B-ALL diagnosed on FCM immunophenotyping is shown in Figure 4.6.

In T-ALL: The expression of the three T-cell lineage antigens (CD3, CD5 and CD7) was analysed in the 33 patients (16 children and 17 adults) (Representative Figure 4.7). The CD5 marker was detected in 82% and 94% cases while CD3 was detected in 53% and 44% cases of children and adults respectively. The CD7 marker was detected in all the cases of children and adults. The expression of CD10 marker was found to be similar in adults and children. The CD34 marker was detected in 18% and 31% cases of children and adults respectively (Table 4.8).

Table 4.8: Expression of lineage specific markers and CD 34 in patients with AL

Type of AL (n=278)	CD markers	% of samples showing CD marker positivity		
		Children (n)	Adults (n)	p-value
AML (n=133) (A=97, C=36	CD13*	85.7 (12/14)	87.7 (43/49)	1.0
	CD33	83.3 (30/36)	89.7 (87/97)	0.37
	CD14*	18.8 (6/32)	21.8 (17/61)	0.80
	MPO*	46.2 (6/13)	22.4 (11/49)	0.15
	CD34	72.2 (26/36)	53.6 (52/97)	0.07
B-ALL (n=112) (A=69, C=43)	CD10	94.2 (65/69)	90.7 (39/43)	0.48
	CD19	98.6 (68/69)	100 (43/43)	1.0
	CD34	39.1 (27/69)	51.2 (22/43)	0.24
T-ALL (n=33) (A=17, C=16)	CD3	52.9 (9/17)	43.8 (7/16)	0.73
	CD5	82.4 (14/17)	93.8 (15/16)	0.60
	CD7*	100 (8/8)	100 (12/12)	-
	CD10	41.2 (7/17)	43.8 (7/16)	1.0
	CD34	17.6 (3/17)	31.3 (5/16)	0.43

Note. Table shows the percentage of positivity of various CD markers in AL patients. Number outside parenthesis indicates percentage, *MPO, CD13 and CD14 expression was not evaluated in all samples.

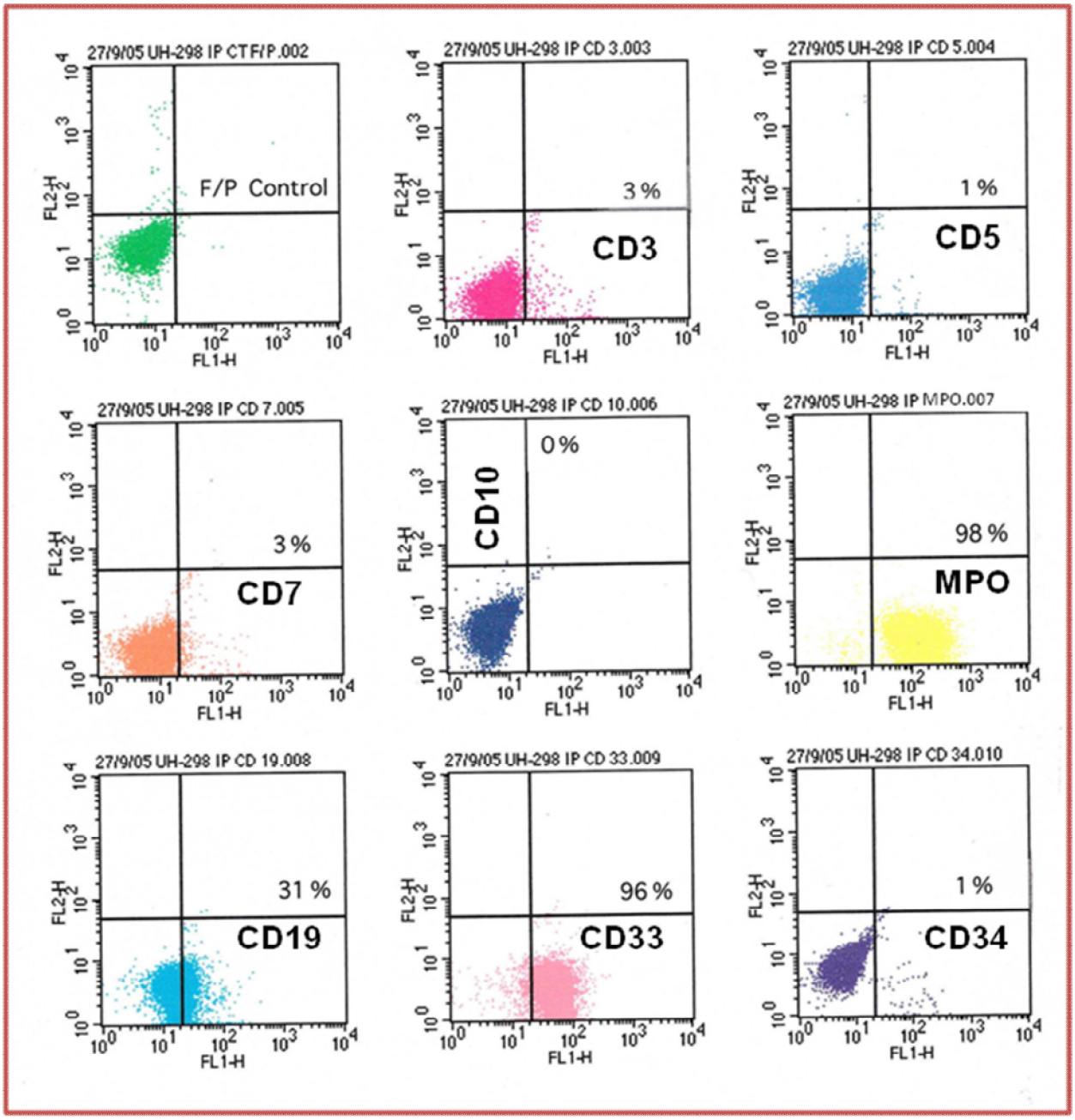


Figure 4.5: Immunophenotypic analysis of AML: A case of AML. Blasts were positive for myeloid marker i.e. CD33 & MPO and negative for CD3, CD5, CD7, CD10, & CD34 markers.

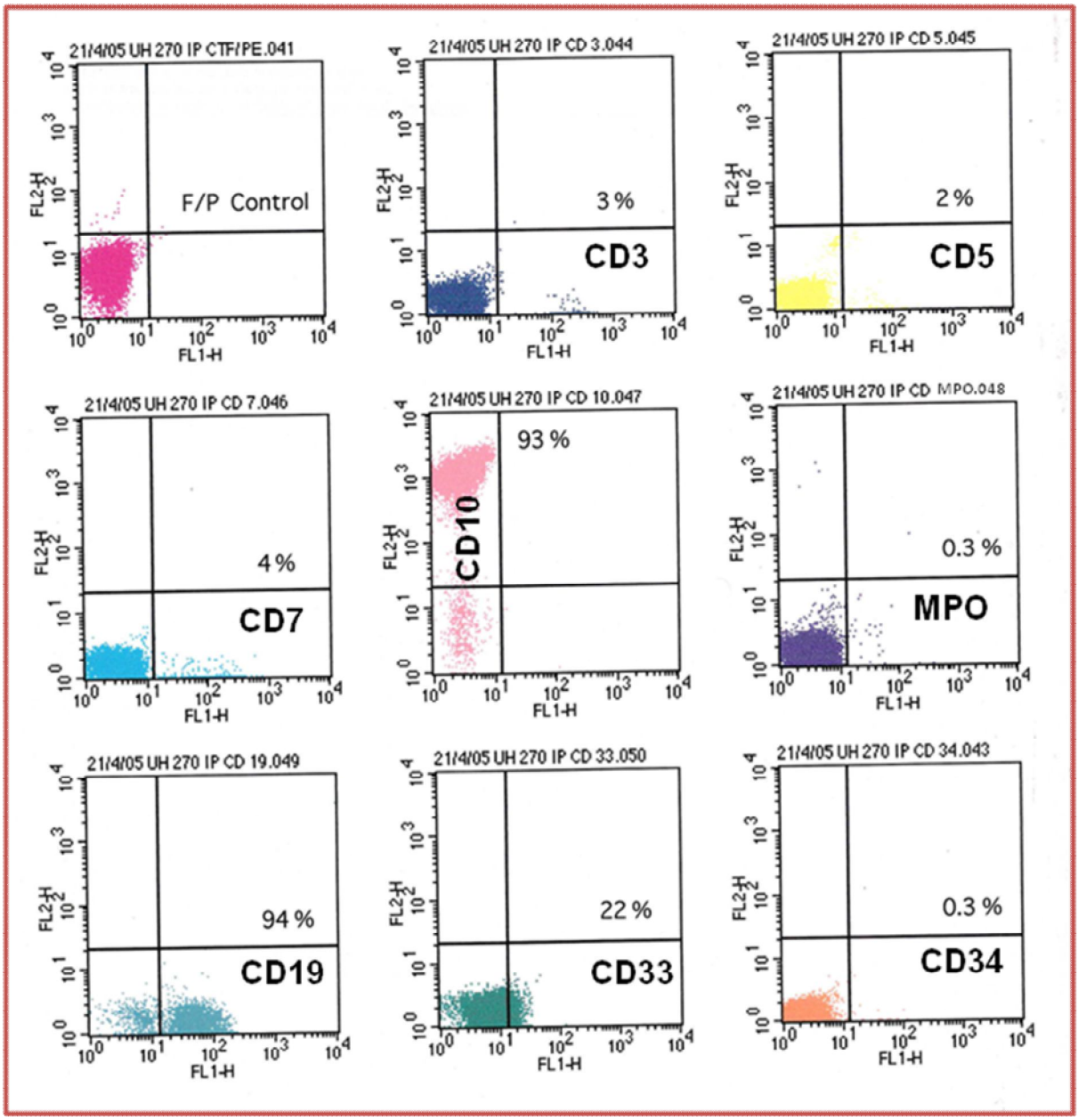


Figure 4.6: Immunophenotypic analysis of B-ALL: A case of B-ALL. Blasts were positive for B-cell marker i.e. CD10 & CD19 and negative for CD3, CD5, CD7, MPO & CD34 markers.

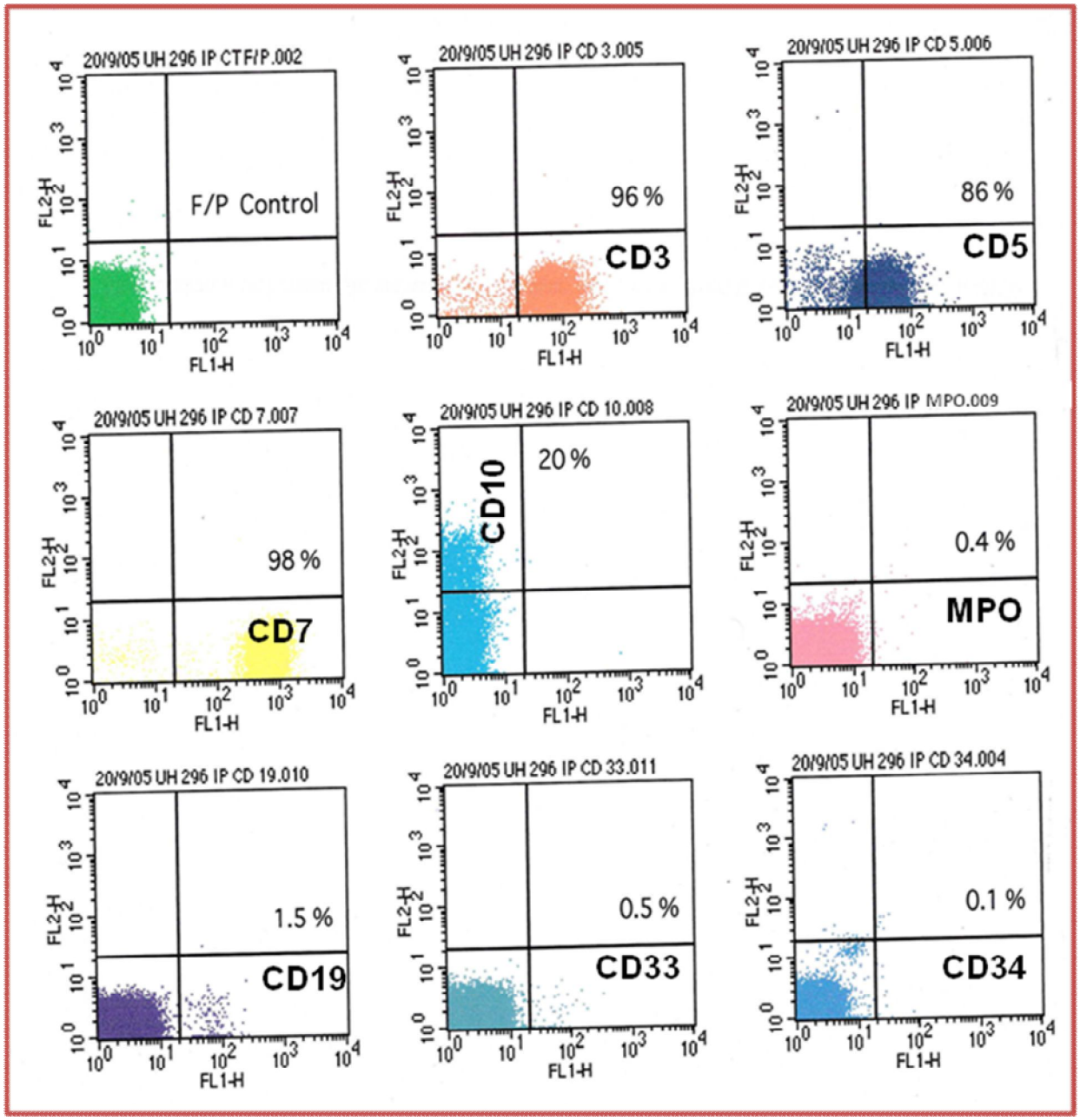


Figure 4.7: Immunophenotypic analysis of T-ALL: A case of T-ALL. Blasts were positive for T-cell marker i.e. CD3, CD5 & CD7 and negative for CD10, CD19, MPO & CD34 markers.

Clinical outcome of AL patients

Analysis of prognostic variables influencing the response to the Induction chemotherapy

In the study, clinical response of the patients was evaluated after completion of induction chemotherapy and was available in 37.4% (104/278) cases. The response rate (CR) in adult and childhood AL patients is given in the Table 4.9. No significant difference was seen in the response rate of children and adults in AML. Interestingly, In B-ALL the response rate was found to be higher in adults (73%) compared to children (58%), although the difference was not significant. In T-ALL, the response rate was found to be higher in children (75%) compared to adults (43%), although this difference was also not significant perhaps due to small sample size.

Table 4.9: Clinical outcome in AL patients

	Acute leukemia (104)				
	Children (51)		**Adults (53)**		
Types of AL	CR% (n=30)	NCR% (n=21)	CR% (n=30)	NCR% (n=23)	P
AML (43)	50 (6/12)	50 (6/12)	51.6 (16/31)	48.4(15/31)	1.00
B-ALL (46)	58.1(18/31)	41.9(13/31)	73.3 (11/15)	26.7 (4/15)	0.35
T-ALL (15)	75.0 (6/8)	25.0 (2/8)	42.9 (3/7)	57.1(4/7)	0.31

Note. CR: complete remission after induction chemotherapy. P-value calculated by $\chi 2$ test. Number outside parenthesis indicates percentage.

Analysis of prognostic variables influencing the response in AL

AML: No significant association was observed for the clinical variables such as gender, fab, Hb, Plt, wbc count, AR presence, LAP, HSM and peripheral blast percentage with the prognosis of AML patients. Among immunophenotypic markers, expression of CD34, stem cell marker was higher in non-responder than responder but it was not statistically significant. However in children expression of CD34, stem cell marker was significantly (marginally) associated with non responders. The CD34 marker was found to be positive in all the cases (100%) of non responders compared to 33% cases of responders (100% vs 33%, p=0.06) but not in adults (47% vs 37%, p=0.72).

Table 4.10: Association of prognostic factors with clinical outcome in AL (CR. vs NCR)

Prognostic features (good vs. poor)	**Types of AL**					
	AML (43)		**B-ALL (46)**		**T-ALL (15)**	
	Child (12)	Adult (31)	Child (31)	Adult (15)	Child (8)	Adult (7)
Gender (female vs. male)	Ns	Ns	Ns	Ns	Ns	Ns
HSM (-ve vs. +ve)	Ns	Ns	Ns	Ns	Ns	Ns
LAP (-ve vs. +ve)	Ns	Ns	Ns	Ns	Ns	Ns
MPO/ PAS# (+ve vs. -ve)	Ns	Ns	Ns	Ns	Ns	Ns
Auer rods (+ve vs. -ve)	Ns	Ns	Ns	Ns	Ns	Ns
CD10 (+ve vs. -ve)	Ns	Ns	Ns	Ns	Ns	Ns
CD34+ (good in ALL/poor in AML)	**0.06***	Ns	Ns	Ns	Ns	Ns
Hb level (<10 vs. >10)	-	Ns	Ns	-	Ns	Ns
Plt (>50×10^9/l vs. <50×10^9/l)	Ns	Ns	Ns	Ns	Ns	Ns
WBC(<50×10^9/l vs. >50×10^9/l)	Ns	Ns	Ns	Ns	Ns	Ns
Peripheral Blast (>50 % vs. <50%)	Ns	Ns	Ns	Ns	-	**0.02***

Note. Table shows the difference in the prognostic features of AL patient between CR and NCR. P-value calculated using $\chi 2$ test. *****Significant p value < 0.05. # = MPO in AML, PAS in B-ALL, ns= Non-significant p value, CR/NCR: Response after induction chemotherapy. - =p-value could not be calculated as the number of sample in other group was none.

B-ALL: No significant difference was found between responders and non responders for the male gender in adult and child. In adults, percentage of males in responders and non responders was 82% and 100% respectively (p=1.0). In children, percentage of males was marginally higher in responders (78%) than non responders (61%) respectively (p=0.43). There was no significant difference between responders and non responders of adult as well as children for other prognostic factors such as Hb level, Plt count WBC count and blast%. No difference was also found between responders and non responders of adult as well as of childhood B-ALL for the presence of HSM, LAP, PAS positivity, CD10 and CD34 expression (Table 4.10).

In T-ALL: In adults, percentage of males in responders and non responders was 100% and 75% respectively (p=1.0). In children, percentage of males in responders and non responders was 83% and 50% respectively (p=1.0). In T-ALL also, no significant difference was found for prognostic factors such as Hb level, Plt count, WBC count, PAS positivity, presence of HSM, LAP and CD10 and CD34 expression. Only percentage of peripheral blast was found to significantly different between responder and non responder of adult T-ALL. Blast percentage (more than 50%) was present in lower number of responder cases (0%) compared to non-responder cases (100%) in adults (p=0.02) (Table 4.10).

Aberrant antigen expression in AL

Frequency of Aberrant expression of lymphoid-associated markers in AML (Ly+ AML) or myeloid associated markers in ALL (My+ ALL)

Two hundred and fifty two cases were evaluated for aberrant expression of antigen. Of the 252 samples, 117 were adults and 135 were children. 110 were AML and 142 were of ALL (110 B-ALL and 32 T-ALL). Out of 252 AL cases, 24% (60/252) showed aberrant expression of antigen. Aberrant expression of myeloid lineage associated antigen (My+ ALL) was seen in 15% (21/142) cases of ALL while aberrant expression of lymphoid lineage associated antigen (Ly+ AML) was seen in 35% (39/110) cases of AML (Table 4.11).

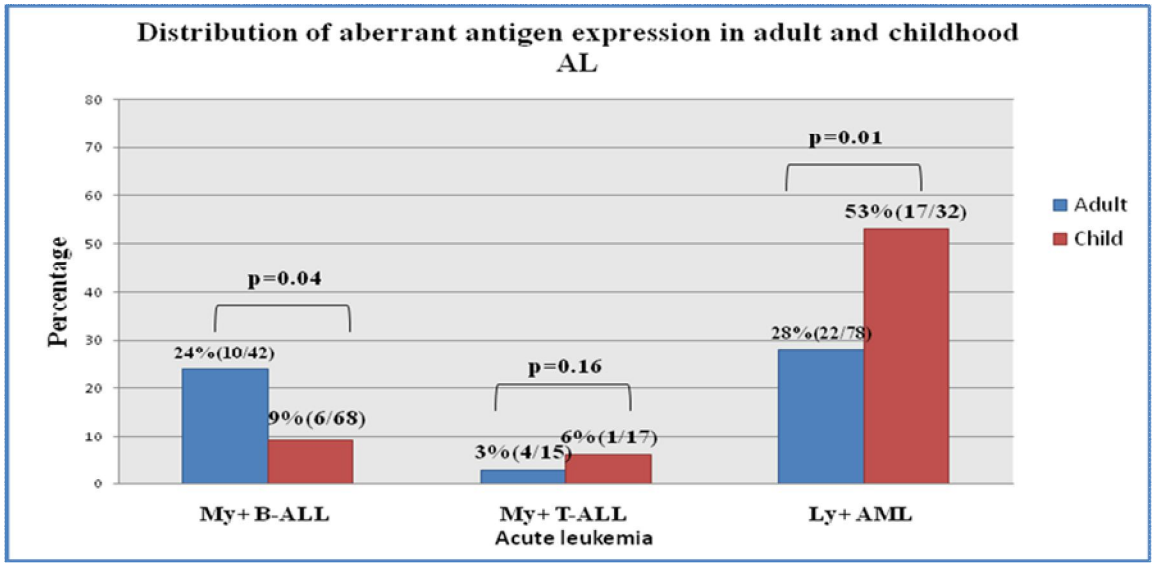
Table 4.11: Detail of AL Patients

Total No. of AL patients (N)	: 252 (110 AML + 110 B-ALL + 32 T- ALL)
Childhood AL	: 117 (32 AML + 68 B-ALL + 17 T- ALL)
Adult AL	: 135 (78 AML + 42 B-ALL + 15 T- ALL)
Aberrant Phenotype	: My+ B-ALL - 14.5% (16/110)
	My+ T-ALL – 15.6% (5/32)
	Ly+ AML – 35.4% (39/110)
	Total – 23.8% (60/252)

Distribution of aberrant phenotype in adult and childhood AL

In B-ALL, aberrant phenotype was found significantly more often in adults (24%, 10/42) than children (9%, 6/68) (p=0.04). In T-ALL, frequency of aberrant phenotype was double in children 3% (4/15) than that of adult 6% (1/17) although this was statistically insignificant (p=0.16). In AML, Overall, Ly+ AML phenotype was seen in 35% (39/110) cases of AML. Children had significantly higher frequency of Ly+ AML phenotype compared to adults (53% vs 28%, p=0.01) (Table 4.12) (Figure 4.8).

Figure 4.8: Frequency distribution of aberrant phenotype in adult and childhood AL



Aberrant phenotypes in AML (Ly+AML) and their correlation with clinical features

Age (Children vs Adults): Aberrant expression of CD19 was found to be the most frequent B-cell marker in both adult and children. Children had marginally higher frequency of CD19 phenotype than adults (34% vs 18%, p=0.08). There was no expression of CD10 in adult while only one case of child AML showed aberrant expression of CD10. Among T-cell marker, only CD7 was found to be aberrantly expressed in both adult and childhood AML cases. Although, aberrant expression of CD7 was higher in children compared to adults but it was not statistically significant (22% vs 14%, p=0.39) (Table 4.12).

Table 4.12: Distribution of aberrant lymphoid marker in AML

Markers		AML (110)		
		Adult % (n=78)	**Childhood %(n=32)**	**p-value**
Lymphoid Marker	**CD3**	0 (0)	0 (0)	-
	CD5	0 (0)	0 (0)	-
	CD7	14.1 (11)	21.9 (7)	0.39
	Total T-cell	14.1 (11)	21.9 (7)	0.39
	CD10	0 (0)	3.1 (1)	0.29
	CD19	17.9 (14)	34.4 (11)	0.08
	Total B-cell	17.9(14)	37.5 (12)	**0.04***
	Overall	28.2 (22)	53.1 (17)	**0.01***

Note. Table shows distribution of aberrant expression of lymphoid marker in AML cases. Expression of B-cell markers was significantly different between adult and childhood AML. *Significant p-value <0.05.

FAB subtypes: FAB subtypes distribution of CD19 in adult AML showed higher frequency in M0/M1 (33%) followed by M5 (27%) and M2 (18%). In childhood AML higher frequency of CD19 was observed in M2 (64%) and M5 (40%). Aberrant expression of CD19 in M2 subtype was marginally higher in children than adult (64% vs 18%, p=0.08). In adult, aberrant expression of CD7 was more common in M2 (21%) followed by M5 (18%) and M0/M1 (17%) subtypes while in children, CD7 was found more commonly in M0/M1 (43%) followed by M5 (40%). All the cases of M3 and M4 subtypes of children and adults were negative for lymphoid markers except for one case of M4 subtype that was positive for CD19 in adult AML (Table 4.13).

Table 4.13: Aberrant expression of CD7 and CD19 in FAB subtypes of AML

Lymphoid Markers		FAB subtypes (AML)						Total
		MO/M1	**M2**	**M3**	**M4**	**M5**	**AML**	
CD7	**A (n)**	17(2/12)	21(6/28)	0(0/5)	0(0/9)	18(2/11)	8 (1/13)	14 (11/78)
	C (n)	43(3/7)	9(1/11)	0(0/2)	0(0/1)	40(2/5)	17(1/6)	22 (7/32)
CD19	**A (n)**	33(4/12)	18(5/28)	0(0/5)	11(1/9)	27(3/11)	8(1/13)	18 (14/78)
	C (n)	14(1/7)	4(7/11)	(0/2)	0(0/1)	40(2/5)	17(1/6)	34(11/32)

A= adult, C= Children, Values outside parenthesis indicated percentage

Correlation of Ly+AML phenotype with clinical features, CD34 marker and clinical outcome

The median age for Ly+ AML was 35 years (17-85) and 10 years (1-14) in adult and children respectively. In adult AML, no significant differences were observed in Ly+ AML versus Ly- AML cases in relation to presenting clinical features including gender (p=0.80), LAP (p=0.22), HSM (p=0.46), Hb level (p=0.82), WBC count (p=0.89), Plt count (p=0.13), peripheral blast (p=0.81), presence of auer rod (p=0.20) and CD34 (p=0.61). Analysis of these prognostic factors in children also showed no association with aberrant phenotypes except CD34. Expression of CD34 in children was found to be significantly higher in Ly+ AML compared to Ly- AML cases (94% vs 53%, p=0.01). In adult AML, the response rate was found to be similar in Ly+ AML and Ly- AML (60% vs 53%, p=0.1). In childhood AML, the response rate was found to be lower in Ly+ AML (33%) as compared to Ly- AML phenotype (50%) however, this difference was also not significant (p=0.1) (Table 4.14).

Table 4.14: Association of the prognostic factors with lymphoid phenotype of AML

	AML (110)					
Prognostic factors	**Adult AML(78)**			**Childhood AML (32)**		
	Ly^+ ALL(22)	**Ly^-ALL(56)**	**P**	**Ly^+ ALL(17)**	**Ly^- ALL(15)**	**P**
Age (median, range)	35 (17-85)	35.5(16-77)	0.93	10(1-14)	8(3-15)	0.84
Gender(M/F)	13/9	35/21	0.80	14/3	12/3	1.0
HSM,%(n)	45.5(10/22)	55.4(31/56)	0.46	29.4(5/17)	33.3 (5/15)	1.0
LAP,%(n)	68.2(15/22)	82.1(46/56)	0.22	58.8(10/17)	40(6/15)	0.47
Hb (median, range)	6.9(2.6-10)	6.2(1.3-8)	0.82	5.6(3.3-10.8)	4.8(3.4-7.8)	0.32
WBC (median, range)	24.1(2.3-244)	34.1(1-312)	0.89	34.9(9.1-252)	34.9(9.1-256)	0.95
Plt (median, range)	20(3-129)	40(1-343)	0.13	23(2-120)	42(5.8-130)	0.21
Blasts (median, range)	65(36-90)	68(10-98)	0.81	75(30-96)	60(17-94)	0.24
AR Presence, %(n)	54.5(12/22)	37.5(12/56)	0.20	58.8(10/17)	53.3(8/15)	1.0
CD34 (+) % (n)	59.1 (13/22)	50(28/56)	0.61	94.1(16/17)	53.1(8/15)	**0.01***
CR/NCR	3/2	10/9	1.0	2/4	2/2	1.0

Note. Table shows expression of CD34 is significantly higher in Ly^+AML compared to Ly^-AML cases in children. CR: complete remission after induction chemotherapy. P-value calculated using $\chi 2$ test. *Significant p value < 0.05. (Age in years, Hb in g/dl while WBC and Plt measured as $\times 10^9$/L).

Aberrant phenotypes in ALL (My+ALL) and their correlation with clinical features

Age (Children vs Adults): Aberrant expression of CD13 myeloid marker was done in only 33 cases of adult and 36 cases of childhood ALL cases. Expression of CD13 was found to be similar in children and adult (9% vs 6%, p=0.66). Since CD13 was not evaluated in all cases it is excluded from further analysis. CD33 marker was evaluated in all the 142 cases of AML. Aberrant expression of CD33 was significantly high in adult cases compared to children (25% vs. 8%, p=0.01) (Table 4.15).

Table 4.15: Distribution of aberrant myeloid marker in ALL

Markers		**ALL**		
		Adult ALL	Childhood ALL	p-value
Myeloid Marker	CD13, %(n)	9.1 (3/33)	5.6 (2/36)	0.66
	CD33, %(n)	24.6 (14/57)	8.2 (7/85)	**0.01**

Note. Table shows aberrant expression of CD13 and CD33 in ALL. Expression of CD33 marker is significantly higher in adult cases compared to childhood ALL. Significant p-value <0.05.

FAB subtypes: Among the FAB subtypes of childhood ALL, aberrant expression of CD33 was similar in L1 (11%) and L2 subtypes (7%). In adult ALL, expression of CD33 was found to be higher in L1 (27%) than L2 (20%), although this difference was not significant (p=0.9) (Table 4.16).

Table 4.16: Aberrant expression of CD33 in FAB subtypes of ALL

Lymphoid Markers		**FAB subtypes (ALL)**			**Total**
		L1	**L2**	**ALL**	
CD33	**A, %(n)**	26.9 (7/26)	20 (5/25)	33.3 (2/6)	24.5(14/57)
	C , %(n)	10.6 (5/47)	6.7(2/30)	0(0/8)	8.2 (7/85)

Note. Table shows aberrant expression of CD33 in FAB subtypes of ALL. No significant difference was observed in the distribution of CD33 expression.

Correlation of My+ALL phenotype with clinical features, CD34 marker and clinical outcome

In childhood as well as in adult ALL, adverse prognostic factors were not found to be associated with aberrant phenotypes except with CD34 expression in adult ALL. In adult CD34 expression was found to be significantly higher in My+ phenotype (71%) compared to My-ALL (35%) (p=0.02). In children, CD34 was found in 57% and 33% cases of My+ALL and My-ALL respectively. No significant difference was found in the clinical outcome of My+ALL and My-ALL phenotype in both adult and childhood ALL. In adult ALL, complete remission rate was higher in My-ALL (66%) compared to My+ALL (33%) (p=0.53). In childhood ALL, CR was achieved in 100% and 61% cases of My+ and My- phenotype respectively (p=0.52). There were 14% (16/110) cases of My+ B-ALL and 16% (5/32) of My+ T-ALL in the study. Association of adverse prognostic factors with aberrant phenotype of B-ALL and T-ALL could not be analyzed because of small number of My+ ALL cases (Table 4.17).

Table 4.17: Association of prognostic factors with myeloid phenotype (CD33) of ALL

Prognostic factors	**Adult ALL(57)**			**Child ALL (85)**		
	My^+ALL(14)	**My^-ALL(43)**	P	**My^+ ALL(7)**	**My^-ALL(78)**	P
Age (median, range)	24(17-57)	23(16-67)	0.84	11(1-15)	8(1-15)	0.38
Gender(M/F)	10/4	13/30	1.0	5/2	57/21	1.0
HSM, %(n)	92.9(13/14)	76.7 (33/43)	0.26	85.7 (6/7)	78.2 (61/78)	1.0
LAP, %(n)	50(7/14)	46.5 (20/43)	1.0	42.9 (3/7)	52.6 (41/78)	0.70
Hb (median, range)	7.1(3.5-2.4)	5.9 (2-11.2)	0.55	6.4 (5.2-7.4)	6.3(2.8-12.8)	0.84
WBC (median, range)	73(9-185)	31 (2.1-240)	0.41	23 (8.9-256)	44(14-395)	0.54
Plt (median, range)	20(6-150)	20 (1-160)	0.47	20(10-90)	25(16-173)	0.88
Blasts (median, range)	89(60-93)	78 (10-96)	0.13	80(70-90)	80(10-99)	0.32
CD34 (+) %(n)	71.4(10/14)	34.9 (15/43)	**0.02**	57.1 (4/7)	33.3(26/78)	0.23
B-ALL/T-ALL	10/4	32/11	1.0	6/1	62/16	1.0
CR/NCR	1/2	12/6	0.53	2/0	22/14	0.52

Note. Table shows expression of CD34 is significantly higher in My^+ALL compared to My^-ALL cases in adults. CR: complete remission after induction chemotherapy. p-value calculated using $\chi 2$ test. Bold represent significant p value < 0.05. Age in years, Hb in g/dl while WBC and Plt were measured in $\times 10^9$/L.

Discussion

In most laboratories in our country, diagnosis of acute leukemia (AL) is based on morphology, cytochemistry, immunophenotyping, cytogenetic and molecular diagnostic studies. In the current study, 289 cases of AL were evaluated, based on FAB morphology 47% (137/289) were diagnosed as AML and 50% (145/289) as ALL while 1.0% (4/289) diagnosed as possible BAL. Based on FCM, 46% cases were of AML and 50% of ALL while MLAL were 2% cases of AL. The frequency of AML reported is slightly higher than the earlier studies reported by Tata Memorial Hospital (Mumbai) [Ghosh *et al.,* 2003; Gujral *et al.,* 2009]. Amongst ALL, B-ALL constituted 77% (112/145) and T-ALL constituted 23% (33/145) of ALL in the current study. In contrast to our reports of T-ALL, very high proportions of T-ALL (40%) have been reported from South India [Rajalekshmy *et al.,* 1997].

Distribution of AL patients: In the present study, most AL in children were of lymphoid (B-ALL) type with peak incidence at 11-15 years age group whereas in adults they were typically myeloid (AML) in origin with peak incidence in the age group of 31-35 yr which is much lower than the median age of western countries (60yrs) [Ries *et al.,* 2006; Deschler *et al.,* 2006]. In general the prevalence of AL is higher among males compared to females [Tyagi *et al.,* 2006; ICMR 2010]. AML accounted for 29% of all AL in children and 62% in adults in the present study which is lower than the USA and Europe studies which report higher incidence of AML upto 80% of AML in adults [Thalhammer-Scherrer *et al.,* 2002; Xie *et al.,* 2003; Deschler *et al.,* 2006]. In contrast to AML, ALL accounts for 70% in children and 38% in adults of total AL cases. In Europe also, ALL accounts for around 80% of AL among children aged 0–14 years [Coebergh *et al.,* 2006]. In ALL, frequency of B-ALL was found to be higher (77%) compared to T-ALL (23%). In childhood and adult ALL, the frequency of B-ALL vs. T-ALL in childhood ALL and adult ALL was 80% vs 20% and 73 vs. 27% respectively. The high frequency of B-ALL in both children and adults is also in accordance with the previous reports [Agarwal *et al.,* 2001; Chen et al., 2004; Bajel *et al.,* 2008]. In contrast to B-ALL, reports of frequency of T-ALL have been inconsistent. Agarwal *et al.,* [2001] and Bajel *et al.,* [2008] have reported low frequency of T-ALL in children i.e. 17% and 22% respectively. Further, Agarwal *et al.,* [2001] did not find any T-ALL cases in adults. Chen *et al.,* [2004] and Khawaja *et al.,* [2005] have also reported low frequency of T-ALL i.e. 24% (in children) and 17% (14% in children, 22% in adults) respectively.

Immunophenotyping

The standard methods for establishing the diagnosis of acute leukemias are cytomorphology and cytochemistry in combination with immunophenotyping. However, immunohistochemical staining done in routine laboratory work has its limits: the analysis may be subjective, reproducibility is limited, and the process is time-consuming. Inter- and intra-observer variability is high because so many factors may interfere with the processing of specimens and interpretation of results. Thus, lack of consensus in quantifying antigen expression and defining positive and negative results in poor reproducibility. Though immunohistochemistry is applicable retrospectively fixed tissues, but fixation might lead to loss of some cells and/or cellular antigenicity. On the contrary, flow cytometry immunophenotyping is rapid and quantification of the percentage of positive cells is more precise because many more cells are evaluated. It allows a more precise definition of individual cell types since the cells of interest are identified by a combination of physical characteristics and the use of multiple antibodies directly conjugated with different fluorochromes. It also has the ability to assess monoclonality through detection of immunoglobulin light chain expression and the results can be available within few hours after receiving the specimen. Further, dead cells may be gated out of the analysis. Weakly expressed surface antigens may also be detected using bright fluorochrome antibodies.

AML: Flow cytometric immunophenotyping still remains the cornerstone method especially in the diagnosis of ALL and AML. It is fast, objective, quantative and amenable to standardization among all other techniques of diagnosing AL. In the current study, CD13 and CD33 were expressed in more than 80% cases of AML. CD33 was detected in 90% and 83% cases of children and adults while CD13 was detected in 88% and 86% cases of children and adults. These findings were similar to other published studies from India as well as abroad [Legrand *et al.,* 2000; Ghosh *et al.,* 2003; Chen *et al.,* 2005]. Overall, CD33 is a much more sensitive marker than CD13 for myeloid lineage, but by the same virtue, it is less specific than CD13. MPO was detected in only 28% (17/62) of cases. The lower positivity in the study was due to smaller number of cases evaluated for MPO positivity using flow cytometry. CD34 is a primary marker that is expressed on progenitor myeloid, lymphoid or stem cells. This marker is present in about 40-60% of AML cases, especially in the cases with less differentiated phenotypes. In the current

study CD34 was detected in 72% and 54% cases of children and adults respectively. Basso *et al.,* [2001] has discussed a number of clinical and methodological factors for the heterogeneity reported in the incidence of CD34+ AML and its association with variable survival rates. Legrand *et al.,* [2000], Chang *et al.,* [2004] and Liu *et al.,* [2007] have reported CD34 expression in 60-65% cases of AML and shows association between CD34+AML and lower response rate following induction therapy. In the present study, no difference was found in the response rate of CD34+ and CD34- adult AML cases (56% vs 46%). However, in children, a marginal significant association was observed towards a lower remission rate in CD34+ (50%) cases compared to CD34- (100%) cases (p=0.06). This confirms the poor prognosis of CD34 marker on the clinical outcome in AML children [Dalal *et al.,* 1997; Raspadori *et al.,* 1997]. The present study suggests that CD34 expression on AML blasts does not play a prognostic role in adults; however, it could influence the clinical outcome in children. Other prognostic factors such as gender, HSM, LAP, and peripheral blast count did not find any clinical relevance in the current study.

B-ALL: In the current study, B-ALL cases were categorized into pro-B-ALL and common ALL as the panel used in the study included only CD19 and CD10. CD19 is stably expressed on all stages of B lineage differentiation. In the present study, almost all the patients showed positivity for CD19 and CD10 (100% and 94%) in adults and children (99% and 91%). Overall, these findings indicate that two markers i.e. CD19 and CD10 may suffice to diagnose pro-B-ALL (CD19+/CD10-) and common B-ALL (CD19+/CD10+) cases. Expression of CD34 was more commonly seen in adults (51%) as compared to children (39%) (p=0.07). In literature higher percentage (62%-76%) of B-ALL cases in adults were reported to be positive for CD34 expression [Cascavilla *et al.,* 1997; Mi *et al.,* 1999]. Several studies in childhood B-ALL have reported a strong association between CD34 expression and favorable prognosis [Pui *et al.,* 1993; Cascavilla *et al.,* 1997]. However, other studies have not confirmed this association [Sperling *et al.,* 1995; Vanhaeke *et al.,* 1995]. In the current study, CD34 positivity was associated with higher response rate in children (44% vs. 31%) and lower response rate in adults (45% vs. 50%) but this difference was not statistically significant.

T-ALL: Normal T lymphoblasts typically express TdT, CD2, CD5, CD7, and lack CD3 surface expression while cCD3 is typically expressed [Cruse *et al.,* 2004]. In the current study, CD7 (100%) was the most commonly detected CD marker followed by CD5 and CD3 in both children

and adults. CD5 was detected in 84% and 94% cases of children and adults respectively while CD3 was detected in 53% and 44% cases of children and adults. Similar to present study, Chen *et al.,* (2005) has also earlier reported that the sensitivity of CD7 (100%) was higher than CD3 (80%). Although, they have reported that the specificity of CD3 was higher (98%) than CD7 (78%). Expression of CD10 was also observed in approximately 40% cases of T-ALL in both children and adults. Similar to our results, Lewis *et al.,* [2006] also report that CD10 positivity was seen in 47% of cases of pre T-ALL cases. Overall, these findings suggest that CD3 or preferably CD5 should be included with CD7 in the panel for immunophenotyping in order to differentiae T-ALL from B-ALL. No significant difference was seen in the expression of CD34 between adults (31%) and children (18%) (p=0.4). In contrast to our report Pituch-Noworolska *et al.,* [2001] has observed CD34 expression in higher percentage of cases (28%) with childhood T-ALL. In the current study, childhood T-ALL samples that were CD34+ had higher response rate as compared to CD34- cases (100% vs. 67%, p=1.0) although the difference was not significant, while in adults, there was a trend toward lower response rate of CD34+ cases as compared to CD34- cases (0% vs. 50%, p=1.0) but this was also not significant probably due to lower number of cases. Vitale et al., [2007] has also reported lower response rate in CD34+ adult T-ALL as compared to CD34 negative T-ALL cases.

The subtype of B-ALL or T-ALL affects prognosis. In B-ALL, response rates were higher in adult than children (73% vs 58%, p=0.35). The relatively good clinical outcome for adults in B-ALL patients compared to children in the current study is unexpected as ALL in elderly people has been reported to have a poor outcome, even with the advent of the newer chemotherapy regimens and the availability of hematopoietic growth factor therapy [Stock *et al.,* 2008; Pui *et al.,* 2011]. Similarly, in comparison to T-ALL adults, remission rates were higher for B-ALL patients (73% vs 43%). This is in contrast to earlier studies that have reported the superior outcome of T-lineage ALL as compared to B-lineage ALL [*Gokbuget et al.,* 2001; Rowe *et al.,* 2007]. This could be due to higher number of patients with B-ALL as compared to T-ALL in the current study. Further, in T-ALL, response rate was higher in children (75%) than adults (43%) but it was not statistically significant (p=0.31).

Aberrant antigen expression in AL

AML: In this current study, aberrant antigen expression was identified in 35% of AML cases. Aberrant expression of lymphoid lineage associated antigen (Ly+ ALL) was seen more often in cases of children (53%) than adults (28%) (p=0.01). In adults, Ly+ AML phenotype was found in 45% cases of AML, which is similar to other reports [Cruse *et al.,* 2005; El-Sissy *et al.,* 2006]. However, in literature the frequency of Ly+ AML in children has been reported to be lower than our study i.e 24% to 35% [Kawai *et al.,* 1995; Shen *et al.,* 2003]. Among lymphoid antigen, CD19 was the most common aberrant lymphoid antigen expression in both adult and children followed by CD7. This is in contrast to other studies which reports CD7 as a more common lymphoid associated antigen as compared to CD19 [Legrand *et al.,* 2000, El-Sissy *et al.,* 2006, Khurram *et al.,* 2010]. CD7 an activation and adhesion molecule, is expressed by T cells, NK cells and stem cells [Cruse *et al.,* 2004]. The WHO states that CD7 may be frequently expressed in AML, but at low intensity [Jaffe *et al.,* 2001]. In the current study CD7 was expressed in 22% cases of AML in children and 11% in adult while CD19 was expressed in 37% and 18% cases of children and adults AML respectively. In the present study, there was no expression of CD3, CD5 and CD10 markers in adult cases. Only one pediatric AML sample expressed CD10 marker. These data support the current WHO immunophenotype for AML and literature which reports low or absent expression of CD3 and CD5 in AML cases [Legrand *et al.,* 2000; Jaffe *et al.,* 2001; Lewis *et al.,* 2006].

Association of some antigens with specific FAB subtypes of AML are well documented in the literature, e.g. the presence of CD19 in AML-M2 with the t(8;21) translocation [Babusikova *et al.,* 1996; Khalidi *et al.,* 1998]. In the current study, CD19 in M2 subtype was found to be marginally higher in children cases compared to adult (64% vs 18%, p=0.08). Further CD7 was mostly confined to FAB M0/M1 (43%) and M5 (40%) in children and in FAB M2 (21%), M5 (18%) and M0/M1 (17%) in adults. These results are in contrast to other literature which supports CD7 expression in early hematopoietic ontogeny in the lesser-differentiated AML subtypes, including FAB M0, M1, and M2 [Tiftik *et al.,* 2004; Cruse *et al.,* 2005; El-Sissy *et al.,* 2006].

Detection of aberrant phenotypes is not only important for accurate diagnosis of AML, but potentially also for AML prognosis. Prognostic factors which correlate with aberrant lymphoid antigen have been reported including CD7 antigen, which has been related to a poor prognosis in AML. The co-expression of this antigen in AML patients indicates an aggressive clinical course and associated with decrease complete remission rate [Kita *et al.,* 1993; Venditti *et al.,* 1998]. In our study, we have found no difference in the treatment outcome of CD7+ and CD7- AML cases neither in adults (p=0.23) nor in children (p=1.0). This could be due to small number of cases positive for CD7 in our study. We found no correlation between the lymphoid antigen displayed on myeloid cells and clinical features in adult AML. However, in children CD34, immature stem cell marker, was found to be significantly associated with the lymphoid antigen expression (94% in Ly+ AML and 53% in Ly- AML). Similarly, Kawai *et al.,* [1995] have also reported that CD34 was more often associated with Ly+ phenotype (91%) as compared to Ly- phenotype (31%) of childhood AML. Apparently, CD34 expression in AML is considered to be a negative prognostic factor for chemotherapy.

ALL: Aberrant expression of myeloid lineage associated antigen (CD33) was seen in 15% cases of ALL. CD33 expression was seen more often in adults (25%) as compared to children (8%) (p=0.01). CD33 is a pan myeloid marker involved in negative selection for human self-regenerating hematopoietic stem cells [Cruse *et al.,* 2004]. Similarly Vitale *et al.,* [2007] reported expression of CD13 and/or CD33 in 24% cases of adult T-ALL more frequently CD13 than CD33. Gujral *et al.,* [2009] also showed CD13 to be more commonly expressed antigen in B-ALL and T-ALL cases as compared to CD33. Conversely Thalhammer-Scherrer *et al.,* [2002] have reported low expression of CD33 in B-ALL (3%) and T-ALL (7%) cases of adults. Whereas Marks *et al.,* [2009] have reported CD33 expression in 30% cases of T-ALL in adults.

In children, My+ phenotype has been reported in 36% and 30% cases of B-ALL and T-ALL respectively [Shen *et al.,* 2003]. In our study, My+ phenotype was found in 9% (6/68) cases of childhood B-ALL which is in contrast to studies that have earlier reported aberrant phenotype in 23% to 27% cases of childhood B-ALL [Ng *et al.,* 2000; Suggs *et al.,* 2007]. Conversely, in this study, CD33 was present in only 1/17 (6%) case of childhood T-ALL. Suggs *et al.,* [2007] have

also reported no expression of CD33 in childhood T-ALL (in less than 13 year). Similarly, Lewis *et al.,* [2006] found only 2 of 16 cases that expressed CD33 in T-ALL cases although age distribution of the study is not known.

The clinical significance of the presence of myeloid antigens on lymphoid leukemias has been widely debated. While different investigators have associated the expression of myeloid antigen with an adverse prognosis for patients with childhood T-ALL [Wiersma *et al.,* 1991; Uckun *et al.,* 1997] and in adult [Drexler *et al.,* 1991; Thomas *et al.,* 1995], others have conflicted it particularly in adult ALL [Yenerel *et al.,* 2002]. In adult ALL, favorable outcome has been reported by Yenerel *et al.,* [2002] for aberrant phenotype. In our study, both adult and childhood ALL patients showed no significant difference in the clinical outcome of My+ ALL and My- ALL phenotype. In adult, CR rate was 33% for the My+ ALL and of 67% for the My-ALL patients (p=0.53). No significant difference was also found between adverse prognostic factors of childhood My+ ALL and My- ALL. Vitale *et al.,* [2007] have also showed no difference between adult My+ and My- phenotype in terms of presenting clinical, hematological and biological features and clinical outcome. Prognostic significance of CD34 expression particularly in adult ALL is controversial [Thomas *et al.,* 1995; Vanhaeke *et al.,* 1995]. Vitale et al., [2007] had found that CD34 positivity lowered the CR rate from 84% to 54%. In contrast to this, Marks *et al.,* [2009] had not found any affect of CD34 positivity on CR rate.

In summary, aberrant expression of lymphoid antigen was more in AML than the aberrant expression of myeloid in ALL. CD19 was the most common lymphoid antigen expressed, most frequent in AML-M0/M1 followed by AML-M5 subtype. Although some of our findings lack statistical strength, mainly owing to the relatively small number of patients included in the study still the quantification of aberrant expression of markers such as CD7, CD19 in AML and CD13, CD33 in ALL patients by flow cytometry certainly be a valuable and indispensible part of diagnosis and prognosis of acute leukemia patients. In conjunction with cytogenetics, immunophenotyping may help in the identification of subsets of patients for whom the best possible results with intensive chemotherapy can be obtained.

Chapter 5
Detection of commonly occurring genetic alteration in acute leukemia

Chapter 5

Detection of commonly occurring genetic alteration in acute leukemia

Introduction

Acute leukemia (AL) develops from malignant transformation of immature hematopoietic cells through a complex, multistep process that requires cooperation of two classes of genetic alterations/mutations [Gilliland and Griffin, 2002]. The class I mutations activate signal transduction pathways that gives proliferation and survival advantage to the hematopoietic cells but do not affect differentiation. In contrast, the class II mutations involve transcriptional regulators of normal hematopoietic differentiation, and result in a block of hematopoietic cell maturation and/or aberrant self-renewal capacity. These mutations primarily serve to impair hematopoietic differentiation [Kelly and Gilliland, 2002; Frohling *et al.,* 2005]. Genetic alterations that are associated with acute leukemia include receptor tyrosine kinase (RTK) FLT3, c-kit (KIT) and Ras signaling pathway that are considered to be class I mutations. The class II mutations comprises chromosomal translocation such as t(8;21), inv(16) and t(15; 17), which affects transcriptional regulators such as core binding factors (CBF), RARα and MLL. Neither class I nor Class II alone is sufficient to cause leukemia since clinical and experimental data have shown that acute leukemia harbor more than one recurring mutations, suggesting a mult-hit model for leukemogenesis [Stubbs *et al.,* 2008]. The genetic analysis of progression from chronic myelogenous leukemia (CML) to acute myelogenous leukemia (AML) also supports the multi-step pathogenesis of acute leukemia.

Chromosomal translocations in acute leukemia

Chromosomal translocations constitute an important parameter to identify prognostically relevant subgroups in acute leukemia. Recurrent translocation that are commonly found in AML include the t(8;21) translocation, resulting in the formation of AML-ETO fusion and the t(15;17) translocation resulting in the PML-RARα fusion gene. Translocation t(8;21) was the first reciprocal translocation in AML that was identified using banding techniques [Rowley, 1973a]. This translocation is strongly associated with FAB M2 subtype [Bennett *et al.,* 1985]. The translocation juxtaposes the AML1 gene on 21q22 with the ETO gene on 8q22 resulting in the

production of a chimeric AML/ETO mRNA and protein [Downing *et al.,* 1993]. Another recurrent translocation t(15;17) which lead to the formation of fusion gene PML/RARα is exclusively associated with acute promyelocytic leukemia (APL) [Grignani *et al.,* 1994]. APL-associated gene rearrangements are characterized by fusing RARα to several different partner genes, of which the most common fusion is PML/RARα resulting from t(15;17). The PML-RARα protein binds to corepressor/histone deacetylase (HDAC) complexes with higher affinity than the wild type RARA, leading to aberrant chromatin acetylation and alterations of chromatin conformation inhibiting the normal transcription of genes regulated by RARα, a nuclear hormone receptors [Segalla *et al.,* 2003]. The Philadelphia chromosome (Ph) t(9;22), the hallmark of CML, results from a translocation between chromosomes 9q34 and 22q11 and fuses the BCR gene on chromosome 22 to the ABL gene on chromosome 9 [Rowley, 1973]. Unlike most other rearrangements in ALL which target transcription factors, BCR-ABL protein is an activated tyrosine kinase molecule that exert its effects by interfering with cellular signal transduction pathways normally involved in the control of cell death, proliferation and cell-cell adhesion [Goga *et al.,* 1995; Cortez *et al.,* 1996].

These chromosomal translocations t(8;21), the t(15;17) and the t(9;22), which typically lead to the formation of fusion genes and their expression as chimeric transcripts and proteins are of clinical relevance in leukemogenesis because they influence the expression of key regulatory genes in the hematopoietic system thereby constitute an important parameter to identify prognostically relevant subgroups in acute leukemia..

Gene mutations in AML

The intensive molecular investigation in recent years has described variety of new molecular mutation which play an important role in molecular diagnostics and estimation of prognosis in acute myeloid leukemia [Buzzai and Licht, 2008; Schlenk and Dohner, 2009]. Leukemia's harbor chromosomal translocations and additional molecular mutations with these translocation drives oncogenic transformation of hematopoietic cells. Genes involved in signal transduction have been the main focus of molecular mutation analyses. Fms- like tyrosine kinase-3 (FLT3) is a class III receptor tyrosine kinase along with KIT, FMS and PDGFR, located on chromosome 13q12 [Rosnet *et al.,* 1993]. Wild type FLT3 is expressed on normal hematopoietic stem cells

and in the majority of AML blast cells [Nakao *et al.,* 1996]. The most common mutation in the FLT3 gene is internal tandem duplication (FLT3/ITD) of the region coding for the juxtamembrane (JM) domain of the FLT3 receptor [Hayakawa *et al.,* 2000]. The mutated FLT3 gene was always transcribed in frame, and coded mutant FLT3 with a long JM domain. In vitro studies have demonstrated that the FLT3 ligand stimulates proliferation and inhibits apoptosis in myeloid leukemic cells [Drexler 1996; Mizuki *et al.,* 2000]. Another mutation reported is missense mutations in the tyrosine kinase domain (TKD) of the FLT3 gene. These mutations also promote constitutive phosphorylation of the receptor [Abu-Duhier *et al.,* 2001]. The most frequent is point mutations and deletions of codons 835/836 in second TKD of the gene.

Another most common mutation in AML that interferes with cell cycle regulation is exon 12 of nucleophosmin (NPM1) gene [Falini *et al.,* 2005]. The frameshift mutation at exon 12 of the NPM1 gene is an alternative leukemogenetic mechanism rather than chromosomal translocations that was recently discovered in 2005 [Falini *et al.,* 2005; Verhaak *et al.,* 2005]. NPM1 is a nucleus-cytoplasm shuttling protein that is ubiquitously expressed and is highly conserved. The NPM1 protein has been shown to contribute to many basic cellular processes such as the regulation of centrosome function, biosynthesis of ribosomes, preventing aggregation of proteins in the nucleolus and also participate in (ARF-P53) tumor suppressor pathway [Olson *et al.,* 1986; Szebeni and Olson*,* 1999; Colombo *et al.,* 2002].

In leukemia and lymphoma, NPM1 is a partner in chromosomal translocations, where the dislocated cytoplasmic nucleophosmin appears to contribute to oncogenesis. Falini et al., 2005 identified NPM1 mutation as an independent positive prognostic factor for attainment of complete remission. Subsequent studies evaluating the clinical outcome of patients with and without NPM1 mutations irrespective of the presence of other genetic rearrangements have yielded somewhat inconsistent results. In some studies, patients harboring NPM1 mutations had a significantly higher complete remission rate [Schnittger *et al.,* 2005; Suzuki *et al.,* 2005], and longer disease-free survival [Thiede *et al.,* 2006], relapse-free survival [Dohner *et al.,* 2005] and event-free survival [Schnittger *et al.,* 2005]. In contrast, other studies revealed no significant differences in complete remission rates [Boissel et al., 2005; Bardet et al., 2006]. Thiede *et al.,* [2006] provided evidence that NPM1 mutations usually precede the acquisition of FLT3-ITDs, suggesting that NPM1 mutations might constitute a primary event in leukemogenesis. About

40% of patients harboring NPM1 mutations also carry FLT3-ITD mutation [Dohner *et al.,* 2005; Schnittger *et al.,* 2005; Thiede *et al.,* 2006]. However, among patients without the FLT3-ITD, NPM1 mutations were associated with higher complete remission rates [Schnittger *et al.,* 2005; Thiede *et al.,* 2006]. Studies particularly in Indian population, taking into account the incidence and prognostic impact of these newly described genetic events in acute leukemia patients are warranted. Therefore, the aim of this objective were to

- To determine the prevalence of the translocation t(9;22) (p210, p190 BCR-ABL fusion transcripts) by multiplex RT-PCR in ALL and AML patients.
- To determine the prevalence of translocation t(8;21) (AML/ETO) and the t(15;17) (PML/RARα) in AML patients by simple RT-PCR.
- To determine the clinical characteristics associated with the above translocation and their relation with the FAB subytpes and immunological markers in AML.
- To determine the prevalence and significance of FLT3 (FLT3/ITD and FLT3/D835) and exon12 NPM1 mutations in AML patients and to investigate their associated biological and clinical characteristics.

Experimental methods

Materials

Agarose, Tris base, EDTA, NaCl, SDS, Triton X-100 and other fine chemicals were purchased from Sigma Chemicals, USA. TRIzol, Taq polymerase, dNTPs, $MgCl_2$, was obtained from Invitrogen, and MBI fermentas, USA. Oligos were synthesized by Microsynth, Switzerland. RNA later, DNA and RNA extraction kit were purchased from Qiagen Sciences, USA and Himedia, India.

Chemicals used

LYSIS BUFFER I: 30mm Tris-Hcl (pH-8), 5mm EDTA, 50 mm Nacl; LYSIS BUFFER II: 75mm Nacl, 2mm EDTA (pH-8); SDS STOCK: 20 gm of SDS dissolved in 80 ml of TDW at 65^0C. Make up volume up to 100 ml; PROTEINASE K: 10 mg dissolved in 1 ml of TDW: 1% ; AGAROSE: 1gm of agarose dissolved 1X TAE buffer.

Selection criteria of patients: As described in chapter 4

Inclusion criteria: As described in chapter 4

Exclusion criteria: As described in chapter 4

Patients' details: As described in chapter 4

Collection of blood samples: As described in chapter 4

Separation of peripheral blood mononuclear cells: As described in chapter-1

Reverse transcription - polymerase chain reaction (RT-PCR) analysis for translocation

Isolation of RNA

Leukemic cells were lysed with TRIZOL (Invitrogen, USA) and RNA was recovered according to manufacturer's instruction before resuspension in 10-20 µl of DEPC water. RNA integrity was checked on a 1.2% formaldehyde-agarose gel. (Figure 5.1)

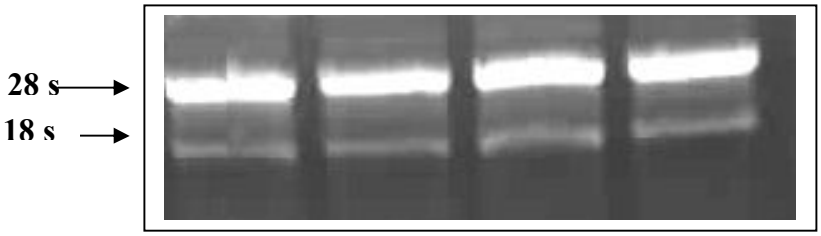


Figure 5.1: Gel picture showing the integrity of RNA on 1.2% formaldehyde-agarose gel.

Synthesis of cDNA

Human complementary DNA (cDNA) was generated by reverse transcription of total RNA extracted from leukemic cells using high capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. Integrity of cDNA was checked by amplification of GAPDH (Figure 5.2).

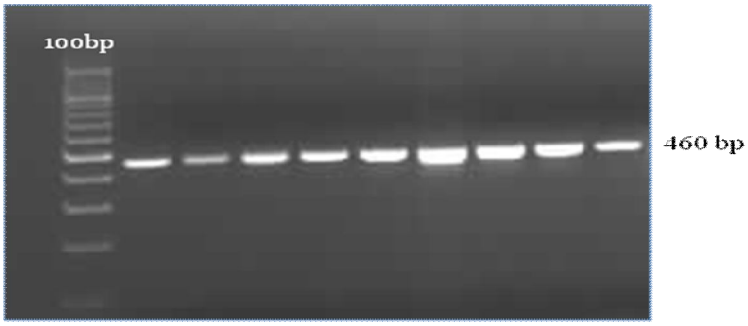


Figure 5.2: Gel picture showing the integrity of cDNA by amplification of GAPDH

Reverse Transcriptase Polymerase chain reaction (RT-PCR)

All polymerase chain reaction (PCR) reactions were performed on a thermal cycler (PTC-200, MJ Research, USA). Amplicons were electrophoresed on agarose gels stained with ethidium bromide (0.5 ug/ml) and visualized on Gel documentation system (Gene Snap, SYNGENE, UK). Positive and negative controls were included in all PCR reactions.

Multiplex RT-PCR for the detection of BCR/ABL Translocation

The BCR/ABL rearrangement was amplified using multiplex RT-PCR using primers described by Cross *et al.,* [1994]. The primers were used for p190 and p210 BCR/ABL protein product translated by e1a2 and b2a2 or b3a2 transcript respectively. The position of primer used in the study with respect to normal BCR and ABL cDNA is shown in the figure 5.3. The PCR products were visualized using agarose gel electrophoresis with ethidium bromide staining. In the multiplex PCR, the bands are of: 808 bp, normal BCR; 481 bp, e1a2 (p190); 385 bp and/or 310 bp, b3a2 and b2a2 (p210), respectively. An amplified product from the BCR gene is detected in those patient who are BCR/ABL negative translocation. The presence of this band indicates that the quality of the RNA and the efficiency of cDNA synthesis were good; absence of this band indicates procedural failure. Negative controls included the PCR-reagent mixture with water instead of cDNA from sample in each experiment. cDNA from K562 cells (p210-positive CML blast crisis cell line) was used as a positive control. Cycling condition and multiplex RT-PCR

protocol is shown in the Table 5.1 and Table 5.2. Multiplex RT PCR amplification primers were custom designed by Microsynth (Switzerland) (Table 5.3).

Figure 5.3: Position of primers used in the study with respect to normal BCR and ABL cDNA

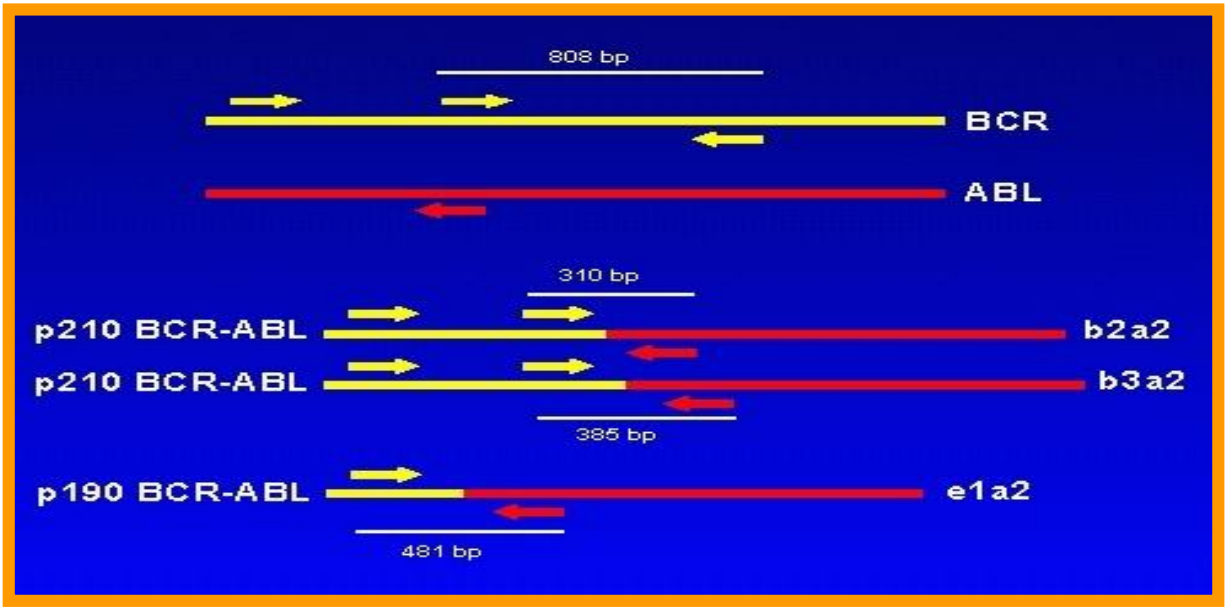


Table 5.1: Cycling regime for amplification of BCR/ABL translocation

Step	Temperature °C	Time (hrs/min/sec)	Remark
1. Initial denaturation	96 °C	00:05:00	One cycle only
2. Denaturation	96 °C	00:01:00	35 cycles
3. Annealing	63 °C	00:00:50	(repeat steps 2, 3, 4)
4. Polymerization	72 °C	00:01:15	
6. Final extension	72 °C	00:10:00	One cycle (final only)
7. Ice	4°C	00:00:00	Indefinite hold

Table 5.2: Multiplex RT-PCR reaction volume for BCR/ABL translocation

Components	Master Stock	Working Stock	Reaction Volume (25µ1)
Water	X		16.8
PCR buffer	10X	1X	2.5
dNTP mix (2.5 mM each)	10 mM	200 µM	0.5
Taq DNA polymerase	5 U/µl	1U/25µl	0.2
$MgCl_2$	50 mM	2 mM	1.0
Primer BCR-C	10 µM	0.2 µM	0.5
B2B	10 µM	0.2 µM	0.5
C5e-	10 µM	0.2 µM	0.5
CA3-	10 µM	0.2 µM	0.5
Template (cDNA)	Y	100 to 300 ng	2.0

Table 5.3: Sequence of Primers used in multiplex RT-PCR for BCR/ABL translocation

Isoform	Name	Primers Sequences	Size (bp)
BCR/ABL (p210)	BCR-B2B	5'-ACAGAATTCCGCTGACCATCAATAAG-3'	310 for b2a2
	ABL-CA3	5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3'	385 for b3a2
BCR/ABL (p190)	BCR-C	5'-ACCGCATGTTCCGGGACAAAG-3'	481 for e1a2
	ABL-C5E	5'-ATAGGATCCTTTGCAACCGGGTCTGAA- 3'	

RT-PCR for the detection AML/ETO and PML/RARα translocation

For the detection of AML/ETO and PML/RAR α fusion gene, the same PCR cyclic condition was run but separately for each of the translocation. The PCR was carried out in a final volume of 25 µl with 1 µl cDNA, 1x PCR buffer, 200 µM dNTP, 1.5 mM $MgCl_2$, 1 µM of each primer pair and 1 unit of Taq polymerase (Invitrogen, California, USA). Samples were subjected to the following PCR condition shown in the table 5.5. A known sample positive for each PML/RAR α and AML/ETO was used as a positive control. Negative controls included the PCR-reagent mixture with water instead of cDNA from sample in each experiment. The sequence of the primers used in the study for the above translocations are shown in Table 5.4.

Table 5.4: Sequence of Primers used for the detection of AML/ETO and PML/RARα translocation

Translocation	Name	Primers Sequences	Size (bp)
AML/ETO	AML	5'-CTACCGCAGCCATGAAGAACC-3	395 bp
	ETO	5'-AGAGGAAGGCCCATTGCTGAA-3	
PML/RARα	PML	5'-CAGTGTACGCCTTCTCCATCA-3	381 bp for bcr1
	RAR	5'-GCTTGTAGATGCGGGGTAGA-3	345 bp for bcr2
	PML	5'-CTGCTGGAGGCTGTGGAC-3	376 bp for bcr3

Table 5.5: Cycling Regime for amplification of AML/ETO and PML/RARα translocation

Step	Temperature °C	Time (hrs/min/sec)	Remark
1. Initial denaturation	95 °C	00:05:00	One cycle (initial only)
2. Denaturation	95 °C	00:01:00	37 cycles (repeat steps 2, 3, 4)
3. Annealing	62 °C	00:00:45	
4. Polymerization	72 °C	00:01:15	
6. Final extension	72 °C	00:10:00	One cycle (final only)
7. Ice	4°C	00:00:00	Indefinite hold

Detection of FLT3 (FLT3/ITD and FLT3/D835) and exon12 NPM1 mutation

Extraction of Genomic DNA

Genomic DNA from acute leukemia patients was extracted by using Himedia kit (Mumbai, India) and stored at -20^{O}C till further analyzed.

Quantification of Genomic DNA

For the quantification of DNA, readings were taken in Nanodrop spectrophotometer. Precisely 1.5µl of the sample was loaded on the pedestal of the instrument. Readings were taken in

specific module for DNA after taking measurement for blank. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is accepted as "pure" for DNA.

Agarose Gel Electrophoresis of Extracted DNA Samples

In order to check the quality of the extracted DNA from blood samples, agarose gel electrophoresis was carried out in a 0.8% agarose gel in TAE buffer. 0.8 gram of agarose was dissolved in 100 ml of 1x TAE buffer and boiled. The solution was cooled to 45-50^0 C and 5-6 ul of EtBr was added in to the solution. Then solution was poured in to the casting tray with a comb. After solidification, gel was placed in electrophoresis tank containing 1% TAE buffer. The DNA samples (5 µl) were mixed with 6X loading dye (1 µl) and loaded into the slot/well of submerged gel. Applying a constant current of 100 mA the gel was run for up to 30 minutes. Gels were visualized under the gel documentation system and images acquired (Figure 5.4).

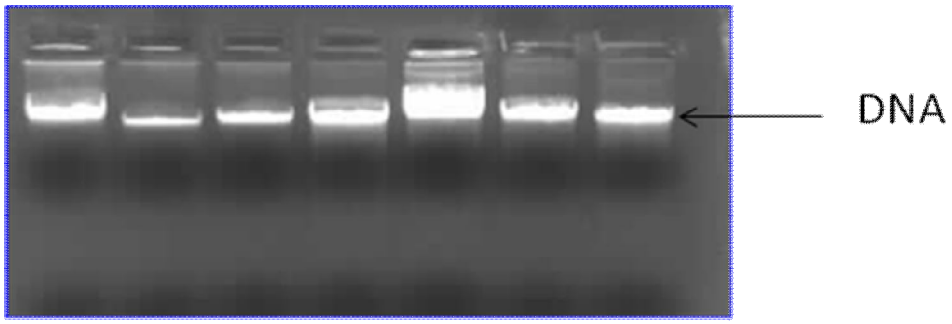


Figure 5.4: Agarose gel picture showing quality of genomic DNA isolated from the subjects

Polymerase chain reaction analysis for FLT3/ITD and FLT3/D835 mutation

For FLT3-ITD and FLT3/D835 mutation, PCR based amplification of genomic DNA was carried out using primers reported earlier in literatures [Nakao *et al.,* 1996; Yamamoto et al. 2001]. PCR for FLT3/ITD mutation was carried out for exon14 (formerly exon11) using Primers 11F-11R which amplify the juxtamembrane domain of the and first part of the TK-1 domain where most of the reported mutation are located. For FLT3/D835 mutation, the FLT3 gene was amplified using the primers which explore the region containing exon 20 where D835 mutation is located. PCR reactions were carried out in a volume of 25µl as mentioned in table 5.6. The amplification reaction was carried out using 5 min of initial denaturation at 95°C, 35 cycles of 30 sec at 95°C,

Annealing (50°C for FLT3/ITD, 64°C for FLT3/D835) for 45s and extension for 30 sec at 72°C. A final extension step at 72°C for 10 min was performed. Ten µl of each PCR product were run on 2% agarose gel and visualized under UV light after EtBr staining. The FLT3 wild-type produced a band at 133 base pairs (bp). Any patient with an additional higher molecular weight band was considered to be positive for an ITD. Samples showing additional bands, indicative of an ITD, were subjected to sequencing.

Table 5.6: PCR reaction volume for FLT3/ITD and FLT3/D835 mutation

Components	Master Stock	Working Stock	Reaction Volume (µl)
Nuclease-free water	X		17.3
PCR buffer	10X	1X	2.5
dNTP mix (2.5 mM each)	10 mM	200 µM	0.5
DNA polymerase	3 U/µl	0.6 U/25µl	0.2
$MgCl_2$	25 mM	1.5 mM	1.5
Forward primer	10 µM	0.2 µM	0.5
Reverse primer	10 µM	0.2 µM	0.5
Template (DNA)	Y	100 to 300 ng	2.0
Total Reaction Volume = 25.0 µl			

RFLP analysis of FLT3/D835 mutation

Restriction digestion of the amplified fragments was carried out by enzyme EcoRV (NEB, USA) which recognizes the sequence **5'-GAT^ATC -3'**

3'-CTA^TAG-5'

The restriction digestion was carried out in 15 µl reaction as mentioned in table 5.7, in a water bath at 37°C, O/N.

RFLP products were loaded on 2.5% agarose gel and subjected to gel electrophoresis in 0.5X TBE buffer, stained with ethidium bromide and visualized under UV. In the presence of a wild-type exon 17 the amplified fragment was digested into two fragments of 68 and 46 bp, easily

distinguishable on gel. Mutations affecting either D835 or I836 amino acids led to the detection of the undigested product of 114 bp, in addition to the two 68 and 46 bp fragments corresponding to the digestion of the wild-type allele.

Table 5.7: Reaction volume of RFLP for the detection of FLT3/D835 mutation

Components	Stock Conc.	Working Conc.	1 Reaction (ul)
Water	1x	1x	3
Buffer	10X	1	1.5
EcoRV (enzyme)	20Units/µl	10Units	0.5
PCR product			10.0

Polymerase chain reaction and capillary electrophoresis for NPM1 exon 12 mutation

For NPM1 mutation analysis, NPM1 exon-12 was amplified by genomic PCR using primers reported by Gale et al. 2008. The reaction mixture contained 10 pmol of each primer, 100 ng of genomic DNA, 200 µM dNTPs, 2.0 mM $Mgcl_2$ and 1 Unit of Taq DNA polymerase (MBI fermentas, UK) in the buffer provided by the manufacturer. Amplification was performed in a thermal cycler (PTC 200; MJ Research, USA), at an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 2 minutes,62°C for 45 seconds, 72°C for 45 seconds, and followed by a hold at 72°C for 10 minutes and a cool down. The 6-FAM labeled PCR products were diluted tenthfold in water, and 0.5µl was mixed with 9.5 µl of HiDi formamide (Applied Biosystems,USA) and 0.25 µl of GeneScan Liz 250 internal size standards (Applied Biosystems, USA) and heated to 95°C for 2 minutes snapchill down on ice. The samples were run on an ABI 3130xl Genetic Analyzer using 16-cm capillaries. Raw data were analyzed with GeneMapper v4.0 software (Applied Biosystems, Inc.). For simplicity, rounded base pair values are used in the text. WT product size was 198 bp. PCR amplification primers were custom designed by Microsynth (Switzerland). Sequence of primer used in the mutation study is shown in table 5.8.

Table 5.8: Sequence of Primers used for the detection of FLT3 and NPM1 gene mutation

Gene	Name	Primers Sequences	Size (bp)
FLT3 /ITD	FLT3 11 F	5'-CAATTTAGGTATGAAAGCC-3'	133 bp
	FLT3 11 R	5'-CAAACTCTAAATTTTCTCT-3'	
FLT3 /D835	FLT3 17F	5'-CCGCCAGGAACGTGCTTG-3'	114 bp
	FLT3 17R	5'-GCAGCCTCACATTGCCCC-3'	
NPM1 (exon12)	NPM1 F	5'-FAM-CTTAACCACATTTCTTTTTTTTTTTTCCAG-3'	198 bp
	NPM1 R	5'-GGACAACATTTATCAAACACGGTAG-3'	

Sequence analysis

For FLT3/ITD and NPM1 mutation analysis, PCR products larger than the wild-type product were sequenced to identify the type and extent of duplication/Insertion. Amplified fragments were cut and extracted from agarose gels using Qiagen mini elute kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Approximately 100 ng purified PCR product was directly sequenced with 3.3 pmol primers as described above with the Big Dye Terminator Cycle Sequencing Kit v3.1. For analysis of FLT3/D835 mutations, undigested band of 114bp was isolated and sequenced as above. Sequence analysis was performed on an ABI 3130xl Sequence Detection System (Applied Biosystems, USA).

Statistical Analysis

For the analysis of categorical variables either Fischer's exact or Pearson's X^2 test was applied. Mann-Whitney Rank Sum Test was used for the differences in the median variable in Hemoglobin level, WBC/ platelet count and peripheral blast percentage. A p-value of ≤ 0.05 was considered statistically significant. All the analysis was performed using SPSS software (version 19).

RESULTS

Prevalence and clinical characteristics of ALL patients with BCR-ABL translocation

Ninety seven ALL patients (71 male, 26 female) were investigated at presentation. There were 55 children (median7 yrs, range 1-15 yrs) and 42 adults (median 25, range 16-67yrs). Eighty-one patients were common ALL (CD10+ve) and sixteen were CD10-ve. Overall, 26 (27%) patients were found to be positive for the translocation: 16 patients were positive for p210 (b3a2/b2a2) and eight were positive for p190 (e1a2) and two patients were positive for both p210 and p190 isoform of BCR/ABL. There was no case of T-ALL positive for this translocation compared to 33% positive in B-ALL (p=0.03). Among B-ALL, BCR/ABL fusion gene was detected in 37% (12/32) cases of adults and in 29% (13/44) cases of children (Table 5.9) (Figure 5.5).

Table 5.9: Distribution of BCR/ABL isoforms in acute leukemia patients

Types of BCR/ABL	ALL(97)		AML(123)
	B-ALL (76)	T-ALL (21)	
p210(b3a2)	8% (6)	0%	2%(2)
p210(b2a2)	13% (10)	0%	5%(6)
p190(e1a2)	11% (8)	0%	2%(3)
(p190+ p210)	1% (1)	0%	0

Note. One case of mixed lineage positive for the p190+p210 isoform was excluded from table

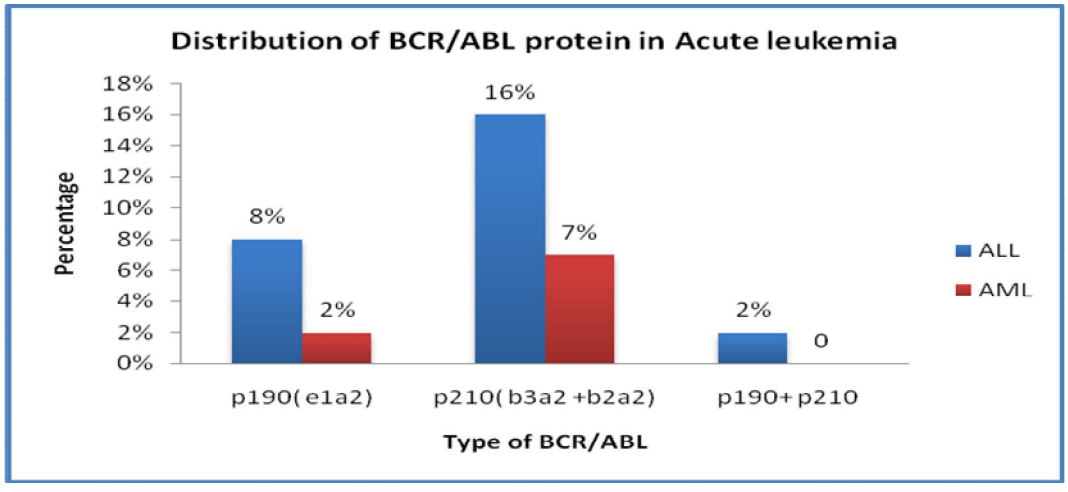


Figure 5.5: Distribution of BCR/ABL Isoforms in acute leukemia patients

Clinical characteristics of B-ALL patients with BCR/ABL translocation

FCM distribution showed that all the BCR/ABL positive cases were of B-ALL, one case was of mixed lineage type while none of the T-ALL cases were positive for BCR/ABL translocation (p=0.003). Since all cases belong to B-ALL type, clinical and hematological features were compared only in B-ALL cases while T-ALL cases were excluded from the analysis. No significant association was found with clinical features except for FAB distribution and WBC count. FAB distribution of BCR/ABL positive cases showed significant differences (p=0.03). BCR/ABL translocation was found in 56% cases of L1 followed by 44% of L2 subtype. The median WBC count was significantly high in BCR/ABL positive cases compared to BCR/ABL negative cases (p=0.04). CR rate were higher in BCR/ABL negative cases (67%) compared to BCR/ABL positive cases (43%) but it was not statistically significant (p=0.38) (Table 5.10).

Table 5.10: Clinical characteristics of B-ALL patients with BCR/ABL translocation

	BCR/ABL$^+$ (25)	**BCR/ABL$^-$(51)**	**p-value**
Gender Male, %(n)	68 (17)	69(35)	
Female, %(n)	32 (8)	31(16)	1.0
Age Child (≤15 yrs), %(n)	52(13)	61(31)	
Adult (>15 yrs), %(n)	48(12)	39(20)	0.62
FAB L1, %(n)	56(14)	45(23)	
L2, %(n)	44(11)	31(16)	
ALL, %(n)	0(0)	24(12)	**0.03***
PAS Positivity, %(n)	60(12/20)	54(24/44)	0.78
LAP, %(n)	56(14/25)	49(25/51)	0.63
HSM, %(n)	76(19/25)	82(42/51)	0.54
Hb g/dl, median (range)	6.1(3.3-9.7)	5.9(2-12.4)	0.82
Plt $\times 10^9$/L, median (range)	20.5(3-160)	20(1-160)	0.47
WBC$\times 10^9$/L median (range)	68.4(5-296)	28(0.9-256)	**0.04***
Blast% median (range)	80(25-96)	80(10-96)	0.33
CR, %(n)	43(3/7)	67(14/21)	0.38

Note. Table shows significantly higher WBC count in BCR/ABL positive cases compared to BCR/ABL negative cases. All categorical variables were compared using the $\chi 2$ test while Continuous variable were compared using Mann-Whitney test. * Significant p value < 0.05. CR: complete remission after induction chemotherapy.

Immunophenotypic characteristics of B-ALL patients with BCR/ABL translocation

There was no significant difference in the expression of immunological markers between BCR/ABL positive and BCR/ABL negative cases. Expression of CD10 was found to be positive in lower number of BCR/ABL$^+$ cases (84% vs 96%, p=0.08) while TdT in higher number of BCR/ABL$^+$ cases (64% vs 33%, p=0.09) compared to BCR/ABL negative (Table 5.11).

Table 5.11: Immunophenotypic characteristics of B-ALL patients with BCR/ABL translocation

Antigen	BCR/ABL$^+$ (25)	BCR/ABL$^-$ (51)	p-value
CD3	0	4	0.55
CD5	0	4	0.29
CD7	4	11	0.41
CD10	84	96	0.08
CD13*	0	9	0.26
CD14	4	2	1.0
CD19	33	67	1.0
CD33	16	14	1.0
CD34	52	53	1.0
HLA-DR*	95	93	1.0
TdT*	64	33	0.09

*Markers were not done in all cases, Number represent the percentage of cases positive for the marker.p-value calculated using $\chi 2$ test

Prevalence and Clinical characteristics of AML patients with Translocation

BCR/ABL translocation in AML: In AML including BAL patients, 123 patients (86 male, 37 female) were screened for the presence of the BCR/ABL translocation at presentation. These include 31 children (median 10 yrs, range 1-15 yrs) and 92 adult (median 30 yrs, range 16-85 yrs). BCR/ABL translocation was observed in 11 (9%) patient of AML: Eight patients were positive for p210 (b3a2/b2a2) and three patients were positive for p190 (e1a2) (Figure 5.6).

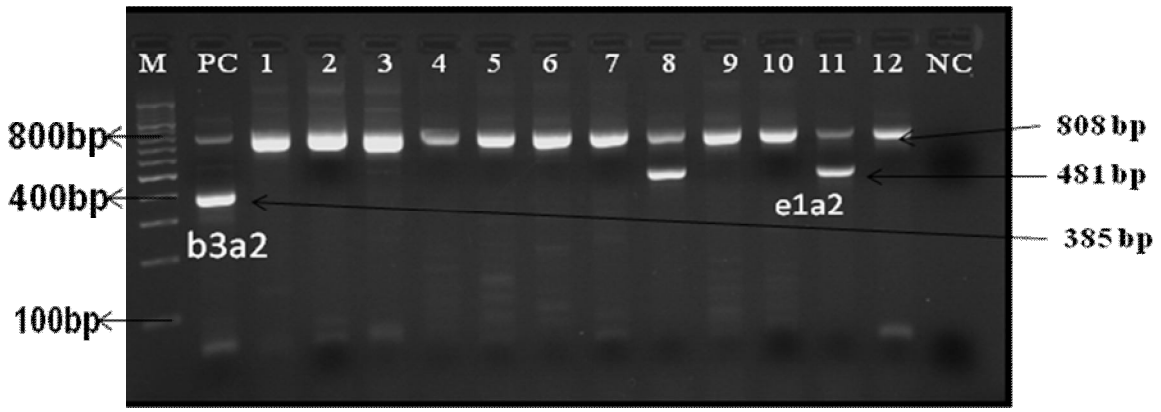


Figure 5.6: Agarose gel analysis of BCR/ABL fusion transcript showing different isoforms. M-100bp ladder; PC-Positive control K562 cells with 385bp representing b3a2 isoform; Lane 8 and Lane 11-samples with 481bp representing e1a2 isoform. Band of 808 bp is of BCR gene BCR representing internal control for cDNA quality. NC-Negative control

BCR/ABL translocation was significantly associated with the presence of LAP (p=0.001), HSM (p=0.01), MPO positivity (p=0.01) and high WBC count (p=0.004). The median WBC count in BCR/ABL positive cases was $127x10^9$/L compared to 26 x 10^9/L in BCR/ABL negative cases. FAB distribution of BCR/ABL positive cases also showed significant differences (p=0.01). BCR/ABL positive cases were higher in M0/M1 (27%) subtype followed by BAL (27%), M2 (18%) subtype. No significant difference was observed in CR rate after induction chemotherapy between BCR/ABL positive and BCR/ABL negative cases (51% vs 33%, p=1.0) (Table 5.13).

Immunophenotypically, AML cases positive for BCR/ABL translocation showed significant higher number of cases positive for CD10 and CD19 marker (p=0.001 and 0.002). TdT expression was also found in higher number of BCR/ABL positive cases compared to negative ones (43% vs 7%, p=0.03). CD34 (immature cell antigen) was also expressed in significantly higher number of cases positive for BCR/ABL translocation (91% vs 59%, p=0.05) (Table 5.12).

PML/RARα Translocation in AML patients: PML/RARα translocation was detected in only 4% (5/123) of patients with AML. Five (71%) of the seven patients classified as acute promyelocytic leukemia (M3) were found to be positive for the PML/RARα translocation. bcr1 isoform constitute 60% (3/5) while bcr3 was 40% (2/5) of PML/RARα fusion gene (Figure 5.7).

Figure 5.7: Agarose gel analysis of PML/RARα fusion transcript showing different isoforms. Ladder-50bp; Lane 3 -Positive control sample of patient used in the study with 381bp representing bcr1 isoform; Lane 6 and Lane 7- samples positive for bcr1 isoform.Lane1- sample positive for bcr2 isoform represented by 345bp band. Lane 2, 4, 5; samples negative for the translocation. NC-Negative control

There was no association seen between PML/RARα translocation and gender, age, sex , MPO positivity, LAP, Hb level, Plt count, peripheral blast at diagnosis. However, the median WBC count was significantly lower in PML/RARα positive cases compared to negative cases ($5x10^9$/L vs 30.8 $x10^9$/L, p=0.003). PML/RARα translocation was also associated with the absence of HSM (p=0.01) (Table 5.13). Cases positive for PML/RARα translocation lack expression of HLA-DR and showed lower expression of CD34 compared to negative cases (p=0.01 and p=0.06 respectively) (Table 5.12).

AML/ETO translocation in AML: AML/ETO transcript was detected in 29 (24%) out of 123 patients screened for this translocation (Figure 5.8). The FAB distribution of AML/ETO positive cases showed significant differences (p=0.05). AML/ETO translocation was found to be detected more often in M4 subtype 53% (8/15) followed M2 subtype 25% (10/40). In addition, one case of M5 subtype and two cases of BAL were also positive for the translocation (Figure 5.9).

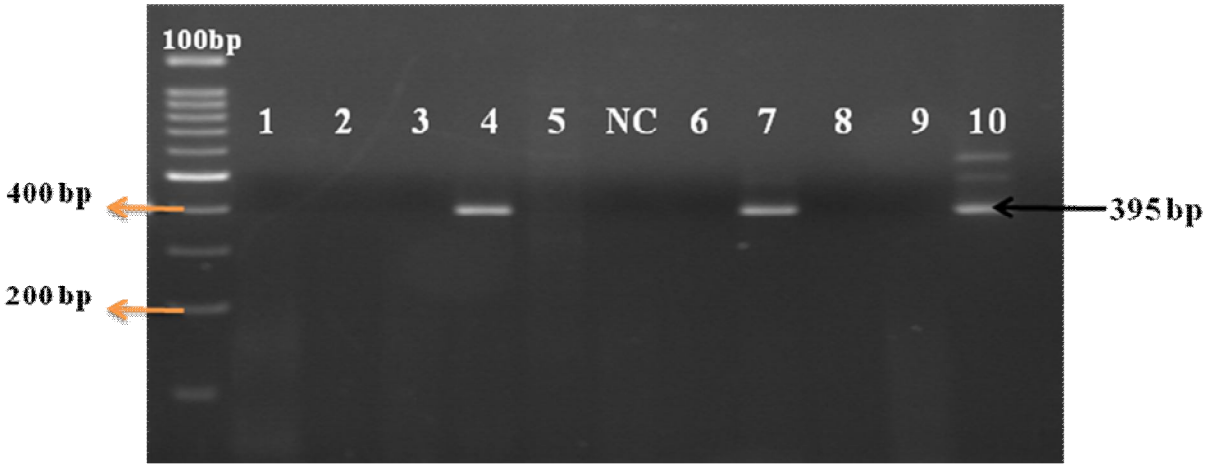
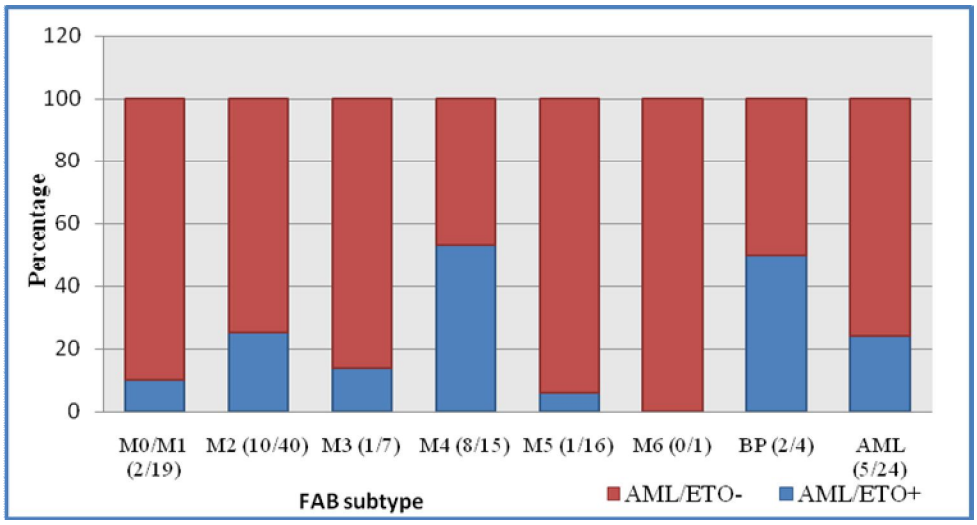


Figure 5.8: Agarose gel analysis of AML/ETO translocation: Ladder-100bp; Lane 4 -Positive control sample of patient used in the study with 395bp; Lane 7 and Lane 10- samples positive for the translocation. Lane 1-3, 5, 6, 8 and 9; samples negative for the translocation. NC-Negative control.

Figure 5.9: Distribution of AML/ETO translocation in AML patients in each FAB Subtype



Out of 29 cases positive for AML/ETO translocation, 34% were of M2 subtype followed by M4 (28%). The AML/ETO translocation was significantly associated with higher level of Hb ($p=0.02$) and with the presence of auer rod ($p=0.005$). Other clinical factors such as presence of LAP, WBC count and lower peripheral blast percentage were marginally associated with the

AML/ETO translocation. CR rate were higher in AML/ETO positive cases (67%) compared to AML/ETO negative cases (47%) but statistically insignificant (p=0.66) (Table 5.13).

AML cases positive for AML/ETO translocation showed significantly lower positivity of CD33 (74% vs 94%, p=0.006) and MPO (8% vs 39%, p=0.04) compared to negative cases. CD34 expression was marginally higher in AML/ETO positive cases than negative cases (78% vs 57%, p=0.07). Aberrant expression of CD7 was absent in translocation positive cases compared to 24% in translocation negative cases (p=0.01) (Table 5.12).

Table 5.12: Immunophenotypic characteristics of AML patients positive for the translocation

Antigen	**BCR/ABL**			**PML/RARα**			**AML/ETO**		
	Positive (n=11)	**Negative (n=112)**	**P**	**Positive (n=5)**	**Negative (n=118)**	**P**	**Positive (n=29)**	**Negative (n=94)**	**P**
CD3	0%	2%	1.0	0%	2%	1.0	4 %	1%	0.41
CD5	9%	0%	0.09	0%	1%	1.0	0 %	1%	1.0
CD7	18%	19%	1.0	0%	58%	0.58	0 %	24%	**0.01**
CD10	45%	3%	**0.001**	0%	7 %	1.0	4 %	8 %	0.67
CD13*	89%	86%	1.0	100%	86%	1.0	90%	86%	1.0
CD14	9 %	13%	1.0	0 %	14%	1.0	5 %	15%	0.45
CD19	64%	18%	**0.002**	0 %	23%	0.58	37%	18 %	0.06
CD33	82%	90%	0.31	80%	90%	0.42	74%	94%	**0.006**
CD34	91%	59%	**0.05**	20%	64%	0.06	78%	57%	0.07
MPO*	62%	29%	0.10	0 %	34%	0.54	8 %	39%	**0.04**
HLA-DR*	87%	71%	0.43	0 %	77 %	**0.01**	70%	74 %	1.0
TdT*	43%	7 %	**0.03**	0%	13%	1.0	12%	12 %	1.0

Note. Table shows significant difference in the expression of CD markers in AML cases positive for translocation. P=p-value calculated using $\chi 2$ test. Significant p value < 0.05. *These markers were not done in all cases.

Total frequency of BCR/ABL, AML/ETO and PML/RAR alpha translocation in AML

Overall in AML, 11 (9%) patients were positive for BCR-ABL fusion gene. The prevalence of AML/ETO fusion transcript was 24% in AML. The prevalence of PML/RARα was 4% in AML perhaps due to lesser number of M3 subtype in the study. Among M3 subytpe, PML/RARα was found in 71% (5/7) cases of AML (Table 5.13).

Table 5.13: Clinical characteristics of AML patients with BCR/ABL, PML/RARα and AML/ETO translocation

	Total	**BCR/ABL$^+$ %(11)**	**BCR/ABL$^-$ %(112)**	**P**	**PML/RARα$^+$ % (5)**	**PML/RARα$^-$ % (118)**	**P**	**AML/ETO$^+$ % (29)**	**AML/ETO$^-$ % (94)**	**P**
Male	86	73 (8)	70 (78)		80 (4)	70 (82)		72 (21)	69(65)	
Female	37	27 (3)	30 (34)	1.0	20 (1)	30 (36)	1.0	28 (8)	31 (29)	0.82
Child(≤15 yrs)	31	36 (4)	24 (27)		20 (1)	25 (30)		31(9)	23 (22)	
Adult(>15 yrs)	92	64 (7)	76 (85)	0.46	80 (4)	75 (88)	1.0	69 (20)	77 (72)	0.46
M0/M1	19	27 (3)	14 (16)		0	16(19)		7 (2)	18 (19)	
M2	40	18 (2)	34 (38)		0	34(40)		34 (10)	32 (30)	
M3	7	0 (0)	6 (7)		100(5)	2(2)		3 (1)	6 (6)	
M4	15	0 (0)	13 (15)		0	13(15)		28 (8)	7 (7)	
M5	16	9 (1)	13 (15)		0	13(16)		3 (1)	16 (15)	
M6	1	0 (0)	1 (1)		0	1(1)		0 (0)	1 (1)	
BAL	4	27 (3)	1 (1)		0	3(4)		7 (2)	2 (2)	
AML(NC)	21	18 (2)	17 (19)	**0.01**	0	18(21)	1.0	17 (5)	17 (16)	**0.05**
MPO %(n)	(82 /93)	78 (7/9)	89 (75/84)	0.28	100 (5/5)	88 (77/88)	1.0	100 (20/20)	85 (62/73)	0.11
AR, %(n)	55	18 (2/11)	47 (53/112)	0.10	40 (2/5)	45 (53/118)	1.0	69 (20/29)	37 (35/94)	**0.005**
LAP, %(n)	34	73 (8/11)	23 (26/112)	0.001	20 (1/5)	28 (33/118)	1.0	14 (4/29)	32 (30/94)	0.06
HSM, %(n)	67	91 (10/11)	51 (57/112)	**0.01**	0 (0/5)	57 (67/118)	**0.01**	45 (13/29)	57 (54/94)	0.28
Hb (g/dl)	6.2(2-13.8)	6.2 (3.9-9)	6.2(2-13.8)	0.71	8.6 (3.9-11.6)	6.2(2-13.8)	0.2	5.1(2.6-10.2)	6.7(2-13.8)	**0.02**
Plt×10^9/L	22(1-343)	22(3-111)	23 (1-343)	0.52	25 (2-150)	22(1-343)	0.8	20(1-211)	23.7 (2-343)	0.38
WBC×10^9/L	28.2(1-428)	127 (14-281)	26 (1-428)	**0.004**	5 (1-131)	30.8(1.4-428)	**0.003**	18.8 (2-267)	42.2 (1-428)	0.06
Blast%	67(10-98)	80(40-90)	64 (10-98)	0.12	42(10-88)	67(17-98)	0.2	60 (21-94)	70 (10-98)	0.08
CR, %(n)	20	33 (1/3)	51(19/37)	1.0	50(2/4)	50 (18/36)	1.0	67(4/6)	47(16/34)	0.66

Note. AML (NC) - AML not Classified, BAL-Biphenotypic acute leukemia. For Hb, Plt, WBC and Blast- values indicated median while values in parenthesis indicate range. All categorical variables were compared using the $\chi 2$ test while Continuous variable were compared using Mann-Whitney test. P indicated p-value. Bold number indicates significant p value < 0.05.CR: complete remission after induction chemotherapy.

Gene mutations in AML

Frequency of FLT3 (ITD/D835) and NPM1mutations

A total of 161 de novo AML patients, including 116 adult (median age: 32, range 16-85) and 45 children (median age: 10, range 1-15) were screened for FLT3 and exon 12 NPM1 mutation. Patients characteristics were shown in Table 5.16. Alterations in FLT3 gene were detected in 25% (40/161) patients. These aberrations include FLT3/ITD in 35 (22%) cases and D835 mutation in 5 cases (3%) (Figure 5.10). None of the samples showed combination of both FLT3/ITD and FLT3/D835 mutation. NPM1 mutation was detected in 34 (21%) of 161 cases.

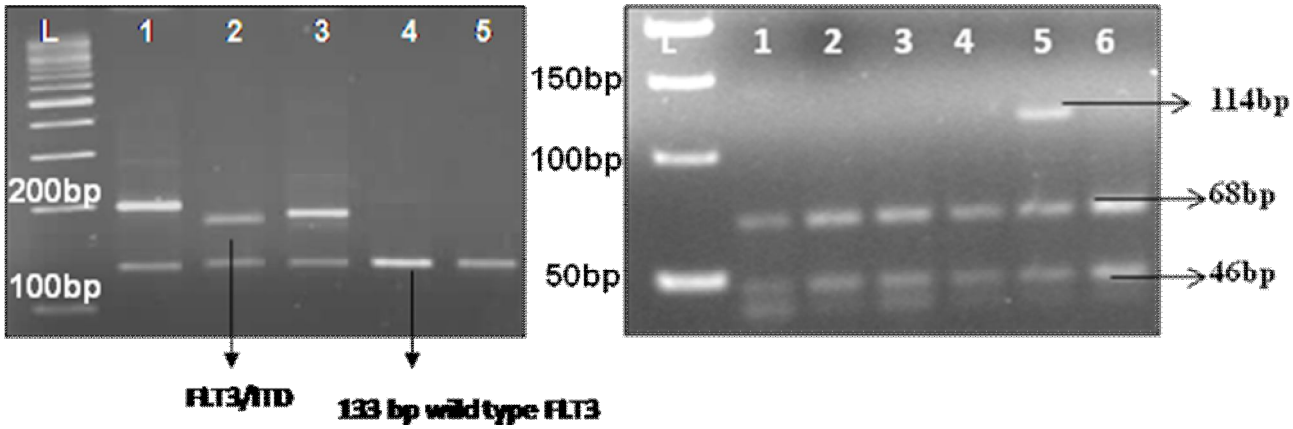


Figure 5.10: Agarose gel analysis of FLT3/ITD and FLT3/D835 mutation: A) PCR Product of FLT3/ITD gene: Ladder-100bp; Lane 4 and 5- samples with 133bp representing wild type FLT3; Lane 1, 2, 3 - samples of patient positive for FLT3/ITD mutation represented by additional band. (B) RFLP FLT3/D835 mutation after digestion after EcoRV enzyme: Ladder-50bp; Lane 5- samples with undigested product of 114bp in addition to the two 68 and 46bp fragments represent FLT3/D835 mutation. Lane 1-4 and 6 – samples negative for FLT3/D835 mutation.

DNA Sequencing Analysis

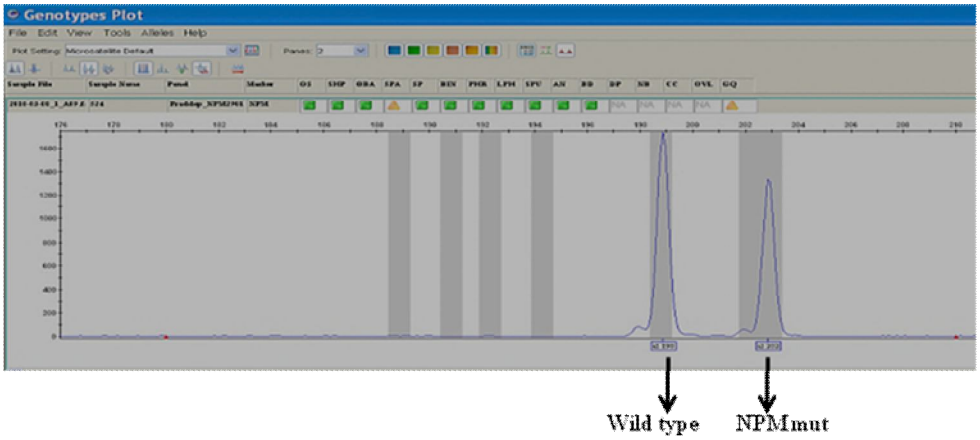
Sequencing analysis was carried out in 10 samples of FLT3/ITD mutation and 8 samples of NPM1 mutation. All the samples sequenced in FLT3/ITD mutation showed different position of duplications. Sequence analysis revealed that all the duplication in FLT3/ITD mutation was in-frame and their length varied from 13 to 59 bp. (Table 5.14A). Four of FLT3/ITD samples show insertion of 5-15 base pairs of nucleotides along with duplication while 5 samples had simple duplication without any insertion or deletion. Two samples with FLT3/D835 mutation were sequenced, both of them showed partial replacement of T nucleotide in place of G nucleotide for the D835 codon resulting in substitution of Asp to Tyr residue (Table 5.14B).

Table 5.14: Sequencing analysis of FLT3/ITD and FLT3/D835 mutation in AML patient

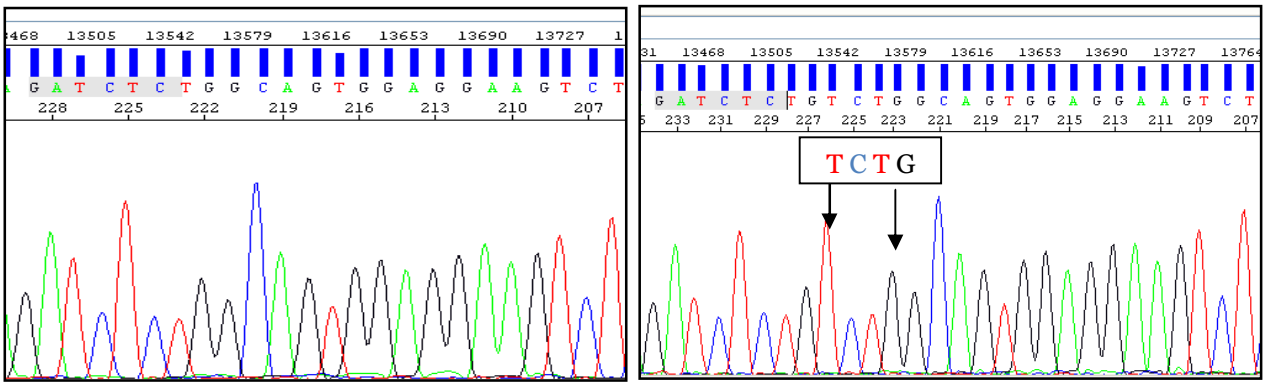
A. Sample ID	**Amino acid sequence of FLT3/ITD mutation**
Wild	QFRYESQLQMVQVTGSSDNEYFYVDFREYEYDLKWEFPRENLEF +1
UH530	QFRYESQLQMVQVTGSSDNEYFYVDFREYE YDLKWEFP **VG***YDLKWEFP* RENLEF
UH532	QFRYESQLQMVQVTGSSDNEYEYVDFREYE YDLKWEFPRENLEF **GPDIL** *YDLKWEFPRENLEF*
UH366	QFRYESQLQMVQVTGSSDNEYFY VDFREYEYDLKWEFPRENLEF **TRLPS** *VDFREYEYDLKWEFPRENLEF*
UH526	QFRYESQLQMVQVTGSSDNEYFYVDFR EYEYDLKWEFPRENLEF *EYEYDLKWEFPRENLEF* **IV** *EYEYDLK*
UH504	QFRYESQLQMVQVTGSS DNEYFYVDFR *DNEYFYVDFR* EYEYDLKWEFPRENLEF
UH514	QFRYESQLQMVQVTGSSDNEYFYV DFREYEYDLKWE FPRENLEF *DFREYEYDLKWE*
UH570	QFRYESQLQMVT *QFRYESQLQMVQVT* SSDNEYFYVDFREYEYDLKWEFPRENLEF
UH384	QFRYESQLQMVQ VTGSSDNEYFYV *VTGSSDNEYFYV* FREYEYDLKWEFPRENLEF
UH558	QFRYESQLQMVQVT GSSDNEYFY *GSSDNEYFY* VDFREYEYDLKWEFPRENLEF
B.	**Nucleotide sequence of FLT3/D835**
Wild	TTG GCT CGA **G**AT ATC ATG AGT GAT
UH153	TTG GCT CGA **T**AT ATC ATG AGT GAT
UH288	TTG GCT CGA **T**AT ATC ATG AGT GAT

Note. (A) Table shows amino acid sequence of FLT3/ITD mutations detected in patients with AML. Duplicated sequences in samples are shown in italics and underlined while insertion sequences are shown in bold. (B) Nucleotide sequence of FLT3/D835 mutation detected in two patients of AML. Sample (UH153 & UH288) showed nucleotide change of T in place of G as underline in the sequence at codon D835 position.

In NPM1 mutation, six sequence variants were observed, all leading to a frame shift in the region encoding the C-terminal of the NPM protein. The most frequent mutation (Type A mutation) was duplication of a TCTG tetra nucleotide found in 3 cases (Table 5.15) (Figure 5.11).



(A) Capilllary Electrophoresis of NPM1 mutated sample



Wild Sequence **Mutant Sequence** (4 bp Insert)

(B) Sequence electropherograms of wild type and mutant sample

Figure 5.11: (A) Capillary Electrophoresis of NPM1 mutation in AML samples. Fluorescent PCR product analysis of exon 12 showed one wild type NPM1 peak at 198 bp and additional peak at 202 bp in patients with mutated NPM1, corresponding to the 4 bp insertion most commonly seen.
(B) Sequence electropherograms showing the NPM1 mutation. Direct sequencing of PCR product from normal and NPM1 mutant samples was done. The Reverse PCR primer was used as a sequencing primer. The boxed tetranucleotides are the insertion sequences between nucleotide 960 and 961 from the transcription start site shown in the mutant sequence.

Table 5.15: Sequencing analysis of NPM1 mutation in AML patient

Sample ID	DNA sequence of exon 12 of NPM1 gene
Wild type	GATCTCTG ---- GCAG TGGAGGAAGTCTCTTTAAGAAAATAG
UH542	GATCTCTG**TCTG**GCAG TGGAGGAAGTCTCTTTAAGAAAATAG
UH545	GATCTCTG**CATG**GCAG TGGAGGAAGTCTCTTTAAGAAAATAG
UH533	GATCTCTG **TTTG**GCAG TGGAGGAAGTCTCTTTAAGAAAATAG
UH581	GATC **CGCT**TCTGGCAG TGGAGGAAGTCTCTTTAAGAAAATAG
UH591	GATC **TATG**TCTGGCAG TGGAGGAAGTCTCTTTAAGAAAATAG
UH595	GATCTCTG**TCTG**GCAG TGGAGGAAGTCTCTTTAAGAAAATAG
UH 576	GATCTCTG**TCTG**GCAG TGGAGGAAGTCTCTTTAAGAAAATAG
UH567	GATCTCTG**TGTG**GCAG TGGAGGAAGTCTCTTTAAGAAAATAG

Note. Table showed the different mutation pattern of NPM1 gene. Inserted nucleotides are in Turquoise. TCTG is the most common 4 bp nucleotide insertion in exon 12 of NPM1 gene.

Correlation of FLT3 and NPM1 mutations with clinical and biological characteristics

FLT3 mutations: The clinico-hematologic features of the patients whose samples were positive for FLT3/ITD mutations were compared with those without FLT3/ITD mutations (Table 5.16). No significant difference in age or gender was found in patients with or without FLT3/ITD mutation. Although the frequency of FLT3/ITD mutations was 18% (8/45) in children (≤15yrs) whereas in adult (above 15yrs) the frequency was higher at 23% (27/116), this difference was not statistically significant (p= 0.52). No significant correlation of the mutation with any of the specific FAB subtype was observed (p=0.85). FLT3/ITD mutations was found more frequently in M1 (33%) followed by M5 (27%) and M2 (23%) FAB subtype. There were also no significant differences in hemoglobin level, platelet count, and percentage of blast cells in the peripheral blood in samples from patients with or without FLT3/D835. The presence of HSM or LAP involvement was not significantly different in patients with or without mutation. The classical factor (WBC) count was significantly higher in samples with FLT3/ITD mutations than those without the mutation. The median WBC count was 60 x 10^9/L (range 1.4 – 428 x 10^9 /L) and 27 x 10^9/L (range 1.4-380 x 10^9/L) respectively (p<0.007). WBC count was also higher for samples with FLT3/D835 mutations 41.6 x 10^9/L but not statistically significant when compared to samples without FLT3/D835 mutations 30.8 x 10^9/L (Table 5.16).

Table 5.16: Clinical characteristics of AML patients with FLT3/ITD and FLT3/D835 mutations

	Total	**FLT3/ITD$^+$ (35)**	**FLT3/ITD$^-$(126)**	**P**	**FLT3/D835$^+$(5)**	**FLT3/D835$^-$(156)**	**P**	**FLT3 %(n)**
Male	110	69 (24)	68(86)		80 (4)	68 (106)		25 (28)
Female	51	31(11)	32 (40)	1.00	20 (1)	32 (50)	1.0	23 (12)
Children	45	23 (8)	29(37)		40 (2)	28 (43)		22 (10)
Adult	116	77 (27)	71 (89)	0.52	60 (3)	72 (113)	0.61	26 (30)
M0/M1	24	17(6)	14(18)		40(2)	14(22)		33 (8)
M2	60	37(13)	37(47)		20(1)	38(59)		23 (14)
M3	9	6(2)	6(7)		0 (0)	6(9)		22 (2)
M4	18	6(2)	13(16)		20(1)	11(17)		17 (3)
M5	26	17(6)	16(20)		20(1)	16(25)		27 (7)
M6	1	0(0)	1(1)		0(0)	1(1)		0 (0)
BAL	3	0(0)	2(3)		0 (0)	2 (3)		0 (0)
AML	20	17(6)	11(14)	0.85	0(0)	13(20)	0.77	30(6)
MPO (+)	121	93(28/30)	88(80/91)	0.51	100(2/2)	89(106/119)	1.0	28(30)
AR present	79	51(18)	48(61)	0.84	20(1)	50(78)	0.36	24 (19)
LAP	46	40(14)	25(32)	0.09	40(2)	28(44)	0.62	35 (16)
HSM	92	46(16)	60(76)	0.12	60 (3)	57(89)	1.0	21 (19)
Hb level	6.2(2-13.8)	6.2 (2-10.4)	6.2 (2.6-13.8)	0.59	5.3(3.6-10.0)	6.2(2-13.8)	0.49	6.2(2.6-13.8)
TLC count	31.5(1-428)	60.2(1.4-428)	26.6(1-380)	**0.007***	41.6(2.5-256)	30.8 (1-428)	0.94	53(1.4-428)
Plt count	22(1-343)	23(2-126)	22 (1-343)	0.55	20(2-191)	22(1-343)	0.75	21.5 (2-191)
Blast (%)	69(10-98)	70(29-96)	69(10-98)	0.75	80(50-90)	69(10-98)	0.56	71 (29-96)
NPM1 mt	34	29 (10)	19(24)	0.24	60(3)	20(31)	0.06	13(38)
CR	24	29 (2/7)	56(22/39)	0.23	100(1)	51(23/45)	1.0	12(3)

Note: Table showed association of clinical variables of AML patients with FLT3 mutation. Data of Hb level, TLC count, Plt count and Blast (%) represent Median, Values in parenthesis indicates range. Data of categorical variable (Male, Female, Adult, child, MPO positivity, LAP, HSM, CR) represent percentage while values in parenthesis indicates number. All categorical variables were compared using the $\chi 2$ test while Continious variable were compared using Mann-Whitney test. *****Significant p value < 0.05.

NPM1 mutations: Comparison of the clinical and laboratory features between the 34 patients with and 127 without NPM1 mutation is summarized in Table 5.17. NPM1 mutation were found more frequently in females (27%) than males (18%), however, this difference was not significant (p=0.21). The NPM1 mutations were found more frequently in adults as compared to children (26% vs 9%, p=0.01). Further stratification of age showed that frequency of NPM1 mutation increased with age, 38% (12/32) being the highest frequency that was observed in AML patient above 45yrs of age (p=0.02) (Figure 5.12). FAB distribution of NPM1 mutation did not show any significant difference (p=0.80). Patients with AML M2 subtype had a higher NPM1 mutation frequency (27%) than other subtypes, but this difference was not statistical significance (p=0.2). NPM1 mutations were significantly associated with higher initial Plt counts (p=0.02) and presence of HSM (p=0.01), but not associated with LAP, auer rod presence, Hb levels and peripheral blast percentages (Table 5.17).

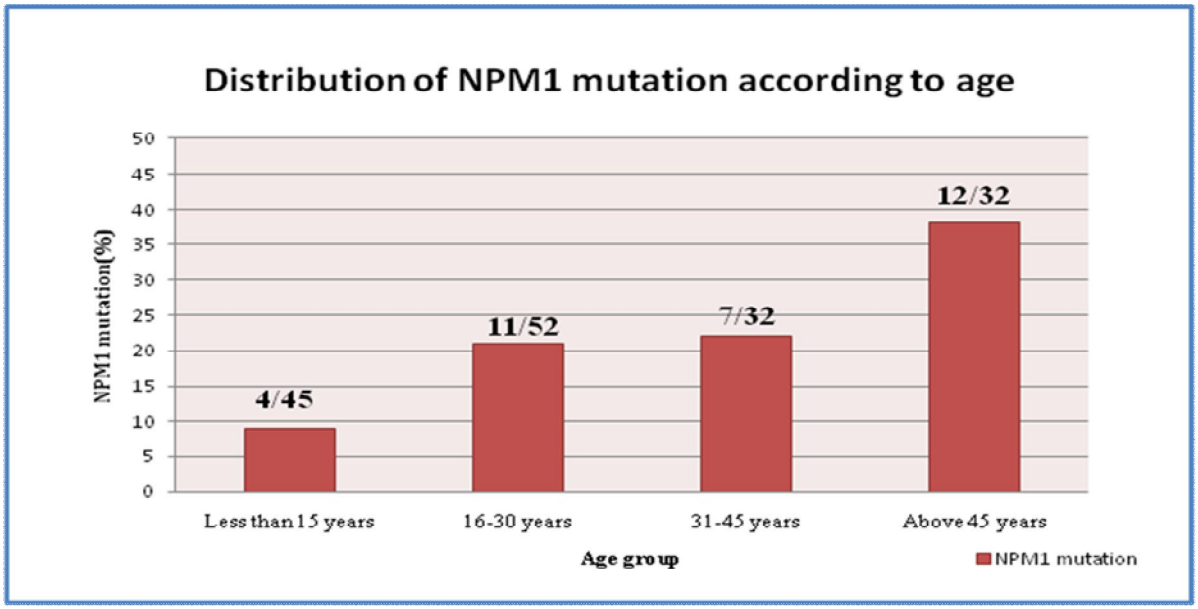


Figure 5.12: Incidence of NPM1 mutation according to age

Note: Figure showed the distribution of NPM1 mutation in AML according to age. Higher percentage of NPM1 mutation was observed in AML cases of above 45 years age.

Table 5.17: Clinical characteristics of AML patients with NPM1 mutations

	Total	NPM1 mut %(34)	NPM1 wild %(127)	NPM1 mut (%)	p-value
Gender Male	110	59(20)	71(90)	18	
Female	51	42(14)	29(37)	27	0.21
Child	45	12(4)	32(41)	9	
Adult	116	88(30)	68(86)	26	**0.01***
FAB M0/M1	24	12(4)	16(20)	17	
M2	60	47(16)	35(44)	27	
M3	9	6(2)	5(7)	22	
M4	18	6(2)	13(16)	11	
M5	26	15(5)	16(21)	19	
M6	1	0 (0)	1(1)	0	
BAL	3	0 (0)	2(3)	0	
AML	20	15(5)	11(15)	25	0.80
MPO Positivity	108	96(24/25)	87(84/96)	22	0.29
AR Present	79	56(19)	47(60)	24	0.44
LAP Present	46	23(8)	30(38)	17	0.52
HSM Present	92	38(13)	62(79)	14	**0.01***
Age	24 (1-85)	32 (8-75)	21 (1-85)	-	**0.005***
Hb level	6.2(2-13.8)	6.6(3-12.2)	6.2(2-13.8)	-	0.53
TLC count	31.5(1-428)	34.7(1.6-380)	31.5(1-480)	-	0.92
Plt count	22(1-343)	46.5(9-343)	20(1-270)	-	**0.02***
Blast (%)	69(10-98)	75(10-95)	68(17-98)	-	0.32
FLT3 mutation	40	38(13)	27(21)	32	**0.04***
Follow up CR	24	73(8/11)	46(16/35)	33	0.17

Note: Table showed association of clinical variable of AML patients with NPM1 mutation. Data of Hb level (g/dl), TLC count ($x10^9$/L), Plt count($x10^9$/L), Age (yrs) and Blast (%) represent Median, Values in parenthesis indicates range. Data of categorical variable (Gender, Adult, child, MPO positivity, LAP, HSM, CR) represent percentage while values in parenthesis indicates number. All categorical variables were compared using the $\chi 2$ test while Continuous variable were compared using Mann-Whitney test. *Significant p value < 0.05.

Immunophenotypic characteristics of FLT3 and NPM1 mutation

FLT3 (ITD and D835) mutated cases had higher expression of CD7 (p=0.04) and marginally lower expression of CD19 (p=0.07). Other markers, such as CD3, CD5, CD10, CD13, CD14, CD33, CD34, MPO, HLA-DR and Tdt were not found to be significantly associated with FLT3 mutation status (Table 5.18). NPM1 mutation was significantly associated with the lower expression of CD34 and HLA-DR (p<0.001 and p<0.001, respectively). The frequency of other markers such as CD3, CD5, CD7 (T-Cell marker), CD10, CD19 (B-cell marker), CD14, CD33, TdT, HLA-DR and MPO did not significantly differ between NPM1 mutated and NPM1 wild cases (Table 5.18).

Table 5.18: Immunophenotypic characteristics of AML patients with FLT3 and NPM1 mutation

Antigen Marker	Total Percentage	FLT3 mutated	FLT3 wild	p-value	NPM1 mutated	NPM1 wild	p-value
CD7	17%	31%	13%	**0.04**	9%	20%	0.35
CD19	22 %	10%	26%	**0.07**	12%	25%	0.10
CD34	60%	57%	62%	0.69	29%	69%	**0.000**
HLA-DR*	71%	82%	67%	0.35	29%	84%	**0.000**

Note: p-value calculated by $\chi 2$ test. Significant p value < 0.05.*These markers were not done in all cases

Correlation of FLT3 and NPM1 mutations with translocation

Data of translocation was available for 120 cases screened for FLT3 and NPM1 mutation. BCR/ABL translocation was observed in 2 (6%) of 33 cases with FLT3 mutation compared to 9 (10%) of 87 cases without FLT3 mutation. In NPM1 mutated cases frequency of BCR/ABL translocation was 4% (1/22) compared to 10% (10/98) of NPM wild cases. PML/RARα translocation was detected in one case of both FLT3 and NPM1 mutation. Frequency of AML/ETO translocation was 12% in FLT3 mutation cases and 28% in FLT3 wild group (Table 5.19).

Table 5.19: Association of FLT3 and NPM1 mutations with translocations

Translocation	FLT3 mut(33)	FLT3 wild (87)	p-value	NPM1 mut(22)	NPM1 wild (98)	p-value
BCR/ABL	6 (2/33)	10 (9/87)	0.72	4 (1/22)	10 (10/98)	0.68
PML/RARα	3 (1/33)	5 (4/87)	1.0	4 (1/22)	4 (4/98)	1.0
AML/ETO	12 (4/33)	28 (24/87)	0.09	14 (3/22)	25 (25/98)	0.27

Discussion

Chromosomal translocations are observed in a significant number of patients with acute lymphoblastic leukaemia. Unlike most other rearrangements in ALL which target transcription factors, BCR-ABL is an activated tyrosine kinase molecule that activates RAS and other signaling pathways. Three major mechanisms have been implicated in the malignant transformation by BCR/ABL, namely altered adhesion to stroma cells and extracellular matrix, constitutively active mitogenic signaling through Ras and MAPK kinase pathway, reduced apoptosis and proteasome-mediated degradation of Abl inhibitory proteins (66, 88-89). Although the hallmark of CML, BCR-ABL is detected in approximately 25% of adults and 3% of children with ALL (Pui et al., 2004).

In the current study, we prospectively studied the prevalence of BCR-ABL fusion gene in 97 ALL patients and found it to be positive in 26 (27%) patients. There was no case of T-ALL positive for this translocation compared to 33% positivity in B-ALL (p=0.03). The prevalence of BCR-ABL in T-cell leukemias varies among studies from 0% to 5% [Westbrook *et al.,* 1992; Lim *et al.,* 1999; Gleissner *et al.,* 2002]. Among B-ALL, BCR/ABL fusion gene was detected in 37% (12/32) cases of adults and in 29% (13/44) cases of children. This prevalence is high particularly in children in comparison with previously published Indian reports that showed the prevalence range from 3% to 8% in children [Siraj *et al.,* 2002; Siraj *et al.,* 2003; Sazawal *et al.,* 2004]. The prevalence of the BCR-ABL fusion gene in adult ALL patients was reported to vary with different Asian countries i.e 8% from Korea [Choi *et al.,* 2011], 18%-27% from Taiwan [Udomsakdi-Auewarakul *et al.,* 2003], 28% from China [Li *et al.,* 2010] and 55% from Pakistan

[Faiz *et al.,* 2011]. However, the data from non-Asian countries disclosed less heterogeneity in prevalence of BCR/ABL in adult ALL patients i.e 19% from Southwest Oncology Group (USA) [Pullarkat *et al.,* 2008], 26% from Italy [Mancini *et al.,* 2005] and 19% from Medical Research Council group (UK) [Moorman *et al.,* 2007]. In AML, 9% (11) patients were found to be positive for the translocation BCR/ABL in including BAL patients. Therefore, only 6.7% AML patients (excluding BAL) were found to be positive for this translocation. Further, in AML BCR/ABL was detected in 6.6% and 6.8% of adult and children cases respectively. In literature the incidences of BCR/ABL is reported to be vary from 0% to 6% of AML cases [Legrand *et al.,* 1998; Paietta *et al.,* 1998; Keung *et al.,* 2004].

In the present study, BCR/ABL translocation was significantly associated with a high WBC count in ALL (p=0.04), an adverse prognosis factor. This is due to increased tyrosine kinase activity of BCR/ABL protein that induces resistance to apoptosis, growth factor interactions and alters cell–cell and cell–matrix interaction inducing proliferation [Bedi *et al.,* 1994]. The CR rate in the present study was not significantly different between BCR/ABL positive and BCR/ABL negative patients. Although a trend toward worst prognosis was observed in ALL i.e CR rate were 43% in BCR/ABL positive patients compared to 67% in BCR/ABL negative patients. In literatures also, BCR/ABL translocation is associated with decrease in CR rate and overall survival in both children and adults ALL [Arico *et al.,* 2000; Gleissner *et al.,* 2002]. Gleissner *et al.,* [2002] found that 68% patient of BCR/ABL positive adult ALL achieved CR compared to 85% of BCR/ABL negative patients, with a higher frequency of early relapses in BCR/ABL positive patients. Further, in patients with acute lymphoblastic leukemia, Radich et al., 1997 have shown that detection of bcr-abl transcripts after allogeneic stem cell transplantation is highly predictive of eventual relapse in acute lymphoblastic leukemia patients. Thus, identification of the type of fusion transcript in a leukemia patient prior to bone marrow or stem cell transplant is essential for assessing minimal residual disease after transplantation.

Genetic Alterations in AML

Chromosomal Translocations: Acute leukemia (AML) is very heterogeneous at the cytogenetic and molecular genetic levels. Over the last 30 years, several specific recurrent chromosome aberrations have been described in AML, both chromosomal aberrations and submicroscopic

gene mutations. The t(8;21) or the corresponding AML/ETO rearrangement is a typical and common abnormality in AML. In the new WHO classification also, AML/ETO is one of the four different characterized types of translocation in the first group of AML with recurrent cytogenetic translocation [Chillon *et al.,* 2001]. In the current study AML/ETO translocation was detected in 24% of AML cases. Age wise distribution of AML/ETO showed children have higher frequency of AML/ETO translocation than adults (29% vs 21%). However, in literature according to conventional karyotyping, the frequency of t(8;21) in adult AML cases vary with countries such as 13% in Japanese and 5% in Australian [Nakase *et al.,* 2000], 16% in Malaysian study [Rosline *et al.,* 2004] and 9% in an Indian study [Ahmad *et al.,* 2009]. In another Indian study, the frequency of detection of AML/ETO by RT-PCR was 28% in AML cases [Sazawal *et al.,* 2009]. The reasons for the discrepancies among studies could be due to sample size, or the technique used either RT-PCR or karyotype analysis. However, it is to be noted that molecular techniques like RT-PCR might increase incidence of translocation by 5–10% as patients lacking t(8;21) gene rearrangement can be detected by RT-PCR. Interestingly, based on morphology the AML/ETO translocation was detected more commonly in AML-M4 (53%) followed by BAL (50%) and M2 (25%) subtype in the current study. This is in contrast to the literature that report strong association with M2 FAB subytype worldwide i.e [Rubnitz *et al.,* 2002; Nishii *et al.,* 2003; Creutzig *et al.,* 2011]. Further in the current study, AML/ETO translocation was significantly associated with the presence of Auer rod and LAP, low level of Hb and WBC count concordant with the report of Cho *et al.,* [2003].

Immunophenotypic analysis showed that CD19 and CD34 were associated with the presence of AML/ETO translocation. This finding is consistent with the previous reports and further strengthens the reliability of CD19 as a marker of AML/ETO translocation [Nakase *et al.,* 2000; Rosline *et al.,* 2004]. Interestingly AML cases with AML/ETO translocation were also associated with the decreased expression of CD7, CD33 and MPO antigen. The presence of AML/ETO translocation is associated with high remission rate and prolonged survival rate [Grimwade *et al.,* 1998; Cho *et al.,* 2003]. In the current study, patients with AML/ETO translocation showed trend toward better outcome to induction chemotherapy. The CR rate were higher in AML/ETO positive group compared to negative group (67% vs 47%, p=0.6).

The t(15;17) or PML/RARα is a characteristic molecular abnormality of acute promyelocytic leukemia (APL) subtype of AML (FAB M3) which is characterized by an excess of immature promyelocytes in the bone marrow that fail to differentiate into mature granulocytes. PML/RARα is causative oncogene of APL that contains most of the PML sequence and large part of RARα including its DNA and nuclear hormone binding domain. The PML/RARα fusion gene has also been shown to play an important role in aberrant recruitment of histone deacetylase, activity similar to that of AML/ETO [Grignani *et al.,* 1998]. Overall, the frequency of PML/RARα in the current study was 5%. However, in APL the frequency of PML/RARα was lower in the current study (71%) as compared to the study of Sazawal *et al.,* [2009] (96%). PML/RARα translocation was associated with low WBC count and absence of HSM. The prognostic significance of PML/RARα could not be determined due to smaller number of patient positive for this translocation.

Gene mutation in AML

Genetic alterations of acute myeloid leukemia (AML) are known to be major determinants of patient response to therapy and outcome besides their role in the pathogenesis. In addition to aberrations that are revealed by conventional karyotyping, submicroscopic lesions such as FLT3 and NPM1 mutation which are undetectable at chromosome level have been described in AML which may confer distinct prognosis [Gilliland *et al.,* 2002; Thiede *et al.,* 2002; Falini *et al.,* 2005]. Both FLT3 and NPM1 mutations have been shown to be the most prevalent somatic alterations in AML, especially in cytogenetically normal AML. In the current study, the frequency of FLT3 and NPM1 mutation was 25% and 21%, respectively, similar to the frequency reported in German (21%, 27%) [Thiede *et al.,* 2006] and in Japanese (22.5.0%, 24.9%) population [Suzuki *et al.,* 2005].

Adult patients usually have a higher prevalence of FLT3 and NPM1 mutation than pediatric patients. The prevalence of FLT3 mutation was 22% in the patients less than 15 yrs of age in the current study which is higher than the frequency range reported by others 9 - 16% [Meshinchi *et al.,* 2001; Liang *et al.,* 2002; Krstovski *et al.,* 2010]. However the frequency of 26% in adults

(≥15yrs) was found to be lower compared to other studies in adult group which range from 29%-39% [Stirewalt *et al.,* 2006; Lee *et al.,* 2007; Seedhouse *et al.,* 2009] but similar to the report of Kayser *et al.,* [2009] and Gale *et al.,* [2008] (23% and 26%). This observation may partially explain why adult AML has a poorer clinical outcome than pediatric AML. In contrast to FLT3 mutation, Children had a significantly lower frequency of NPM1 mutations than adults (p=0.01). The frequency of NPM1 was 9% in children and 26% in adult respectively in the current study. Similarly, Ahmad *et al.,* 2009 reports that adults had higher frequency of NPM1 mutation than children in Indian population (23.6 vs 2.5%). We also observed a significant trend of increased frequency of NPM1 mutation with age in adult patients. The mutation occurred more often in AML patients more than 45 years age (p=0.02).

Certain associations between the two gene mutations and clinical characteristics have been reported in the literature. We found a significantly increased leukocyte count in patients with FLT3/ITD (p=0.007) but not with FLT3/D835 and NPM1 mutation. FLT3/ITD mutation leads to constitutively activated FLT3 protein which causes proliferation, inhibits apoptosis, and suppresses differentiation of leukemic cells. It is reported that ligand independent constitutive activation of FLT3 induced by ITD mutation could activate some downstream signal molecules including mitogenactivated protein (MAP) kinase, signal transducer and activator of transcription 5 (STAT5), and serinethreonine kinases Akt, which contribute to cell proliferation and survival advantages [Hayakawa *et al.,* 2000; Kiyoi *et al.,* 2002; Rocnik *et al.,* 2006]. This may be the reason for higher WBC count in these cases. This also supports the observation reported in earlier studies that FLT3/ITD but not the FLT3/D835 mutation is associated with higher WBC count in AML patients [Wang *et al.,* 2005; Colovic *et al.,* 2007]. Similarly, NPM1 mutations were particularly associated with specific clinical factors; for instance, a higher platelet count (p=0.02) and absence of hepatosplenomegaly (p=0.01). Thiede *et al.,* [2006] also observed that blasts with NPM1 mutation might retain a certain capacity for thrombocytic differentiation as demonstrated by in vitro experiments of Hsu *et al.,* [2003]. In accordance with our study findings, Dohner *et al.,* [2005] and Falini *et al.,* [2005] also found correlation of NPM1 mutated cases with platelet counts.

FLT3 mutation was not equally distributed in the FAB subtypes, higher frequency was found in patients with M1 (33%) followed by M5 (27%). Zheng *et al.,* [2004] reported that FLT3/ITD blocked granulocytic differentiation through suppression of CCAAT/enhance binding protein alpha (C/EBPα) expression in 32D cells transfected with FLT3/ITD. This could be the reason that FLT3 aberrant activation blocked the differentiation of myeloblastic cells, most probably granulocytic differentiation. Meanwhile, a higher frequency of NPM1 mutation was found in patients with M2 subtype and lower frequency in M4 and M5 subtype which is in contrast to the earlier studies that suggest NPM1 mutation is more frequent in myelomonocytic and monocytic leukemia [Suzuki *et al.,* 2005; Thiede *et al.,* 2006; Huang *et al.,* 2008].

FLT3/ITD patients did not display a unique immunophenotype, a significant association of FLT3/ITD mutation with aberrant expression of CD7 and CD19 marker was found. An earlier study by Rausei-Mills *et al.,* [2008] has also shown the association of co-expression of CD7 antigen with FLT3/ITD mutation. In the current study, NPM1 mutation was associated with the lack of CD34 expression (29 vs 69%, $p<0.001$). In several European studies, NPM1 mutation in AML was also associated with lack of CD34 expression [Dohner *et al.,* 2005; Thiede *et al.,* 2006]. Similar report of CD34 absence has also been reported from South Asian countries [Chou *et al.,* 2006; Boonthimat *et al.,* 2008]. All these studies including ours are in agreement with CD34 expression as an indicator for poor response to induction chemotherapy.

Several earlier studies have also reported a poor clinical outcome of FLT3/ITD patients, conflicting results have been reported by others [Sheikh et al., 2003; Scholl *et al.,* 2008, Santos *et al.,* 2011]. The CR rates were lower for patients with FLT3/ITD mutation (29%) compared to without this mutation (56%) but this was not statistically significant. NPM1 mutation too did not affect the CR rate significantly in the current study. Though the CR rates were higher in the patient with NPM1 mutation compared to those without (73% vs 46%) this was statistically insignificant ($p=0.17$). Although this contradicts earlier studies that stresses the prognostic importance of NPM1 mutation [Dohner *et al.,* 2005; Suzuki *et al.,* 2005; Thiede *et al.,* 2006; Schlenk *et al.,* 2008] however, recent studies have failed to show such associations including

ours [Wandt *et al.,* 2008; Konoplev *et al.,* 2009]. This may be attributable to several factors including the populations analyzed in this study was heterogeneous with respect to age, relatively small number of follow up in the study and differences in the treatment regimen from other studies.

Further, in concurrence with other report, there was no significant association of NPM1 mutation with translocation AML/ETO, PML/RARα and BCR/ABL in the present study [Braoudaki *et al.,* 2010; Chou *et al.,* 2006]. However, the NPM1 mutations were observed more frequently in AML/ETO (14%) positive cases than PML/RARα translocation (4%). Similarly, FLT3 mutation was found in 12% cases of AML/ETO positive cases compared to only 3% cases of PML/RARα. Interestingly, It has been reported that 41% cases of t(15;17) translocation were associated with FLT3 mutations [Sritana *et al,* 2008]. These results support the observation that besides chromosomal translocation and gene mutations additional mutations are required for the pathogenesis of acute myeloid leukemia.

Chapter 6
Study of Multidrug resistance genes in acute leukemia

Chapter 6

Study of Multidrug resistance genes in acute leukemia

Introduction

Drug resistance is a multifactorial phenomenon and several mechanisms have been recognized for clinical resistance to chemotherapy in solid tumors as well as in hematologic malignancies. There are several mechanisms of cellular drug resistance; these include: 1. Multidrug resistance proteins. 2. Intracellular entrapment of drug 3. Drug detoxification by glutathione and its transferases. 4. Drug efflux. 5. Molecular target. 6. DNA repair. 7. Apoptosis and its regulation. However in leukemia, cellular drug resistance on leukemic cells is mainly conferred by two mechanism i.e expression of drug resistance genes and activation of anti-apoptotic mechanism [Van den Heuvel-Eibrink *et al.,* 2000; Gu *et al.,* 2002].

One of the innate resistance mechanisms of leukemic stem cells is the expression of one or more ATP-binding cassette (ABC) transporters. The ATP-binding cassette (ABC) genes represent the largest family of transmembrane (TM) proteins. The superfamily of ABC proteins includes 48 genes encoding for export pumps membrane receptors and ion channel regulators. These proteins bind ATP and use the energy to drive the transport of various molecules across all cell membranes [Dean *et al.,* 1995]. The multidrug resistance gene (MDR1) and multidrug resistance–associated protein (MRP1) are members of the ATP-binding cassette (ABC) superfamily of transporter proteins that plays an important role in clinical drug resistance. The protein encoded by the MDR1 gene is P-glycoprotein (Pgp), which has been suggested to be an independent adverse prognostic factor for response and survival in hematologic malignancies [Marie *et al.,* 1996]. However, clinical investigations into the role of MDR1 gene in acute leukemia have yielded equivocal results [Tsimberidou *et al.,* 2002; Benderra *et al.,* 2005]. Like MDR1, another ABC MDR-associated protein known as MRP1 also functions in drug transport and demonstrated to transport glutathione conjugates of many toxic compounds. MRP1 is overexpressed or amplified in many resistant tumor cell lines. The family of MRPs also belongs

to the ATP-binding cassette superfamily of transporters. In acute leukemia the expression of MRP1 has been shown to be correlated with the increased expression of the drug metabolizing gene GSTP1 [Den Boer *et al.,* 1999; Plasschaert *et al.,* 2005].

Other member of ABC family of transport proteins include BCRP (breast cancer resistance protein) also known as MXR, ABCP, or ABCG2, a 655–amino acid protein encoded by the BCRP gene located on chromosome 4q22 [Allikmets *et al.,* 1998]. BCRP, so called because it was first described in resistant human breast cancer cells [Doyle et al., 1998]. BCRP is also a member of ABC family but more biologically complex than Pgp and MRP-1. First, BCRP is an ABC half-transporter that requires multimerization for function [Maliepaard et al., 2001]. Secondly, BCRP substrate specificity depends on mutations that alter amino acid 482 of the protein [Honjo et al., 2001]. The in vitro drug-resistant profile of BCRP-positive cell lines shows cross-resistant to mitoxantrone, daunorubicin, doxorubicin, bisantrene, and topotecan but remained sensitive to vinblastine and paclitaxel [Litman et al., 2000]. In clinical studies, BCRP mRNA expression has been found to be expressed at variable levels in blast cells from de novo AML patients [Ross et al., 2000], however no concrete evidence of association with clinical outcome so for which warranted more investigations to determine the relation of BCRP expression with treatment outcome in leukemia. Other mechanisms of multidrug resistance in leukemia that have attracted the most attention are those associated with altered apoptotic response and non–P-gp/MDR1 drug efflux or drug entrapment through vault proteins. The major vault protein /lung resistance protein (LRP) was initially described in non–small-cell lung cancer cell lines that lacked P-gp. LRP is found to be associated with intrinsic resistance to doxorubicin, vincristine and platinum compounds in drug resistant cell lines [Izquierdo *et al.,* 1996].

Chemotherapeutic modalities kill leukemic cells primarily through apoptosis therefore altered expressions of p53 and BCL-2 genes in the apoptotic pathway may contribute to drug resistance [Jia *et al.,* 1999]. Similarly, mutations in the p53 gene found in many breast cancer cell lines affect response to DNA damage resulting from microtubule inhibitors and other therapy [Chekhun *et al.,* 2007]. Altered expression of the antiapoptosis gene Survivin (BIRC5) also affects drug

resistance. Survivin appears to regulate tumor cell resistance to certain anticancer agents and ionizing radiation. Survivin is a member of inhibitor of apoptosis protein (IAP) family, which inhibits apoptosis through a pathway different from the BCL-2 family. Survivin is identified as uniformly expressed in cancer, but not in normal cells [Velculescu *et al.,* 1999].

With the advent of better chemotherapy and supportive therapy care in the past decade, clinical outcome has improved considerably for adult patients with both acute myeloid leukemia (AML) as well as acute lymphoblastic leukemia (ALL). However, leukemic cells from adults are intrinsically more resistant to drugs commonly used in induction chemotherapy as compared to those from pediatric patients. The reason could be due to unfavorable karyotype, poor treatment tolerance and over expression of multi drug resistant genes in adults [Jeha *et al.,* 2003; Pulte *et al.,* 2008].

The aim of this objective were to

- To investigate the expression of genes related to both drug resistance (MDR1, MRP1, LRP, BCRP, GSTP1 and DHFR) and apoptosis (p53, BCL-2, Survivin) in patients with AL.
- To correlate the expression of these genes with clinical findings, response to the induction chemotherapy and immunophenotypic characteristics of the patients.
- To correlate the expression of these genes with gene mutations such as FLT3 and NPM1 and with the presence of fusion genes.

Experimental Methods

Materials

Agarose, Tris base, EDTA, other fine chemicals were purchased from Sigma Chemicals, USA. Platinum Taq polymerase, dNTPs, MgCl2, was obtained from Invitrogen and MBI fermentas USA. TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression probes were purchased from Applied Biosystems (USA). RNA later from Ambion and RNA extraction kit were purchased from Qiagen Sciences USA and Himedia, India.

Selection criteria of patients: As described in chapter 4

Inclusion criteria: As described in chapter 4

Exclusion criteria: As described in chapter 4. Patients of acute promyelocytic leukemia (M3) were also excluded from this objective.

Patients' details

The present study included 145 patients (85 Adults and 60 children) of acute leukemia (AL) diagnosed between 2008 and 2010 at the Department of Hematology, Safdarjung Hospital, New Delhi. The diagnosis of acute leukemia was made on routinely stained bone marrow aspiration/biopsies and peripheral blood smears and evaluated according to the French-American-British (FAB) criteria. Patients of acute promyelocytic leukemia (M3) and therapy-related AML were also excluded from the study. Peripheral blood samples/bone marrow aspirates obtained from these patients were collected in EDTA/heparin for the study. All in vitro procedures were performed according to protocols approved by the Ethical Committee of the Institute and Safdarjung Hospital. Clinical data, including age, gender, whole blood cell count, hemoglobin, platelets count and presence of organomegaly were collected at the time of diagnosis.

Collection of samples: As described in chapter-4

Immunophenotyping: Immunophenotyping studies were carried out at the time of diagnosis as described in chapter-4.

Treatment Protocol and Response to Induction Chemotherapy: The details of the treatment protocol have been described in chapter-1.

RNA Isolation

Leukemic cells were lysed with TRIZOL (Invitrogen) and RNA was recovered according to manufacturer's instruction before resuspension in 10-20 µl of DEPC water. The quality and quantity of the RNA samples were determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and Nano-drop ND-1000 Full –spectrum UV/Vis spectrophotometer. Samples having RNA integrity Number (RIN) 6 and above were selected for real time experiment.

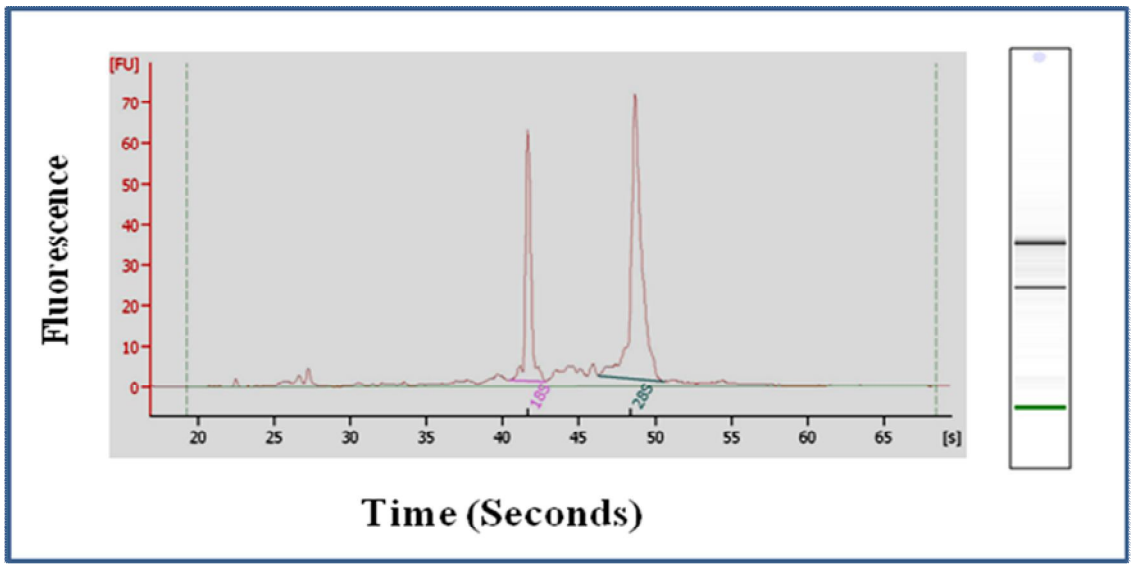


Figure 6.1: Electropherogram of acute leukemia sample showing RNA quality

Synthesis of cDNA: As described in chapter-5.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The mRNA levels of drug resistance gene MDR1, MRP1, LRP, BCRP, GSTP1, DHFR and apoptotic genes p53, BCL-2, Survivin were measured by quantitative real time PCR. The qPCR analysis was performed with the ABI PRISM 7000 Sequence Detection System by using TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression probes (Applied Biosystems,USA). The expression level of each target genes was calculated by normalizing it to

a house keeping gene TBP (TATA box binding protein). The primer pairs and TaqMan probes are 5-labeled with a 6-carboxyfluorescein (FAM) reporter and 3-labeled with a quencher (Applied Biosystems). Briefly, each reaction (25 µl total volume) contained 12.5µl of 2x TaqMan Universal PCR Master Mix (Applied Biosystems), 1.25µl of 20x Assay on Demand Gene Specific Mix containing the TaqMan probe and primers, 1.25µl of 20x Assay on Demand Endogenous control, 5µl of the cDNA sample (corresponding to 33ng of RNA) and 5µl of DNase, RNase free water.

$\Delta\Delta C_T$ Method was used to analyze the Real Time RT-PCR data [Livak et al., 2001]. Results of Real Time RT-PCR data is presented as C_T values where C_T is defined as the threshold PCR cycle number at which an amplified product is first detected. There is an inverse correlation between C_T and amount of target: lower target amounts correspond to higher C_T values and vice versa. The average C_T was calculated for both genes of interest and endogenous control and the dC_T was determined as the mean of the replicates C_T values for genes of interest minus the mean of the replicates of the C_T values for endogenous control. The $\Delta\Delta C_T$ was calculated by the formula $\Delta\Delta C_T = \Delta C_T$ of leukemic sample $-\Delta C_T$ of calibrator. The N-fold differential expression of the genes for a sample compared with the calibrator was expressed as $2^{-\Delta\Delta C_T}$. Using the $2^{-\Delta\Delta C_T}$ method, the data presented as the fold change in the target gene expression in leukemia normalized to an internal control gene and relative to the calibrator. In this study, human bone marrow total RNA from Clontech (Mountain View, CA, USA) was used as a calibrator.

Statistical Analysis

Statistical analysis was carried out using nonparametric methods. The association between the variables analyzed and gene expression levels was evaluated by non-parametric test the Mann–Whitney test for two groups and the Kruskal–Wallis test for more than two groups. The Spearman rank correlation was used to determine correlation among drug resistance and antiapoptoic genes and other continuous variables such as Hb level, WBC count, Platelet count and peripheral blast percentage. A p value of ≤ 0.05 was considered statistically significant. All the analysis was performed using SPSS software (version 19).

Results

Patient characteristics

A total of 145 AL patients were studied. There were 85 adult (45AML and 40 ALL) and 60 children (25 AML and 35 ALL). Of the 40 adult ALL patients, 27 were of B-ALL & 13 were of T-ALL while in children, 30 were B-ALL and 5 were of T-ALL. Clinical characteristics of AML and ALL patients were shown in Table 6.1. FLT3/ITD gene mutation was detected in 27% (12/45) of adult AML and in 20% (5/25) of childhood AML samples. In AML, NPM1 mutation was detected in 24% cases of adult while in 8% cases of children. In adult, six samples of AML were positive for AML/ETO fusion gene while BCR/ABL fusion gene was detected in 27% (11/40) samples of ALL compared to only 4% (2/45) in AML samples. In children, only 5 AML patients and 3 ALL patients were positive for BCR/ABL translocation.

Table 6.1: Demographic, clinical and hematological data of patients with AL

	Adults(85)		**Children(60)**	
Variables	**AML(N=45)**	**ALL(N=40)**	**AML(N=25)**	**ALL(N=35)**
Age Median (range)	35(19-85)	25(19-67)	8(3-14)	7(1-15)
Sex Males Females	20(44.4) 25(55.5)	30(75) 10(25)	3(12) 22(88)	23(66) 12(34)
WBC count ($\times 10^9$/l) Median (Range)	28.2(0.8-312)	61.1(0.8-275)	56(9.8-252)	30(2.6-275)
Hb (g/dl) Median (Range)	7.2(2.6-11.6)	6.4(3-10)	5(4-8.2)	6.9(3.4-10.3)
Plt count ($\times 10^9$/l) Median (Range)	22(2-228)	20(3.0-160)	32(5-130)	37(3-173)
Peripheral Blast (%) Median (Range)	69(25-92)	82(25-94)	74(28-96)	80(25-90)
FAB Morphology n (%)	M0/M1:14(31.1) M2:17(37.7) M4:8(17.8) M5:3(6.6) NC:3(6.6)	L1:16 (40) L2:19 (47.5) NC:5(12.5) T-ALL:13(32.5) B-ALL:27(67.5)	M0/M1:11(44) M2:6(24) M3:2(8) M4:0(0) M5:4(16) NC:2(8)	L1:27 (77) L2: 6(17) NC:2(6) T-ALL:5(14) B-ALL:30(86)
CD34 positive, n (%)	23(51.1)	18(45.0)	19(83)	18(51)
HSM, n (%)	17(37.7)	32(80)	21(84)	29(83)
LAP, n (%)	12(26.6)	19(47.5)	17(68)	21(60)
AML-ETO, n (%)	6(13.3)	-	2(8)	-
BCR-ABL, n (%)	2(4.4)	11(27.5)	3(12)	5(14)
FLT3 mutation, n (%)	12(26.6)	-	5(20)	-
NPM1 mutation, n (%)	11(24.4)	-	2(8)	-
Follow up CR	16(53.3)	18(60)	12/17(71)	22/27(81)

NC: Not classified, [a] CR : Complete remission evaluated after induction chemotherapy

Note. Data of Hb level, TLC count, Plt count and Blast (%) represent by median, Values in parenthesis indicates range. Data of categorical variable (Gender, Adult, child, MPO positivity, LAP, HSM, CR) represent number while values in parenthesis indicates percentage.

Expression of drug resistance and apoptotic genes in acute leukemia

Adults: Expression level of genes involved in drug resistance and apoptosis in AL samples is summarized in Table 6.2. The expression of LRP, MRP1, GSTP1, BCL-2 and p53 did not differ significantly between AML and ALL patients at the time of diagnosis in adult patients. However expression level of DHFR was significantly higher in ALL compared to AML patients (mean ± SD: 1.10 ± 1.30 vs. 0.49 ± 0.26 fold, p=0.006). The expression level of MDR1 was significantly higher in AML compared to ALL patients (p=0.001) whereas expression levels of BCRP and Survivin were higher in ALL patients (p=0.0003 and p=0.0001).

Children: In children, expression level of LRP and Survivin gene did not differ significantly between AML and ALL patients at the time of diagnosis. However, expression levels of BCL-2, MDR1, GSTP1 and MRP1 were significantly higher in AML than ALL samples while expression of DHFR, p53 and BCRP were significantly lower in AML compared to ALL samples.

Table 6.2: Expression of drug resistance and apoptotic genes in acute leukemia patients

Genes	Gene Expression level (Mean ± SD)					
	Adults (85)			Children (60)		
	AML(45)	ALL (40)	p-value	AML(25)	ALL (35)	p-value
BCL-2	2.71 ± 1.77	3.22 ± 3.06	0.98	4.38±1.82	3.34±2.49	**0.03**
DHFR	0.49 ± 0.26	1.10 ± 1.30	**0.006**	0.53±0.2	0.92±0.82	**0.01**
MDR1	2.26 ± 2.65	0.86 ± 1.12	**0.001**	2.35±2.25	0.97±0.83	**0.01**
GSTP1	1.45 ± 1.23	1.59 ± 1.66	0.61	1.50±0.86	1.27±1.37	**0.02**
P53	1.33 ± 0.61	1.70 ± 1.56	0.08	1.09±0.33	1.38±0.57	**0.03**
BCRP	0.06 ± 0.11	0.14 ± 0.14	**0.0003**	0.10±0.17	0.21±0.39	**0.03**
LRP	1.29 ± 2.66	0.82 ± 0.86	0.32	1.13±0.67	0.86±0.62	0.14
MRP1	2.59 ± 3.13	2.54 ± 1.66	0.12	3.26±2.31	1.40±0.96	**0.001**
Survivin	0.13 ± 0.13	0.38 ± 0.31	**0.0001**	0.20±0.16	0.25±0.19	0.24

Note. Expression level of gene was expressed as fold change relative to normal bone marrow (reference). Values represent mean ± SD. P-value is calculated using Mann–Whitney test. Bold values are significant p value ≤ 0.05.

Correlation between drug resistance genes, apoptotic genes and clinical features in AML patients

Adults: Analysis of potential interrelationship among drug resistance genes revealed that the mRNA levels of LRP positively correlated with MRP1 (r_s=0.44, p=0.016) and DHFR expression (r_s=0.41, p=0.02) (Table 6.3). Similarly, a significant correlation was observed between BCL-2 and MDR1 (r_s=0.38, p=0.03) and BCL-2 and GSTP1 (r_s=0.48, p=0.008). A positive correlation was also observed between Survivin and BCRP (r_s=0.46, p=0.01). Further, LRP and GSTP1 expression showed a positive correlation with white blood cell (WBC) counts (r_s=0.39, p=0.03 and r_s=0.39, p=0.03) whereas expression of BCL-2 was highly correlated with the peripheral blast percentage in AML patients (r_s=0.47, p=0.009) (Table 6.3).

Table 6.3: Correlation between expression of drug resistance and apoptotic genes among themselves and with clinical variables in adult patients with AML

	BCL2	DHFR	MDR1	GSTP1	P53	BCRP	LRP	MRP1	Survivin
BCL2	-	0.02	0.38*	0.48**	0.06	0.24	-0.09	-0.0	0.24
DHFR	0.02	-	-0.21	0.36	0.23	0.14	0.41*	0.20	0.36
MDR1	0.38*	-0.21	-	-0.16	-0.16	0.06	-0.28	-0.33	-0.14
GSTP1	0.48**	0.36	-0.16	-	0.32	.00	0.25	0.30	0.19
P53	0.06	0.23	-0.16	0.32	-	-0.27	0.15	0.07	0.01
BCRP	0.24	0.14	0.06	.00	-0.27	-	-0.07	-0.12	0.46*
LRP	-0.09	0.41*	-0.28	0.25	0.15	-0.07	-	0.44*	-0.08
MRP1	-.00	0.20	-0.33	0.30	0.07	-0.12	0.44*	-	-0.24
Survivin	0.24	0.36	-0.14	0.19	0.01	0.46*	-0.08	-0.24	-
Age	-0.02	-0.32	-0.12	-0.08	-0.05	-0.06	0.04	0.04	-0.09
Hb	0.13	-.17	-.04	-.05	0.09	0.10	-0.16	-.25	-.00
Plt	-0.24	0.14	-0.21	.00	-0.16	-0.07	0.32	.024	0.16
WBC	0.00	-0.14	-0.14	0.39[a]	0.07	-0.23	0.39*	0.25	-0.21
Blast	0.47**	-0.17	0.24	0.21	-0.02	-0.02	-0.15	0.22	-0.13

Note. Correlation was tested with Spearman's rank correlation test. Values in the table represent spearman's correlation coefficient (r). * Correlation is significant at p=0.05 level, ** Correlation is significant at p=0.01 level.

No significant association was observed between gene expression level and clinical variables such as AR presence, LAP, HSM and NPM1 gene mutation. However, BCL-2 expression was significantly higher in FAB M0/M1 subtype compared to other subtypes ($p<0.001$). The expression levels of GSTP1 and p53 were higher in AML patients with FLT3/ITD gene mutation compared to patients without mutation ($p=0.02$ and $p=0.002$) (Table 6.4). However, expression of MDR1 was higher in samples negative for FLT3/ITD gene mutation ($p=0.007$). Correlations between immunophenotypic data and gene expression showed significant association of MDR1 expression with CD34 marker ($p=0.002$) (Table 6.4). Significant association was also found between CD34 and GSTP1 ($p=0.01$), CD34 and Survivin ($p=0.01$) and CD7 and p53 expression ($p=0.01$) (Table 6.4). No association was observed between other CD markers (CD3, CD5, CD10, CD13, CD33, MPO and HLA-DR) and expression of target genes.

Table 6.4: Association between expression of the drug resistance and apoptotic genes with clinical features in adult patients with AML

	MDR1	GSTP1	LRP	BCL2	P53	Survivin
FAB subtype [a]	2.63±1.89	1.64±1.12	1.43±3.46	3.92± 2.29	1.03±0.36	0.09±0.08
M0-M1/M2-M5	1.53±1.66	1.42±1.34	1.29±2.39	2.18 ± 1.21	1.00±0.73	0.16±0.15
	p=0.07	p=0.06	p=0.06	**p<0.001**	p=0.8	p=0.09
	2.26±1.98	1.45±1.24	0.62±0.30	2.33±0.87	1.41 ± 0.85	0.06±0.08
CD7+/CD7-	2.38±2.93	1.45±1.28	1.54±3.18	2.91±2.05	0.89 ± 0.45	0.16 ± 0.17
	p=0.68	p=0.76	p=1.00	p=0.55	**p=0.01**	p=0.06
	1.02±0.34	0.75±0.69	0.83±0.07	1.08±0.13	0.51 ±0.14	0.14±0.02
CD14+/CD14-	2.45±2.75	1.51±1.28	1.32±2.82	2.65±1.79	1.07 ± 0.63	0.16±0.01
	p=1.0	p=0.31	p=0.25	p=0.09	**p=0.03**	p=0.1
	3.48 ± 3.02	1.11 ± 0.96	0.69±0.50	2.77±2.16	1.02±0.73	0.08 ± 0.07
CD34+/CD34-	0.99±1.35	1.81 ± 1.39	1.91±3.71	2.65±1.30	1.03±0.47	0.18 ±0.16
	p=0.002	**p=0.01**	p=0.39	p=0.70	p=0.51	**p=0.01**
	2.95±2.85	1.29 ± 1.08	1.88±3.64	2.48±1.58	0.95± 0.66	0.16±0.08
$FLT3^{wild}/FLT3^{mut}$	0.60±0.61	1.88 ± 1.57	1.07±2.27	2.81±1.88	1.18 ± 0.43	0.18± 0.10
	p=0.007	**p=0.02**	p=0.48	p=0.62	p=0.02	p=0.1

Note. Expression level of gene was expressed as fold change relative to normal bone marrow (reference). Values represent mean ± SD. P-value is calculated using Mann–Whitney test except for FAB. [a] p-value is calculated using Kruskal Wallis test in FAB subtype variable. Bold values are significant p value ≤ 0.05. DHFR, MRP1 and BCRP were excluded from the table because no significant association was observed with any of the clinical features analyzed.

Children: In children, correlations between the expressions of the examined resistance genes were for BCL-2 with MDR1 (r_s=0.50, p=0.01), BCRP (r_s=0.60, p=0.001), LRP (r_s=0.46, p=0.01) and MDR1 with BCRP (r_s=0.60, p=0.002) and LRP (r_s=0.76, p<0.001). Similarly, significant correlation was also found between DHFR and GSTP1 (r_s=0.68, p<0.001). Further, a positive correlation was also observed for Hb level with BCRP (r_s=0.55, p=0.004) and Survivin(r_s=0.52, p=0.008). Plt level were also positively correlated with the expression of DHFR(r_s=0.63, p=0.001) and GSTP1(r_s=0.55, p=0.004). However negative correlation was also observed between Plt level and MRP1 expression (r_s=-0.43, p=0.02) (Table 6.5).

Table 6.5: Correlation between expression of drug resistance and apoptotic genes among themselves and with clinical variables in children with AML

	BCL2	**DHFR**	**MDR1**	**GSTP1**	**P53**	**BCRP**	**LRP**	**MRP1**	**Survivin**
BCL2	-	0.00	0.50**	-0.26	-0.04	0.63**	0.44*	0.05	-0.37
DHFR	0.00	-	0.13	0.68**	0.25	-0.15	0.30	-0.18	0.39
MDR1	0.50**	0.13	-	0.25	0.34	0.60**	0.76**	-0.03	0.32
GSTP1	-0.26	0.68**	0.25	-	0.29	-0.03	0.27	-0.07	0.33
P53	-0.04	0.25	0.34	0.29	-	0.06	0.03	0.07	0.30
BCRP	0.63**	-0.15	0.60**	-0.03	0.06	-	0.38	0.21	0.44*
LRP	0.44*	0.30	0.76**	0.27	0.03	0.38	-	-0.00	0.35
MRP1	-0.05	-0.18	-0.03	-0.07	0.07	0.21	-0.00	-	-0.00
Survivin	0.37	0.39	0.32	0.33	0.30	0.44*	0.35	-0.00	-
Hb	0.13	-0.12	0.22	0.15	0.31	0.55**	-0.06	0.02	0.52*
Plt	-0.26	0.63**	-0.02	0.55**	0.25	-0.17	0.09	-0.43*	0.24

Note. Correlation was tested with Spearman's rank correlation test. Values in the table represent spearman's correlation coefficient (r). * Correlation is significant at p=0.05 level, ** Correlation is significant at p=0.01 level. Data not shown for age, wbc count and blast percentage as no significant correlation was found.

Correlation with antigen expression showed significant association of CD10 with higher expression of BCL-2 (p=0.02) and BCRP (p=0.02), CD33 with lower expression of MDR1 (p=0.02) and LRP (p=0.02). We also observed significant association of low DHFR expression with CD34 positivity. Similarly immature marker, TdT was also found to be associated with lower expression of GSTP1 (p=0.03) and DHFR (p=0.03). Correlation with genetic rearrangements shows significant association of BCR/ABL with higher expression of LRP and BCL-2). We did not find any significant association of FLT3 mutation with the expression of target genes. However, NPM1 mutation was associated with higher expression of both MDR1 (p=0.009), GSTP1 (p=0.005) and DHFR (p=0.01) (Table 6.6)

Table 6.6: Association between expression of the drug resistance and apoptotic genes with clinical features in children with AML

	MDR1	**GSTP1**	**LRP**	**BCL2**	**DHFR**	**BCRP**	**MRP1**
CD10+/CD10-	5.40±0.14 1.92±2.16 p=0.06	1.42±0.70 1.51±0.93 p=1.0	1.34±0.28 1.14±0.72 p=0.15	7.17±0.70 4.31±1.64 p=**0.02**	0.38±0.07 0.56±0.20 p=0.19	0.60±0.07 0.06±0.09 p=**0.02**	4.59±0.14 3.25±2.47 p=0.19
CD33+/CD33-	1.71±1.58 7.62±1.41 p=**0.02**	1.45±0.93 2.11±0.14 p=0.15	0.96±0.29 3.16±0.07 p=**0.02**	4.39±1.70 6.32±0.28 p=0.12	0.54±0.20 0.66±0.70 p=0.41	0.10±0.17 0.26±0.07 p=0.08	3.31±2.49 3.98±0.21 p=0.58
CD34+/CD34-	2.56±2.39 0.64±0.48 p=0.06	1.40±0.87 2.02±1.02 p=0.19	1.16±0.75 1.14±0.27 p=0.41	4.81±1.61 3.36±2.31 p=0.96	0.51±0.20 0.71±0.10 p=**0.05**	0.13±0.19 0.04±0.03 p=0.96	3.64±2.46 2.08±1.60 p=0.10
MPO+/MPO-	1.66±2.32 2.68±2.21 p=0.09	1.71±0.81 1.40±0.89 p=0.29	0.99±0.36 1.19±0.77 p=0.95	3.66±2.32 4.71±1.49 p=0.18	0.51±0.17 0.54±0.22 p=0.72	0.15±0.27 0.08±0.09 p=0.23	5.37±2.37 2.27±1.51 p=**0.002**
TdT+/ TdT-	0.45±0.07 3.10±3.43 p=0.07	0.42±0.14 1.63±0.99 p=**0.03**	0.79±0.14 1.43±0.88 p=0.07	4.05±0.21 5.14±1.39 p=0.55	0.22±0.14 0.65±0.16 p=**0.03**	0.07±0.02 0.07±0.10 p=0.55	1.47±0.21 1.88±1.17 p=1.0
HLA-DR+/HLA-DR-	2.69±2.85 0.87±0.70 p=0.39	1.21±0.58 1.15±0.14 p=0.86	1.28±0.88 1.15±0.42 p=0.86	4.61±1.70 5.34±0.28 p=0.49	0.46±0.15 0.66±0.14 p=0.12	0.15±0.21 0.07±0.02 p=0.61	3.93±2.69 0.70±0.21 p=**0.02**
LAP+/ LAP-	2.18±2.31 2.17±2.22 p=0.64	1.57±1.02 1.34±0.39 p=0.90	1.25±0.75 0.87±0.33 p=0.11	4.30±1.76 4.54±2.06 p=0.81	0.59±0.21 0.41±0.13 p=**0.03**	0.06±0.08 0.21±0.25 p=0.26	2.86±2.59 4.11±1.35 p=**0.01**
HSM+ /HSM-	2.30±2.38 2.63±1.55 p=0.37	1.54±0.93 1.27±0.38 p=0.88	1.29±0.70 0.74±0.13 p=**0.05**	4.48±1.28 3.86±1.86 p=0.50	0.56±0.20 0.40±0.16 p=0.12	1.07±0.18 0.11±0.11 p=0.29	3.17±2.40 3.73±1.96 p=0.26
BCR-ABL$^+$ /BCR-ABL	4.44±1.66 2.06±2.19 p=0.06	1.21±0.62 1.54±0.90 p=0.50	1.36±0.20 1.10±0.70 p=**0.05**	6.27±1.63 4.12±1.72 p=**0.05**	0.43±0.11 0.54±0.21 p=0.33	0.41±0.33 0.06±0.08 p=0.06	3.52±1.85 3.22±1.40 p=0.55
NPM1+/NPM1-	5.41±2.69 1.77±1.66 p=**0.009**	2.76±0.76 1.26±0.66 p=**0.005**	2.06±1.27 0.95±0.29 p=0.07	4.97±1.59 4.27±1.87 p=0.41	0.77±0.15 0.49±0.18 p=**0.01**	0.13±0.15 0.10±0.17 p=0.79	2.60±1.60 3.38±2.43 p=0.50

Note. Expression level of gene was expressed as fold change relative to normal bone marrow (reference). Values represent mean ± SD. P-value is calculated using Mann–Whitney test. Bold values are significant p value ≤ 0.05. Survivin and p53 genes were excluded from the table because no significant association was observed with any of the clinical features analyzed.

Correlation between drug resistance, apoptotic genes and clinical features in ALL patients

Adults: A significant correlation was observed between mRNA level of DHFR, GSTP1, MDR1, p53, Survivin and BCRP with r_s ranging from 0.57 to 0.91 (Table 6.7). Correlation with clinical variables was also calculated. A positive correlation was observed between BCL-2 and platelet count ($r_{s=}$0.50, p=0.02) whereas negative correlation was seen between WBC count and MDR1 ($r_{s=}$-0.52 p=0.01) and Hb level and MRP1 ($r_{s=}$-0.44, p=0.04) (Table 6.7). Further, significant association was found between LRP gene and female sex (p<0.0001), LRP and B-ALL patients (p=0.04) and LRP and BCR/ABL positive patients (p=0.004). Relationship with surface antigen expression revealed significant association between CD19 expression and LRP (p=0.002) and CD19 expression and BCL-2 (p=0.006) whereas expression of CD10 was associated with lower expression of MDR1 (p=0.004) and p53 (p=0.002) (Table 6.8).

Table 6.7: Correlation between expression of drug resistance and apoptotic genes among themselves and with clinical variables in adult patients with ALL

	BCL2	**DHFR**	**MDR1**	**GSTP1**	**P53**	**BCRP**	**LRP**	**MRP1**	**Survivin**
BCL2	-	.00	0.27	0.12	-0.14	-0.14	0.21	-0.20	-0.12
DHFR	.00	-	0.24	0.72**	0.83**	0.68**	-0.10	0.12	0.91**
MDR1	0.27	0.24	-	0.28	0.30	0.32	0.31	0.06	0.24
GSTP1	0.12	0.72**	0.28	-	0.77**	0.65**	0.15	0.12	0.57**
P53	-0.14	0.83**	0.30	0.77**		0.72**	0.06	0.12	0.84**
BCRP	-0.14	0.68**	0.32	0.65**	0.72**	-	0.12	0.24	0.65**
LRP	0.21	-0.10	0.31	0.15	0.06	0.12	-	0.06	-0.21
MRP1	.038	0.59	0.79	0.57	0.59	0.29	0.78	-	0.41
Survivin	-0.12	0.91**	0.24	0.57**	0.84**	0.65**	-0.21	0.18	-
Hb	0.14	0.02	0.23	0.0	-0.01	-0.27	0.08	-0.44*	-0.04
Plt	0.50*	-0.29	-0.08	-0.15	-0.41	-0.32	-0.13	-0.29	-0.26
WBC	-0.17	-0.30	-0.52*	-0.14	-0.22	-0.20	.00	0.09	0.33

Note. Correlation was tested with Spearman's rank correlation test. Values in the table represent spearman's correlation coefficient (r). * Correlation is significant at p= 0.05 level, ** Correlation is significant at p= 0.01 level.

Table 6.8: Association between expression of the drug resistance and apoptotic genes with clinical features in adult patients with ALL

	MDR1	LRP	BCL2	P53	Survivin
Male/Female	0.81±1.06	0.43 ± 0.49	3.42±3.48	1.82±1.78	0.41±0.34
	1.34±1.27	1.98 ± 0 .69	2.64±0.92	1.35±0.36	0.28±0.14
	p=0.08	**p=0.001**	p=0.80	p=0.57	p=0.57
B-ALL/T- ALL	0.70±0.91	1.13 ± 0.90	2.93±1.59	1.33±0.50	0.32±0.21
	1.19±1.46	0.17 ± 0.09	3.83±4.93	2.49±2.53	0.51±0.42
	p=0.74	**p<0.0001**	p=0.12	p=0.57	p=0.33
CD7 +/CD7 -	1.07±1.35	0.56±0.95	3.64 ± 4.98	2.54±2.49	0.49±0.39
	0.75±1.01	0.94±0.81	2.86 ± 1.64	1.30±0.53	0.33±0.25
	p=0.87	**p=0.04**	p=0.1	p=0.39	p=0.30
CD10+/CD10-	0.38 ± 0.35	0.93±0.81	2.78±1.80	1.07 ± 0.46	0.30±0.27
	1.57± 1.48	0.65±0.94	3.89±4.30	2.65± 2.10	0.54±0.35
	p=0.004	p=0.40	p=0.82	**p=0.002**	p=0.1
CD19+/CD19-	0.93±1.23	1.06 ± 0.90	3.61± 2.97	1.35±0.50	0.36±0.25
	0.66±0.77	0.18 ± 0.09	2.20 ± 3.18	2.62±2.74	0.43±0.42
	p=0.42	**p=0.002**	**p=0.006**	p=0.91	p=0.96
HLA-DR+/	1.07±1.40	1.02±1.01	3.97 ± 3.18	1.38±0.34	0.35±0.23
HLA-DR -	0.43±0.45	0.55±0.23	1.79 ± 2.06	2.70±2.43	0.55±0.37
	p=0.12	p=0.09	**p=0.003**	p=0.42	p=0.11
BCR-ABL$^+$/ BCR-ABL$^-$	0.57±0.46	1.23± 0.72 0.66	2.54±0.90	1.39±0.69	0.31±0.30
	0.96±1.28	± 0.87 **p=0.004**	3.48±3.53	1.82±1.78	0.40±0.31
	p=0.73		p=0.64	p=0.62	p=0.21

Note. Expression level of gene was expressed as fold change relative to normal bone marrow (reference). Values represent mean ± SD. P-value is calculated using Mann–Whitney test. Bold values are significant p value ≤ 0.05. MRP1, BCRP, DHFR, and GSTP1 genes were excluded from the table because no significant association was observed with any of the clinical features analyzed.

Children: In ALL children, significant correlation was also observed for BCL-2 with MDR1 (r_s=0.58, p<0.001), GSTP1 (r_s=0.44, p=0.008), p53 (r_s=0.45, p=0.006), LRP (r_s=0.54, p=0.001) and GSTP1 with DHFR (r_s=0.69, p<0.001) and LRP (r_s=0.43, p=0.009). A negative correlation was also observed between MRP1 and MDR1 ((r_s=0.35, p=0.03). The expression levels of the resistance genes were not significantly associated with the age of the patients except that of Survivin (r_s=0.34, p=0.04). DHFR and Survivin expression showed positive correlation with WBC count while BCL-2 and MDR1 showed negative association with WBC count. A negative

correlation was also found for peripheral blast percentage with BCL-2 (r_s=0.51, p=0.002) and LRP (r_s=0.45, p=0.008). However, MRP1 expression positively correlated with the expression blast percentage (r_s=0.42, p=0.01) (Table 6.9). No significant association was observed between gene expression level and clinical variables such as sex, FAB, HSM and with B and T ALL. However, association with surface antigen expression analysis shows significant association of CD34 marker with the higher expression of BCL-2 (p=0.05) and lower expression of DHFR (p=0.03). A significant association was also seen between CD3 and DHFR gene expression (p=0.007). Further, expression of MDR1 and Survivin was lower in BCR/ABL positive cases compared to BCR/ABL negative cases (p=0.05 and p=0.05) (Table 6.10).

Table 6.9: Correlation between expression of drug resistance and apoptotic genes among themselves and with clinical variables in children with ALL

	BCL2	**DHFR**	**MDR1**	**GSTP1**	**P53**	**BCRP**	**LRP**	**MRP1**	**Survivin**
BCL2	-	0.01	0.58**	0.44**	0.45**	-0.25	0.54**	-0.15	-0.10
DHFR	0.012	-	-0.19	0.69**	0.29	0.15	-0.02	0.19	0.73**
MDR1	0.58**	-0.19	-	0.08	0.08	-0.07	-0.09	-0.35*	0.003
GSTP1	0.44**	0.69**	-0.08	-	0.29	-0.09	0.43**	0.13	0.31
P53	0.45**	0.29	0.08	0.29	-	0.02	0.30	0.27	0.35*
BCRP	-0.25	0.15	-0.07	-0.09	0.02	-	-0.08	0.17	0.07
LRP	0.54**	-0.02	-0.09	0.43	0.30	-0.08	-	0.32	-0.22
MRP1	-0.15	0.19	-0.35*	0.13	0.27	0.17	0.32	-	0.23
Survivin	-0.10	0.73**	0.003	0.31	0.35*	0.07	-0.22	0.23	-
Age	-0.31	0.14	-0.12	-0.15	-0.07	0.14	-0.27	0.08	0.34*
Hb	-0.17	-0.05	-0.29	-0.08	0.45**	-0.18	-0.30	-0.05	-0.03
Plt	-0.31	-0.19	-0.24	-0.27	-0.18	0.11	0.003	0.43**	-0.17
WBC	-0.35*	0.52**	-0.43**	0.16	-0.01	-0.09	-0.22	0.13	0.37*
Blast	-0.51**	0.07	-0.09	-0.27	-0.14	0.33	-0.45**	0.42*	0.26

Note. Correlation was tested with Spearman's rank correlation test. Values in the table represent spearman's correlation coefficient (r). * Correlation is significant at p=0.05 level, ** Correlation is significant at p= 0.01 level.

Table 6.10: Association between expression of the drug resistance and apoptotic genes with clinical features in children with ALL

	MDR1	LRP	BCL2	P53	Survivin	DHFR	MRP1
CD3+/ CD3-	0.79±0.15 0.99±0.88 p=0.79	1.48±0.73 0.78±0.57 p=0.06	4.70±3.78 3.16±2.30 p=0.35	1.43±0.35 1.38±0.60 p=0.79	0.20±0.04 0.26±0.20 p=0.77	1.71±0.42 0.82±0.80 **p=0.007**	1.84±0.39 1.34±1.10 p=0.08
CD34+/ CD34-	1.19±1.02 0.73±0.49 p=0.26	1.02±0.54 0.69±0.67 p=0.12	4.09±2.47 2.54±2.31 **p=0.05**	1.42±0.61 1.34±0.54 p=0.59	0.21±0.10 0.31±0.25 p=0.32	0.64±0.31 1.21±1.07 **p=0.03**	1.27±0.74 1.54±1.15 p=0.66
Peroxidase stain	0.82±0.55 0.68±0.63 p=0.9	0.91±0.61 0.76±0.73 p=0.4	2.94±2.2 3.65±2.81 p=0.5	1.27±0.54 1.84±0.60 **p=0.031**	0.21±0.14 0.41±0.31 **p=0.043**	0.80±0.50 1.48±1.49 p=0.18	1.44±1.08 1.41±0.48 p=0.79
HLADR+/ HLA-DR-	1.14±0.87 0.64±0.68 p=0.39	0.84±0.61 0.07±0.06 **p=0.005**	3.91±2.61 1.03±0.51 p=0.06	1.42±1.56 0.84±0.93 p=0.17	0.25±0.14 0.39±0.59 p=0.39	0.84±0.50 1.71±2.62 p=0.43	1.04±0.44 1.27±1.06 p=0.94
LAP+/ LAP-	1.01±0.92 0.91±0.70 p=0.89	0.94±0.69 0.74±0.50 p=0.38	3.61±2.55 2.92±2.43 p=0.29	1.43±0.56 1.32±0.61 p=0.31	0.21±0.10 0.32±0.27 p=0.42	0.86±0.69 1.01±1.09 p=0.54	1.70±1.04 0.94±0.59 **p=0.005**
$BCRABL^{+}/BCRABL^{-}$	0.43±0.39 1.06±0.85 **p=0.05**	0.63±0.69 0.90±0.61 p=0.34	1.72±1.23 3.61±2.55 p=0.10	1.01±1.43 1.44±0.58 p=0.06	0.13±0.06 0.27±0.20 **p=0.05**	0.55±0.31 0.98±0.86 p=0.17	1.10±0.60 1.45±1.00 p=0.60

Note. Expression level of gene was expressed as fold change relative to normal bone marrow (reference). Values represent mean ± SD. P-value is calculated using Mann–Whitney test. Bold values are significant p value < 0.05. BCRP and GSTP1 genes were excluded from the table because no significant association was observed with any of the clinical features analyzed.

Correlation between drug resistance, apoptotic genes and outcome of Induction chemotherapy

To assess the clinical relevance of the studied genes, expression levels were correlated with response to induction chemotherapy. Clinical and follow up data were available for 60 adult (30 AML and 30 ALL) and 44 children (17AML and 27ALL) after the completion of induction chemotherapy.

Gene expression and correlation with induction chemotherapy in AML

Adults: In Adults, complete response (CR) was achieved in 53% (16/30) of patients while incomplete response was observed in 47% (14/30) of patients. The expression of MDR1 (3.56 ± 2.23) and BCL-2 (3.43 ± 1.77) was significantly higher in patients who did not responded to induction chemotherapy compared to those who did (1.71 ± 3.09, p=0.001 and 2.19 ± 1.06, p=0.02) (Figure 6.2). However, no significant difference in the expression of DHFR, GSTP1, p53, BCRP, LRP, MRP1, and Survivin was found in AML patients (Table 6.11).

Children: In children CR was achieved in 12 patients while incomplete response was observed in 5 patients. We found no significant difference in the expression of drug resistance and apoptotic genes expression between CR and NR group (Table 6.11)

Table 6.11: Relationship between gene expression at diagnosis and response to induction chemotherapy in AML patients

Genes	**Gene Expression level in AML (Mean ± SD)**					
	Adults(30)		**p-value**	**Children(17)**		**p-value**
	CR (16)	NR (14)		CR (12)	NR (5)	
BCL-2	2.19 ± 1.06	3.43 ± 1.77	**0.02***	3.98±2.03	5.21±1.70	0.24
DHFR	0.49 ± 0.28	0.58 ± 0.26	0.38	0.54±0.26	0.55±0.12	0.91
MDR1	1.71 ± 3.09	3.56 ± 2.23	**0.001***	2.35±2.03	2.15±0.51	1.0
GSTP1	1.59 ± 1.05	1.67 ± 1.37	0.77	1.79±1.10	0.92±0.13	0.09
P53	1.04 ± 0.39	0.93 ± 0.51	0.24	1.12±0.40	1.23±0.20	0.46
BCRP	0.06 ± 0.12	0.07 ± 0.08	0.53	0.13±0.22	0.02±0.02	0.24
LRP	2.31 ± 4.30	0.79 ± 0.65	0.40	1.03±0.27	1.04±0.24	0.75
MRP1	3.21 ± 4.05	1.77 ± 1.55	0.58	2.25±1.42	1.78±0.52	0.75
Survivin	0.13 ± 0.07	0.15 ± 0.09	0.45	0.19±0.16	0.27±0.13	0.24

Note. Expression level of gene was expressed as fold change relative to normal bone marrow (reference). CR: complete remission, NR: Non-responder after induction chemotherapy. Values represent mean ± SD. P-value is calculated using Mann–Whitney test. *****Bold values are significant p value ≤ 0.05.

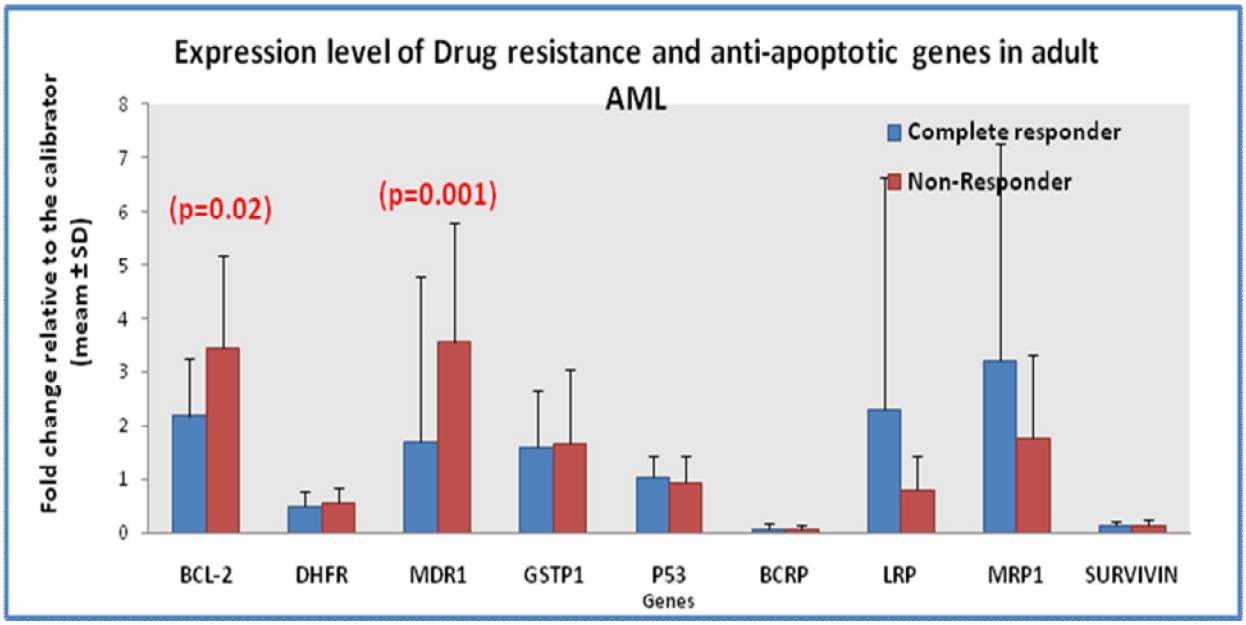


Figure 6.2: Expression level of drug resistance and apoptosis genes in adult AML patients who achieved remission (CR) and who did not achieve remission after receiving full induction therapy (NR). Expression level of gene was expressed as fold change relative to normal bone marrow.

Gene expression and correlation with induction chemotherapy in ALL

Adults: The clinical response to induction chemotherapy was evaluated in 30 patients of ALL. On completion of induction chemotherapy, 18(60%) out of 30 patients achieved CR. The expression of only MRP1 gene was found to be significantly higher in patients who did not responded to induction chemotherapy compare to those who did (p=0.007) (Figure 6.3).

Table 6.12: Relationship between gene expression at diagnosis and response to induction chemotherapy in ALL patients

Genes	Gene Expression level in ALL (Mean ± SD)					
	Adults(30)			Children(27)		
	CR(18)	NR(12)	p-value	CR(22)	NR(5)	p-value
BCL-2	3.38 ± 2.73	2.00 ± 1.35	0.30	2.82±2.26	4.09±2.24	0.22
DHFR	1.14 ± 1.49	1.28 ± 1.47	0.93	0.91±0.53	0.64±0.48	0.26
MDR1	0.70 ± 0 .66	0.26 ± 0.14	0.14	0.83±0.60	0.79±0.38	0.70
GSTP1	1.31 ± 1.23	1.14 ± 1.22	0.49	1.31±1.53	0.83±0.57	0.79
P53	1.87 ± 1.63	1.73 ± 2.06	0.30	1.32±1.48	1.54±0.91	0.49
BCRP	0.12 ± 0.10	0.12 ± 0.09	0.93	0.29±0.48	0.03±0.009	**0.025***
LRP	0.56 ± 0.56	0.66 ± 0.69	0.86	0.93±0.63	1.02±0.71	0.75
MRP1	1.48 ± 0.90	2.84 ± 1.39	**0.007***	1.57±1.10	1.11±0.50	0.61
Survivin	0.40 ± 0.30	0.33 ± 0.34	0.55	0.24±0.14	0.19±0.11	0.53

Note. Expression level of gene was expressed as fold change relative to normal bone marrow (reference). CR: complete remission, NR: Non-responder after induction chemotherapy. P-value is calculated using Mann–Whitney test. *Bold values are significant p value ≤0.05.

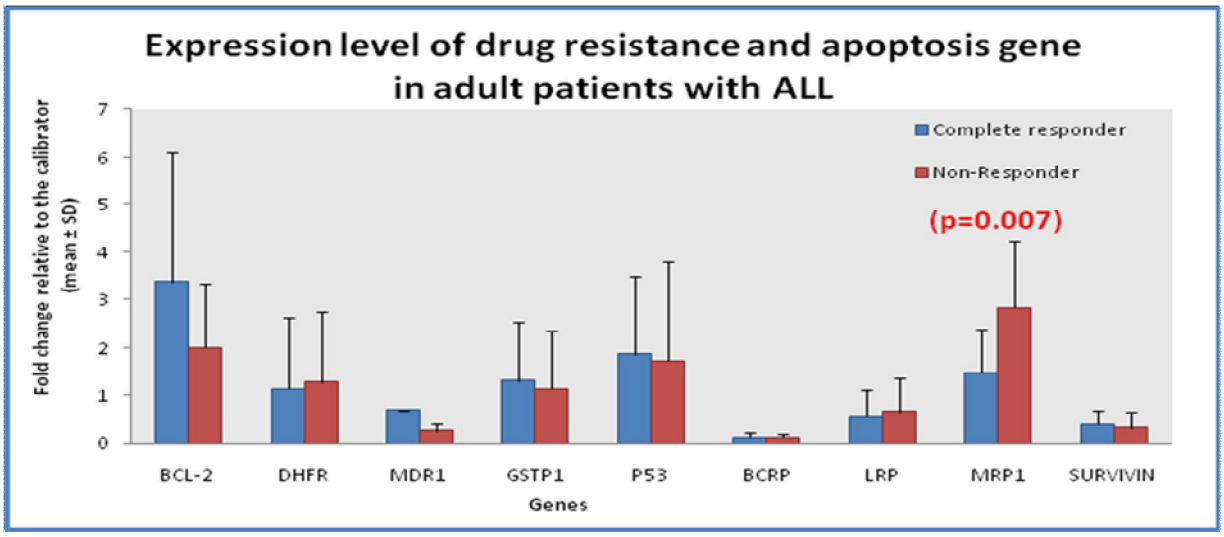


Figure 6.3: Expression level of drug resistance and apoptosis genes in adult ALL patients who achieved remission after induction therapy (CR) and who did not achieve remission after receiving full induction therapy (NR). Expression level of gene was expressed as fold change relative to normal bone marrow.

Children: In children, twenty seven patients received standard induction chemotherapy. On completion of induction chemotherapy, 22 (81%) out of 30 patients achieved CR. The expression of only BCRP gene was significantly different between CR and NR group. BCRP expression was higher in patients who responded to induction chemotherapy than those who did not (0.29 ± 0.48 vs 0.03 ± 0.009, p=0.025). However, no significant association was found between response to induction chemotherapy and expression of other genes such as BCL-2, DHFR, MDR1, GSTP1, p53, LRP, MRP1, and Survivin (Figure 6.4).

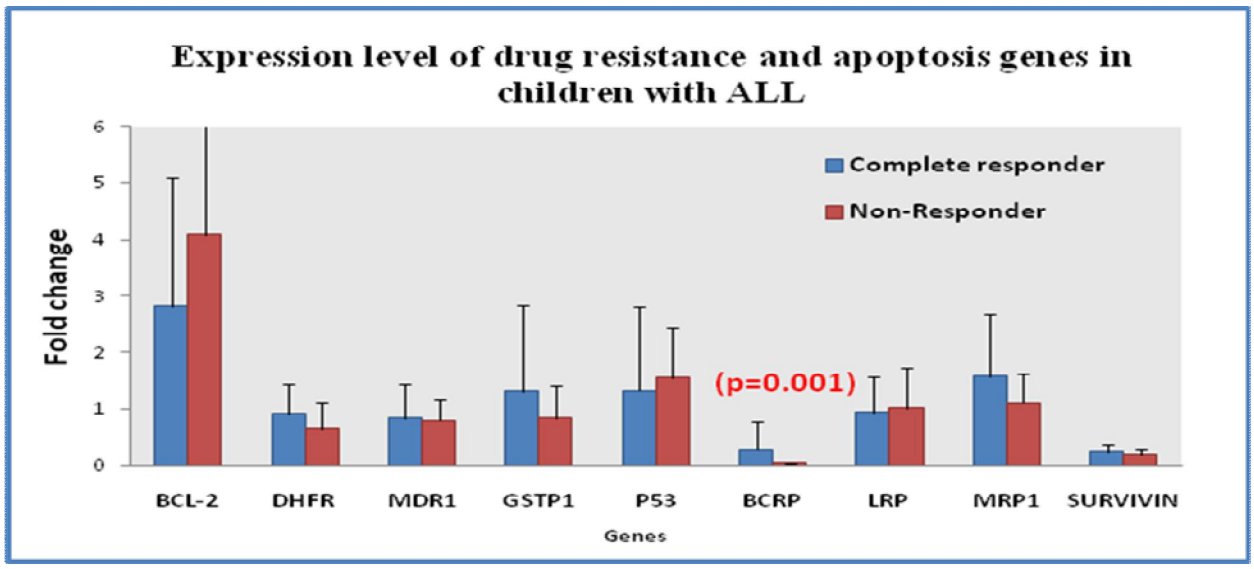


Figure 6.4: Expression level of drug resistance and apoptosis genes in ALL children who achieved remission after induction therapy (CR) and who did not achieve remission after receiving full induction therapy (NR)

Discussion

Drug resistance is a major obstacle and an important mechanism to explain the failure of chemotherapy in patients with leukemia. Deregulation of genes associated with drug resistance and with the apoptosis pathway are the most widely characterized drug resistance mechanisms in leukemia. Analysis of the correlation of expression levels among the genes under study by the Spearman correlation coefficient showed a significant positive correlation among all of them, suggesting their joint activation in both childhood and adult acute leukemia. The pattern of correlation of the expression of drug resistance and apoptotic genes in ALL was quite distinct and more heterogeneous as compared to AML.

Expression of drug resistance and apoptosis genes in patients with AML

One of the major obstacles to successful treatment is the drug resistance of leukemic cells to chemotherapeutic agents. An accepted mechanism of drug resistance is reduced intracellular accumulation and altered metabolism of cytotoxic drugs. In many instances this is mediated by increased expression of the MDR1 gene product: pgp acts as a molecular pump that extrudes out a wide variety of drugs [van den *et al.,* 2000]. This lowers the intracellular drug concentration at the target and results in drug resistance. MDR1 has been the most extensively studied drug resistance gene in acute leukemia. However, its role as a prognostic factor is still controversial. Over expression of MDR1 protein in AML has been associated with poor response to chemotherapy and reduced patient's survival [Izquierdo *et al.,* 1996; Wuchter *et al.,* 1999; Benderra *et al*., 2005]. However, other studies have failed to confirm this association [Tsimberidou *et al*., 2002]. In the current study, the expression of MDR1 gene at the time of diagnosis was significantly higher in non responder as compared to responder in adult patients only. Furthermore, a significant association of MDR1 expression with the adverse prognostic marker, CD34, was found. It has been suggested earlier that overexpression of MDR1 in CD34 positive samples may be the reason for the adverse prognosis of this marker in AML [Benderra *et al.,* 2005; van den Heuvel-Eibrink *et al*., 2007].

Interestingly, we also observed an association of FLT3 gene mutation with low expression of MDR1 and but with higher p53 and GSTP1 expression. Both FLT3 mutations and MDR1 expression have independently been associated with worse survival outcome in AML but no significant correlation has been reported between them [Galimberti *et al.,* 2002]. This seems to be even more interesting as patients with FLT3 mutations were less frequently MDR1 positive, possibly because of a loss of the MDR1 phenotype under increased proliferative capacity, as reported by Smeets et al (1999). In contrast to the prognostic value of MDR1 expression in AML, other drug resistance genes included in the current study such as MRP1, LRP, BCRP and GSTP1 expression showed no significant difference in responder and non responder AML patients. Recent literature on expression of MRP1, LRP, and BCRP mRNA at diagnosis has also found no significant association of these genes with response rates. However, they were found to be unfavorable prognostic factor for overall survival [van den Heuvel-Eibrink *et al.,* 2007; Uggla *et al.,* 2005].

Regarding the expression of apoptotic genes, AML patients who did not respond to induction chemotherapy showed higher expression of BCL-2 compared to responders. Earlier reports have shown that constitutive BCL-2 expression in adult AML correlates with response to induction chemotherapy [Karakas *et al.,* 1998; Wuchter *et al.,* 1999]. AML patients with high BCL-2 mRNA expression at diagnosis achieved lower response rates as compared to those with no or low BCL-2 expression in these studies. As a potent inhibitor of apoptosis, BCL-2 has a proven oncogenic potential and is able to protect leukemic blasts from apoptosis induced by chemotherapy.

Expression of drug resistance and apoptosis genes in patients with ALL

The only drug resistance gene that was significantly different between responder and non responder adult patients with ALL was MRP1. However to our knowledge, only few studies have addressed the role of MRP1 in adult ALL [Tsimberidou *et al.,* 2002; Tafuri *et al.,* 2002; Plasschaert et al., 2003]. MRP1 expression has been reported to be higher in adult ALL as compared to childhood ALL. However, an association with clinical outcome was found only in

children [Plasschaert *et al.,* 2005]. Moreover MRP1 expression was found to be higher in T-ALL as compared to B-ALL in the current study though it was not significant. Expression of MRP1 protein is reported to be higher in normal T cells as compared to B-cells, so it is possible that over expression in our study may be lineage specific and not related to drug resistance [Legrand *et al.,* 1996]. MRP1, unlike MDR1, transports negatively charged natural-product drugs and drugs that have been modified by glutathione, conjugation, glucosylation, sulfation, and glucuronylation. The clinical impact of MRP1 expression in ALL is also controversial, with some reporting it to be involved in drug resistance while others have found it to be lacking prognostic significance [Zhou *et al.,* 1995; van der Kolk *et al.,* 2001].

Another intriguing finding in adult AL was the significant difference in the expression level of DHFR in AML and ALL patients. DHFR gene was significantly higher in ALL as compared to AML patients. Mouse, hamster and human methotrexate-resistant cell lines have been described with increased levels of DHFR activity due to gene amplification. MTX acts as a tight-binding inhibitor of DHFR and is the most widely used antimetabolite in pediatric malignancies, being especially effective in the treatment of ALL. It is now well established that an important mechanism of resistance of cells to MTX is an increase in DHFR activity due to amplification of the DHFR gene [Goker et al., 1995].

In childhood ALL patients, the drug resistance gene that was significantly different between responder and non responder patients was BCRP. Children who did not respond to induction chemotherapy showed lower expression of BCRP compared to responders. However, in literature conflicting results have been reported about the BCRP. Sauerbrey *et al.,* [2002] in a study of 67 children, reported lower expression of BCRP with no clinical significance. In another study significant association was observed between higher expression level of BCRP and the presence of complete clinical response [Cortez *et al.,* 2009]. BCRP gene is expressed in childhood ALL but with wide variation and contradicting results on childhood ALL. BCRP is more biologically complex than Pgp and MRP-1. BCRP is an ABC half-transporter that requires

multimerization for function [Maliepaard et al, 2001a]. In normal hematopoietic cells, BCRP is expressed in pluripotent stem cells and sharply down-regulated at the point of commitment to lineage-specific development [Zhou *et al.,* 2001; Scharenberg *et al.,* 2002]. However, in the present study we did not observe any association between immature marker, CD34 and BCRP expression neither in adults nor in children with ALL.

Despite profound research, the clinical importance of drug resistance related genes in AL remains controversial. The comparison of data is hampered by the lack in standardized detection techniques, the heterogeneity in patient groups (e.g. pooled data of ALL and AML, initial and relapse samples, adults and children) and differences in treatment protocols. In addition, differences in the methods and design of each study, the characteristics of the patients studied the use of different levels of gene expression as cut-off points and housekeeping genes and especially the small number of samples evaluated may also be responsible for these controversial results in the literatures. Detailed information on the clinical relevance of MDR-related efflux pumps is needed before the potential of transporter-specific modulators can be studied.

Chapter 7
Role of Xenobiotic metabolizing (Drug metabolizing) genes and p53 codon 72 polymorphism in acute leukemia

Chapter 7

Role of Xenobiotic metabolizing (Drug metabolizing) genes and p53 codon 72 polymorphism in acute leukemia

Introduction

Acute leukemia (AL) is a multifactorial disease resulting from complex interactions between genetic and environmental factors. Possible risk factor includes exposure to carcinogens present in the environment [Preston *et al.,* 1994; Nordlinder and Jarvholm, 1997]. The etiology of AL appears to be multifactorial and the exposure to benzene, ionizing radiation, and cytotoxic therapy has been implicated as the risk factors in the pathogenesis of AL. Every individual is considered to be exposed to benzene either from vehicle exhaust emission or from active and passive smoking [Groves *et al.*, 1994; Infante-Rivard *et al.,* 1999]. Exposure to pesticides and alcohol are other environmental risk factors that are found to be inadequately and inconsistently associated with the development of AL. Carcinogens present in these environmental factors may cause DNA damage at the level of hematopoietic progenitors, which is an essential prerequisite for the development of AL.

Xenobiotic metabolic enzymes constitute the primary defense against these exposures. Genetic polymorphism in xenobiotic metabolizing enzymes seems to play an important role in determining individual susceptibility to these carcinogens. Primary candidates for gene-environment interaction study in the carcinogenesis are enzymes that are involved in the activation and detoxification of xenobiotic compounds. Genetic polymorphisms have been identified in many of these enzymes and these may be responsible for variation in the enzymatic activity thereby leading to interindividual and interethnic variation in the metabolism of carcinogens [Taningher *et al.,* 1999]. Environmental procarcinogens are activated by phase I enzymes [cytochrome P-450s (CYPs), alcohol dehydrogenase (ALDH) and epoxide hydroxylase (EPHX)] converting them into reactive intermediates which are than detoxified by phase II enzymes [glutathione S-transferases (GST), sulfotransferase (SULT) and N-acetyltransferase (NAT)] [Hein 2002; Nazar-Stewart *et al.,* 2003; Sparks *et al.,* 2004].

Genetic polymorphism in the critical cell-cycle checkpoint control genes may also predispose to cancer. The polymorphism, related to changes in the function of the p53 protein is strongly associated with increased risk of developing certain tumors [Wang *et al.,* 1999; Ignaszak-Szczepaniak *et al.,* 2006; Damin *et al.,* 2006]**.** Benzopyerene diol epoxide, an intermediate product of polycyclic aromatic hydrocarbon present in the automobile exhaust and tobacco smoke is able to inactivate the p53 oncogene by forming benzopyrene diol epoxide-DNA adducts which are associated with decrease repair capability and reduced apoptotic potential [Dong *et al.,* 2004]. Further, p53 mutations have been associated with reduced repair and enhanced cytotoxicity in cell damaged by benzo(a)pyrene diol epoxide-DNA adducts [Wani *et al.,* 2000]. The coordinated expression and regulation of phase I and II enzymes determines the outcome of the carcinogen exposure. In addition interaction of polymorphisms in p53 gene with various phase I and phase II enzymes has also been associated with increased risk of carcinogenesis [Miller *et al.,* 2002; Quinones *et al.,* 2006].

In our earlier pilot study on adult AML, we did not find any association of GSTs, p53 and EPHX1 polymorphism with AML [Chauhan et al., 2011]. The effect of a possible interaction between p53 and GSTs, EPHX1 and GSTs, EPHX1and p53, was also investigated further in combined analysis. However, the two loci gene-gene interaction did not impart any statistical significant risk for adult AML. These finding suggests that etiology of adult AML cannot be explained by polymorphism at single locus perhaps due to complexity involved in the metabolism of diverse xenobiotic compounds. Therefore for a complex disease like leukemia, single gene association studies provide limited information compared to multigenic approaches in predicting risk. Moreover, the risk conferred by a single gene may not be substantial due to its low-penetrance and heterogeneous nature of cancer. High order gene-gene interaction in multigenic approach allows more precise delineation of the risk groups. Two of the data mining approaches used in the literatures lately include classification and regression trees (CART) and multifactor dimensionality reduction (MDR). Both these analytical approaches are model free and non-parametric methods of estimating non-linear interactions with low false-positives for two or more loci even on relatively small sample size [Ritchie *et al.,* 2003]. In a study on sporadic breast cancer, MDR identified high order interaction in three different estrogen-

metabolizing genes COMT, CYP1B1, and CYP1A1 in the absence of any independent main effect [Ritchie *et al.,* 2001]. Similarly in ever smokers, the combination of CCNH, ERCC6 and RAD23B identified by CART analysis was associated with the highest bladder cancer risk, although individually these genes were associated with modest risk [Chen *et al.,* 2007]. These studies indicate epistasis effects of the genes in modifying cancer risk even in absence of their main effects. Chan *et al.,* [2010] CART analysis on a cohort of pediatric patients with ALL, revealed significant interactions between xenobiotic and folate pathway genes that reflect the complex nature of leukaemogenesis. However, most studies on single gene approach of xenobiotic metabolism in ALL and AML have reported inconsistent results.

The aim of this objective were to

- To examine the association of eight polymorphisms in six xenobiotic/drug metabolizing genes (GSTM1, GSTT1, GSTP1, CYP1A1, EPHX1, and SULT1A1) and p53 codon 72 gene polymorphism with acute leukemia risk.
- To explore the high order gene-gene interactions of the above genes in modulating the acute leukemia risk using classification and regression trees (CART) and multifactor dimensionality reduction (MDR) analysis method.

Experimental methods

Materials

Agarose, Tris base, EDTA, NaCl, SDS, Triton X-100 and other fine chemicals were purchased from Sigma Chemicals, USA. *Taq* polymerase, dNTPs, MgCl2, was obtained from Invitrogen and MBI fermentas USA. Oligos were synthesized by Microsynth, Switzerland. RNA later, DNA and RNA extraction kit were purchased from Qiagen Sciences, USA and Himedia, India.

Chemicals used

LYSIS BUFFER I: 30mm Tris-Hcl (Ph-8), 5mm EDTA, 50 Mm Nacl; LYSIS BUFFER II: 75mm Nacl, 2mm EDTA (Ph-8); SDS STOCK: 20 gm of SDS dissolved in 80 ml of TDW at 65^0C. Make up volume up to 100 ml ; PROTEINASE K: 10 mg dissolved in 1 ml of TDW:1% ; AGAROSE: 1gm of agarose dissolved 1% TAE buffer.

Selection criteria of patients: As described in chapter 4

Inclusion criteria: As described in chapter 4

Exclusion criteria: As described in chapter 4

Patients' details: The study was conducted in samples of 230 patients (159 males, 71 females) of AL diagnosed between 2005 and 2009 at the Department of Hematology, Safdarjung Hospital, New Delhi. Peripheral blood samples from 199 (134 males, 65 females) healthy volunteers were used as controls. The diagnosis of acute leukemia was made on routinely stained bone marrow aspiration/biopsies and peripheral blood smears and evaluated according to the French-American-British (FAB) criteria. All in vitro procedures were performed according to protocols approved by the Ethical Committee of the Institute and Safdarjung Hospital. Clinical data, including age, gender, whole blood cell count, hemoglobin, platelets count and presence of organomegaly were collected at the time of diagnosis.

Collection of blood samples: As described in chapter 4

DNA extraction: As described in chapter-5

Genotyping protocol:

Genotyping of GSTM1 and GSTT1 by Multiplex PCR

A multiplex PCR method was used to detect the presence or absence of the GSTM1 and GSTT1 genes in the genomic DNA samples (Table 7.1). This method had both GST primers sets in the same PCR reaction and included a third primer set for β-globin as internal control to ensure proper functioning of PCR. The PCR was carried out for an initial activation step at 94^0C for 4 min, 20 cycles of denaturation at 93^0C for 1 min; annealing at 60^0C for 1 min; 72^0C for 1 min and in addition with these there were 15 cycles of denaturation at 93^0C for 1 min; annealing at 50^0C for 1 min; 72^0C for 1 min and a final extension at 72^0C for 10 min. The PCR products were electrophoresed in 2.5% agarose gels containing ethidium bromide, prepared and run in 0.5X TBE buffer. The absence of 459 bp band indicates GSTT1 null and the absence of 219bp indicates GSTM1 null genotypes (Figure 7.1).

Table 7.1: Multiplex PCR for genotyping of GSTM1 and GSTT1 polymorphism

Components	Master Stock	Working Stock	Reaction l (25µl)
Nuclease-free water (25 µl)	X		15.3
PCR buffer	10X	1X	2.5
$MgCl_2$	25 mM	1.0 mM	1.0
dNTP mix (2.5 mM each)	25 mM	0.2 mM	0.2
GSTM1 (Forward primer)	10 µM	0.25 µM	0.625
GSTM1 (Reverse primer)	10 µM	0.25 µm	0.625
GSTT1 (Forward primer)	10 µM	0.25 µM	0.625
GSTT1 (Reverse primer)	10 µM	0.25 µm	0.625
β-Globin (Forward primer)	10 µM	0.25 µM	0.625
β-Globin (Reverse primer)	10 µM	0.25 µm	0.625
Taq DNA polymerase	5 U/µl	1.25U	0.25
Template (DNA)	Y	100 to 300 ng	2.0

Genotyping of GSTP1, EPHX1, CYP1A1 and SULT1A1

Polymorphism in GSTP1, EPHX1 Exon3 and Exon4, CYP1A12A, CYP1A12C and SULT1A1 were genotyped using PCR-RFLP (Polymerase chain reaction-Restriction Fragment length polymorphism) method. Standard PCR were performed on PTC-200 (MJ Research, USA). The PCR reaction were performed in a volume of 25µl with a final concentration of 1X PCR Buffer (MBI Fermentas), 1.5mM $Mgcl_2$, 200 µM dNTPs, 2.0 mM , 1 Unit of Taq DNA polymerase and 100-300 ng of DNA Template. Negative controls were included in all PCR-runs to prevent misjudging following contamination of samples. PCR amplification consist of 35 cycles of denaturation at 94^0C for 45s; annealing at depend on gene for 45s; 72^0C for 45s followed by a final extension at 72^0C for 10 min. PCR products were loaded on 2.5% agarose gel and subjected to gel electrophoresis in 0.5X TBE buffer, stained with ethidium bromide and visualized under UV. Detail of single-nucleotide polymorphisms (SNPs) selected for the study is summarized in Table 7.2. Sequence of the primer and their annealing temperatures are given in the Table 7.3.

Table 7.2: Detail of the single-nucleotide polymorphisms (SNPs) selected for the study

Gene	Chr[a]	SNP	Loc	Polymorphism	
				Nucleotide	Codon
GSTT1	22q11.23	GSTT1	Gene	Presence>Null	Deletion
GSTM1	1p13.3	GSTM1	Gene	Presence>Null	Deletion
GSTP1	11q13	rs1695	Exon 5	313A>G	Ile105Val
P53	17p13.1	rs1042522	Exon4	215C>G	Arg72Pro
EPHX1	1q42.1	rs2234922	Exon 4	418A>G	His139Arg
EPHX1	1q42.1	rs1051740	Exon 3	339T>C	Tyr113His
CYP1A1	15q24.1	rs4646903[c]	3'UTR	6235T>C	NA[b]
CYP1A1	15q24.1	rs1048943[d]	Exon 7	2454A>G	Ile462Val
SULT1A1	16p12.1	rs9282861	Exon 7	638G>A	Arg213His

[a]Chromosomal position is based on NCBI Build.
[b]Not Applicable

Table7.3: Sequence of primers used in the study

Gene	**Primer sequence**	**T^0C**	**PCR (bp)**
GSTT1	5'-TTCCTTACTGGTCCTCACATCTC-3' 5'-TCACCGGATCATGGCCAGCA-3'	-	459
GSTM1	5'-GAACTCCCTGAAAAGCTAAAGC-3' 5'- GTTGGGCTCAAATATACGGTGG-3'	-	219
GSTP1	5'-CCAGTGACTGTGTGTTGATC-3' 5'-CAACCCTGGTGCAGATGCTC-3'	62	189
P53	5'-TTGCCGTCCCAAGCAATGGATGA-3' 5'-TCTGGGAAGGGACAGAAGATGAC-3'	60	199
EPHX1 Exon 4	5'-ACATCCACTTCATCCACGT-3' 5'-ATGCCTCTGAGAAGCCAT-3'	56	210
EPHX1 Exon 3	5'-GATCGATAAGTTCCGTTTCACC-3' 5'-ATCCTTAGTCTTGAAGTGAGGAT-3'	52	162
CYP1A12A	5'-TAGGAGTCTTGTCTCATGCCT-3' 5'-CAGTGAAGAGGTGTAGCCGCT-3'	61	340
CYP1A12C	5'- GAAAGGCTGGGTCCACCCTCT -3' 5'-CCAGGAAGAGAAAGACCTCCCAGCGGGCCA-3'	63	333
SULT1A1	5'-AGTTGGCTCTGCAGGGTTTCT-3' 5'-ACCACGAAGTCCACGGTCTC-3'	59	200

RFLP analysis of GSTP1, EPHX1 Exon3, EPHX1 Exon4, CYP1A12A, CYP1A12C and SULT1A1 polymorphism

Restriction digestion of the amplified fragments was carried out for the above polymorphism in a water bath (Table 7.4). Heat inactivation of enzyme was done at 80^0C for 20 minutes after completion of incubation with enzyme. Restriction enzymes that cleave the DNA specifically for different alleles were used (Table 7.5) and the alleles of each specific sample can be observed as a specific band pattern on the gel (Figure 7.2-7.8). The genotyping results were confirmed by repeated analysis of approximately 10% of all samples randomly chosen.

Table 7.4: Standard protocol used for the RFLP experiment

COMPONENTS	STOCK CONC.	WORKING CONC.	1 REACTION (µl)
Water			3
Buffer	10X	1	1.5
Enzyme*	10Units/µl	5Units	0.5
PCR product			10.0
* Enzymes are specific for each polymorphism given in the table 7.5			

Table 7.5: Detail of the RFLP enzymes used for each polymorphism

Gene	Enzyme	Site	Incubation Condition	PCR	RFLP product (bp)	
					Homo wild	Homo variant
GSTP1	BsmA1	5'-GTCTC^-3' 3'-CAGAG^-5'	55^0C for 8 hrs	189	189	148+41
P53	BstUI	5'-CG^CG-3' 3'-GC^GC-5'	37^0C overnight	199	113+86	199
EPHX1 ex4	RsaI	5'-GT^AC-3' 3'CA^TG-5'	37^0C for 4hrs	210	210	164 + 46
EPHX1 ex3	EcoRV	5'-GAT^ATC-3' 3'CTA^TAG-5'	37^0C overnight	162	140+22	162
CYP1A12A	MspI	5'-CC^GG-3' 3'GG^CC-5'	37^0C overnight	340	340	220+140
CYP1A12C	NcoI	5'-C^CATGG-3' 3'GGTAC^C-5'	37^0C overnight	333	69+32+232	69 + 264
SULT1A1	HhaI	5'-GCG^C-3' 3'C^GCG-5'	37^0C for 3hrs	200	160+40	200

Statistical analysis

Associations between genetic variants and AL were assessed by odds ratios and 95% confidence intervals using a conditional logistic regression model adjusted for age and sex. The test for Hardy-Weinberg equilibrium (HWE) was determined by χ^2 test / Fisher's exact test (two tailed). Demographic variables associated with smoking, alcohol consumption, dietary habits and occupational information were not available in all the patients therefore data could not be analyzed on these variables. All analysis was performed using Stata 8.0 software. For two genes, EPHX1 (exon3 and exon4) and CYP1A1 (2A and 2C), pairwise linkage disequilibrium (LD) estimation and expectation-maximization (EM)-based haplotype association analysis were performed using Haploview v.4.0 software.

1) Classification and regression trees (CART) analysis

The non-parametric classification and regression tree analysis was performed to detect high order gene-gene interactions using the CART Software (version 6.0, Salford Systems) [Steinberg and Colla, 1997]. CART is a binary recursive partitioning method that creates a decision tree which describes how well each genotype or environmental factor variable predicts class (eg. acute leukemia case-control status). The CART model selects the variable used to split each branch and the split point. Splitting rules are used to stratify data into subsets of individuals, which are represented in the CART decision tree as nodes. The tree building process continues until the terminal nodes have no subsequent statistically significant splits or they reach a pre-specified minimum size (10 subjects in AML and 5 in ALL for this study). Optimal tree was determined by reducing overfitting trees using one standard error (1-SE) rule and repeating 10 fold cross validation. The p-value and corresponding OR with 95%CI for each terminal node were calculated using Fisher's exact test.

2) Multifactor dimensionality reduction (MDR) analysis

The MDR software (version 2.0 beta) was also used to analyze the gene-gene interaction in the study [Hahn et al., 2003]. Briefly, MDR classifies multi-locus genotypes into high-risk and low-risk groups. This new one-dimensional variable can be evaluated for its ability to classify and predict disease status using cross-validation and permutation testing that minimizes false positive results by multiple examinations of the data. Ten-fold cross-validation and 1000 fold

permutation testing was used in this MDR analysis. The fitness of an MDR model was assessed by estimating the maximum values of cross validation consistency (CVC) and testing accuracy (TA). Statistical significance of the best model of each order was determined using 1000 fold permutation testing and p-value, obtained for each testing accuracy and CVC. MDR models were considered statistically significant at the 0.05 level.

3) False Positive Report Probability (FPRP)

To estimate the false positive report probability (FPRP) and to evaluate robustness of the significant findings of the study, we used the Bayesian approach described by Wacholder *et al.,* [2004]. The method requires prior probabilities that the genetic variant and disease association is real. We set a fairly wider range of prior probabilities (0.0001 to 0.25) with an estimated statistical power to detect an OR of 1.5 and 2.0 and α level equal to the observed p-value with FPRP cut point set at 0.5.

4) Interaction entropy graphs

Hierarchical interaction graphs were built to interpret and visualize the independent effects and interactive relationships between the polymorphisms of the best model of ALL. Interaction graphs use entropy estimates as described by Jakulin and Bratko, [2003] for determining the gain in information about a class variable (e.g. case–control status) from merging two variables together over that provided by the variables independently. This measure of entropy is useful for building interaction graphs that facilitate the interpretation of the relationship between variables. Interaction graphs are comprised of a node for each variable with pairwise connections between them. The percentage of entropy removed (i.e. information gain) by each variable is visualized for each node. The percentage of entropy removed for each pairwise Cartesian product of variables is visualized for each connection. Positive entropy (plotted in green) indicates non-linear interaction while negative entropy (plotted in red) indicates redundancy. Interaction entropy analysis was performed using the Orange software package [Demsar and Zupan, 2004].

Result

Characteristics of the AL patients

The main clinical and hematologic characteristics of the patients included in the study are summarized in Table 7.6. All 230 patients with acute leukemia who were included in this study were ≥15years old (mean ± S.D at diagnosis was 27.1 ± 15.2 years, range 10-82) (except 8 cases in AML and 4 cases in ALL were in the range of 10-14yrs). Pediatric patients with AL below 10 years of age were excluded from this study. There were 131 AML and 99 ALL (75 B-ALL& 24 T-ALL) patients. The median age of the patients with AML was 29 years (range 10-80 years) while in ALL, the median age was 18 years (range 10-67 years). Comparison between hematological and clinical data showed that the median percentage of leukemic blast cell in peripheral blood was 70% in AML and 80% in ALL patients. M2 was found to be the most common FAB subtype (46/131, 35%) followed by M0/M1 subtype in AML. The median age of the controls was 39 years (range 10-82). Controls had no history of cancer and were not related to the patients. Controls were matched to the cases on the basis of age (±5 years) and gender. All the patients and controls were from the north India.

Table 7.6: Demographic, clinical and hematological data of patients with acute leukemia

Variables	AML (N=131)	ALL (N=99)	Control (199)
Sex (%)Males	89 (67.93)	70 (70.7)	134 (67.3)
Females	42 (32.06)	29 (29.2)	65(32.6)
Age Median (range)	29 (10-80)	18 (10-67)	40 (10-82)
WBC($\times 10^9$/l) median (Range)	20 (1-343)	35 (1-178)	
Hb (g/dl) median (Range)	6.2 (2-13.8)	5.9 (2-13.8)	
Plt count ($\times 10^9$/l) median (Range)	34 (1-428)	39 (0.9-370)	
Peripheral Blast (%) median (Range)	70 (10-98)	80 (8-99)	
FAB Morphology † n(%)	M0/M1: 20 (15.2) M2: 46 (35.1) M3: 7(5.3) M4: 18 (13.7) M5: 20 (15.2) M6: 1(1.0) BP: 3(2.2) NC*: 16 (12.2)	L1:40 L2:41 NC: 18	
CD34 positive, n (%)	73/125 (58.4)	46 (46.4)	
Auer rod positive, n (%)	61 (46.5)	-	
Hepatosplenomegaly, n (%)	75 (57.25)	77 (77.7)	
Lymphadenopathy, n (%)	36 (27.48)	47 (47.4)	
CR/NCR, n (%)	26/35 (74.2)	21/30(70%)	

*NC- Not classified, AML-Acute myeloid leukemia, ALL- Acute lymphoid leukemia.
† FAB- French-American-British

Risk Associated with Individual SNPs

The main effect of each polymorphism was assessed for the risk of AML and ALL using conditional logistic regression model adjusted for age and sex. Two of the analyzed SNPs EPHX1 exon3 and CYP1A12C in control ($\chi 2$ =13.3, p=0.0002 & χ^2=9.01, p=0.002) and EPHX1 exon3 in ALL ($\chi 2$=6.21, p=0.01) showed deviations from HWE. No association was observed between AML risk and any of the nine SNPs studied (Table 7.7). However in ALL, CYP1A12A heterozygous genotype was associated with a significant increased risk (OR=2.02;95% CI=1.14-

.3.58,p=0.01) while GSTM1 imparted a 45% lesser risk (OR=0.55;95%CI=0.31-0.96,p=0.03) (Table 7.8). Furthermore when the data was analyzed for dominant and recessive model for all the polymorphisms, only CYP1A12A was associated with significant risk for ALL in dominant model (OR=2.11;95%CI=1.22-3.65,p=0.007) (Table 7.9). Haplotype analysis further supported the increased risk associated with CYP1A12A variant in ALL. We found increased risk for haplotype with variant CYP1A1-CA haplotype compared to the most common CYP1A1-TA haplotype in ALL (OR=1.97;95%CI=1.26-3.09,p=0.002) (Table 7.11). Pair-wise LD analysis suggested evidence of modest LD between CYP1A12A and CYP1A12C polymorphism in acute leukemia (D= 0.61 in AML and 0.56 in ALL) and showed poor correlation (r^2= 0.13 in AML and 0.15 in ALL) with each other. Haplotype generated with EPHX1 SNP did not exhibit any significant association with AML and ALL risk (Table 7.10 & 7.11).

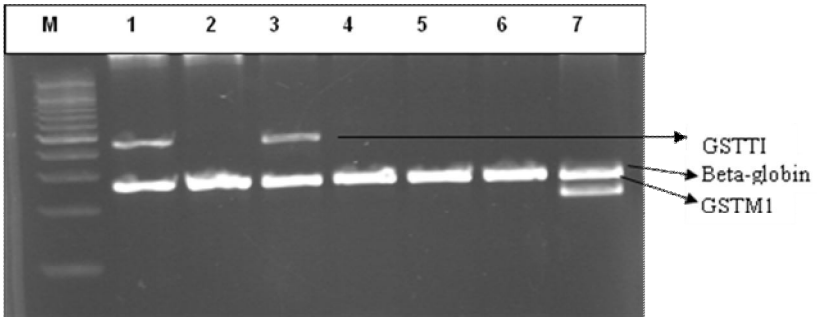


Figure 7.1: Agarose gel picture showing multiplex PCR for GST polymorphism. Lane 1 and 3-samples with 459 bp represent wild type GSTT1; Lane 7-sample with 219bp represent GSTM1 gene; The presence of 267bp in all lanes represent β-globin gene used as internal control. M-100bp ladder

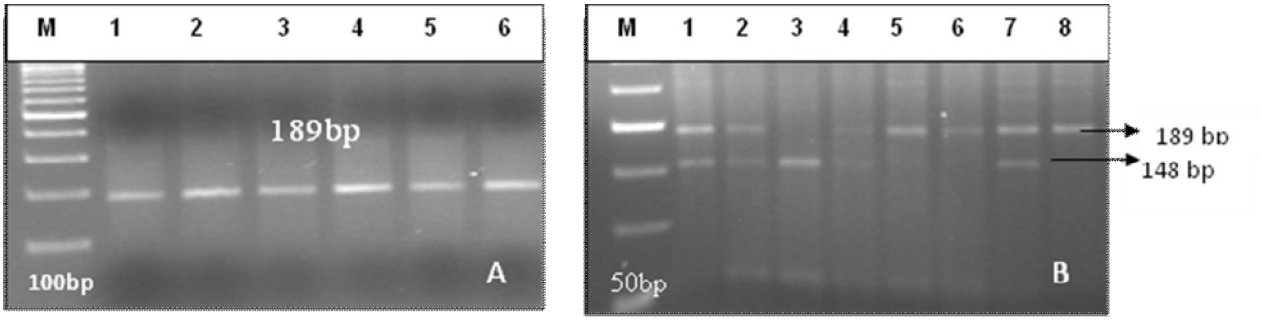


Figure 7.2: Agarose gel picture showing RFLP products of GSTP1 gene: A- showing PCR amplification of GSTP1 gene (189bp). B-RFLP of GSTP1 PCR product; Lane 5,6,8-samples with 189 bp represent wild type Ile/Ile allele (AA genotype); Lane1,2.4 and 7-sample with 189 bp and 148 bp represent heterozygous Ile/Val allele (AG genotype); Lane 3 - sample with 148bp and 41 bp not visible represent homozygous Val/Val allele (GG genotype). M-100 & 50bp ladder.

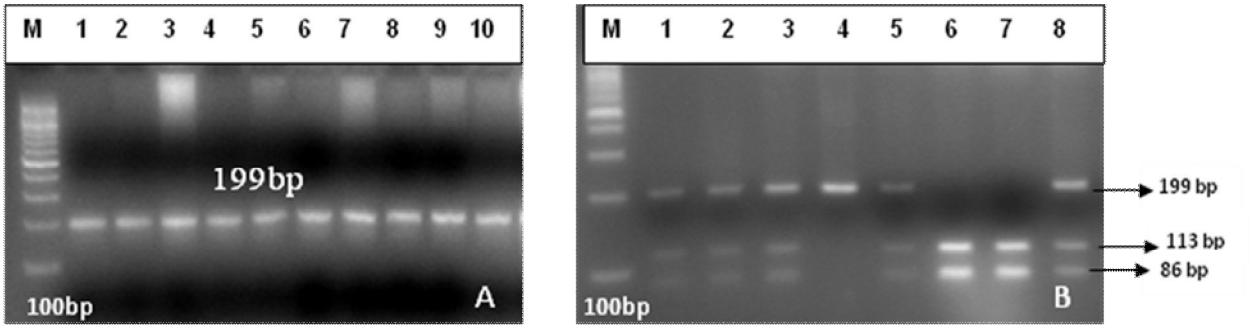


Figure 7.3: Agarose gel picture showing RFLP products of P53 codon 72 polymorphism: A- showing PCR amplification of exon 4 of p53 gene. B-RFLP of p53 PCR product; Lane 4-sample with 199 bp represent homozygous Pro/Pro allele (GG genotype); Lane 1-3 & 5-8 - samples with all three bands (199bp, 113bp, 86bp) represent heterozygous Arg/Pro allele (GC genotype); Lane 6 and 7- samples with two band (113 bp, 86 bp) represent homozygous Arg/Arg allele (CC genotype) . M-100bp ladder.

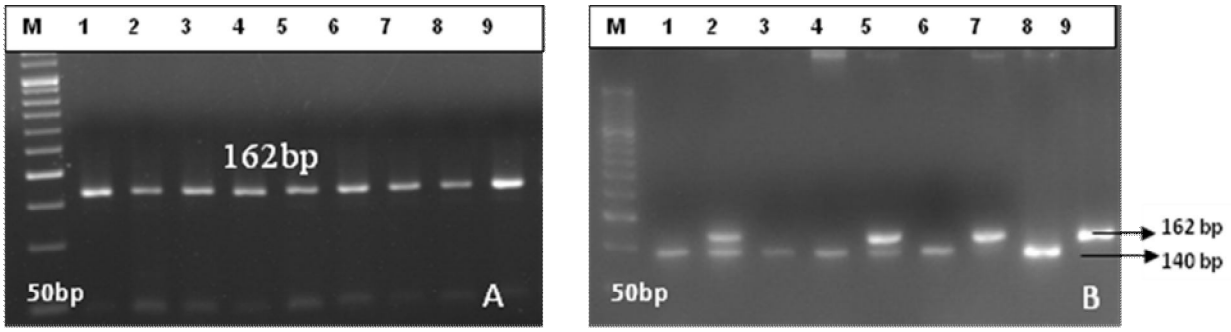


Figure 7.4: Agarose gel picture showing RFLP products of EPHX1 exon3 polymorphism: A- showing PCR amplification of exon4 of EPHX1 gene. B-RFLP of EPHX1 exon 3 PCR product; Lane 1,3,4,6,8- samples with two band (140bp and 22bp not visible) represent homozygous Tyr/Tyr allele (TT genotype); Lane 2,5-samples with all three bands (162bp, 140bp and 22bp not visible) represent heterozygous Tyr/His allele (TC genotype); Lane 7,9- samples with only one band (162bp) represent homozygous His/His allele (CC genotype) M-50bp ladder.

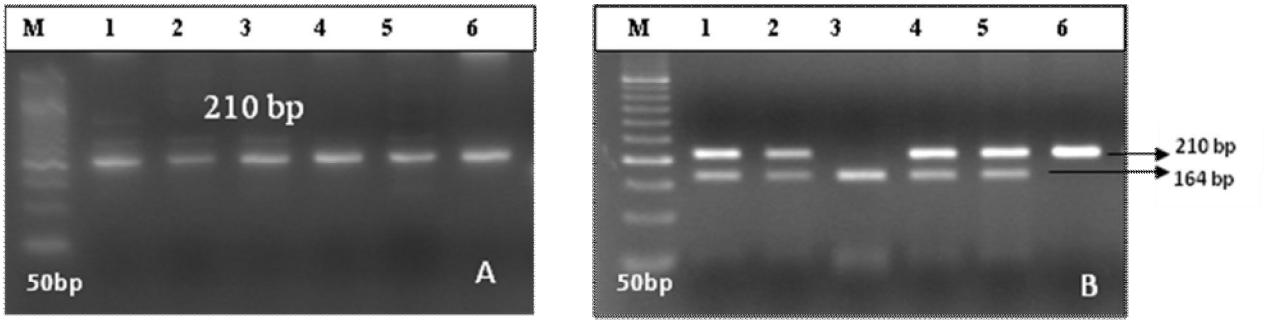


Figure 7.5: Agarose gel picture showing RFLP products of EPHX1 exon4 polymorphism: A- showing PCR amplification of exon4 of EPHX1 gene. B-RFLP of EPHX1 exon 4 PCR poduct; Lane 6-sample with single band (210bp) represent wild homozygous His/His allele (AA); Lane 1,2,3,4-samples with all three bands (210bp,164bp and 46bp not visible) represent heterozygous His/Arg allele (AG genotype); Lane 3- samples with two band (164 bp and 46bp not visible) represent homozygous Arg/Arg allele(GG genotype) M-50bp ladder.

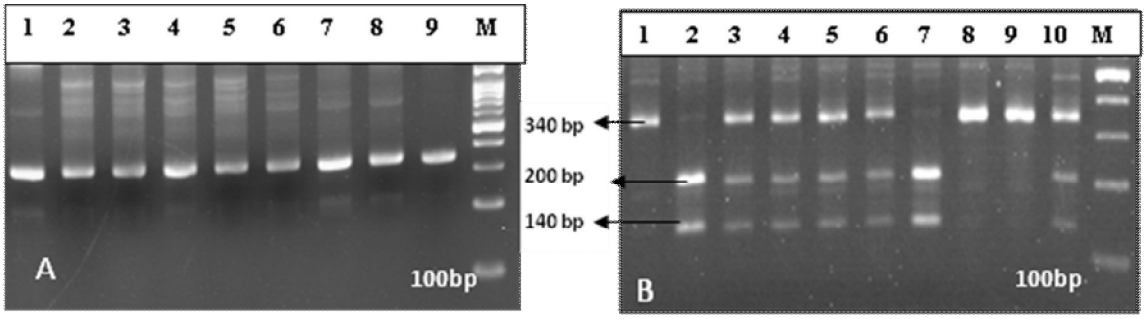


Figure 7.6: Agarose gel picture showing RFLP products of CYP1A12A polymorphism: A- showing PCR amplification of CYP1A12A. B-RFLP of CYP1A12A PCR; Lane 1,8,9-samples with single band (340bp) represent wild type TT genotype; Lane 3-6,10-samples with all three band (340bp, 200bp ,140bp) represent heterozygous TC genotype; Lane 2,7- samples with two band (200bp and 140bp) represent homozygous CC genotype. M-100bp ladder.

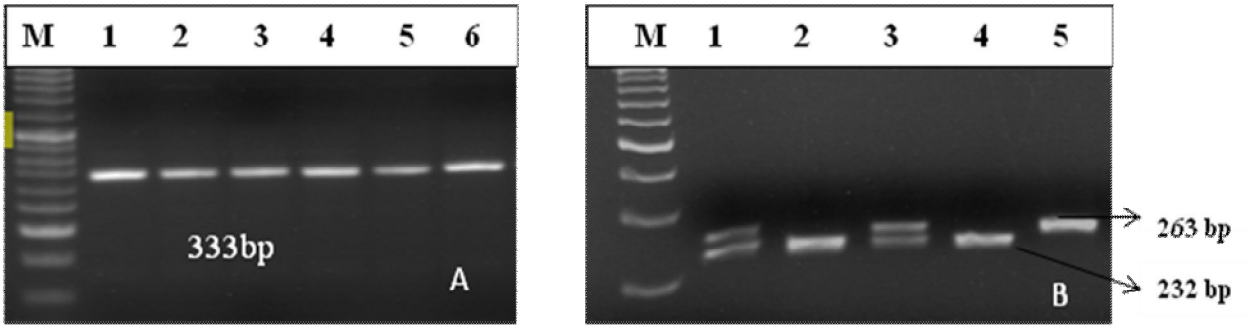


Figure 7.7: Agarose gel picture showing RFLP products of CYP1A12C polymorphism: A- showing PCR amplification of CYP1A12C. B-RFLP of CYP1A12C PCR; Lane 2,4-samples with single band (232bp) represent wild type Ile/Ile allele; Lane 5-sample with 263bp represent homozygous variant Val/Val allele. Lane 1,3- samples represent heterozygous Ile/Val allele , M-100bp ladder.

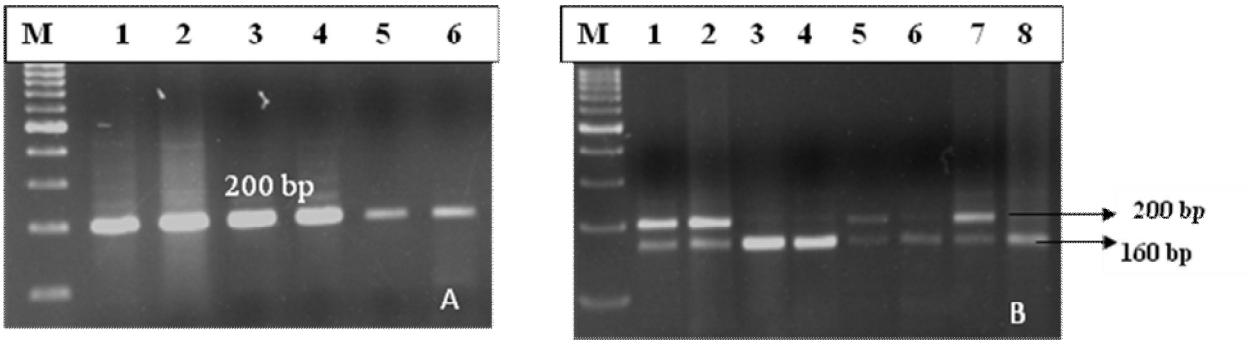


Figure 7.8: Agarose gel picture showing RFLP products of SULT1A1 polymorphism: A- showing PCR amplification of SULT1A1 gene. B-RFLP of SULT1A1 PCR; Lane 1,2,5,7-samples with all three bands (200bp, 160bp, 40bp not visible) represent heterozygous Arg/His allele (GA genotype); Lane 3,4,6,8-sample with two band (160bp and 40bp not visible) represent the homozygous Arg/Arg allele. The undigested product (200bp) which represents His/His allele is not shown in the gel. M-100bp ladder.

Table 7.7: Association of the genotypes of six xenobiotic metabolizing genes and p53 codon 72 polymorphism with AML

		Controls (199)	AML (131)		
Gene	**Genotype**	**n (%)**	**n (%)**	**OR (95%C.I.)***	**p-value**
GSTT1	Wild	152 (76.38)	104(79.39)	Ref	-
	Null	47(23.61)	27(20.61)	0.64(0.40-1.03)	0.06
GSTM1	Wild	104(52.26)	81(61.83)	Ref	-
	Null	95(47.73)	50(38.17)	0.68(0.39-1.19)	0.1
GSTP1	AA	103(51.75)	66(50.38)	Ref	-
	AG	79(39.69)	55(41.98)	1.19(0.73-1.95)	0.4
	GG	17(8.54)	10(7.63)	1.16(0.47-2.85)	0.7
p53	GG	51(25.62)	38(29.0)	Ref	-
	GC	112(56.28)	71(54.19)	0.89(0.52-1.53)	0.6
	CC	36(18.09)	22(16.79)	0.96(0.47-1.96)	0.9
EPHX1 Exon 4	AA	127(63.81)	81(61.83)	Ref	-
	AG	64(32.16)	43(32.82)	0.83(0.50-1.38)	0.4
	GG	8(4.02)	7(5.34)	1.31(0.44-3.88)	0.6
EPHX1 Exon 3	TT	87(43.71)	54(41.22)	Ref	-
	TC	70(35.17)	55(41.98)	1.27(0.76-2.13)	0.3
	CC	42(21.10)	22(16.79)	1.05(0.54-2.04)	0.8
CYP1A12A	TT	108(54.27)	65(49.61)	Ref	-
	TC	78(39.19)	57(43.51)	1.16(0.71-1.90)	0.5
	CC	13(6.53)	9(6.87)	1.13(0.44-2.87)	0.2
CYP1A12C	AA	156(78.39)	97(74.04)	Ref	-
	AG	35(17.58)	31(23.66)	1.27(0.70-2.30)	0.4
	GG	8(4.02)	3(2.29)	0.65(0.14-2.90)	0.5
SULT1A1	GG	135(67.83)	86(65.64)	Ref	-
	GA	54(27.13)	42(32.06)	1.32(0.78-2.23)	0.2
	AA	10(5.02)	3(2.29)	0.42(0.11-1.62)	0.2

Note. AML: Acute myeloid leukemia, Ref: Reference Category, *OR (95%C.I.)= Odds ratio (95% confidence intervals) adjusted for age and gender calculated using conditional logistic regression analysis.

Table 7.8: Association of the genotypes of six xenobiotic metabolizing genes and p53 codon 72 polymorphism with ALL

		Controls (199)	ALL (99)		
Gene	**Genotype**	**n (%)**	**n (%)**	**OR (95%C.I.)***	**P-value**
GSTT1	Wild	152 (76.38)	71(71.71)	Ref	-
	Null	47(23.61)	28(28.28)	0.98(0.55-1.77)	0.9
GSTM1	Wild	104(52.26)	63(63.63)	Ref	-
	Null	95(47.73)	36(36.36)	0.55(0.31-0.96)	**0.03***
GSTP1	AA	103(51.75)	45(45.45)	Ref	-
	AG	79(39.69)	45(45.45)	1.53(0.87-2.68)	0.1
	GG	17(8.54)	9(9.09)	1.53(0.57-4.12)	0.3
p53	GG	51(25.62)	28(28.28)	Ref	-
	GC	112(56.28)	43(43.43)	0.58(0.29-1.15)	0.1
	CC	36(18.09)	28(28.28)	1.29(0.60-2.77)	0.4
EPHX1 Exon 4	AA	127(63.81)	60(60.0)	Ref	-
	AG	64(32.16)	37(37.37)	1.06(0.60-1.88)	0.8
	GG	8(4.02)	2(2.02)	0.49(0.9-2.65)	0.4
EPHX1 Exon 3	TT	87(43.71)	40 (40.40)	Ref	-
	TC	70(35.17)	36(36.36)	1.14(0.63-2.06)	0.6
	CC	42(21.10)	23(23.23)	1.21(0.61-2.06)	0.5
CYP1A12A	TT	108(54.27)	35(35.35)	Ref	-
	TC	78(39.19)	53(53.53)	2.02(1.14-3.58)	**0.01***
	CC	13(6.53)	11(11.11)	2.64(0.95-7.32)	0.06
CYP1A12C	AA	156(78.39)	69(69.69)	Ref	-
	AG	35(17.58)	25(25.25)	1.30(0.69-2.44)	0.4
	GG	8(4.02)	5(5.05)	1.94(0.51-7.37)	0.3
SULT1A1	GG	135(67.83)	66(66.66)	Ref	-
	GA	54(27.13)	30(30.30)	1.12(0.62-2.02)	0.6
	AA	10(5.02)	3(3.03)	0.52(0.13-2.08)	0.3

Note. ALL: Acute lymphoblastic leukemia, Ref: Reference Category, *OR (95%C.I.)= Odds ratio (95% confidence intervals) adjusted for age and gender calculated using conditional logistic regression analysis. *Bold values are significant p value < 0.05.

Table 7.9: Genotype representation and associations of polymorphism under dominant and recessive model in acute leukemia

				AML	ALL
Gene	**Model**	**Effect**	**Reference**	**OR(95%CI);P**	**OR (95%CI); P**
p53	Dominant	GC+ CC	GG	0.90(0.54-1.52);0.71	0.78(0.42-1.45);0.44
	Recessive	CC	GG + GC	1.03(0.55-1.92);0.90	1.79(0.94-3.42); 0.07
GSTP1	Dominant	AG+GG	AA	1.19(0.74-1.90);0.46	1.53(0.89-2.62); 0.12
	Recessive	GG	AA+AG	1.07(0.47-2.54);0.87	1.48(0.24-3.19); 0.65
EPHX1 Exon 4	Dominant	AG+GG	AA	0.88(0.54-1.43);0.62	1.00(0.57-1.75); 0.98
	Recessive	GG	AA+AG	1.40(0.48-4.06);0.53	0.48(0.09-2.54); 0.39
EPHX1 Exon 3	Dominant	TC+CC	TT	1.20(0.75-1.94);0.43	1.17(0.69-1.98); 0.55
	Recessive	CC	TT+TC	0.93(0.51-1.70);0.82	1.14(0.61-2.12); 0.67
CYP1A12A	Dominant	TC+CC	TT	1.15(0.72-1.85);0.54	2.11(1.22-3.65); **0.007**
	Recessive	CC	TT+TC	1.05(0.42-2.61);0.90	1.91(0.72-5.02); 0.18
CYP1A12C	Dominant	AG+GG	AA	1.17(0.67-2.06);0.57	1.38(0.76-2.50); 0.28
	Recessive	GG	AA+AG	0.62(0.14-2.73);0.53	1.82(0.48-6.86); 0.37
SULT1A1	Dominant	GA+AA	GG	1.13(0.69-1.85);0.60	1.01(0.57-1.78); 0.95
	Recessive	AA	GG+GA	0.41(0.10-1.56);0.19	0.50(0.12-1.98); 0.33

Note. CYP1A12A was associated with significant risk for ALL in dominant model. OR(95%CI);P represent Odds ratio (95% confidence intervals);p-value adjusted for age and gender. Bold values are significant p value < 0.05.

Table 7.10: Frequency distribution of the CYP1A1 and EPHX1 haplotype in AML

Haplotype	**Control (398) n (%)**	**AML (262)n (%)**	**χ^2**	**p-value**	**OR (95%CI);P**
CYP1A12A/2C					
TA	281(70.94)	172(64.96)	1.82	0.1	Ref
CA	66(16.24)	53(20.92)	1.44	0.2	1.31(0.85-2.01);0.2
CG	38(9.89)	22(7.71)	0.26	0.6	0.94(0.52-1.70);0.8
TG	13(2.93)	15(6.41)	2.42	0.1	1.88(0.82-4.32);0.1
EPHX1 Ex 4/Ex 3					
AT	185(46.34)	124(47.72)	0.03	0.8	Ref
AC	133(33.56)	81(30.52)	0.40	0.5	0.90(0.62-1.32);0.6
GT	59(14.97)	39(14.49)	0.005	0.9	0.98(0.60-1.60);1.0
GC	21(5.13)	18(7.26)	0.60	0.4	1.27(0.62-2.62);0.4

Note. No haplotype was found to be significantly associated with AML risk. Ref- Reference category, χ^2 chi-square test. OR(95%CI);P represent Odds ratio (95% confidence intervals);P-value.

Table 7.11: Frequency distribution of the CYP1A1 and EPHX1 haplotype in ALL

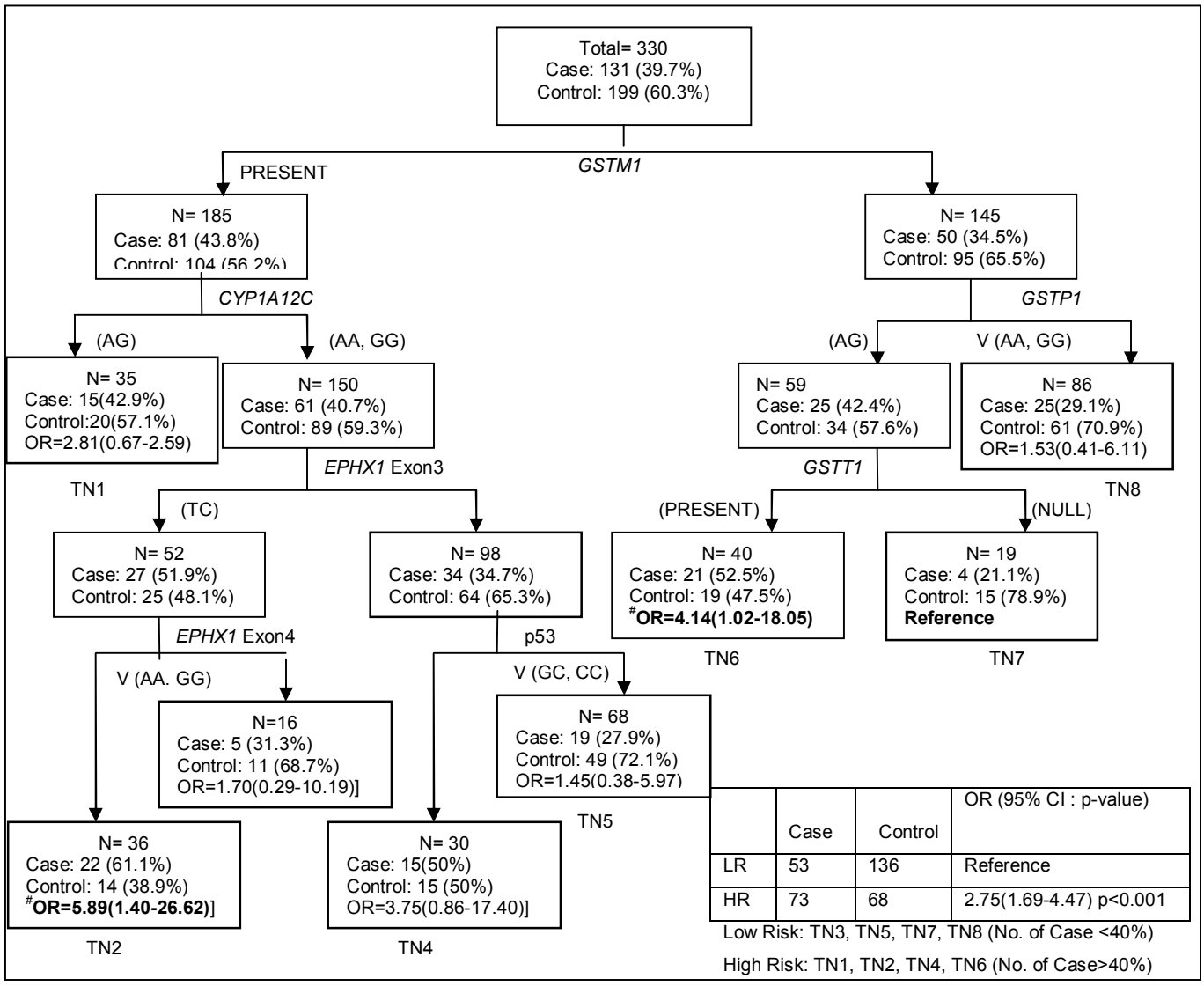
Haplotype	Control (398) n (%)	ALL (188) n (%)	χ^2	p-value	OR(95%CI);P
CYP1A12A/2C					
TA	281(70.94)	112(56.10)	11.37	0.0007	Ref
CA	66(16.24)	51(26.22)	6.86	0.008	1.97(1.26-3.09);**0.002**
CG	38(9.89)	24(11.66)	1.04	0.3	1.54(0.85-2.78);0.13
TG	13(2.93)	11(6.02)	1.62	0.2	2.30(0.91-5.79);0.05
EPHX1 Ex 4/Ex 3					
AT	185(46.34)	86(43.06)	0.48	0.4	Ref
AC	133(33.56)	71(36.23)	0.34	0.5	1.13(0.75-1.69);0.5
GT	59(14.97)	30(15.53)	0.009	0.9	1.07(0.62-1.83);0.7
GC	21(5.13)	11(5.18)	0.02	0.8	1.17(0.50-2.72)0.6

Note. CYP1A1-CA haplotype was found to be significantly associated with ALL risk. Ref; Reference category, χ^2 chi-square test. OR(95%CI);P represent Odds ratio (95% confidence intervals);P-value.

Classification and Regression Tree (CART) analysis

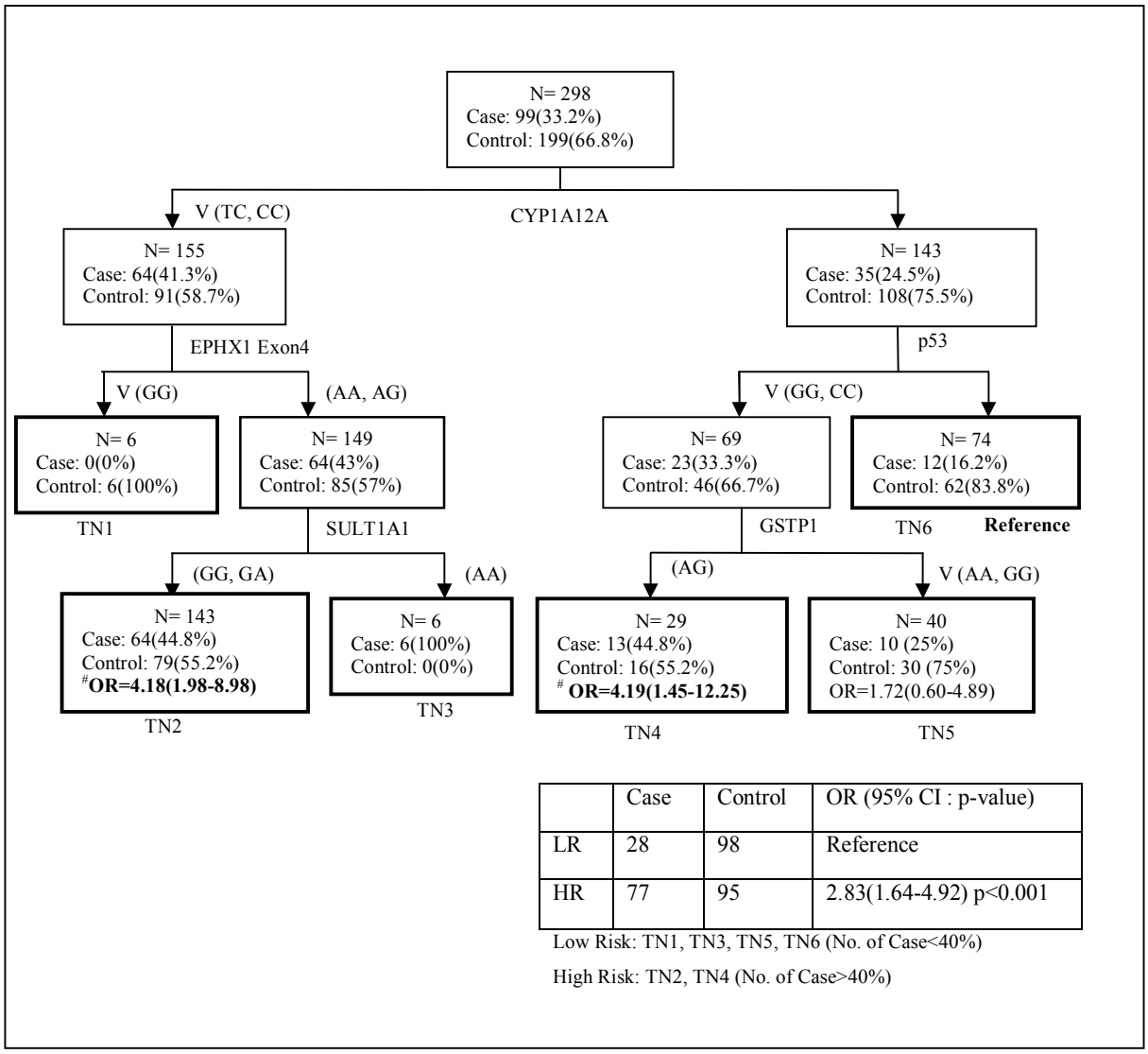
In CART analysis, the decision tree identified GSTM1 and CYP1A12A as the main splitters in AML and ALL respectively. The prediction success of the testing set in AML and ALL for cases was 52.67% in AML and 64.65% in ALL. The final tree had 8 and 6 terminal nodes in AML and ALL respectively (Figure 7.9 & 7.10). The risk of the terminal nodes in the two trees was estimated in reference to the terminal node consisting of the least percentage of cases. In AML the highest risk was observed for combination of GSTM1 present, CYP1A12C AA or GG, EPHX1 exon3 TC and EPHX1 exon4 AA or GG genotypes in terminal node TN2 (OR=5.89;95%CI=1.40-26.62,p=0.01) (Figure 7.9). In ALL highest risk was observed for combination of CYP1A12A TT, p53 GG or CC and GSTP1 AG genotypes in terminal node TN 4 (OR=4.19;95%CI=1.45-12.25,p=0.004) (Figure 7.10). Further we pooled the terminal node based on case ratio into low (<40%) and high risk group (>40%) in AML and ALL. Compared with low risk group terminal node, high risk groups were associated with significantly increased AML and ALL risk respectively (OR=2.75;95%CI=1.69-4.47,p<0.001) (OR=2.83;95%CI=1.64-4.92,p<0.001) (Figure 7.9 &7.10).

Figure 7.9: Classification and regression tree (CART) model for polymorphism of xenobiotic metabolizing genes and p53 codon 72 gene polymorphism in AML



Note. Classification and regression tree analysis of risk predictors in AML cases. Nodes of the classification tree are formed by recursive splits of AML case/control status by predictor variables. The numbers within each node indicate the number of control subjects/number of case patients. Within each terminal node, the odds ratio (with 95% confidence interval) of AML is shown for that node with respect to the reference node (TN7). Polymorphism used for splitting is displayed under each node. OR(95%C.I.)= Odds ratio (95% confidence intervals), V: Variant genotype, TN: Terminal Node, #p value <0.05.

Figure 7.10: Classification and regression tree (CART) model for polymorphism of xenobiotic metabolizing genes and p53 codon 72 gene polymorphism in ALL



Note. Classification and regression tree analysis of risk predictors in ALL cases. Nodes of the classification tree are formed by recursive splits of ALL case/control status by predictor variables. The numbers within each node indicate the number of control subjects/number of case patients. Within each terminal node, the odds ratio (with 95% confidence interval) of AML is shown for that node with respect to the reference node (TN6). Polymorphism used for splitting is displayed under each node. OR(95%C.I.)= Odds ratio (95% confidence intervals), V: Variant genotype, TN: Terminal Node, #p value <0.05.

Multifactor dimensionality reduction (MDR) analysis

The MDR analysis was done to analyze for the high order gene-gene interaction. Table 7.12 summarizes the results of MDR analysis for the best model of each order in ALL and AML along with their testing accuracy (TA) and cross-validation consistency (CVC). GSTM1 and CYP1A12A were the best one loci models with testing accuracy of 51.85% (CVC=9/10) and 59.46% (CVC=10/10) in AML and ALL respectively. Though both factor were insignificant in empirical observations of p-values based on permutation testing. Four loci model in AML (p53, EPHX1 exon3, CYP1A12A and SULT1A1) and in ALL (GSTP1, p53, EPHX1 exon3 and CYP1A12A) had TA of 58.08% (CVC=10/10) and 63.50% (CVC=10/10) respectively. The TA was statistically significant only for the four loci model in ALL, with p= 0.009 obtained from permutation testing. Other 2 and 3 loci best models in AML and ALL had lower CV and TA value than four loci model and were insignificant in permutation testing. Therefore, the four loci model was chosen as the best model in ALL only.

Table 7.12: Multifactor dimensionality reduction model for gene-gene interaction in AL

	Locus	Model	p value (χ^2test)	TA	p-value*	CVC	p-value*
AML	1st order	GSTM1	p=0.08	0.5185	0.69	9/10	0.46
	2nd order	p53, EPHX1 Exon3	p=0.009	0.4437	0.99	4/10	0.98
	3rd order	CYP1A12A, CYP1A12C, SULT1A1	p< 0.0001	0.4677	0.96	4/10	0.98
	4th order	p53, EPHX1 Exon3, CYP1A12A, SULT1A1	p< 0.0001	0.5808	0.14	10/10	0.28
ALL	1st order	CYP1A12A	p=0.0021	0.5946	0.12	10/10	0.32
	2nd order	CYP1A12A, SULT1A1	p=0.0002	0.5468	0.50	4/10	0.99
	3rd order	CYP1A12A, CYP1A12C, SULT1A1	p< 0.0001	0.5440	0.52	6/10	0.87
	4th order†	GSTP1, p53 EPHX1 Exon3, CYP1A12A	p< 0.0001	0.6350	**0.009**	10/10	0.32

Note. *1,000-fold permutation test. † Best models selected with maximum cross-validation consistency (CVC) and maximum testing accuracy (TA). Bold values represent significant value ≤0.05.

False positive report probability (FPRP)

The FPRP was estimated for the statistically significant associations of the present study obtained from logistic regression analysis, haplotype analysis and MDR analysis (Table 7.13). CYP1A12A alone and in dominant model of inheritance showed reliable association at high probability of (0.5-0.1) for detecting OR=1.5 and 2.0 (Table 7.13). Risk haplotype of CYP1A1-CA showed true association till prior probability of 0.1 and 0.01 for OR=1.5 and 2.0. Four loci best model of ALL showed good reliability even at very low prior probability (10^{-4}) for detecting an OR of 2.0 (Table 7.13).

Table 7.13: False positive report probability and Odds ratio for significant finding in ALL

		OR (95% CI) p value	OR=1.5	Prior Probability						OR=2.0	Prior Probability					
			Power	0.5	0.25	0.1	0.01	0.001	10^{-4}	Power	0.5	0.25	0.1	0.01	0.001	10^{-4}
Logistic regression	CYP1A12A	2.02(1.14-3.58 p=0.01	0.154	**0.094**	**0.238**	**0.484**	0.912	0.990	0.999	0.486	**0.032**	**0.090**	**0.229**	0.765	0.971	0.997
	GSTM1	0.55(0.31-0.96) p=0.03	0.249	**0.124**	**0.299**	0.561	0.934	0.993	0.999	0.631	**0.053**	**0.144**	**0.335**	0.847	0.982	0.998
Genetic model	Dominant CYP1A12A	2.11(1.22-3.65) p=0.007	0.111	**0.064**	**0.170**	**0.380**	0.871	0.986	0.999	0.424	**0.018**	**0.051**	**0.138**	0.639	0.947	0.994
Haplotype	Haplotype CYP1A12A-2C	1.97(1.26-3.09) p=0.002	0.118	**0.026**	**0.074**	**0.194**	0.726	0.964	0.996	0.526	**0.006**	**0.018**	**0.051**	**0.372**	0.857	0.984
MDR (Best model)	GSTP1, P53, EPHX1 Ex3 CYP1A12A	9.22(4.89-17.3) p < 0.0001	0.000	**0.001**	**0.002**	**0.006**	**0.060**	**0.391**	0.865	0.000	**0.000**	**0.000**	**0.000**	**0.001**	**0.005**	**0.051**

Note: Prior probabilities ranging from 0.5 to 10^{-4}, with the estimated statistical power to detect an OR of 1.5 & 2.0 with α level equal to the observed p-value. Bold type indicates the FPRP for the most likely prior probabilities i.e. a noteworthy association at the 0.5 FPRP cut point. OR(95%CI);P represent Odds ratio (95% confidence intervals);P-value

Interaction entropy graphs

Finally, the interaction entropy graph was constructed to decipher the relationships in the four loci MDR model of ALL (Figure 7.11). CYP1A12A was the key gene polymorphism in the model associated with entropy of 2.59%. p53 also showed strong main effect with entropy removal of 1.42%. A synergistic interaction was also observed between these two, as the combination removed additional entropy of 2.46%. Though small percentage of entropy is associated with GSTP1 (0.28%) and EPHX1 exon3 (0.008%) individually, their interaction removed a substantial 1.98% of entropy suggesting a non-additive relationship between the two. The overall two way interactions between all the polymorphisms in the model were mostly non-additive in nature.

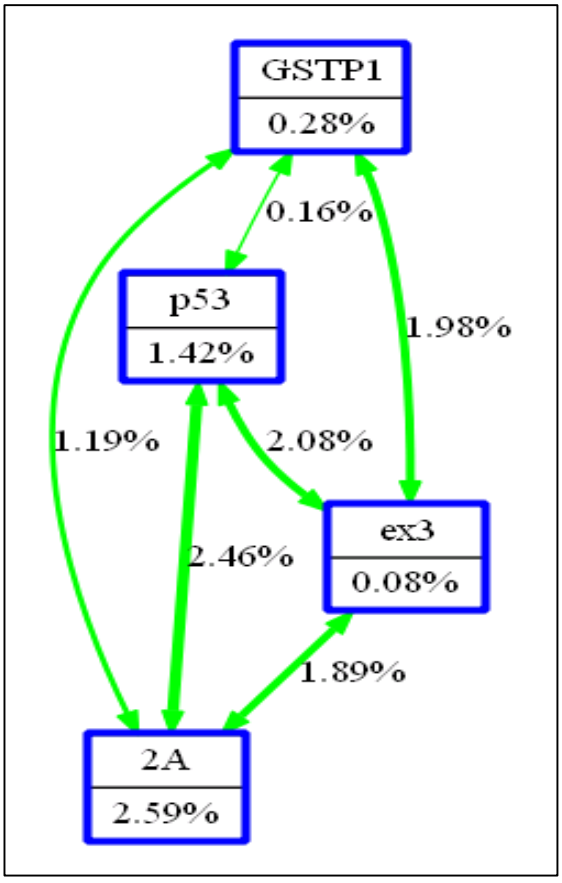


Figure 7.11: Interaction entropy graph for the four polymorphism of the Best model in acute lymphoblastic leukemia (ALL). The interaction model describes the percentage of the entropy (information gain) removed by each variable and by each pair wise combination of attributes (interaction effect: represented by connections). A positive interaction is observed between all the polymorphism in the model. Labels: Ex3: EPHX1 Exon3, 2A: CYP1A12A.

Discussion

The pathogenesis of AL is very heterogenous and complex with regard to clinical features and acquired genetic alterations which are unlikely to be predisposed by single genetic factor. Taking these factors into consideration, employment of more comprehensive approaches to assess the interaction of multiple genes and high order gene-gene interaction in acute leukemia predisposition is indispensable.

The present study investigated the association of genetic polymorphism in six xenobiotic genes and p53 codon 72 polymorphism with the risk of acute leukemia by multifaceted analytical approach. No association was observed for any of the polymorphism in six xenobiotic genes and p53 with AML risk in LR analysis. However, in ALL, GSTM1 null genotype was found to be associated with significantly reduced risk while CYP1A12A heterozygous genotype conferred higher risk of ALL. The effect was further confirmed in haplotype analysis. Cytochrome P450 (CYP) enzymes that encodes an aromatic hydrocarbon hydroxylase plays a key role in the phase I xenobiotic metabolism of drugs and environmental carcinogens. The enzyme catalyzes the oxidation of poly aromatic hydrocarbons (PAH) such as benzo [a] pyrene (B(a)P) to benzo [a] pyrene -diol-epoxide (BPDE) [Bartsch *et al.,* 2000]. Two most common polymorphism reported in CYP1A1 gene are CYP1A12A (6235T>C) present in the 3' end of the gene and CYP1A12C in exon7 producing an Isoleucine to Valine substitution at amino acid 462. These two polymorphic alleles are often but not always in linkage disequilibrium [Wormhoudt et al., 1999]. Functional studies for these SNPs have predicted an increased catalytic activity and higher levels of hydrophobic DNA adducts [Alexandrov *et al.,* 2002]. Several studies have suggested increased risk associated with these variant alleles in solid cancers particularly those associated with tobacco use [Bartsch *et al.,* 2000; Sam *et al.,* 2008]. Expressions of CYP family enzymes is also reported in human myeloblastic and lymphoid cell lines, probably contributing to the carcinogenesis of hematopoietic cells [Nagai *et al.,* 2002]. However, findings in hematological malignancies remain inconclusive. Association of CYP1A12A with the risk of childhood and adult ALL has been reported in literature [Sinnett *et al.,* 2000; Joseph *et al.,* 2004; Gallegos-Arreola *et al.,* 2004]. However, no significant association has been observed in adult AL [Aydin-Sayitoglu *et al.,* 2006; Bolufer *et al.,* 2007].

In addition, CART and MDR method were used in association analyses due to its ability of detecting the gene-gene interaction, which is not available in the traditional chi-square association analysis methods. Both CART and MDR analysis identified GSTM1 and CYP1A12A as most important single locus factors in AML and ALL risk respectively, although the results did not show statistical significance in MDR analysis. Interaction of GSTM1, CYP1A12C, EPHX1 exon3 and EPHX1 exon4 in AML and interaction of CYP1A12A, p53 and GSTP1 in ALL identified by CART method reflects the complexity of xenobiotic metabolism in acute leukemia. The results are biological plausible and are consistent with the epidemiological evidences from phase I and phase II gene interaction studies in other cancers [Talseth *et al.,* 2006; Zienolddiny *et al.,* 2008]. Interaction between CYP1A1 and GST polymorphism has been reported in acute leukemia [Krajinovic *et al.,* 1999; Sinnett *et al.,* 2000; DAlo *et al.,* 2004; Majumdar *et al.,* 2008] and presence of p53 codon 72 polymorphism further augments the risk as reported in lung and prostate cancer [Miller *et al.,* 2000; Ritchie *et al.,* 2003]. These observations suggest that bioactivation of phase I metabolites such as reactive benzo(a)pyrene diol epoxide by GSTs initiate a tumoral process which becomes more conducive to carcinogenesis with further involvement of p53 gene.

Association of GST null genotype with cancer risk is probably related to an inability to metabolize carcinogens, such as benzo(a)pyrene. Studies from U.K, Iran and India have linked GSTT1 and GSTM1 null genotype with risk of both AML and ALL [Rollinson *et al.,* 2000; Saadat and Saadat, 2000; Majumdar *et al.,* 2008]. However in the present study GSTM1 null genotype was found to confer reduced risk to ALL probably implying a dual role of GSTM1 in the metabolism of a wide range of xenobiotics. GSTM1 enzyme is primarily a detoxifying enzyme. However, sometimes the detoxification reaction leads to activation of reactive intermediates, from phase I pathway, similar to the activation of dihalomethanes and other chlorinated hydrocarbons by GSTT1 enzyme [Shimada *et al.,* 1996] which might contribute to the carcinogenesis process. However several factors preclude about such an association in the present study. First such an association with GSTM1 enzyme is not supported by other studies in hematological malignancies [Aydin-Sayitoglu *et al.,* 2006; Bolufer *et al.,* 2007; Majumdar et al.,

2008]. Secondly, there is lack of information on environmental factors in the study which might modify the risk associated with the gene. Finally the small sample size of the study probably reflects instability of our estimate.

MDR analysis identified four locus model of GSTP1, p53, EPHX1 exon3 and CYP1A12A to be significantly associated with the ALL risk. However, none of the SNPs except CYP1A12A were statistically significant in single locus analysis in logistic regression indicating that epistasis effects of these four factors could modulate the risk of ALL. Even after applying a method for the estimation of false positive results, four locus model of ALL remained a plausibly true association even when considering a very low prior probability of 0.0001 (OR= 2.0). Further, interaction entropy algorithm helped us to interpret the nature of interaction of the 4 loci model revealing strong synergistic interaction of p53 with EPHX1 exon3 and CYP1A12A polymorphism. This observation indicated that variations in biotransformation activities of both phase I and phase II enzymes in coordination with p53 activity may regulate the effect of DNA toxic metabolites and determine susceptibility to carcinogen exposure.

Mutlifacted analytic approaches like CART and MDR have been used in previous studies to explore the high order gene-gene interaction even with small sample size. Yang H *et al.,* [2008] applied CART on set of 147 patients with oral premalignant lesion and 147 controls and identified some biologically informative interactions among double stranded break DNA repair genes. Similarly, H.H. Chung *et al.,* [2006] applied MDR to analyze the role of seven SNPs in response to chemotherapy in 36 patients of cervical carcinoma. In this study both CART and MDR approach showed consistency at one and two order interaction levels, however results differed at higher order. In CART analysis, interaction of GSTM1, CYP1A12C, EPHX1 exon3 and EPHX1 exon4 polymorphism was found to be a significant predictor for the risk of AML. MDR analysis showed that a combination of GSTP1, p53, EPHX1 exon3 and CYP1A12A genes was the best four loci model in predicting the risk for ALL. The results are of interest, yet these are empirical observation of gene-gene interaction with leukemia. Validations in independent large populations need to be done to confirm our observation.

Two SNPs in our study (EPHX1 Exon3 and CYP1A12C) were not in HWE in control population. Deviation from HWE may be indicative of genotyping error, though genotyping was repeated on 10% of the samples and concordance was observed. HWE violation seems to be a chance finding or it is most likely due to population admixture. In addition, overfitting of data due to the small sample size in the current study may limit the prediction ability of MDR, particularly for the four loci model. In CART analysis, prediction accuracy of the best model was only 52.67% and 64.65% in testing set of ALL and AML cases respectively. Such low prediction success implies that the results should be interpreted with caution. The incorporation of environmental factors may have improved the results.

The results of the study are quiet promising but functional analysis is warranted to confirm the biological plausibility of the complex gene-gene interactions identified. The study provides further support to a multi-genetic approach and taking consideration of complex high-order interactions in cancer association studies.

Chapter 8

Conclusions and Future Scope of Work

Chapter 8

Conclusions and Future Scope of Work

Objective 1: Confirmation of diagnosis and typing of acute leukemia using Flow Cytometer

The aim of this objective was to determine the prevalence of AML and ALL and the frequency of expression of cross lineage marker and their association with clinical prognostic factors. Of the 289 cases included in the current study, AML accounted for 46% of AL, ALL accounts 50% (B-ALL- 39% and T-ALL-11%) while MLAL were 2% cases of AL.

AML: M2 was found to be the most common FAB subtypes in adult and children. CD13 and CD33 were the most commonly expressed myeloid marker in the study. No difference was found in the response rate in CD34+ and CD34- group in adult AML. However, in children a marginal significant association was observed towards a lower remission rate in CD34+ (50%) cases compared to CD34- (100%) cases (p=0.06). thus, confirming the negative influence of this marker on the clinical outcome. Among clinical features, lymphadenopathy (LAP) was more common in adults than children (80% vs 56%, p=0.007). Median level of Hb was also higher in adults than children (p=0.006). In AML remission rates in adult and children was similar (52% vs 50%). Aberrant antigen expression was identified in 35% of AML cases in the study. Aberrant expression of lymphoid lineage associated antigen (Ly+ AML) was seen more often in cases of children (53%) than adults (28%) (p=0.01). CD19 was the most common aberrant lymphoid antigen expression in both adult and children followed by CD7. In children CD34, immature stem cell marker, was found to be significantly associated with the lymphoid antigen expression (94% in Ly+ AML and 53% in Ly- AML).

ALL: L1 subtype was more common than L2 subtype in both children and adults. In B-ALL, L1 and L2 FAB subtypes were equally common in both children and adults. Interestingly, in adult T-ALL, the prevalence of L1 (66%) was almost double that of L2 (33%) subtype. Antigen marker, CD19 and CD10 may suffice to diagnose pro-B-ALL (CD19+/CD10-) and common B-

ALL (CD19+/CD10+) cases. In B-ALL**,** CD34 expression was more commonly detected in adults (51%) as compared to children (39%) (p=0.07). However, it did not have any correlation with clinical response. In T-ALL CD7 expression was detected in all cases (100%). LAP was more often associated with T-ALL than B-ALL cases. In adult T-ALL, higher percentage of blast (>50%) in the peripheral blood was significantly associated with poorer clinical outcome (p=0.02). CD33 expression was seen more often in cases of adults (25%) than children (8%) (p=0.01). In adult ALL, CD34 was significantly associated with My+ ALL (71%) than My-ALL (35%) (p=0.02).

Objective 2: Detection of commonly occurring genetic alteration in acute leukemia

The objective of this work was to study the prevalence and prognostic significance of commonly reported genetic alteration in acute leukemia. In this study the frequency of commonly occurring translocations (BCR/ABL, AML/ETO and PML/RARα) and gene mutations (FLT3 and NPM1) were analyzed in patient with AL.

ALL: The prevalence of BCR-ABL translocation was 27% in ALL patients. On further stratification, there was no case of T-ALL positive for this translocation compared to 33% positive in B-ALL (p=0.03). BCR/ABL translocation was significantly associated with adverse prognosis factors WBC count (p=0.04) and FAB subtype L1 (p=0.03). Though the CR rates were 43% in BCR/ABL positive patients compared to 67% in BCR/ABL negative patients it was not statistically significant.

AML: The frequency of BCR/ABL, AML/ETO and PML/RARα translocation was 7%, 24% and 5% respectively in AML cases. Further, AML/ETO translocation was significantly associated with the presence of Auer rod and LAP, low level of Hb and WBC count in the study. Immunophenotypically, AML/ETO translocation cases were associated with the expression of CD19 and CD34, more frequently observed in FAB M4 subtype cases.

The frequency of FLT3 and NPM1 mutations were 25% and 21% respectively in the study. Adult patients showed higher frequency of FLT3 and NPM1 mutation than pediatric patients. FLT3/ITD mutation was associated with higher WBC count while NPM1 mutation was

associated with a higher platelet count (p=0.02) and absence of hepatosplenomegaly (p=0.01). A significant association of FLT3/ITD mutation with aberrant expression of CD7 and CD19 marker was found in the study. NPM1 mutation samples lacked expression of hematopoietic stem cell markers CD34 (p<0.001). We also a found higher frequency of FLT3 mutation in NPM1 mutation cases or vice versa. Both FLT3 and NPM1 mutations were observed more frequently in AML/ETO positive cases than PML/RARα translocation cases. These results support the observation that besides chromosomal translocation and gene mutations additional mutations are required for the pathogenesis of acute myeloid leukemia.

Objective 3: Study of Multidrug resistance genes in acute leukemia

The major cause for treatment failure in acute leukemia (AL) is pre-existent or acquired resistance to chemotherapy. The results of several studies that reported about the correlation between clinical resistance and the expression of these resistance proteins induce some arguments. In this study we aimed to investigate the expression of drug resistance (MDR1, MRP1, LRP, BCRP, GSTP1, DHFR) and apoptotic genes (p53, BCL-2, Survivin) in acute leukemias and compare them with clinical and hematological findings and response to induction chemotherapy.

AML: Expression of MDR1 gene was significantly higher in non responders as compared to responders in adult patients. Furthermore, a significant association of MDR1 expression with the adverse prognostic marker, CD34, was also found in adult patients. We also observed an association of FLT3 gene mutation with low expression of MDR1 but with higher p53 and GSTP1 expression in adults. Expression of anti-apoptotic gene BCL-2 was also higher in non responders compared to responders in adults. In children, strong correlation was observed between LRP and MDR1 gene. NPM1 gene mutation was significantly associated with higher expression of both MDR1 and GSTP1. In contrast to adults, the study did not show a clinical relevance of any of the drug resistance and apoptotic genes in childhood AML.

ALL: In adults, MRP1 expression was found to be higher in T-ALL as compared to B-ALL. Further, the expression of MRP1 gene was found to be significantly higher in non responders compared to responders. In children, drug resistance gene that was significantly different between responders and non responders was BCRP.

Objective 4: Role of Xenobiotic metabolizing (Drug metabolizing) genes and p53 codon 72 polymorphism in acute leukemia

A major part of carcinogenic substances require metabolic activation by enzymes to be genotoxic, and inherited variations in carcinogens metabolizing genes may alter enzyme activity and subsequently carcinogens activation or deactivation. Based on this knowledge we investigated the association of genetic polymorphism in six xenobiotic genes and p53 codon 72 polymorphism with the risk of acute leukemia by multifaceted analytical approach.

ALL: GSTM1 null genotype confered reduced risk to ALL probably implying a dual role of GSTM1 in the metabolism of a wide range of xenobiotics. The study showed increased risk for ALL among those carrying CYP1A12A heterozygous genotype. Further, CYP1A12A was associated with significant risk for ALL in dominant model. Haplotype analysis further supported the increased risk associated with CYP1A12A (CYP1A1-CA) variant in ALL. While exploring non-linear interacting through CART analysis, patients carrying the combination of CYP1A12A, p53 and GSTP1 gene showed highest risk for ALL. Four locus model of GSTP1, p53, EPHX1 exon3 and CYP1A12A was the best model in predicting the ALL risk by MDR analysis. This four loci model showed good reliability in false positive report probability analysis. Interaction entropy graph showed that CYP1A12A was the key gene polymorphism in the model associated with the maximum entropy.

AML: In AML the highest risk was observed for combination of GSTM1 present, CYP1A12C AA or GG, EPHX1 exon3 TC and EPHX1 exon4 AA or GG genotypes by CART analysis. Four loci model (p53, EPHX1 exon3, CYP1A12A and SULT1A1) was the best model in MDR analysis in predicting risk in AML but lost its significance in permutation testing of the model.

Future Scope of the Thesis

The complexity and biologic heterogeneity of acute leukemia pose significant challenges in understanding the pathogenesis of the disease. This study has been performed to investigate the frequencies and the prognostic relevance of previously known genetic events in North Indian population. The work has provided more insight into the role of genetic factors in acute leukemia etiology. Given the importance of co-operation between recurrent chromosomal translocation and genetic mutation in the study, future work should be carried out to involve more molecular markers to clearly understand the complexity of leukemogenesis. The study also attempted to unravel the mechanisms responsible for multidrug resistant phenomenon in acute leukemia, however heterogeneity of the patients group hampers the results. Future work needs to be done on large number of patients and other mechanisms to explain this multifactorial process. In addition the study also showed that there are genetic variants in drug metabolizing genes that affect inter-individual risk to acute leukemia. However, the incorporation of environmental factors and functional analysis is warranted in future studies to confirm the biological plausibility of the complex gene-gene interactions identified in the study.

References

References

Abu-Duhier FM, Goodeve AC, Wilson GA, Care RS, Peake IR, Reilly JT. Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. Br J Haematol. 2001 Jun;113(4):983-988.

Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC. Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. Lancet. 1998 Mar 21;351(9106):882-883.

Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, Pincus MR, Sardana M, Henderson CJ, Wolf CR, Davis RJ, Ronai Z. Regulation of JNK signaling by GSTp. EMBO J. 1999 Mar 1;18(5):1321-1334.

Adolfsson J, Månsson R, Buza-Vidas N, Hultquist A, Liuba K, Jensen CT, Bryder D, Yang L, Borge OJ, Thoren LA, Anderson K, Sitnicka E, Sasaki Y, Sigvardsson M, Jacobsen SE. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell. 2005 Apr 22;121(2):295-306.

Advani S, Pai S, Venzon D, Adde M, Kurkure PK, Nair CN, Sirohi B, Banavali SD, Hawaldar R, Kolhatkar BB, Vats T, Magrath I. Acute lymphoblastic leukemia in India: an analysis of prognostic factors using a single treatment regimen. Ann Oncol. 1999 Feb;10(2):167-176.

Agarwal V, Dabadghao S, Misra MN, Nityanand S. Immunophenotypic subsets in acute lymphoblastic leukemia from a single centre in North India: correlation with outcome to induction chemotherapy. Indian J Cancer. 2001 Jun-Dec;38(2-4):85-91.

Agundez JA. Cytochrome P450 gene polymorphism and cancer. Curr Drug Metab. 2004 Jun;5(3):211-224.

Ahmad F, Mandava S, Das BR. Analysis of FLT3-ITD and FLT3-Asp835 mutations in de novo acute myeloid leukemia: evaluation of incidence, distribution pattern, correlation with cytogenetics and characterization of internal tandem duplication from Indian population. Cancer Invest. 2010 Jan;28(1):63-73.

Ahmad F, Mandava S, Das BR. Mutations of NPM1 gene in de novo acute myeloid leukaemia: determination of incidence, distribution pattern and identification of two novel mutations in Indian population. Hematol Oncol. 2009 Jun;27(2):90-97.

Akashi K, Traver D, Kondo M, Weissman IL. Lymphoid development from hematopoietic stem cells. Int J Hematol. 1999 Jun;69(4):217-226.

Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000 Mar 9;404(6774):193-197.

Alexandrov K, Cascorbi I, Rojas M, Bouvier G, Kriek E, Bartsch H. CYP1A1 and GSTM1 genotypes affect benzo[a]pyrene DNA adducts in smokers' lung: comparison with aromatic/hydrophobic adduct formation. Carcinogenesis. 2002 Dec;23(12):1969-1977.

Ali-Osman F, Akande O, Antoun G, Mao JX, Buolamwini J. Molecular cloning, characterization, and expression in Escherichia coli of full-length cDNAs of three human glutathione S-transferase Pi gene variants. Evidence for differential catalytic activity of the encoded proteins. J Biol Chem. 1997 Apr 11;272(15):10004-10012.

Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res. 1998 Dec 1;58(23):5337-5339.

Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med. 1997 Aug;3(8):917-921.

Amson RB, Nemani M, Roperch JP, Israeli D, Bougueleret L, Le Gall I, Medhioub M, Linares-Cruz G, Lethrosne F, Pasturaud P, Piouffre L, Prieur S, Susini L, Alvaro V, Millasseau P, Guidicelli C, Bui H, Massart C, Cazes L, Dufour F, Bruzzoni-Giovanelli H, Owadi H, Hennion C, Charpak G, Telerman A, et al. Isolation of 10 differentially expressed cDNAs in p53-induced apoptosis: activation of the vertebrate homologue of the drosophila seven in absentia gene. Proc Natl Acad Sci U S A. 1996 Apr 30;93(9):3953-3957.

Arber DA, Brunning RD, Le Beau MM, Falini B, Vardiman JW, Porwit A, Thiele J, Bloomfield CD. Acute myeloid leukaemia with recurrent genetic abnormalities. In: WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. 4th edition. pp. 110-123. Swerdlow SH, Campo E, Harris NL et al. (eds.). International Agency for Research on Cancer (IARC), Lyon, France, 2008.

Arber DA, Glackin C, Lowe G, Medeiros LJ, Slovak ML. Presence of t(8;21)(q22;q22) in myeloperoxidase-positive, myeloid surface antigen-negative acute myeloid leukemia.. Am J Clin Pathol. 1997 Jan;107(1):68-73.

Arico M, Valsecchi MG, Camitta B, Schrappe M, Chessells J, Baruchel A, Gaynon P, Silverman L, Janka-Schaub G, Kamps W, Pui CH, Masera G.Outcome of treatment in children with Philadelphia chromosome-positive acute lymphoblastic leukemia. N Engl J Med. 2000 Apr 6;342(14):998-1006.

Attarbaschi A, Mann G, König M, Steiner M, Strehl S, Schreiberhuber A, Schneider B, Meyer C, Marschalek R, Borkhardt A, Pickl WF, Lion T, Gadner H, Haas OA, Dworzak MN. Mixed lineage leukemia-rearranged childhood pro-B and CD10-negative pre-B acute lymphoblastic leukemia constitute a distinct clinical entity. Clin Cancer Res. 2006 May 15;12(10):2988-2994.

Aydin-Sayitoglu M, Hatirnaz O, Erensoy N, Ozbek U. Role of CYP2D6, CYP1A1, CYP2E1, GSTT1, and GSTM1 genes in the susceptibility to acute leukemias. Am J Hematol. 2006 Mar;81(3):162-170.

Babusikova O, Glasová M, Koníková E, Kusenda J. Leukemia-associated phenotypes: their characteristics and incidence in acute leukemia. Neoplasma. 1996;43(6):367-372.

Bae SC, Ogawa E, Maruyama M, Oka H, Satake M, Shigesada K, Jenkins NA, Gilbert DJ, Copeland NG, Ito Y. PEBP2 alpha B/mouse AML1 consists of multiple isoforms that possess differential transactivation potentials. Mol Cell Biol. 1994 May;14(5):3242-3252.

Bajel A, George B, Mathews V, Viswabandya A, Kavitha ML, Srivastava A, Chandy M. Treatment of children with acute lymphoblastic leukemia in India using a BFM protocol. Pediatr Blood Cancer. 2008 Nov;51(5):621-625.

Bardet V, Costa LD, Elie C, Malinge S, Demur C, Tamburini J, Lefebvre PC, Witz F, Lioure B, Jourdan E, Pigneux A, Ifrah N, Attal M, Dreyfus F, Mayeux P, Lacombe C, Bennaceur-Griscelli A, Bernard OA, Bouscary D, Récher C; GOELAMS: Groupe Ouest Est d'etude des Leucémies et des Autres Maladies du Sang. Nucleophosmin status may influence the therapeutic decision in de novo acute myeloid leukemia with normal karyotype. Leukemia. 2006 Sep;20(9):1644-1660.

Bartsch H, Nair U, Risch A, Rojas M, Wikman H, Alexandrov K. Genetic polymorphism of CYP genes, alone or in combination, as a risk modifier of tobacco-related cancers. Cancer Epidemiol Biomarkers Prev. 2000 Jan;9(1):3-28.

Basso G, Lanza F, Orfao A, Moretti S, Castoldi G. Clinical and biological significance of CD34 expression in acute leukemia. J Biol Regul Homeost Agents. 2001 Jan-Mar;15(1):68-78.

Bedi A, Zehnbauer BA, Barber JP, Sharkis SJ, Jones RJ. Inhibition of apoptosis by BCR-ABL in chronic myeloid leukemia. Blood. 1994 Apr 15;83(8):2038-2044.

Benderra Z, Faussat AM, Sayada L, Perrot JY, Chaoui D, Marie JP, Legrand O. Breast cancer resistance protein and P-glycoprotein in 149 adult acute myeloid leukemias. Clin Cancer Res. 2004 Dec 1;10(23):7896-7902.

Benderra Z, Faussat AM, Sayada L, Perrot JY, Tang R, Chaoui D, Morjani H, Marzac C, Marie JP, Legrand O. MRP3, BCRP, and P-glycoprotein activities are prognostic factors in adult acute myeloid leukemia. Clin Cancer Res. 2005 Nov 1;11(21):7764-7772.

Bene MC, Bernier M, Casasnovas RO, Castoldi G, Doekharan D, van der Holt B, Knapp W, Lemez P, Ludwig WD, Matutes E, Orfao A, Schoch C, Sperling C, van't Veer MB. Acute myeloid leukaemia M0: haematological, immunophenotypic and cytogenetic characteristics and their prognostic significance: an analysis in 241 patients. Br J Haematol. 2001 Jun;113(3):737-745.

Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, van't Veer MB. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia. 1995 Oct;9(10):1783-1786.

Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol. 1976 Aug;33(4):451-458.

Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C.Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med. 1985 Oct;103(4):620-625.

Bhatia S, Neglia JP. Epidemiology of childhood acute myelogenous leukemia. J Pediatr Hematol Oncol. 1995 May;17(2):94-100.

Boissel N, Renneville A, Biggio V, Philippe N, Thomas X, Cayuela JM, Terre C, Tigaud I, Castaigne S, Raffoux E, De Botton S, Fenaux P, Dombret H, Preudhomme C. Prevalence, clinical profile, and prognosis of NPM mutations in AML with normal karyotype. Blood. 2005 Nov 15;106(10):3618-3620.

Bolhuis H, van Veen HW, Poolman B, Driessen AJ, Konings WN. Mechanisms of multidrug transporters. FEMS Microbiol Rev. 1997 Aug;21(1):55-84.

Bolufer P, Collado M, Barragán E, Cervera J, Calasanz MJ, Colomer D, Roman-Gómez J, Sanz MA. The potential effect of gender in combination with common genetic polymorphisms of drug-metabolizing enzymes on the risk of developing acute leukemia. Haematologica. 2007 Mar;92(3):308-314.

Boonthimat C, Thongnoppakhun W, Auewarakul CU Nucleophosmin mutation in Southeast Asian acute myeloid leukemia: eight novel variants, FLT3 coexistence and prognostic impact of NPM1/FLT3 mutations.. Haematologica. 2008 Oct;93(10):1565-1569.

Boucheix C, David B, Sebban C, Racadot E, Bené MC, Bernard A, Campos L, Jouault H, Sigaux F, Lepage E, et al. Immunophenotype of adult acute lymphoblastic leukemia, clinical parameters, and outcome: an analysis of a prospective trial including 562 tested patients (LALA87). French Group on Therapy for Adult Acute Lymphoblastic Leukemia. Blood. 1994 Sep 1;84(5):1603-1612.

Braoudaki M, Papathanassiou C, Katsibardi K, Tourkadoni N, Karamolegou K, Tzortzatou-Stathopoulou F. The frequency of NPM1 mutations in childhood acute myeloid leukemia. J Hematol Oncol. 2010 Oct 27;3:41.

Brown P, McIntyre E, Rau R, Meshinchi S, Lacayo N, Dahl G, Alonzo TA, Chang M, Arceci RJ, Small D. The incidence and clinical significance of nucleophosmin mutations in childhood AML. Blood. 2007 Aug 1;110(3):979-985.

Buzzai M, Licht JD. New molecular concepts and targets in acute myeloid leukemia. Curr Opin Hematol. 2008 Mar;15(2):82-87

Casasnovas RO, Slimane FK, Garand R, Faure GC, Campos L, Deneys V, Bernier M, Falkenrodt A, Lecalvez G, Maynadié M, Béné MC. Immunological classification of acute myeloblastic leukemias: relevance to patient outcome. Leukemia. 2003 Mar;17(3):515-527.

Cascavilla N, Musto P, D'Arena G, Ladogana S, Matera R, Carotenuto M. Adult and childhood acute lymphoblastic leukemia: clinico-biological differences based on CD34 antigen expression. Haematologica. 1997 Jan-Feb;82(1):31-37.

Castell JV, Donato MT, Gómez-Lechón MJ. Metabolism and bioactivation of toxicants in the lung. The in vitro cellular approach. Exp Toxicol Pathol. 2005 Jul;57 Suppl 1:189-204.

Chan JY, Ugrasena DG, Lum DW, Lu Y, Yeoh AE. Xenobiotic and folate pathway gene polymorphisms and risk of childhood acute lymphoblastic leukaemia in Javanese children. Hematol Oncol. 2011 Sep;29(3):116-123.

Chang H, Salma F, Yi QL, Patterson B, Brien B, Minden MD. Prognostic relevance of immunophenotyping in 379 patients with acute myeloid leukemia. Leuk Res. 2004 Jan;28(1):43-48.

Chaudhary PM, Mechetner EB, Roninson IB. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. Blood. 1992 Dec 1;80(11):2735-2739.

Chauhan PS, Ihsan R, Yadav DS, Mishra AK, Bhushan B, Soni A, Kaushal M, Devi TR, Saluja S, Gupta DK, Mittal V, Saxena S, Kapur S. Association of glutathione S-transferase, EPHX, and p53 codon 72 gene polymorphisms with adult acute myeloid leukemia. DNA Cell Biol. 2011 Jan;30(1):39-46.

Chauncey TR. Drug resistance mechanisms in acute leukemia. Curr Opin Oncol. 2001 Jan;13(1):21-26.

Chekhun VF, Lukyanova NY, Kovalchuk O, Tryndyak VP, Pogribny IP. Epigenetic profiling of multidrug-resistant human MCF-7 breast adenocarcinoma cells reveals novel hyper- and hypomethylated targets. Mol Cancer Ther. 2007 Mar;6(3):1089-1098.

Chen BG, Zhang L, Li BL, Luo WD. Sensitivity and specificity analysis of the lineage related antibodies in acute leukemia immunophenotyping by flow cytometry. Zhongguo Shi Yan Xue Ye Xue Za Zhi. 2005 Apr;13(2):329-331

Chen M, Kamat AM, Huang M, Grossman HB, Dinney CP, Lerner SP, Wu X, Gu J. High-order interactions among genetic polymorphisms in nucleotide excision repair pathway genes and smoking in modulating bladder cancer risk. Carcinogenesis. 2007 Oct;28(10):2160-2165.

Chen W, Rassidakis GZ, Medeiros LJ. Nucleophosmin gene mutations in acute myeloid leukemia. Arch Pathol Lab Med. 2006 Nov;130(11):1687-1692.

Chen YH, Tang YM, Shen HQ, Song H, Yang SL, Shi SW, Qian BQ, Xu WQ, Ning BT. The expression of CD19 in 210 cases of childhood acute leukemia and its significance. Zhonghua Er Ke Za Zhi. 2004 Mar;42(3):188-191.

Chen YN, Mickley LA, Schwartz AM, Acton EM, Hwang JL, Fojo AT. Characterization of adriamycin-resistant human breast cancer cells which display overexpression of a novel resistance-related membrane protein. J Biol Chem. 1990 Jun 15;265(17):10073-10080.

Cheson BD, Bennett JM, Kopecky KJ, Büchner T, Willman CL, Estey EH, Schiffer CA, Doehner H, Tallman MS, Lister TA, Lo-Coco F, Willemze R, Biondi A, Hiddemann W, Larson RA, Löwenberg B, Sanz MA, Head DR, Ohno R, Bloomfield CD; International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J Clin Oncol. 2003 Dec 15;21(24):4642-4649.

Chillon CM, García-Sanz R, Balanzategui A, Ramos F, Fernández-Calvo J, Rodríguez MJ, Rodríguez-Salazar MI, Corrales A, Calmuntia MJ, Orfão A, González M, San Miguel JF. Molecular characterization of acute myeloblastic leukemia according to the new WHO classification: a different distribution in Central-West Spain. Haematologica. 2001 Feb;86(2):162-166.

Cho EK, Bang SM, Ahn JY, Yoo SM, Park PW, Seo YH, Shin DB, Lee JH. Prognostic value of AML 1/ETO fusion transcripts in patients with acute myelogenous leukemia. Korean J Intern Med. 2003 Mar;18(1):13-20.

Choi HJ, Kim HR, Shin MG, Kook H, Kim HJ, Shin JH, Suh SP, Ryang DW. Spectra of chromosomal aberrations in 325 leukemia patients and implications for the development of new molecular detection systems. J Korean Med Sci. 2011 Jul;26(7):886-892.

Chou WC, Tang JL, Lin LI, Yao M, Tsay W, Chen CY, Wu SJ, Huang CF, Chiou RJ, Tseng MH, Lin DT, Lin KH, Chen YC, Tien HF. Nucleophosmin mutations in de novo acute myeloid leukemia: the age-dependent incidences and the stability during disease evolution. Cancer Res. 2006 Mar 15;66(6):3310-3316.

Choudhary C, Schwäble J, Brandts C, Tickenbrock L, Sargin B, Kindler T, Fischer T, Berdel WE, Müller-Tidow C, Serve H. AML-associated Flt3 kinase domain mutations show signal transduction differences compared with Flt3 ITD mutations. Blood. 2005 Jul 1;106(1):265-273.

Chugani DC, Rome LH, Kedersha NL. Evidence that vault ribonucleoprotein particles localize to the nuclear pore complex. J Cell Sci. 1993 Sep;106 (Pt 1):23-29.

Chung HH, Kim MK, Kim JW, Park NH, Song YS, Kang SB, Lee HP. XRCC1 R399Q polymorphism is associated with response to platinum-based neoadjuvant chemotherapy in bulky cervical cancer. Gynecol Oncol. 2006 Dec;103(3):1031-1037.

Coebergh JW, Reedijk AM, de Vries E, Martos C, Jakab Z, Steliarova-Foucher E, Kamps WA. Leukaemia incidence and survival in children and adolescents in Europe during 1978-1997. Report from the Automated Childhood Cancer Information System project. Eur J Cancer. 2006 Sep;42(13):2019-2036.

Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science. 1992 Dec 4;258(5088):1650-1654.

Colombo E, Marine JC, Danovi D, Falini B, Pelicci PG. Nucleophosmin regulates the stability and transcriptional activity of p53. Nat Cell Biol. 2002 Jul;4(7):529-533.

Colovic N, Tosic N, Aveic S, Djuric M, Milic N, Bumbasirevic V, Colovic M, Pavlovic S. Importance of early detection and follow-up of FLT3 mutations in patients with acute myeloid leukemia. Ann Hematol. 2007 Oct;86(10):741-747.

Consolini R, Legitimo A, Rondelli R, Guguelmi C, Barisone E, Lippi A, Cantù-Rajnoldi A, Aricò M, Conter V, Cocito MG, Putti MC, Pession A, Masera G, Biondi A, Basso G. Clinical relevance of CD10 expression in childhood ALL. The Italian Association for Pediatric Hematology and Oncology (AIEOP). Haematologica. 1998 Nov;83(11):967-973.

Copelan EA, McGuire EA. The biology and treatment of acute lymphoblastic leukemia in adults. Blood. 1995 Mar 1;85(5):1151-1168.

Cortez D, Stoica G, Pierce JH, Pendergast AM. The BCR-ABL tyrosine kinase inhibits apoptosis by activating a Ras-dependent signaling pathway. Oncogene. 1996 Dec 19;13(12):2589-2594.

Cortez MA, Scrideli CA, Yunes JA, Valera ET, Toledo SR, Pavoni-Ferreira PC, Lee ML, Petrilli AS, Brandalise SR, Tone LG. mRNA expression profile of multidrug resistance genes in childhood acute lymphoblastic leukemia. Low expression levels associated with a higher risk of toxic death. Pediatr Blood Cancer. 2009 Dec;53(6):996-1004.

Cote ML, Kardia SL, Wenzlaff AS, Land SJ, Schwartz AG. Combinations of glutathione S-transferase genotypes and risk of early-onset lung cancer in Caucasians and African Americans: a population-based study. Carcinogenesis. 2005 Apr;26(4):811-819.

Creutzig U, Zimmermann M, Bourquin JP, Dworzak MN, von Neuhoff C, Sander A, Schrauder A, Teigler-Schlegel A, Stary J, Corbacioglu S, Reinhardt D. Second induction with high-dose cytarabine and mitoxantrone: different impact on pediatric AML patients with t(8;21) and with inv(16). Blood. 2011 Nov 17;118(20):5409-5415.

Cross NC, Melo JV, Feng L, Goldman JM. An optimized multiplex polymerase chain reaction (PCR) for detection of BCR-ABL fusion mRNAs in haematological disorders. Leukemia. 1994 Jan;8(1):186-189.

Cruse JM, Lewis RE, Pierce S, Lam J, Tadros Y. Aberrant expression of CD7, CD56, and CD79a antigens in acute myeloid leukemias. Exp Mol Pathol. 2005 Aug;79(1):39-41.

Cruse, J., Lewis, R.E., Wang, H., 2004. Immunology Guidebook. Elsevier, San Diego.

Czuczman MS, Dodge RK, Stewart CC, Frankel SR, Davey FR, Powell BL, Szatrowski TP, Schiffer CA, Larson RA, Bloomfield CD. Value of immunophenotype in intensively treated adult acute lymphoblastic leukemia: cancer and leukemia Group B study 8364. Blood. 1999 Jun 1;93(11):3931-3939.

Dalal BI, Wu V, Barnett MJ, Horsman DE, Spinelli JJ, Naiman SC, Shepherd JD, Nantel SH, Reece DE, Sutherland HJ, Klingemann HG, Phillips GL.Induction failure in de novo acute myelogenous leukemia is associated with expression of high levels of CD34 antigen by the leukemic blasts. Leuk Lymphoma. 1997 Jul;26(3-4):299-306.

DAlo F, Voso MT, Guidi F, Massini G, Scardocci A, Sica S, Pagano L, Hohaus S, Leone G. Polymorphisms of CYP1A1 and glutathione S-transferase and susceptibility to adult acute myeloid leukemia. Haematologica. 2004 Jun;89(6):664-670.

Dai Z, Quackenbush RC, Courtney KD, Grove M, Cortez D, Reuther GW, Pendergast AM.Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway.Genes Dev. 1998 May 15;12(10):1415-1424.

Damiani D, Tiribelli M, Calistri E, Geromin A, Chiarvesio A, Michelutti A, Cavallin M, Fanin R. The prognostic value of P-glycoprotein (ABCB) and breast cancer resistance protein (ABCG2) in adults with de novo acute myeloid leukemia with normal karyotype. Haematologica. 2006 Jun;91(6):825-828.

Damin AP, Frazzon AP, Damin DC, Roehe A, Hermes V, Zettler C, Alexandre CO. Evidence for an association of TP53 codon 72 polymorphism with breast cancer risk. Cancer Detect Prev. 2006;30(6):523-529.

de The H, Vivanco-Ruiz MM, Tiollais P, Stunnenberg H, Dejean A. Identification of a retinoic acid responsive element in the retinoic acid receptor beta gene. Nature. 1990 Jan 11;343(6254):177-180.

Dean M, Allikmets R. Evolution of ATP-binding cassette transporter genes. Curr Opin Genet Dev. 1995 Dec;5(6):779-785.

Deguchi K, Gilliland DG. Cooperativity between mutations in tyrosine kinases and in hematopoietic transcription factors in AML. Leukemia. 2002 Apr;16(4):740-744.

Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. Blood. 2000 Nov 15;96(10):3343-3356.

Demsar J, Zupan B. Orange: From Experimental Machine Learning to Interactive Data Mining. White Paper, Faculty of Computer and Information Science, University of Ljublijana, Ljublijana, Slovenia. Omnipress, Madison, WI 2004.

Den Boer ML, Pieters R, Kazemier KM, Janka-Schaub GE, Henze G, Creutzig U, Kaspers GJ, Kearns PR, Hall AG, Pearson AD, Veerman AJ. Different expression of glutathione S-transferase alpha, mu and pi in childhood acute lymphoblastic and myeloid leukaemia. Br J Haematol. 1999 Feb;104(2):321-327.

Deschler B, Lubbert M. Acute myeloid leukemia: epidemiology and etiology. Cancer. 2006 Nov 1;107(9):2099-2107.

Dohner K, Schlenk RF, Habdank M, Scholl C, Rücker FG, Corbacioglu A, Bullinger L, Fröhling S, Döhner H. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood. 2005 Dec 1;106(12):3740-3746.

Dong H, Bonala RR, Suzuki N, Johnson F, Grollman AP, Shibutani S. Mutagenic potential of benzo[a]pyrene-derived DNA adducts positioned in codon 273 of the human P53 gene. Biochemistry. 2004 Dec 21;43(50):15922-15928.

Douer D, Santillana S, Ramezani L, Samanez C, Slovak ML, Lee MS, Watkins K, Williams T, Vallejos C.Acute promyelocytic leukaemia in patients originating in Latin America is associated with an increased frequency of the bcr1 subtype of the PML/RARalpha fusion gene. r J Haematol. 2003 Aug;122(4):563-570.

Downing JR, Head DR, Curcio-Brint AM, Hulshof MG, Motroni TA, Raimondi SC, Carroll AJ, Drabkin HA, Willman C, Theil KS, et al. An AML1/ETO fusion transcript is consistently detected by RNA-based polymerase chain reaction in acute myelogenous leukemia containing the (8;21)(q22;q22) translocation. Blood. 1993 Jun 1;81(11):2860-2865.

Downing JR. The AML1-ETO chimaeric transcription factor in acute myeloid leukaemia: biology and clinical significance. Br J Haematol. 1999 Aug;106(2):296-308.

Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A. 1998 Dec 22;95(26):15665-15670.

Drexler HG, Thiel E, Ludwig WD. Review of the incidence and clinical relevance of myeloid antigen-positive acute lymphoblastic leukemia. Leukemia. 1991 Aug;5(8):637-645.

Dunphy CH. Comprehensive review of adult acute myelogenous leukemia: cytomorphological, enzyme cytochemical, flow cytometric immunophenotypic, and cytogenetic findings. J Clin Lab Anal. 1999;13(1):19-26.

Dutta P, Sazawal S, Kumar R, Saxena R.Does acute promyelocytic leukemia in Indian patients have biology different from the West? Indian J Pathol Microbiol. 2008 Jul-Sep;51(3):437-439.

Eckelman BP, Salvesen GS, Scott FL. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. EMBO Rep. 2006 Oct;7(10):988-994.

El-Sissy AH, El-Mashari MA, Bassuni WY, El-Swaayed AF. Aberrant lymphoid antigen expression in acute myeloid leukemia in Saudi Arabia. J Egypt Natl Canc Inst. 2006 Sep;18(3):244-249.

Ewart GD, Howells AJ. ABC transporters involved in transport of eye pigment precursors in Drosophila melanogaster. Methods Enzymol. 1998;292:213-224.

Faiz M, Iqbal QJ, Qureshi A. High prevalence of BCR-ABL fusion transcripts with poor prognostic impact among adult ALL patients: report from Pakistan. Asia Pac J Clin Oncol. 2011 Mar;7(1):47-55.

Falini B, Bolli N, Shan J, Martelli MP, Liso A, Pucciarini A, Bigerna B, Pasqualucci L, Mannucci R, Rosati R, Gorello P, Diverio D, Roti G, Tiacci E, Cazzaniga G, Biondi A, Schnittger S, Haferlach T, Hiddemann W, Martelli MF, Gu W, Mecucci C, Nicoletti I. Both carboxy-terminus NES motif and mutated tryptophan(s) are crucial for aberrant nuclear export of nucleophosmin leukemic mutants in NPMc+ AML. Blood. 2006 Jun 1;107(11):4514-23.

Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, La Starza R, Diverio D, Colombo E, Santucci A, Bigerna B, Pacini R, Pucciarini A, Liso A, Vignetti M, Fazi P, Meani N, Pettirossi V, Saglio G, Mandelli F, Lo-Coco F, Pelicci PG, Martelli MF; GIMEMA Acute Leukemia Working Party. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005 Jan 20;352(3):254-266.

Ferlay J, Shin HR, Bray F, Forman D, Mathers CD, Parkin D. GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10. Lyon, France: International Agency for Research on Cancer; Year. Available at: http://globocan.iarc.fr. 2010. Last accessed 8/17/2010.

Ferrandina G, Legge F, Martinelli E, Ranelletti FO, Zannoni GF, Lauriola L, Gessi M, Gallotta V, Scambia G. Survivin expression in ovarian cancer and its correlation with clinico-pathological, surgical and apoptosis-related parameters. Br J Cancer. 2005 Jan 31;92(2):271-277.

Flens MJ, Zaman GJ, van der Valk P, Izquierdo MA, Schroeijers AB, Scheffer GL, van der Groep P, de Haas M, Meijer CJ, Scheper RJ. Tissue distribution of the multidrug resistance protein. Am J Pathol. 1996 Apr;148(4):1237-1247.

Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM, Pastan I. Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci U S A. 1987 Jan;84(1):265-269.

Fojo T, Bates S. Strategies for reversing drug resistance. Oncogene. 2003 Oct 20;22(47):7512-7523.

Frohling S, Scholl C, Gilliland DG, Levine RL. Genetics of myeloid malignancies: pathogenetic and clinical implications. J Clin Oncol. 2005 Sep 10;23(26):6285-6295.

Fujimaki S, Funato T, Harigae H, Fujiwara J, Kameoka J, Meguro K, Kaku M, Sasaki T. Quantitative analysis of a MDR1 transcript for prediction of drug resistance in acute leukemia. Clin Chem. 2002 Jun;48(6 Pt 1):811-817.

Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK, Linch DC; Medical Research Council Adult Leukaemia Working Party.The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia.Blood. 2008 Mar 1;111(5):2776-2784.

Galimberti S, Rossi A, Palumbo GA, Morabito F, Guerrini F, Vincelli I, Fazzi R, Santini V, Petrini M. FLT3 mutations do not influence MDR-1 gene expression in acute myeloid leukemia. Anticancer Res. 2003 Jul-Aug;23(4):3419-3426.

Gallegos-Arreola MP, Batista-González CM, Delgado-Lamas JL, Figuera LE, Puebla-Pérez AM, Arnaud-López L, Peralta-Leal V, Ramírez-Jirano LJ, Zúñiga-González GM. Cytochrome P4501A1 polymorphism is associated with susceptibility to acute lymphoblastic leukemia in adult Mexican patients. Blood Cells Mol Dis. 2004 Nov-Dec;33(3):326-329.

Garte S, Gaspari L, Alexandrie AK, Ambrosone C, Autrup H, Autrup JL, Baranova H, Bathum L, Benhamou S, Boffetta P, Bouchardy C, Breskvar K, et al. Metabolic gene polymorphism frequencies in control populations. Cancer Epidemiol Biomarkers Prev. 2001 Dec;10(12):1239-1248.

Ghosh S, Shinde SC, Kumaran GS, Sapre RS, Dhond SR, Badrinath Y, Ansari R, Kumar A, Mahadik S, Chougule AB, Nair CN. Haematologic and immunophenotypic profile of acute myeloid leukemia: an experience of Tata Memorial Hospital. Indian J Cancer. 2003 Apr-Jun;40(2):71-76.

Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood. 2002 Sep 1;100(5):1532-1542.

Gilliland DG, Jordan CT, Felix CA. The molecular basis of leukemia. Hematology Am Soc Hematol Educ Program. 2004:80-97.

Glass DC, Gray CN, Jolley DJ, Gibbons C, Sim MR, Fritschi L, Adams GG, Bisby JA, Manuell R. Leukemia risk associated with low-level benzene exposure. Epidemiology. 2003 Sep;14(5):569-77.

Glatt H, Engelke CE, Pabel U, Teubner W, Jones AL, Coughtrie MW, Andrae U, Falany CN, Meinl W. Sulfotransferases: genetics and role in toxicology. Toxicol Lett. 2000 Mar 15;112-113:341-348.

Gleissner B, Gökbuget N, Bartram CR, Janssen B, Rieder H, Janssen JW, Fonatsch C, Heyll A, Voliotis D, Beck J, Lipp T, Munzert G, Maurer J, Hoelzer D, Thiel E; German Multicenter Trials of Adult Acute Lymphoblastic Leukemia Study Group. Leading prognostic relevance of the BCR-ABL translocation in adult acute B-lineage lymphoblastic leukemia: a prospective study of the German Multicenter Trial Group and confirmed polymerase chain reaction analysis. Blood. 2002 Mar 1;99(5):1536-1543.

Goga A, McLaughlin J, Afar DE, Saffran DC, Witte ON. Alternative signals to RAS for hematopoietic transformation by the BCR-ABL oncogene. Cell. 1995 Sep 22;82(6):981-988.

Gokbuget N, Arnold R, Buechner Th, et al. Intensification of induction and consolidation improves only subgroups of adult ALL: Analysis of 1200 patients in GMALL study 05/93 [abstract]. Blood. 2001;98:802a.

Goker E, Waltham M, Kheradpour A, Trippett T, Mazumdar M, Elisseyeff Y, Schnieders B, Steinherz P, Tan C, Berman E, et al. Amplification of the dihydrofolate reductase gene is a mechanism of acquired resistance to methotrexate in patients with acute lymphoblastic leukemia and is correlated with p53 gene mutations. Blood. 1995 Jul 15;86(2):677-684.

Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer. 2002 Jan;2(1):48-58.

Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V, Cioce M, Fanelli M, Ruthardt M, Ferrara FF, Zamir I, Seiser C, Grignani F, Lazar MA, Minucci S, Pelicci PG. Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature. 1998 Feb 19;391(6669):815-818.

Grignani F, Fagioli M, Alcalay M, Longo L, Pandolfi PP, Donti E, Biondi A, Lo Coco F, Grignani F, Pelicci PG. Acute promyelocytic leukemia: from genetics to treatment. Blood. 1994 Jan 1;83(1):10-25

Grignani F, Ferrucci PF, Testa U, Talamo G, Fagioli M, Alcalay M, Mencarelli A, Grignani F, Peschle C, Nicoletti I, et al. The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell. 1993 Aug 13;74(3):423-431.

Grimwade D, Howe K, Langabeer S, Davies L, Oliver F, Walker H, Swirsky D, Wheatley K, Goldstone A, Burnett A, Solomon E. Establishing the presence of the t(15;17) in suspected acute promyelocytic leukaemia: cytogenetic, molecular and PML immunofluorescence assessment of patients entered into the M.R.C. ATRA trial. M.R.C. Adult Leukaemia Working Party. Br J Haematol. 1996 Sep;94(3):557-573.

Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, Rees J, Hann I, Stevens R, Burnett A, Goldstone A. The importance of diagnostic cytogenetics on outcome in AML: analysis of

1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood. 1998 Oct 1;92(7):2322-2333.

Grisendi S, Mecucci C, Falini B, Pandolfi PP. Nucleophosmin and cancer. Nat Rev Cancer. 2006 Jul;6(7):493-496.

Groves FD, Linet MS, Devesa SS. Epidemiology of human leukemia. Curr Opin Hematol. 1994 Jul;1(4):321-326

Grundler R, Miething C, Thiede C, Peschel C, Duyster J. FLT3-ITD and tyrosine kinase domain mutants induce 2 distinct phenotypes in a murine bone marrow transplantation model. Blood. 2005 Jun 15;105(12):4792-4799.

Gu L, Findley HW, Zhou M. MDM2 induces NF-kappaB/p65 expression transcriptionally through Sp1-binding sites: a novel, p53-independent role of MDM2 in doxorubicin resistance in acute lymphoblastic leukemia. Blood. 2002 May 1;99(9):3367-3375

Gujral S, Badrinath Y, Kumar A, Subramanian PG, Raje G, Jain H, Pais A, Amre Kadam PS, Banavali SD, Arora B, Kumar P, Hari Menon VG, Kurkure PA, Parikh PM, Mahadik S, Chogule AB, Shinde SC, Nair CN. Immunophenotypic profile of acute leukemia: critical analysis and insights gained at a tertiary care center in India. Cytometry B Clin Cytom. 2009 May;76(3):199-205.

Gurbuxani S, Lacorte JM, Raina V, Arya LS, Pepin D, Sazawal S, Marie JP, Bhargava M. Detection of BCR-ABL transcripts in acute lymphoblastic leukemia in Indian patients. Leuk Res. 1998 Jan;22(1):77-80.

Haase D, Feuring-Buske M, Konemann S, Fonatsch C, Troff C, Verbeek W, Pekrun A, Hiddemann W, Wörmann B.Evidence for malignant transformation in acute myeloid leukemia at the level of early hematopoietic stem cells by cytogenetic analysis of CD34+ subpopulations. Blood. 1995 Oct 15;86(8):2906-2912.

Haferlach C, Mecucci C, Schnittger S, Kohlmann A, Mancini M, Cuneo A, Testoni N, Rege-Cambrin G, Santucci A, Vignetti M, Fazi P, Martelli MP, Haferlach T, Falini B. AML with mutated NPM1 carrying a normal or aberrant karyotype show overlapping biologic, pathologic, immunophenotypic, and prognostic features. Blood. 2009 Oct 1;114(14):3024-3032.

Hahn LW, Ritchie MD, Moore J.H. 2003. Multifactor dimensionality reduction software for detecting gene-gene and gene-environment interactions. Bioinformatics 12:376-382.

Hakansson P, Nilsson B, Andersson A, Lassen C, Gullberg U, Fioretos T. Gene expression analysis of BCR/ABL1-dependent transcriptional response reveals enrichment for genes involved in negative feedback regulation. Genes Chromosomes Cancer. 2008 Apr;47(4):267-275.

Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, Bloomfield CD. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol. 1999 Dec;17(12):3835-3849.

Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood. 1994 Sep 1;84(5):1361-1392.

Hayakawa F, Towatari M, Kiyoi H, Tanimoto M, Kitamura T, Saito H, Naoe T. Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. Oncogene. 2000 Feb 3;19(5):624-631.

Hayes JD, McLellan LI. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radic Res. 1999 Oct;31(4):273-300.

Hein DW. Molecular genetics and function of NAT1 and NAT2: role in aromatic amine metabolism and carcinogenesis. Mutat Res. 2002 Sep 30;506-507:65-77.

Helleberg C, Knudsen H, Hansen PB, Nikolajsen K, Kjaersgaard E, Ralfkiaer E, Johnsen HE.CD34+ megakaryoblastic leukaemic cells are CD38-, but CD61+ and glycophorin A+: improved criteria for diagnosis of AML-M7? Leukemia. 1997 Jun;11(6):830-834.

Herrera JE, Savkur R, Olson MO. The ribonuclease activity of nucleolar protein B23. Nucleic Acids Res. 1995 Oct 11;23(19):3974-3979.

Hiebert SW, Downing JR, Lenny N, Meyers S. Transcriptional regulation by the t(8;21) fusion protein, AML-1/ETO. Curr Top Microbiol Immunol. 1996;211:253-258.

Honjo Y, Hrycyna CA, Yan QW, Medina-Pérez WY, Robey RW, van de Laar A, Litman T, Dean M, Bates SE. Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. Cancer Res. 2001 Sep 15;61(18):6635-6639.

Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA, Edwards BK (eds). SEER Cancer Statistics Review, 1975-2008. based on November 2010 SEER data submission, posted to the SEER web site, 2011.

Hsu CY, Yung BY. Involvement of nucleophosmin/B23 in TPA-induced megakaryocytic differentiation of K562 cells. Br J Cancer. 2003 Oct 6;89(7):1320-1326.

Huang Q, Chen W, Gaal KK, Slovak ML, Stein A, Weiss LM.A rapid, one step assay for simultaneous detection of FLT3/ITD and NPM1 mutations in AML with normal cytogenetics. Br J Haematol. 2008 Jul;142(3):489-492. Munoz L, Aventín A, Villamor N, Juncà J, Acebedo G, Domingo A, Rozman M, Torres JP, Tormo M, Nomdedéu JF. Immunophenotypic findings in acute myeloid leukemia with FLT3 internal tandem duplication. Haematologica. 2003 Jun;88(6):637-645.

Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE, Higgins CF. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature. 1990 Jul 26;346(6282):362-365.

Ignaszak-Szczepaniak M, Horst-Sikorska W, Sawicka J, Kaczmarek M, Slomski R. The TP53 codon 72 polymorphism and predisposition to adrenocortical cancer in Polish patients. Oncol Rep. 2006 Jul;16(1):65-71.

Indian Council of Medical Research. National Cancer Registry Programme, three year report of population based cancer registries 2006-2008: National Cancer Registry Programme (ICMR), Bangalore, 2010.

Infante-Rivard C, Labuda D, Krajinovic M, Sinnett D. Risk of childhood leukemia associated with exposure to pesticides and with gene polymorphisms. Epidemiology. 1999 Sep;10(5):481-487.

Infante-Rivard C, Mathonnet G, Sinnett D. Risk of childhood leukemia associated with diagnostic irradiation and polymorphisms in DNA repair genes. Environ Health Perspect. 2000 Jun;108(6):495-498.

Insinga A, Monestiroli S, Ronzoni S, Carbone R, Pearson M, Pruneri G, Viale G, Appella E, Pelicci P, Minucci S. Impairment of p53 acetylation, stability and function by an oncogenic transcription factor. EMBO J. 2004 Mar 10;23(5):1144-54.

Izquierdo MA, Scheffer GL, Flens MJ, Giaccone G, Broxterman HJ, Meijer CJ, van der Valk P, Scheper RJ. Broad distribution of the multidrug resistance-related vault lung resistance protein in normal human tissues and tumors. Am J Pathol. 1996 Mar;148(3):877-887.

Izquierdo MA, Shoemaker RH, Flens MJ, Scheffer GL, Wu L, Prather TR, Scheper RJ. Overlapping phenotypes of multidrug resistance among panels of human cancer-cell lines. Int J Cancer. 1996 Jan 17;65(2):230-237.

Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 2003 Mar;33 Suppl:245-254.

Jaffe ES, Harris NL, Stein H, Vardiman JW, (eds). World Health Organization Classification of Tumors: Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2001.

Jakulin A, Bratko I. Analyzing attribute dependencies. In Lavrac,N., Gamberger,D., Blockeel,H. and Todorovski,L. (eds) PKDD 2003, Vol. 2838, Springer-Verlag, Cavtat, Croatia, 2003.

Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. Cancer Res. 1996 Mar 1;56(5):988-994.

Jeha S. Who should be treating adolescents and young adults with acute lymphoblastic leukaemia? Eur J Cancer. 2003 Dec;39(18):2579-2583.

Jia L, Macey MG, Yin Y, Newland AC, Kelsey SM. Subcellular distribution and redistribution of Bcl-2 family proteins in human leukemia cells undergoing apoptosis. Blood. 1999 Apr 1;93(7):2353-2359.

Joseph T, Kusumakumary P, Chacko P, Abraham A, Radhakrishna Pillai M. Genetic polymorphism of CYP1A1, CYP2D6, GSTM1 and GSTT1 and susceptibility to acute lymphoblastic leukaemia in Indian children. Pediatr Blood Cancer. 2004 Oct;43(5):560-567.

Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta. 1976 Nov 11;455(1):152-162.

Kamashev D, Vitoux D, De Thé H. PML-RARA-RXR oligomers mediate retinoid and rexinoid/cAMP cross-talk in acute promyelocytic leukemia cell differentiation. J Exp Med. 2004 Apr 19;199(8):1163-1174.

Kamesaki S, Kamesaki H, Jorgensen TJ, Tanizawa A, Pommier Y, Cossman J. bcl-2 protein inhibits etoposide-induced apoptosis through its effects on events subsequent to topoisomerase II-induced DNA strand breaks and their repair. Cancer Res. 1993 Sep 15;53(18):4251-4256.

Kanerva J, Tiirikainen M, Mäkipernaa A, Riikonen P, Möttönen M, Salmi TT, Krusius T, Saarinen-Pihkala UM. Multiple drug resistance mediated by P-glycoprotein is not a major factor in a slow response to therapy in childhood ALL. Pediatr Hematol Oncol. 1998 Jan-Feb;15(1):11-21.

Karakas T, Maurer U, Weidmann E, Miething CC, Hoelzer D, Bergmann L. High expression of bcl-2 mRNA as a determinant of poor prognosis in acute myeloid leukemia. Ann Oncol. 1998 Feb;9(2):159-165.

Kaufmann SH, Karp JE, Svingen PA, Krajewski S, Burke PJ, Gore SD, Reed JC. Elevated expression of the apoptotic regulator Mcl-1 at the time of leukemic relapse. Blood. 1998 Feb 1;91(3):991-1000.

Kawai S, Zha Z, Yamamoto Y, Shimizu H, Fujimoto T. Clinical significance of childhood acute myeloid leukemias expressing lymphoid-associated antigens. Pediatr Hematol Oncol. 1995 Sep-Oct;12(5):463-469.

Kayser S, Schlenk RF, Londono MC, Breitenbuecher F, Wittke K, Du J, Groner S, Späth D, Krauter J, Ganser A, Döhner H, Fischer T, Döhner K; German-Austrian AML Study Group (AMLSG). Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. Blood. 2009 Sep 17;114(12):2386-2392.

Kelly LM, Gilliland DG. Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet. 2002;3:179-198.

Keung YK, Beaty M, Powell BL, Molnar I, Buss D, Pettenati M. Philadelphia chromosome positive myelodysplastic syndrome and acute myeloid leukemia-retrospective study and review of literature. Leuk Res. 2004 Jun;28(6):579-586.

Khalidi HS, Chang KL, Medeiros LJ, Brynes RK, Slovak ML, Murata-Collins JL, Arber DA. Acute lymphoblastic leukemia. Survey of immunophenotype, French-American-British classification, frequency of myeloid antigen expression, and karyotypic abnormalities in 210 pediatric and adult cases. Am J Clin Pathol. 1999 Apr;111(4):467-476.

Khalidi HS, Medeiros LJ, Chang KL, Brynes RK, Slovak ML, Arber DA. The immunophenotype of adult acute myeloid leukemia: high frequency of lymphoid antigen expression and comparison of immunophenotype, French-American-British classification, and karyotypic abnormalities. Am J Clin Pathol. 1998 Feb;109(2):211-220.

Khawaja MR, Allana SS, Akbarali AN, Adil SN, Khurshid M, Pervez S. Flow cytometric and demographic analysis of t cell acute lymphoblastic leukemia in Pakistani population. J Ayub Med Coll Abbottabad. 2005 Oct-Dec;17(4):3-8.
Khurram MM, Jafri SA, Mannan A, Nadeem A, Jamal A. Frequency of aberrant expression of CD markers in cases of acute leukemia. Medical Journal of Islamic World Academy of Sciences.2010;18(2):55-60.

Kita K, Miwa H, Nakase K, Kawakami K, Kobayashi T, Shirakawa S, Tanaka I, Ohta C, Tsutani H, Oguma S, et al. Clinical importance of CD7 expression in acute myelocytic leukemia. The Japan Cooperative Group of Leukemia/Lymphoma. Blood. 1993 May 1;81(9):2399-2405.

Kitazono M, Sumizawa T, Takebayashi Y, Chen ZS, Furukawa T, Nagayama S, Tani A, Takao S, Aikou T, Akiyama S. Multidrug resistance and the lung resistance-related protein in human colon carcinoma SW-620 cells. J Natl Cancer Inst. 1999 Oct 6;91(19):1647-1653.

Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, Asou N, Kuriyama K, Jinnai I, Shimazaki C, Akiyama H, Saito K, Oh H, Motoji T, Omoto E, Saito H, Ohno R, Ueda R. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood. 1999 May 1;93(9):3074-3080.

Kiyoi H, Ohno R, Ueda R, Saito H, Naoe T. Mechanism of constitutive activation of FLT3 with internal tandem duplication in the juxtamembrane domain. Oncogene. 2002 Apr 11;21(16):2555-2563.

Koehler M, Behm FG, Shuster J, Crist W, Borowitz M, Look AT, Head D, Carroll AJ, Land V, Steuber P, et al. Transitional pre-B-cell acute lymphoblastic leukemia of childhood is associated with favorable prognostic clinical features and an excellent outcome: a Pediatric Oncology Group study. Leukemia. 1993 Dec;7(12):2064-2068.

Konoplev S, Huang X, Drabkin HA, Koeppen H, Jones D, Kantarjian HM, Garcia-Manero G, Chen W, Medeiros LJ, Bueso-Ramos CE. Cytoplasmic localization of nucleophosmin in bone marrow blasts of acute myeloid leukemia patients is not completely concordant with NPM1 mutation and is not predictive of prognosis. Cancer. 2009 Oct 15;115(20):4737-4744.

Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, Walker H, Wheatley K, Bowen DT, Burnett AK, Goldstone AH, Linch DC. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001 Sep 15;98(6):1752-1759.

Krajinovic M, Labuda D, Richer C, Karimi S, Sinnett D. Susceptibility to childhood acute lymphoblastic leukemia: influence of CYP1A1, CYP2D6, GSTM1, and GSTT1 genetic polymorphisms. Blood. 1999 Mar 1;93(5):1496-1501.

Krstovski N, Tosic N, Janic D, Dokmanovic L, Kuzmanovic M, Spasovski V, Pavlovic S. Incidence of FLT3 and nucleophosmin gene mutations in childhood acute myeloid leukemia: Serbian experience and the review of the literature. Med Oncol. 2010 Sep;27(3):640-645.

Lautier D, Canitrot Y, Deeley RG, Cole SP.Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. Biochem Pharmacol. 1996 Oct 11;52(7):967-977.

Lebailly P, Willett EV, Moorman AV, Roman E, Cartwright R, Morgan GJ, Wild CP. Genetic polymorphisms in microsomal epoxide hydrolase and susceptibility to adult acute myeloid leukaemia with defined cytogenetic abnormalities. Br J Haematol. 2002 Mar;116(3):587-594.

Lee JN, Kim HR, Shin JH, Joo YD. Prevalence of FLT3 internal tandem duplication in adult acute myelogenous leukemia. Korean J Lab Med. 2007 Aug;27(4):237-243.

Legrand O, Perrot JY, Baudard M, Cordier A, Lautier R, Simonin G, Zittoun R, Casadevall N, Marie JP. The immunophenotype of 177 adults with acute myeloid leukemia: proposal of a prognostic score. Blood. 2000 Aug 1;96(3):870-877.

Legrand O, Perrot JY, Simonin G, Baudard M, Cadiou M, Blanc C, Ramond S, Viguié F, Marie JP, Zittoun R. Adult biphenotypic acute leukaemia: an entity with poor prognosis which is related to unfavourable cytogenetics and P-glycoprotein over-expression. Br J Haematol. 1998 Jan;100(1):147-155.

Legrand O, Perrot JY, Tang R, Simonin G, Gurbuxani S, Zittoun R, Marie JP. Expression of the multidrug resistance-associated protein (MRP) mRNA and protein in normal peripheral blood and bone marrow haemopoietic cells. Br J Haematol. 1996 Jul;94(1):23-33.

Lewis RE, Cruse JM, Sanders CM, Webb RN, Tillman BF, Beason KL, Lam J, Koehler J.The immunophenotype of pre-TALL/LBL revisited. Exp Mol Pathol. 2006 Oct;81(2):162-165.

Li C, Wu Z, Liu M, Pazgier M, Lu W. Chemically synthesized human survivin does not inhibit caspase-3. Protein Sci. 2008 Sep;17(9):1624-1629.

Li Y, Zou D, Zhao Y, Mi Y, Wang J, Qiu L. Clinical characteristics and outcomes of adults with Philadelphia chromosome positive and/or bcr-abl positive acute lymphoblastic leukemia: a single center study from China. Leuk Lymphoma. 2010 Mar;51(3):488-496.

Liang DC, Shih LY, Hung IJ, Yang CP, Chen SH, Jaing TH, Liu HC, Chang WH. Clinical relevance of internal tandem duplication of the FLT3 gene in childhood acute myeloid leukemia. Cancer. 2002 Jun 15;94(12):3292-3298.

Lim LC, Heng KK, Vellupillai M, Tan LT, Boey BC, Lau LC, How GF. Molecular and phenotypic spectrum of de novo Philadelphia positive acute leukemia. Int J Mol Med. 1999 Dec;4(6):665-667.

Litman T, Brangi M, Hudson E, Fetsch P, Abati A, Ross DD, Miyake K, Resau JH, Bates SE. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J Cell Sci. 2000 Jun;113 (Pt 11):2011-2021.

Liu YR, Wang YZ, Chen SS, Chang Y, Fu JY, Li LD, Wang H, Yu H, Jiang B, Huang XJ. Analysis of immunophenotype and leukemia associated immunophenotype in 610 patients with acute myeloid leukemia. Zhonghua Xue Ye Xue Za Zhi. 2007 Nov;28(11):731-736.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-408.

Lockhart AC, Tirona RG, Kim RB. Pharmacogenetics of ATP-binding cassette transporters in cancer and chemotherapy. Mol Cancer Ther. 2003 Jul;2(7):685-698.

Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, Housman DE, Jacks T. p53 status and the efficacy of cancer therapy in vivo. Science. 1994 Nov 4;266(5186):807-810.

Mahjoubi F, Golalipour M, Ghavamzadeh A, Alimoghaddam K. Expression of MRP1 gene in acute leukemia. Sao Paulo Med J. 2008 May 1;126(3):172-179.

Mahoney MC, Moysich KB, McCarthy PL Jr, et al. The Chernobyl childhood leukemia study: background & lessons learned. Environ Health. 2004 Nov 8;3(1):12.

Majumdar S, Mondal BC, Ghosh M, Dey S, Mukhopadhyay A, Chandra S, Dasgupta UB. Association of cytochrome P450, glutathione S-transferase and N-acetyl transferase 2 gene polymorphisms with incidence of acute myeloid leukemia. Eur J Cancer Prev. 2008 Apr;17(2):125-132.

Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg AC, Schinkel AH, van De Vijver MJ, Scheper RJ, Schellens JH. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res. 2001 Apr 15;61(8):3458-3464.

Maliepaard M, van Gastelen MA, Tohgo A, Hausheer FH, van Waardenburg RC, de Jong LA, Pluim D, Beijnen JH, Schellens JH. Circumvention of breast cancer resistance protein (BCRP)-mediated resistance to camptothecins in vitro using non-substrate drugs or the BCRP inhibitor GF120918. Clin Cancer Res. 2001 Apr;7(4):935-941.

Mancini M, Scappaticci D, Cimino G, Nanni M, Derme V, Elia L, Tafuri A, Vignetti M, Vitale A, Cuneo A, Castoldi G, Saglio G, Pane F, Mecucci C, Camera A, Specchia G, Tedeschi A, Di Raimondo F, Fioritoni G, Fabbiano F, Marmont F, Ferrara F, Cascavilla N, Todeschini G, Nobile F, Kropp MG, Leoni P, Tabilio A, Luppi M, Annino L, Mandelli F, Foà R. A comprehensive genetic classification of adult acute lymphoblastic leukemia (ALL): analysis of the GIMEMA 0496 protocol. Blood. 2005 May 1;105(9):3434-3441.

Marie JP, Zhou DC, Gurbuxani S, Legrand O, Zittoun R. MDR1/P-glycoprotein in haematological neoplasms. Eur J Cancer. 1996 Jun;32A(6):1034-1038.

Marks DI, Paietta EM, Moorman AV, Richards SM, Buck G, DeWald G, Ferrando A, Fielding AK, Goldstone AH, Ketterling RP, Litzow MR, Luger SM, McMillan AK, Mansour MR, Rowe JM, Tallman MS, Lazarus HM. T-cell acute lymphoblastic leukemia in adults: clinical features, immunophenotype, cytogenetics, and outcome from the large randomized prospective trial (UKALL XII/ECOG 2993). Blood. 2009 Dec 10;114(25):5136-5145.

McCarver DG, Hines RN. The ontogeny of human drug-metabolizing enzymes: phase II conjugation enzymes and regulatory mechanisms. J Pharmacol Exp Ther. 2002 Feb;300(2):361-366.

McKenna SL, Padua RA. Multidrug resistance in leukaemia. Br J Haematol. 1997 Mar;96(4):659-674.

Melo JV, Deininger MW. Biology of chronic myelogenous leukemia--signaling pathways of initiation and transformation. Hematol Oncol Clin North Am. 2004 Jun;18(3):545-568.

Melo RA, de Vasconcellos JF, Melo FC, Machado CG, Lacerda TM, Souto FR.PML-RARalpha fusion gene transcripts and biological features in acute promyelocytic leukemia patients. Clin Lab Haematol. 2006 Apr;28(2):126-129.

Meshinchi S, Woods WG, Stirewalt DL, Sweetser DA, Buckley JD, Tjoa TK, Bernstein ID, Radich JP. Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. Blood. 2001 Jan 1;97(1):89-94.

Metcalf D. Lineage commitment and maturation in hematopoietic cells: the case for extrinsic regulation. Blood. 1998 Jul 15;92(2):345-347;

Mi Y, Bian S, Chen G. Study on the expression of myeloid markers and CD34 antigen in adult acute lymphoblastic leukemia. Zhonghua Xue Ye Xue Za Zhi. 1999 Feb;20(2):76-78.

Michieli M, Damiani D, Ermacora A, Masolini P, Raspadori D, Visani G, Scheper RJ, Baccarani M. P-glycoprotein, lung resistance-related protein and multidrug resistance associated protein in de novo acute non-lymphocytic leukaemias: biological and clinical implications. Br J Haematol. 1999 Feb;104(2):328-335.

Miller DP, Liu G, De Vivo I, Lynch TJ, Wain JC, Su L, Christiani DC. Combinations of the variant genotypes of GSTP1, GSTM1, and p53 are associated with an increased lung cancer risk. Cancer Res. 2002 May 15;62(10):2819-2823.

Miller KB, Daonst PR. Clinical Manifestation of Acute Myeloid Leukemia. In: Hoffman R, et al, editor. Haematology Basic Principles and Practice. 3rd edn. Pennsylvania: Churchill Livingstone; 2000.

Mitra S, Misra C, Singh RK, Panda CK, Roychoudhury S. Association of specific genotype and haplotype of p53 gene with cervical cancer in India. J Clin Pathol. 2005 Jan;58(1):26-31.

Miyata M, Kudo G, Lee YH, Yang TJ, Gelboin HV, Fernandez-Salguero P, Kimura S, Gonzalez FJ. Targeted disruption of the microsomal epoxide hydrolase gene. Microsomal epoxide hydrolase is required for the carcinogenic activity of 7,12-dimethylbenz[a]anthracene. J Biol Chem. 1999 Aug 20;274(34):23963-23968.

Mizuki M, Fenski R, Halfter H, Matsumura I, Schmidt R, Müller C, Grüning W, Kratz-Albers K, Serve S, Steur C, Büchner T, Kienast J, Kanakura Y, Berdel WE, Serve H. Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. Blood. 2000 Dec 1;96(12):3907-3914.

Moorman AV, Harrison CJ, Buck GA, Richards SM, Secker-Walker LM, Martineau M, Vance GH, Cherry AM, Higgins RR, Fielding AK, Foroni L, Paietta E, Tallman MS, Litzow MR, Wiernik PH, Rowe JM, Goldstone AH, Dewald GW; Adult Leukaemia Working Party, Medical Research Council/National Cancer Research Institute. Karyotype is an independent prognostic factor in adult acute lymphoblastic leukemia (ALL): analysis of cytogenetic data from patients treated on the Medical Research Council (MRC) UKALLXII/Eastern Cooperative Oncology Group (ECOG) 2993 trial. Blood. 2007 Apr 15;109(8):3189-3197.

Mori Y, Yoshimoto G, Kumano T, Miyamoto T, Iino T, Takenaka K, Iwasaki H, Harada N, Kinukawa N, Nagafuji K, Teshima T, Shimoda K, Akashi K, Harada M. Distinctive expression of myelomonocytic markers and down-regulation of CD34 in acute myelogenous leukaemia with FLT3 tandem duplication and nucleophosmin mutation. Eur J Haematol. 2007 Jul;79(1):17-24.

Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. Blood Rev. 2004 Jun;18(2):115-136.

Nagai F, Hiyoshi Y, Sugimachi K, Tamura HO. Cytochrome P450 (CYP) expression in human myeloblastic and lymphoid cell lines. Biol Pharm Bull. 2002 Mar;25(3):383-385.

Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, Sonoda Y, Fujimoto T, Misawa S. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia. 1996 Dec;10(12):1911-1918.

Nakase K, Bradstock K, Sartor M, Gottlieb D, Byth K, Kita K, Shiku H, Kamada N. Geographic heterogeneity of cellular characteristics of acute myeloid leukemia: a comparative study of Australian and Japanese adult cases. Leukemia. 2000 Jan;14(1):163-168.

Nazar-Stewart V, Vaughan TL, Stapleton P, Van Loo J, Nicol-Blades B, Eaton DL. A population-based study of glutathione S-transferase M1, T1 and P1 genotypes and risk for lung cancer. Lung Cancer. 2003 Jun;40(3):247-258.

Ng SM, Ariffin WA, Lin HP, Chan LL, Chin YM. Clinical features and treatment outcome of children with myeloid antigen coexpression in B-lineage acute lymphoblastic leukemia: a study of 151 Malaysian children. J Trop Pediatr. 2000 Apr;46(2):73-78.

Nishii K, Usui E, Katayama N, Lorenzo F 5th, Nakase K, Kobayashi T, Miwa H, Mizutani M, Tanaka I, Nasu K, Dohy H, Kyo T, Taniwaki M, Ueda T, Kita K, Shiku H. Characteristics of t(8;21) acute myeloid leukemia (AML) with additional chromosomal abnormality: concomitant trisomy 4 may constitute a distinctive subtype of t(8;21) AML. Leukemia. 2003 Apr;17(4):731-737.

Nishimura Y, Ohkubo T, Furuichi Y, Umekawa H. Tryptophans 286 and 288 in the C-terminal region of protein B23.1 are important for its nucleolar localization. Biosci Biotechnol Biochem. 2002 Oct;66(10):2239-2242.

Nordlinder R, Jarvholm B. Environmental exposure to gasoline and leukemia in children and young adults--an ecology study. Int Arch Occup Environ Health. 1997;70(1):57-60.

Nowell S, Ambrosone CB, Ozawa S, MacLeod SL, Mrackova G, Williams S, Plaxco J, Kadlubar FF, Lang NP. Relationship of phenol sulfotransferase activity (SULT1A1) genotype to sulfotransferase phenotype in platelet cytosol. Pharmacogenetics. 2000 Dec;10(9):789-797.

Nucifora G, Rowley JD. AML1 and the 8;21 and 3;21 translocations in acute and chronic myeloid leukemia. Blood. 1995 Jul 1;86(1):1-14.

O'Connor SM, Boneva RS. Infectious etiologies of childhood leukemia: plausibility and challenges to proof. Environ Health Perspect. 2007 Jan;115(1):146-150.

Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell. 1996 Jan 26;84(2):321-330.

Olson MO, Wallace MO, Herrera AH, Marshall-Carlson L, Hunt RC. Preribosomal ribonucleoprotein particles are a major component of a nucleolar matrix fraction. Biochemistry. 1986 Jan 28;25(2):484-491.

Ormerod MG, O'Neill C, Robertson D, Kelland LR, Harrap KR. cis-Diamminedichloroplatinum(II)-induced cell death through apoptosis in sensitive and resistant human ovarian carcinoma cell lines. Cancer Chemother Pharmacol. 1996;37(5):463-471.

Paietta E, Racevskis J, Bennett JM, Neuberg D, Cassileth PA, Rowe JM, Wiernik PH.Biologic heterogeneity in Philadelphia chromosome-positive acute leukemia with myeloid morphology: the Eastern Cooperative Oncology Group experience. Leukemia. 1998 Dec;12(12):1881-1885.

Pennati M, Folini M, Zaffaroni N. Targeting survivin in cancer therapy: fulfilled promises and open questions. Carcinogenesis. 2007 Jun;28(6):1133-1139.

Perera FP. Molecular epidemiology: insights into cancer susceptibility, risk assessment, and prevention. J Natl Cancer Inst. 1996 Apr 17;88(8):496-509.

Pietsch EC, Humbey O, Murphy ME. Polymorphisms in the p53 pathway. Oncogene. 2006 Mar 13;25(11):1602-1611.

Pituch-Noworolska A. Biological properties and sensitivity to induction therapy of differentiated cells expressing atypical immunophenotype in acute leukemia of children. Folia Med Cracov. 2001;42(3):5-80.

Plasschaert SL, de Bont ES, Boezen M, vander Kolk DM, Daenen SM, Faber KN, Kamps WA, de Vries EG, Vellenga E. Expression of multidrug resistance-associated proteins predicts prognosis in childhood and adult acute lymphoblastic leukemia. Clin Cancer Res. 2005 Dec 15;11(24 Pt 1):8661-8668.

Plasschaert SL, Vellenga E, de Bont ES, van der Kolk DM, Veerman AJ, Sluiter WJ, Daenen SM, de Vries EG, Kamps WA. High functional P-glycoprotein activity is more often present in T-cell acute lymphoblastic leukaemic cells in adults than in children. Leuk Lymphoma. 2003 Jan;44(1):85-95.

Pogoda JM, Nichols PW, Preston-Martin S. Alcohol consumption and risk of adult-onset acute myeloid leukemia: results from a Los Angeles County case-control study. Leuk Res. 2004 Sep;28(9):927-31.

Pogoda JM, Preston-Martin S, Nichols PW, Ross RK.Smoking and risk of acute myeloid leukemia: results from a Los Angeles County case-control study. Am J Epidemiol. 2002 Mar 15;155(6):546-553.

Potapova O, Haghighi A, Bost F, Liu C, Birrer MJ, Gjerset R, Mercola D. The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. J Biol Chem. 1997 May 30;272(22):14041-14044.

Preston DL, Kusumi S, Tomonaga M, Izumi S, Ron E, Kuramoto A, Kamada N, Dohy H, Matsuo T, Matsui T [corrected to Matsuo T, et al. Cancer incidence in atomic bomb survivors. Part III. Leukemia, lymphoma and multiple myeloma, 1950-1987. Radiat Res. 1994 Feb;137(2 Suppl):S68-97.

Pui CH, Behm FG, Crist WM. Clinical and biologic relevance of immunologic marker studies in childhood acute lymphoblastic leukemia. Blood. 1993 Jul 15;82(2):343-362.

Pui CH, Crist WM. Treatment of childhood leukemias. Curr Opin Oncol. 1995 Jan;7(1):36-44.

Pui CH, Pei D, Campana D, Bowman WP, Sandlund JT, Kaste SC, Ribeiro RC, Rubnitz JE, Coustan-Smith E, Jeha S, Cheng C, Metzger ML, Bhojwani D, Inaba H, Raimondi SC, Onciu M, Howard SC, Leung W, Downing JR, Evans WE, Relling MV.Improved prognosis for older adolescents with acute lymphoblastic leukemia. J Clin Oncol. 2011 Feb 1;29(4):386-391.

Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia.N Engl J Med. 2004 Apr 8;350(15):1535-48.

Pui CH, Raimondi SC, Hancock ML, Rivera GK, Ribeiro RC, Mahmoud HH, Sandlund JT, Crist WM, Behm FG. Immunologic, cytogenetic, and clinical characterization of childhood acute lymphoblastic leukemia with the t(1;19) (q23; p13) or its derivative. J Clin Oncol. 1994 Dec;12(12):2601-2606.

Puil L, Liu J, Gish G, Mbamalu G, Bowtell D, Pelicci PG, Arlinghaus R, Pawson T. Bcr-Abl oncoproteins bind directly to activators of the Ras signalling pathway. EMBO J. 1994 Feb 15;13(4):764-773.

Pullarkat V, Slovak ML, Kopecky KJ, Forman SJ, Appelbaum FR. Impact of cytogenetics on the outcome of adult acute lymphoblastic leukemia: results of Southwest Oncology Group 9400 study. Blood. 2008 Mar 1;111(5):2563-72.

Pulte D, Gondos A, Brenner H. Improvements in survival of adults diagnosed with acute myeloblastic leukemia in the early 21st century. Haematologica. 2008 Apr;93(4):594-600.

Quinones LA, Irarrázabal CE, Rojas CR, Orellana CE, Acevedo C, Huidobro C, Varela NE, Cáceres DD. Joint effect among p53, CYP1A1, GSTM1 polymorphism combinations and smoking on prostate cancer risk: an exploratory genotype-environment interaction study. Asian J Androl. 2006 May;8(3):349-355.

Raaijmakers HG, Izquierdo MA, Lokhorst HM, de Leeuw C, Belien JA, Bloem AC, Dekker AW, Scheper RJ, Sonneveld P. Lung-resistance-related protein expression is a negative predictive factor for response to conventional low but not to intensified dose alkylating chemotherapy in multiple myeloma. Blood. 1998 Feb 1;91(3):1029-1036.

Radich J, Gehly G, Lee A, Avery R, Bryant E, Edmands S, Gooley T, Kessler P, Kirk J, Ladne P, Thomas ED, Appelbaum FR.Detection of bcr-abl transcripts in Philadelphia chromosome-positive acute lymphoblastic leukemia after marrow transplantation. Blood. 1997 Apr 1;89(7):2602-9.

Rajalekshmy KR, Abitha AR, Pramila R, Gnanasagar T, Shanta V. Immunophenotypic analysis of T-cell acute lymphoblastic leukaemia in Madras, India. Leuk Res. 1997 Feb;21(2):119-24.

Ramakers-van Woerden NL, Beverloo HB, Veerman AJ, Camitta BM, Loonen AH, van Wering ER, Slater RM, Harbott J, den Boer ML, Ludwig WD, Haas OA, Janka-Schaub GE, Pieters R. In vitro drug-resistance profile in infant acute lymphoblastic leukemia in relation to age, MLL rearrangements and immunophenotype. Leukemia. 2004 Mar;18(3):521-529.

Raspadori D, Lauria F, Ventura MA, Rondelli D, Visani G, de Vivo A, Tura S. Incidence and prognostic relevance of CD34 expression in acute myeloblastic leukemia: analysis of 141 cases. Leuk Res. 1997 Jul;21(7):603-607.

Rausei-Mills V, Chang KL, Gaal KK, Weiss LM, Huang Q. Aberrant expression of CD7 in myeloblasts is highly associated with de novo acute myeloid leukemias with FLT3/ITD mutation. Am J Clin Pathol. 2008 Apr;129(4):624-629.

Ries LAG, Harkins D, Krapcho M et al (2006) SEER cancer statistics review, 1975–2003. National Cancer Institute, Bethesda, Maryland.

Rigato I, Pascolo L, Fernetti C, Ostrow JD, Tiribelli C. The human multidrug-resistance-associated protein MRP1 mediates ATP-dependent transport of unconjugated bilirubin. Biochem J. 2004 Oct 15;383(Pt 2):335-341.

Ritchie MD, Hahn LW, Moore JH. Power of multifactor dimensionality reduction for detecting gene-gene interactions in the presence of genotyping error, missing data, phenocopy, and genetic heterogeneity. Genet Epidemiol. 2003 Feb;24(2):150-157.

Ritchie MD, Hahn LW, Roodi N, Bailey LR, Dupont WD, Parl FF, Moore JH. Multifactor-dimensionality reduction reveals high-order interactions among estrogen-metabolism genes in sporadic breast cancer. Am J Hum Genet. 2001 Jul;69(1):138-147.

Robak T, Wierzbowska A. Current and emerging therapies for acute myeloid leukemia. Clin Ther. 2009;31 Pt 2:2349-2370.

Robey RW, Medina-Pérez WY, Nishiyama K, Lahusen T, Miyake K, Litman T, Senderowicz AM, Ross DD, Bates SE. Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. Clin Cancer Res. 2001 Jan;7(1):145-152.

Rocnik JL, Okabe R, Yu JC, Lee BH, Giese N, Schenkein DP, Gilliland DG. Roles of tyrosine 589 and 591 in STAT5 activation and transformation mediated by FLT3-ITD. Blood. 2006 Aug 15;108(4):1339-45.

Rogounovitch TI, Saenko VA, Ashizawa K, Sedliarou IA, Namba H, Abrosimov AY, Lushnikov EF, Roumiantsev PO, Konova MV, Petoukhova NS, Tchebotareva IV, Ivanov VK, Chekin SY, Bogdanova TI, Tronko MD, Tsyb AF, Thomas GA, Yamashita S. TP53 codon 72 polymorphism in radiation-associated human papillary thyroid cancer. Oncol Rep. 2006 Apr;15(4):949-956.

Rollinson S, Roddam P, Kane E, Roman E, Cartwright R, Jack A, Morgan GJ. Polymorphic variation within the glutathione S-transferase genes and risk of adult acute leukaemia. Carcinogenesis. 2000 Jan;21(1):43-47.

Rosline H, Narazah M. Y, Illunihayati I, Isa M.N, Baba AA. The Detection of AML1/ETO Fusion Transcript in Acute Myeloid Leukaemia in Universiti Sains Malaysia Hospital. Asian jounal of Molecular and Biotechnology, 2004 12(1):49-52.

Rosnet O, Stephenson D, Mattei MG, Marchetto S, Shibuya M, Chapman VM, Birnbaum D. Close physical linkage of the FLT1 and FLT3 genes on chromosome 13 in man and chromosome 5 in mouse. Oncogene. 1993 Jan;8(1):173-179.

Ross DD, Karp JE, Chen TT, Doyle LA. Expression of breast cancer resistance protein in blast cells from patients with acute leukemia. Blood. 2000 Jul 1;96(1):365-368.

Rothe G, Schmitz G. Consensus protocol for the flow cytometric immunophenotyping of hematopoietic malignancies. Working Group on Flow Cytometry and Image Analysis. Leukemia. 1996 May;10(5):877-895.

Rowe JM, Buck G, Burnett AK, Chopra R, Wiernik PH, Richards SM, Lazarus HM, Franklin IM, Litzow MR, Ciobanu N, Prentice HG, Durrant J, Tallman MS, Goldstone AH; ECOG; MRC/NCRI Adult Leukemia Working Party. Induction therapy for adults with acute lymphoblastic leukemia: results of more than 1500 patients from the international ALL trial: MRC UKALL XII/ECOG E2993.Blood. 2005 Dec 1;106(12):3760-3767.

Rowley JD. Identificaton of a translocation with quinacrine fluorescence in a patient with acute leukemia. Ann Genet. 1973a Jun;16(2):109-112

Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature. 1973 Jun 1;243(5405):290-293

Rubnitz JE, Raimondi SC, Halbert AR, Tong X, Srivastava DK, Razzouk BI, Pui CH, Downing JR, Ribeiro RC, Behm FG. Characteristics and outcome of t(8;21)-positive childhood acute myeloid leukemia: a single institution's experience. Leukemia. 2002 Oct;16(10):2072-2077.

Ryberg D, Skaug V, Hewer A, Phillips DH, Harries LW, Wolf CR, Ogreid D, Ulvik A, Vu P, Haugen A. Genotypes of glutathione transferase M1 and P1 and their significance for lung DNA adduct levels and cancer risk. Carcinogenesis. 1997 Jul;18(7):1285-1289.

Saadat I, Saadat M. The glutathione S-transferase mu polymorphism and susceptibility to acute lymphocytic leukemia. Cancer Lett. 2000 Sep 29;158(1):43-45.

Sam SS, Thomas V, Reddy SK, Surianarayanan G, Chandrasekaran A. CYP1A1 polymorphisms and the risk of upper aerodigestive tract cancers in an Indian population. Head Neck. 2008 Dec;30(12):1566-1574.

Santos FP, Jones D, Qiao W, Cortes JE, Ravandi F, Estey EE, Verma D, Kantarjian H, Borthakur G.Prognostic value of FLT3 mutations among different cytogenetic subgroups in acute myeloid leukemia. Cancer. 2011 May 15;117(10):2145-2155.

Sauerbrey A, Sell W, Steinbach D, Voigt A, Zintl F. Expression of the BCRP gene ABCG2/MXR/ABCP) in childhood acute lymphoblastic leukaemia. Br J Haematol. 2002 Jul;118(1):147-150.

Sazawal S, Bhatia K, Gutierrez MI, Saxena R, Arya LS, Bhargava M. Paucity of TEL-AML 1 translocation, by multiplex RT-PCR, in B-lineage acute lymphoblastic leukemia (ALL) in Indian patients. Am J Hematol. 2004 May;76(1):80-82.

Sazawal S, Kumar B, Hasan SK, Dutta P, Kumar R, Chaubey R, Mir R, Saxena R. Haematological & molecular profile of acute myelogenous leukaemia in India. Indian J Med Res. 2009 Mar;129(3):256-261.

Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood. 2002 Jan 15;99(2):507-512.

Scheper RJ, Broxterman HJ, Scheffer GL, Kaaijk P, Dalton WS, van Heijningen TH, van Kalken CK, Slovak ML, de Vries EG, van der Valk P, et al. Overexpression of a M(r) 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res. 1993 Apr 1;53(7):1475-1479.

Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L, Habdank M, Späth D, Morgan M, Benner A, Schlegelberger B, Heil G, Ganser A, Döhner H; German-Austrian Acute Myeloid Leukemia Study Group. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008 May 1;358(18):1909-1918.

Schlenk RF, Döhner K. Impact of new prognostic markers in treatment decisions in acute myeloid leukemia. Curr Opin Hematol. 2009 Mar;16(2):98-104.

Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, Löffler H, Sauerland CM, Serve H, Büchner T, Haferlach T, Hiddemann W. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood. 2002 Jul 1;100(1):59-66.

Schnittger S, Schoch C, Kern W, Mecucci C, Tschulik C, Martelli MF, Haferlach T, Hiddemann W, Falini B. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood. 2005 Dec 1;106(12):3733-3739.

Scholl S, Theuer C, Scheble V, Kunert C, Heller A, Mügge LO, Fricke HJ, Höffken K, Wedding U. Clinical impact of nucleophosmin mutations and Flt3 internal tandem duplications in patients older than 60 yr with acute myeloid leukaemia. Eur J Haematol. 2008 Mar;80(3):208-215.

Seedhouse CH, Pallis M, Grundy M, Shang S, Russell NH. FLT3-ITD expression levels and their effect on STAT5 in AML with and without NPM mutations. Br J Haematol. 2009 Dec;147(5):653-661.

SEER Cancer Statistic Review, 1973-1999. National Cancer Institute, Bethesda, MD, 2000. p.467.

Segalla S, Rinaldi L, Kilstrup-Nielsen C, Badaracco G, Minucci S, Pelicci PG, Landsberger N. Retinoic acid receptor alpha fusion to PML affects its transcriptional and chromatin-remodeling properties. Mol Cell Biol. 2003 Dec;23(23):8795-8808.

Sheikhha MH, Awan A, Tobal K, Liu Yin JA. Prognostic significance of FLT3 ITD and D835 mutations in AML patients. Hematol J. 2003;4(1):41-46.

Shen HQ, Tang YM, Yang SL, Qian BQ, Song H, Shi SW, Xu WQ. Immunophenotyping of 222 children with acute leukemia by multi-color flow cytometry. Zhonghua Er Ke Za Zhi. 2003 May;41(5):334-337.

Shimada A, Taki T, Kubota C, Tawa A, Horibe K, Tsuchida M, Hanada R, Tsukimoto I, Hayashi Y. No nucleophosmin mutations in pediatric acute myeloid leukemia with normal karyotype: a study of the Japanese Childhood AML Cooperative Study Group. Leukemia. 2007 Jun;21(6):1307.

Shimada T, Yamazaki H, Oda Y, Hiratsuka A, Watabe T, Guengerich FP. Activation and inactivation of carcinogenic dihaloalkanes and other compounds by glutathione S-transferase 5-5 in Salmonella typhimurium tester strain NM5004. Chem Res Toxicol. 1996 Jan-Feb;9(1):333-340.

Silveira Vda S, Canalle R, Scrideli CA, Queiroz RG, Tone LG. Role of the CYP2D6, EPHX1, MPO, and NQO1 genes in the susceptibility to acute lymphoblastic leukemia in Brazilian children. Environ Mol Mutagen. 2010 Jan;51(1):48-56.

Sinnett D, Krajinovic M, Labuda D. Genetic susceptibility to childhood acute lymphoblastic leukemia. Leuk Lymphoma. 2000 Aug;38(5-6):447-462.

Siraj AK, Kamat S, Gutiérrez MI, Banavali S, Timpson G, Sazawal S, Bhargava M, Advani S, Adde M, Magrath I, Bhatia K. Frequencies of the major subgroups of precursor B-cell acute lymphoblastic leukemia in Indian children differ from the West. Leukemia. 2003 Jun;17(6):1192-1193.

Siraj AK, Ozbek U, Sazawal S, Sirma S, Timson G, Al-Nasser A, Bhargava M, El Solh H, Bhatia K, Gutiérrez MI. Preclinical validation of a monochrome real-time multiplex assay for translocations in childhood acute lymphoblastic leukemia. Clin Cancer Res. 2002 Dec;8(12):3832-3840.

Slovak ML, Ho JP, Cole SP, Deeley RG, Greenberger L, de Vries EG, Broxterman HJ, Scheffer GL, Scheper RJ. The LRP gene encoding a major vault protein associated with drug resistance maps proximal to MRP on chromosome 16: evidence that chromosome breakage plays a key role in MRP or LRP gene amplification. Cancer Res. 1995 Oct 1;55(19):4214-4219.

Smith M, Barnett M, Bassan R, Gatta G, Tondini C, Kern W. Adult acute myeloid leukaemia. Crit Rev Oncol Hematol. 2004 Jun;50(3):197-222.

Soupir CP, Vergilio JA, Dal Cin P, Muzikansky A, Kantarjian H, Jones D, Hasserjian RP. Philadelphia chromosome-positive acute myeloid leukemia: a rare aggressive leukemia with clinicopathologic features distinct from chronic myeloid leukemia in myeloid blast crisis. Am J Clin Pathol. 2007 Apr;127(4):642-650.

Sparks R, Ulrich CM, Bigler J, Tworoger SS, Yasui Y, Rajan KB, Porter P, Stanczyk FZ, Ballard-Barbash R, Yuan X, Lin MG, McVarish L, Aiello EJ, McTiernan A. UDP-glucuronosyltransferase and sulfotransferase polymorphisms, sex hormone concentrations, and tumor receptor status in breast cancer patients. Breast Cancer Res. 2004;6(5):R488-498.

Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. Nat Rev Cancer. 2002 Jul;2(7):502-513.

Sperling C, Büchner T, Creutzig U, Ritter J, Harbott J, Fonatsch C, Sauerland C, Mielcarek M, Maschmeyer G, Löffler H, et al. Clinical, morphologic, cytogenetic and prognostic implications of CD34 expression in childhood and adult de novo AML. Leuk Lymphoma. 1995 May;17(5-6):417-426.

Sritana N, Auewarakul CU.KIT and FLT3 receptor tyrosine kinase mutations in acute myeloid leukemia with favorable cytogenetics: two novel mutations and selective occurrence in leukemia subtypes and age groups. Exp Mol Pathol. 2008 Dec;85(3):227-231.

Stam RW, van den Heuvel-Eibrink MM, den Boer ML, Ebus ME, Janka-Schaub GE, Allen JD, Pieters R. Multidrug resistance genes in infant acute lymphoblastic leukemia: Ara-C is not a substrate for the breast cancer resistance protein. Leukemia. 2004 Jan;18(1):78-83.

Stavrovskaya AA. Cellular mechanisms of multidrug resistance of tumor cells. Biochemistry (Mosc). 2000 Jan;65(1):95-106.

Steinbach D, Sell W, Voigt A, Hermann J, Zintl F, Sauerbrey A. BCRP gene expression is associated with a poor response to remission induction therapy in childhood acute myeloid leukemia. Leukemia. 2002 Aug;16(8):1443-1447.

Steinberg D, Colla P, P. CART- classification andregression trees. San Diego: Salford Systems. 1997.

Stelzer GT, Goodpasture L. Use of multiparameter flow cytometry and immunophenotyping for the diagnosis and classification of acute myeloid leukemia. In: Stewart CC, Nicholson JKA, eds. Immunophenotyping. New York, NY:Wiley- Liss; 2000:215–238.

Stirewalt DL, Kopecky KJ, Meshinchi S, Engel JH, Pogosova-Agadjanyan EL, Linsley J, Slovak ML, Willman CL, Radich JP. Size of FLT3 internal tandem duplication has prognostic significance in patients with acute myeloid leukemiaBlood. 2006 May 1;107(9):3724-3726.

Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer. 2003 Sep;3(9):650-665

Stock W, La M, Sanford B, Bloomfield CD, Vardiman JW, Gaynon P, Larson RA, Nachman J; Children's Cancer Group; Cancer and Leukemia Group B studies. What determines the outcomes for adolescents and young adults with acute lymphoblastic leukemia treated on cooperative group protocols? A comparison of Children's Cancer Group and Cancer and Leukemia Group B studies. Blood. 2008 Sep 1;112(5):1646-1654.

Stubbs MC, Kim YM, Krivtsov AV, Wright RD, Feng Z, Agarwal J, Kung AL, Armstrong SA. MLL-AF9 and FLT3 cooperation in acute myelogenous leukemia: development of a model for rapid therapeutic assessment. Leukemia. 2008 Jan;22(1):66-77.

Sudhakar N, Rajalekshmy KR, Rajkumar T, Nancy KN. RT-PCR and real-time PCR analysis of E2A-PBX1, TEL-AML1, mBCR-ABL and MLL-AF4 fusion gene transcripts in de novo B-lineage acute lymphoblastic leukaemia patients in south India. J Genet. 2011 Aug;90(2):349-353.

Suggs JL, Cruse JM, Lewis RE. Aberrant myeloid marker expression in precursor B-cell and T-cell leukemias. Exp Mol Pathol. 2007 Dec;83(3):471-473.

Suzuki T, Kiyoi H, Ozeki K, Tomita A, Yamaji S, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Ueda R, Kinoshita T, Emi N, Naoe T. Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood. 2005 Oct 15;106(8):2854-2861.

Szebeni A, Olson MO. Nucleolar protein B23 has molecular chaperone activities. Protein Sci. 1999 Apr;8(4):905-912.

T, Kiyoi H, Ozeki K, Tomita A, Yamaji S, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Ueda R, Kinoshita T, Emi N, Naoe T. Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood. 2005 Oct 15;106(8):2854-2861.

Tafuri A, Gregorj C, Petrucci MT, Ricciardi MR, Mancini M, Cimino G, Mecucci C, Tedeschi A, Fioritoni G, Ferrara F, Di Raimondo F, Gallo E, Liso V, Fabbiano F, Cascavilla N, Pizzolo G, Camera A, Pane F, Lanza F, Cilloni D, Annino L, Vitale A, Vegna ML, Vignetti M, Foà R, Mandelli F; GIMEMA Group. MDR1 protein expression is an independent predictor of complete remission in newly diagnosed adult acute lymphoblastic leukemia. Blood. 2002 Aug 1;100(3):974-981.

Takai N, Miyazaki T, Nishida M, Nasu K, Miyakawa I. Survivin expression correlates with clinical stage, histological grade, invasive behavior and survival rate in endometrial carcinoma. Cancer Lett. 2002 Oct 8;184(1):105-116.

Talseth BA, Meldrum C, Suchy J, Kurzawski G, Lubinski J, Scott RJ. Genetic polymorphisms in xenobiotic clearance genes and their influence on disease expression in hereditary nonpolyposis colorectal cancer patients. Cancer Epidemiol Biomarkers Prev. 2006 Nov;15(11):2307-2310.

Taningher M, Malacarne D, Izzotti A, Ugolini D, Parodi S. Drug metabolism polymorphisms as modulators of cancer susceptibility. Mutat Res. 1999 May;436(3):227-261.

Thalhammer-Scherrer R, Mitterbauer G, Simonitsch I, Jaeger U, Lechner K, Schneider B, Fonatsch C, Schwarzinger I. The immunophenotype of 325 adult acute leukemias: relationship to morphologic and molecular classification and proposal for a minimal screening program highly predictive for lineage discrimination. Am J Clin Pathol. 2002 Mar;117(3):380-389.

Thiede C, Creutzig E, Reinhardt D, Ehninger G, Creutzig U. Different types of NPM1 mutations in children and adults: evidence for an effect of patient age on the prevalence of the TCTG-tandem duplication in NPM1-exon 12. Leukemia. 2007 Feb;21(2):366-370.

Thiede C, Koch S, Creutzig E, Steudel C, Illmer T, Schaich M, Ehninger G. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood. 2006 May 15;107(10):4011-4020.

Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U, Platzbecker U, Wermke M, Bornhäuser M, Ritter M, Neubauer A, Ehninger G, Illmer T. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002 Jun 15;99(12):4326-4335.

Thomas X, Archimbaud E, Charrin C, Magaud JP, Fiere D. CD34 expression is associated with major adverse prognostic factors in adult acute lymphoblastic leukemia. Leukemia. 1995 Feb;9(2):249-253.

Tiftik N, Bolaman Z, Batun S, Ayyildiz O, Isikdogan A, Kadikoylu G, Muftuoglu E. The importance of CD7 and CD56 antigens in acute leukaemias. Int J Clin Pract. 2004 Feb;58(2):149-152.

Townsend DM, Tew KD. The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene. 2003 Oct 20;22(47):7369-7375.

Toyama T, Zhang Z, Nishio M, Hamaguchi M, Kondo N, Iwase H, Iwata H, Takahashi S, Yamashita H, Fujii Y. Association of TP53 codon 72 polymorphism and the outcome of adjuvant therapy in breast cancer patients. Breast Cancer Res. 2007;9(3):R34.

Traweek ST, Slovak ML, Nademanee AP, Brynes RK, Niland JC, Forman SJ.Myelodysplasia and acute myeloid leukemia occurring after autologous bone marrow transplantation for lymphoma. Leuk Lymphoma. 1996 Feb;20(5-6):365-372.

Tsimberidou AM, Paterakis G, Androutsos G, Anagnostopoulos N, Galanopoulos A, Kalmantis T, Meletis J, Rombos Y, Sagriotis A, Symeonidis A, Tiniakou M, Zoumbos N, Yataganas X. Evaluation of the clinical relevance of the expression and function of P-glycoprotein, multidrug resistance protein and lung resistance protein in patients with primary acute myelogenous leukemia. Leuk Res. 2002 Feb;26(2):143-154.

Tyagi BB, Manoharan N, Raina V. Childhood Cancer Incidence in Delhi, 1996-2000 Indian journal of medical & paediatric oncology vol. 2006; 27(4):13-18.

Uckun FM, Sather HN, Gaynon PS, Arthur DC, Trigg ME, Tubergen DG, Nachman J, Steinherz PG, Sensel MG, Reaman GH. Clinical features and treatment outcome of children with myeloid antigen

positive acute lymphoblastic leukemia: a report from the Children's Cancer Group. Blood. 1997 Jul 1;90(1):28-35.

Udomsakdi-Auewarakul C, Promsuwicha O, Tocharoentanaphol C, Munhketvit C, Pattanapanyasat K, Issaragrisil S. Immunophenotypes and outcome of Philadelphia chromosome-positive and -negative Thai adult acute lymphoblastic leukemia. Int J Hematol. 2003 Nov;78(4):337-343.

Uggla B, Ståhl E, Wågsäter D, Paul C, Karlsson MG, Sirsjö A, Tidefelt U. BCRP mRNA expression v. clinical outcome in 40 adult AML patients. Leuk Res. 2005 Feb;29(2):141-146.

van den Heuvel-Eibrink MM, Sonneveld P, Pieters R. The prognostic significance of membrane transport-associated multidrug resistance (MDR) proteins in leukemia. Int J Clin Pharmacol Ther. 2000 Mar;38(3):94-110.

van den Heuvel-Eibrink MM, van der Holt B, Burnett AK, Knauf WU, Fey MF, Verhoef GE, Vellenga E, Ossenkoppele GJ, Löwenberg B, Sonneveld P. CD34-related coexpression of MDR1 and BCRP indicates a clinically resistant phenotype in patients with acute myeloid leukemia (AML) of older age. Ann Hematol. 2007 May;86(5):329-337.

van der Kolk DM, de Vries EG, Noordhoek L, van den Berg E, van der Pol MA, Müller M, Vellenga E. Activity and expression of the multidrug resistance proteins P-glycoprotein, MRP1, MRP2, MRP3 and MRP5 in de novo and relapsed acute myeloid leukemia. Leukemia. 2001 Oct;15(10):1544-1553.

van der Pol MA, Broxterman HJ, Pater JM, Feller N, van der Maas M, Weijers GW, Scheffer GL, Allen JD, Scheper RJ, van Loevezijn A, Ossenkoppele GJ, Schuurhuis GJ. Function of the ABC transporters, P-glycoprotein, multidrug resistance protein and breast cancer resistance protein, in minimal residual disease in acute myeloid leukemia. Haematologica. 2003 Feb;88(2):134-147.

Vanhaeke DR, Béné MC, Garand R, Faure GC. Expression and long-term prognostic value of CD34 in childhood and adult acute lymphoblastic leukemia. Leuk Lymphoma. 1995 Dec;20(1-2):137-142.

Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002 Oct 1;100(7):2292-2302.

Velculescu VE, Madden SL, Zhang L, Lash AE, Yu J, Rago C, Lal A, Wang CJ, Beaudry GA, Ciriello KM, Cook BP, Dufault MR, Ferguson AT, Gao Y, He TC, Hermeking H, Hiraldo SK, Hwang PM, Lopez MA, Luderer HF, Mathews B, Petroziello JM, Polyak K, Zawel L, Kinzler KW, et al. Analysis of human transcriptomes. Nat Genet. 1999 Dec;23(4):387-398.

Venditti A, Del Poeta G, Buccisano F, Tamburini A, Cox MC, Stasi R, Bruno A, Aronica G, Maffei L, Suppo G, Simone MD, Forte L, Cordero V, Postorino M, Tufilli V, Isacchi G, Masi M, Papa G, Amadori S. Minimally differentiated acute myeloid leukemia (AML-M0): comparison of 25 cases with other French-American-British subtypes. Blood. 1997 Jan 15;89(2):621-629

Venditti A, Del Poeta G, Buccisano F, Tamburini A, Cox-Froncillo MC, Aronica G, Bruno A, Del Moro B, Epiceno AM, Battaglia A, Forte L, Postorino M, Cordero V, Santinelli S, Amadori S. Prognostic

relevance of the expression of Tdt and CD7 in 335 cases of acute myeloid leukemia. Leukemia. 1998 Jul;12(7):1056-1063.

Verhaak RG, Goudswaard CS, van Putten W, Bijl MA, Sanders MA, Hugens W, Uitterlinden AG, Erpelinck CA, Delwel R, Löwenberg B, Valk PJ. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. Blood. 2005 Dec 1;106(12):3747-3754

Vitale A, Guarini A, Ariola C, Meloni G, Perbellini O, Pizzuti M, De Gregoris C, Mettivier V, Pastorini A, Pizzolo G, Vignetti M, Mandelli F, Foà R. Absence of prognostic impact of CD13 and/or CD33 antigen expression in adult acute lymphoblastic leukemia. Results of the GIMEMA ALL 0496 trial. Haematologica. 2007 Mar;92(3):342-348.

Volk EL, Farley KM, Wu Y, Li F, Robey RW, Schneider E. Overexpression of wild-type breast cancer resistance protein mediates methotrexate resistance. Cancer Res. 2002 Sep 1;62(17):5035-5040.

Wacholder S, Chanock S, Garcia-Closas M, El Ghormli L, Rothman N. 2004. Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. J Natl Cancer Inst 17:434-442.

Wandt H, Schäkel U, Kroschinsky F, Prange-Krex G, Mohr B, Thiede C, Pascheberg U, Soucek S, Schaich M, Ehninger G. MLD according to the WHO classification in AML has no correlation with age and no independent prognostic relevance as analyzed in 1766 patients. Blood. 2008 Feb 15;111(4):1855-1861.

Wang L, Lin D, Zhang X, Chen S, Wang M, Wang J. Analysis of FLT3 internal tandem duplication and D835 mutations in Chinese acute leukemia patients. Leuk Res. 2005 Dec;29(12):1393-1398.

Wang YC, Lee HS, Chen SK, Chang YY, Chen CY. Prognostic significance of p53 codon 72 polymorphism in lung carcinomas. Eur J Cancer. 1999 Feb;35(2):226-230.

Wani MA, Zhu Q, El-Mahdy M, Venkatachalam S, Wani AA. Enhanced sensitivity to anti-benzo(a)pyrene-diol-epoxide DNA damage correlates with decreased global genomic repair attributable to abrogated p53 function in human cells. Cancer Res. 2000 Apr 15;60(8):2273-2280.

Weinstein HJ. Acute Myeloid Leukemia. In: Childhood Leukemias. Pui CH (eds). UK: Cambridge University Press; 1999. 322-335.

Westbrook CA, Hooberman AL, Spino C, Dodge RK, Larson RA, Davey F, Wurster-Hill DH, Sobol RE, Schiffer C, Bloomfield CD. Clinical significance of the BCR-ABL fusion gene in adult acute lymphoblastic leukemia: a Cancer and Leukemia Group B Study (8762). Blood. 1992 Dec 15;80(12):2983-2990.

Westendorf JJ, Yamamoto CM, Lenny N, Downing JR, Selsted ME, Hiebert SW. The t(8;21) fusion product, AML-1-ETO, associates with C/EBP-alpha, inhibits C/EBP-alpha-dependent transcription, and blocks granulocytic differentiation. Mol Cell Biol. 1998 Jan;18(1):322-333.

Wiersma SR, Ortega J, Sobel E, Weinberg KI. Clinical importance of myeloid-antigen expression in acute lymphoblastic leukemia of childhood. N Engl J Med. 1991 Mar 21;324(12):800-808.

Wognum AW, Eaves AC, Thomas TE. Identification and isolation of hematopoietic stem cells. Arch Med Res. 2003 Nov-Dec;34(6):461-475.

Worch J, Tickenbrock L, Schwäble J, Steffen B, Cauvet T, Mlody B, Buerger H, Koeffler HP, Berdel WE, Serve H, Müller-Tidow C The serine-threonine kinase MNK1 is post-translationally stabilized by PML-RARalpha and regulates differentiation of hematopoietic cells.. Oncogene. 2004 Dec 9;23(57):9162-9172.

Wormhoudt LW, Commandeur JN, Vermeulen NP. Genetic polymorphisms of human N-acetyltransferase, cytochrome P450, glutathione-S-transferase, and epoxide hydrolase enzymes: relevance to xenobiotic metabolism and toxicity. Crit Rev Toxicol. 1999 Jan;29(1):59-124.

Wu X, Zhao H, Amos CI, Shete S, Makan N, Hong WK, Kadlubar FF, Spitz MR. p53 Genotypes and Haplotypes Associated With Lung Cancer Susceptibility and Ethnicity. J Natl Cancer Inst. 2002 May 1;94(9):681-690.

Wuchter C, Karawajew L, Ruppert V, Büchner T, Schoch C, Haferlach T, Ratei R, Dörken B, Ludwig WD. Clinical significance of CD95, Bcl-2 and Bax expression and CD95 function in adult de novo acute myeloid leukemia in context of P-glycoprotein function, maturation stage, and cytogenetics. Leukemia. 1999 Dec;13(12):1943-1953.

Xie Y, Davies SM, Xiang Y, Robison LL, Ross JA. Trends in leukemia incidence and survival in the United States (1973-1998). Cancer. 2003 May 1;97(9):2229-2235.

Yamamoto JF, Goodman MT Patterns of leukemia incidence in the United States by subtype and demographic characteristics, 1997-2002.. Cancer Causes Control. 2008 May;19(4):379-390.

Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Saito K, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Saito H, Ueda R, Ohno R, Naoe T. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001 Apr 15;97(8):2434-2439.

Yang H, Lippman SM, Huang M, Jack Lee J, Wang W, Spitz MR, Wu X. Genetic polymorphisms in double-strand break DNA repair genes associated with risk of oral premalignant lesions. Eur J Cancer. 2008 Jul;44(11):1603-1611.

Yenerel MN, Atamer T, Yavuz AS, Kucukkaya R, Besisik S, Aktan M, Keskin H, Nalcaci M, Sargin D, Pekcelen Y, Dincol G. Myeloid antigen expression provides favorable outcome in patients with adult acute lymphoblastic leukemia: a single-center study. Ann Hematol. 2002 Sep;81(9):498-503.

Zahm SH. Childhood leukemia and pesticides. Epidemiology. 1999 Sep;10(5):473-475.

Zhang JB, Sun Y, Dong J, Liu LX, Ning F. Expression of lung resistance protein and multidrug resistance-associated protein in naive childhood acute leukemia and their clinical significance. Ai Zheng. 2005 Aug;24(8):1015-1017.

Zheng R, Friedman AD, Levis M, Li L, Weir EG, Small D. Internal tandem duplication mutation of FLT3 blocks myeloid differentiation through suppression of C/EBPalpha expression. Blood. 2004 Mar 1;103(5):1883-1890.

Zheng R, Small D. Mutant FLT3 signaling contributes to a block in myeloid differentiation. Leuk Lymphoma. 2005 Dec;46(12):1679-1687.

Zhou DC, Zittoun R, Marie JP. Expression of multidrug resistance-associated protein (MRP) and multidrug resistance (MDR1) genes in acute myeloid leukemia. Leukemia. 1995 Oct;9(10):1661-1666.

Zhou L, Jing Y, Styblo M, Chen Z, Waxman S. Glutathione-S-transferase pi inhibits As2O3-induced apoptosis in lymphoma cells: involvement of hydrogen peroxide catabolism. Blood. 2005 Feb 1;105(3):1198-203.

Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med. 2001 Sep;7(9):1028-1034.

Zienolddiny S, Campa D, Lind H, Ryberg D, Skaug V, Stangeland LB, Canzian F, Haugen A. A comprehensive analysis of phase I and phase II metabolism gene polymorphisms and risk of non-small cell lung cancer in smokers. Carcinogenesis. 2008 Jun;29(6):1164-1169.

Zipursky A, Poon A, Doyle J. Leukemia in Down syndrome: a review. Pediatr Hematol Oncol. 1992 Apr-Jun;9(2):139-149.

Appendix

Appendix

Table 1: List of Flowcytometric antibodies used for Immunophenotyping of AL

S.NO.	ANTIBODIES	CAT. NO.	COMPANY
1.	**CD3 FITC**	555332	BD Pharmingen
2.	**CD5 FITC**	555352	BD Pharmingen
3.	**CD7 FITC**	555360	BD Pharmingen
4.	**CD10 PE**	555375	BD Pharmingen
5.	**CD19 FITC**	555412	BD Pharmingen
6.	**CD13PE**	555394	BD Pharmingen
7.	**CD14FITC**	555397	BD Pharmingen
8.	**CD33 FITC**	555626	BD Pharmingen
9.	**CD34 FITC**	555821	BD Pharmingen
10.	**MPO-FITC**	340580	BD Pharmingen
11.	**HLA-DR FITC**	555811	BD Pharmingen
12.	**TdT -FITC**	550756	BD Pharmingen
13.	**CD45-PE**	555483	BD Pharmingen
14.	**Isotype Control FITC**	555748	BD Pharmingen
15.	**Isotype Control PE**	555749	BD Pharmingen

Table 2: List of Assay-on-Demand TaqMan gene expression probes used for Real Time RT-PCR

S.no.	Gene Symbol	Assay ID	COMPANY
1	**Human TBP (endogenous control)**	4333769F	Applied Biosystems
2	**BCL-2**	Hs00608023_m1	Applied Biosystems
3	**P53**	Hs00153349_m1	Applied Biosystems
4	**DHFR**	Hs00758822_s1	Applied Biosystems
5	**GST-PI**	Hs00168310_m1	Applied Biosystems
6	**MDR1**	Hs00184491_m1	Applied Biosystems
7	**MRP1**	Hs01561518_m1	Applied Biosystems
8	**LRP**	Hs00250049_m1	Applied Biosystems
9	**BCRP**	Hs00184979_m1	Applied Biosystems
10	**Survivin**	Hs00153353_m1	Applied Biosystems

Preparation of reagents

Preparation of 1.2% Formaldehyde-agarose gel (For 50 ml)

Add 0.6 gm Agarose in 37 ml of DEPC H_2O. Boil in microwave for 3-5 minute at 700-800 w. Cool at RT and add 5.0 ml of 10X MOPS Buffer + 8.0 ml of 37% Formaldehyde. Shake vigorously and pour in the casting plate. (Gel will solidify within 30-45 min.)

Preparation of RNA Sample for loading (20µl)

Add solution of Formamide (10µl), Formaldehyde (4µl), DEPC H_2O (2µl), 10X MOPS (2µl), 6x gel lading buffer(2µl), Etbr (0.1 µl) to 1 µg of RNA . Heat it at 650C for 10 minutes. Immediately Plunge the sample tube in ice. Load the 20µl of RNA sample into the Formaldehyde-agarose gel.

Phosphate Buffer Saline (PBS) (1 litre) pH=7.4

8gm of Sodium Chloride (NaCl), 2gm of Potassium Chloride (KCl), 1.44gm of Disodium hydrogen phosphate (Na_2HPO_4) and 0.2gm of Potassium dihydrogen orthophosphate (KH_2PO_4), were dissolved in 800ml of ddH_2O. pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15lb/ sq.in for 20 minutes and stored at room temperature.

Paraformaldehyde (PF) pH=7.4

4% Stock solution (100 ml) : 4 gms of paraformaldehyde + 50 ml of distilled water + 10 ml of 10x PBS + few drops of 2M NaOH was heated on magnetic stirrer in a fume hood. When the solution became clear it was removed from the heat and after cooling pH was adjusted to 7.2. Finally volume was adjusted with distilled water to 100 ml.

1% Working solution (10 ml): 2.5 ml of Stock PF (4%) + 7.5 ml of 1x PBS.

1M Tris: 121.1gm of Tris base was dissolved in 800ml of double distilled water and pH set (6.8, 7.4, 8.0) with concentrated HCl. Volume was made up to 1liter and autoclaved.

0.5M EDTA: 186.1gm of disodium EDTA.-2H_2O was added in 800ml of double distilled water, stirred vigorously on a stirrer, pH set to 8.0 with NaOH (~20 gm of NaOH pellets) and volume made up to1liter and autoclaved.

3M sodium acetate: 204.5gm of $C_2H_3O_2Na$. 3H_2O was dissolved in 400ml of ddH_2O,
pH set to 5.3 with glacial acetic acid, volume made up to 500 ml and autoclaved.

10% SDS: 10gm of electrophoresis grade SDS was dissolved in 70ml of ddH_2O, heated at 60°C to dissolve and the volume made up to 100ml.

Ethidium Bromide (10 mg/ml): 10mg of ethidium bromide was dissolved in 1ml ddH_2O, stored in opaque bottle.

Calcium Chloride (0.1 M): 1.47gm of $CaCl_2.2H_2O$ was dissolved in 100ml of ddH_2O and sterilized by autoclaving.

DEPC water: 0.1% diethylpyrocarbonate was added to 1ltr double distilled water in a fume hood and mixed well. After incubating it for 1hr at 37°C it was autoclaved.

10 X TAE buffer (Tris acetate, EDTA): 4.84gm of Tris base in 80ml of ddH_2O was dissolved and 1.2ml of glacial acetic acid and 2ml of 0.5 EDTA pH 8.0 were added. Final volume was made up to 100ml.

DNA loading dye (6X): 0.2gm bromophenol blue, 0.2gm of xylene cyanol and 30ml of glycerol were dissolved and volume set to 100 by autoclaved ddH_2O.

List of Publications

List of Publications

1. **Chauhan PS**, Ihsan R, Mishra AK, Yadav DS, Saluja S, Mittal V, Saxena S, Kapur S. High order interactions of xenobiotic metabolizing genes and p53 codon 72 polymorphism in acute leukemia **(Accepted for Publication in Environ Mol. Mutagen 2012)**.

2. Ihsan R, **Chauhan PS**, Mishra AK, Yadav DS, Kaushal M, Sharma JD, Zomawia E, Verma Y, Kapur S, Saxena S. Multiple Analytical approaches reveal distinct gene-environment interactions in smokers and non smokers in Lung Cancer. **Plos One 2011;6(12):e29431. Epub 2011 Dec 19.**

3. **Chauhan PS**, Bhushan B, Singh LC, Mishra AK, Saluja S, Mittal V, Gupta DK, Kapur S. Expression of genes related to multiple drug resistance and apoptosis in acute leukemia: response to induction chemotherapy. **Exp Mol Pathol. 2011 Oct 19;92(1):44-49.**

4. **Chauhan PS**, Ihsan R, Yadav DS, Mishra AK, Bhushan B, Soni A, Kaushal M, Devi TR, Saluja S, Gupta DK, Mittal V, Saxena S, Kapur S. Association of Glutathione S-Transferase, EPHX, and p53 codon 72 Gene Polymorphisms with Adult Acute Myeloid Leukemia. **DNA Cell Biol. 2011 Jan;30(1):39-46.**

5. **Chauhan PS**, Bhushan B, Mishra AK, Singh LC, Saluja S, Verma S, Gupta DK, Mittal V, Chaudhry S, Kapur S. Mutation of FLT3 gene in acute myeloid leukemia with normal cytogenetics and its association with clinical and immunophenotypic features. **Med Oncol. 2011 Jun;28(2):544-51.**

6. Yadav DS, Devi TR, Ihsan R, Mishra AK, Kaushal M, **Chauhan PS,** Bagadi SA, Sharma J, Zamoawia E, Verma Y, Nandkumar A, Saxena S, Kapur S. Polymorphisms of glutathione-S-transferase genes and the risk of aerodigestive tract cancers in the Northeast Indian population. **Genet Test Mol Biomarkers. 2010 Oct;14(5):715-23.**

7. Thoudam RD, Yadav DS, Mishra AK, Kaushal M, Ihsan R, Chattopadhyay I, **Chauhan PS,** Sarma J, Zomawia E, Verma Y, Nandkumar A, Mahanta J, Phukan R, Kapur S, Saxena S. Distribution of glutathione S-transferase T1 and M1 genes polymorphisms in North East Indians: a potential report. **Genet Test Mol Biomarkers. 2010 Apr;14(2):163-169.**

8. Bhushan B, **Chauhan PS**, Saluja S, Verma S, Mishra AK, Siddiqui S, Kapur S. Aberrant phenotypes in childhood and adult acute leukemia and its association with adverse prognostic factors and clinical outcome. **Clin Exp Med. 2010 Mar;10(1):33-40.**

Participations

1. Presented poster entitled "Association between polymorphism of xenobiotic-metabolizing genes and the risk of acute leukemia". **Chauhan PS**, Ihsan R, Mishra AK, Yadav DS, Khanna A, Saluja S, Mittal V, Saxena S, Kapur S. AACR's first Conference in India "New Horizons in Cancer Research: Biology to Prevention to Therapy" held at Gurgaon, Delhi from December 13-16, 2011.

2. Attended the workshop on "12th Indo-US Workshop on Flow Cytometry in Clinical Research" organized by The Cytometry Society (India) at Panjab university from 10th to 12th October 2011.

3. Presented poster entitled "Glutathione S-transferase and Microsomal Epoxide Hydrolase Gene Polymorphisms and Risk of Acute myeloid leukemia".**Chauhan PS,** Ihsan R, Mishra AK, Bhushan B, Kaushal M, Yadav DS, Devi TR, Soni A, Chattopadhyay I, Saxena S, Kapur S. First international conference "Hematologic Malignancies: Bridging the Gap 2010" Singapore City, Singapore, Feb 5-7, 2010.

4. Attended the workshop on "Hands-on Basic Flow Cytometry Course" organized by Centre for Cellular and Molecular Platforms, NCBS-TIFR, Bangalore from 17th to 21st December 2009.

5. Attended the National workshop on "Molecular Cytogenetics: Cancer Cytogenetics (solid tissue) by FISH" organized by Department of Reproductive Biology AIIMS, from August 24 to 29, 2009 at New Delhi, India.

6. Attended the "Special course on Leukemia / Lymphoma Immunophenotyping" organized by from Flow Cytometry Training Centre, BD Biosciences Pvt.Ltd, from 1st to 3rd July 2009 at Gurgaon, Haryana, India.

7. Presented poster entitled "Expression of Ki67 as a prognostic indicator in Acute leukemia". **Chauhan PS**, Bhushan B, Verma S, Saluja S, Sharma M, Mishra AK, Mittal V, Kabra M, Bhasin S, Gupta DK, **Kapur S.** National Conference on Emerging Trends in Life Sciences Research, BITS, Pilani , Rajasthan from March 6th & 7th, 2009.

8. Attended the Fourth Workshop on "Genetic Epidemiological Methods for Dissection of Complex Human Traits" organized by TCG-ISI Centre for Population genomics (CpG) from Feb 23-28, 2009 at Kolkatta.

9. Presented poster entitled "Immunophenotypic and clinical findings in adult Acute Myeloid Leukemia with FLT3 Internal Tandem duplication"**Chauhan PS**, Bhushan B, Verma S, Saluja S, Sharma M, Mishra AK, Mittal V, Kabra M, Kapur S.. HUGO's 13th Human Genome Meeting, Hyderabad, September 27 - 30, 2008.

10. Presented poster entitled "Aberrant expression of lineage associated antigens in acute leukemia: A flow cytometric study". **Chauhan PS**, Bhushan B, Verma S, Saluja S, Kapur S. 27th Annual convention of Indian association for cancer research, Ahmedabad, February 6th – 9th, 2008.

11. Attended the "National Workshop on Microarray Technology" held on 16th - 18th April 2007 organized by Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi, India.

12. Attended the workshop on "Screening Tests for Bleeding Disorders" held on 6th May 2006 in Haematology Division, Department of Medicine, VMMC & Safdarjung Hospital, New Delhi, India.

13. Presented poster entitled "Prevalence of Glutathione-S-transferase (GST) polymorphisms in tobacco-associated malignancies in high risk Northeast Indian population". Yadav DS, Devi TR, Ihsan R, **Chauhan PS**, Chattopadhyay I, Kataki AC, Sharma J, Zamoawia E, Verma Y, Kapur S, Saxena S. International symposium on cancer Biology at National Institute of Immunology, New Delhi, November 12-14, 2007.

Brief Biography of the Candidate

Biography of the Candidate

Name **Pradeep Singh Chauhan**

Date of Birth **12th January 1982**

Postal Address Lab. No.611,
Tumor Biology Lab,
National Institute of Pathology (ICMR),
Culture, Institute of Pathology,
Safdarjung Hospital Campus,
Post Box 4909, New Delhi-110029, India.

Phone 09910338033, 011-26198402-06
Fax 011-26198401
E-mail address pradeepchauhan23@gmail.com

Education Qualification

Examination passed	University/ Board	Year	Subjects Studied	Percentage
D.S.S.E (X^{th})	C.B.S.E New Delhi	1997	English, Hindi, Math, Social Science, Science	61%
D.S.S.C.E (X^{th})	C.B.S.E New Delhi	1999	English, Math, Physics, Chemistry, Zoology	77%
B.Sc (GRP. B)	Delhi University New Delhi	2003	Zoology, Botany, Chemistry	67%
M.Sc (Biochemistry)	Jamia Hamdard New Delhi	2005	Microbiology, Immunology, Biotechnology, Biostatistics, Clinical Biochemistry, Molecular Biology,	73%

Fellowship Awards

- Qualified **CSIR- UGC/ NET** (National Eligibility Test), **December -2005** for **CSIR – JRF** (Junior Research Fellowship) in Life Sciences discipline.
- Qualified Indian Council of Medical Research-Junior Research Fellow, July-2005, ICMR-JRF in Life Sciences discipline
- Qualified Graduate Aptitude Test Exam (GATE, 92.04 percentile), 2005

Research Experience

- Pursuing Doctoral Program from January 2005 onwards at IOP as CSIR-JRF.
- Pursuing Doctoral Program from August 2008 onwards at IOP as CSIR-SRF.
- Awarded **independent senior research fellowship** from Indian Council of Medical Research in December 2011 to work at National Institute of Pathology, New Delhi.

Workshop/Training Received

- Attended the workshop on "12th Indo-US Workshop on Flow Cytometry in Clinical Research" organized by The Cytometry Society (India) at Panjab university from 10th to 12th October 2011.
- Attended the workshop on "Hands-on Basic Flow Cytometry Course" organized by Centre for Cellular and Molecular Platforms, NCBS-TIFR, Bangalore from 17th to 21st December 2009.
- Attended the National workshop on "Molecular Cytogenetics: Cancer Cytogenetics (solid tissue) by FISH" organized by Department of Reproductive Biology AIIMS, from August 24 to 29, 2009 at New Delhi, India.
- Attended the "Special course on Leukemia / Lymphoma Immunophenotyping" organized by from Flow Cytometry Training Centre, BD Biosciences Pvt.Ltd, from 1st to 3rd July 2009 at Gurgaon, Haryana, India.
- Attended the Fourth Workshop on "Genetic Epidemiological Methods for Dissection of Complex Human Traits" organized by TCG-ISI Centre for Population genomics (CpG) from Feb 23-28, 2009 at Kolkatta.
- Attended the "National Workshop on Microarray Technology" held on 16th - 18th April 2007 organized by Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi, India.
- Attended the workshop on "Screening Tests for Bleeding Disorders" held on 6th May 2006 in Haematology Division, Department of Medicine, VMMC & Safdarjung Hospital, New Delhi, India.

Conference/Symposium Attended

- **Chauhan PS**, Ihsan R, Mishra AK, Yadav DS, Khanna A, Saluja S, Mittal V, Saxena S, Kapur S. Poster presented on "Association between polymorphism of xenobiotic-metabolizing genes and the risk of acute leukemia". AACR's first Conference in India "New Horizons in Cancer Research: Biology to Prevention to Therapy" held at Gurgaon, Delhi from December 13-16, 2011.

- Ihsan R, **Chauhan PS**, Singh L.C, Zomawia E, Sarma J , Kapur S, Saxena S. "**Gene expression profile of non-small cell lung cancer in high risk population from North**". AACR's first Conference in India "New Horizons in Cancer Research: Biology to Prevention to Therapy" held at Gurgaon, Delhi from December 13-16, 2011.
- **Chauhan PS,** Ihsan R, Mishra AK, Bhushan B, Kaushal M, Yadav DS, Devi TR, Soni A, Chattopadhyay I, Saxena S, Kapur S. "Glutathione S-transferase and Microsomal Epoxide Hydrolase Gene Polymorphisms and Risk of Acute myeloid leukemia". First international conference "Hematologic Malignancies: Bridging the Gap 2010" Singapore City, Singapore, Feb 5-7, 2010.
- **Chauhan PS**, Bhushan B, Verma S, Saluja S, Sharma M, Mishra AK, Mittal V,Kabra M, Bhasin S, Gupta DK, **Kapur S.** "Expression of Ki67 as a prognostic indicator in Acute leukemia". National Conference on Emerging Trends in Life Sciences Research, BITS, Pilani , Rajasthan from March 6th & 7th, 2009.
- **Chauhan PS**, Bhushan B, Verma S, Saluja S, Sharma M, Mishra AK, Mittal V, Kabra M, Kapur S. "Immunophenotypic and clinical findings in adult Acute Myeloid Leukemia with FLT3 Internal Tandem duplication". HUGO's 13th Human Genome Meeting, Hyderabad, September 27 - 30, 2008.
- Bhushan B, **Chauhan PS**, Saluja S, Mishra AK, Bhasin S, Gupta DK, Siddiqui S, Kapur S. "NF-kB signaling pathway in acute leukemia: A study on expression of cell survival and proliferative genes by Real Time RT-PCR". HUGO's 13th Human Genome Meeting, Hyderabad, September 27 - 30, 2008.
- **Chauhan PS**, Bhushan B, Verma S, Saluja S, Kapur S. "Aberrant expression of lineage associated antigens in acute leukemia: A flow cytometric study". 27th Annual convention of Indian association for cancer research, Ahmedabad, February 6th – 9th, 2008.
- Yadav DS, Devi TR, Ihsan R, **Chauhan PS**, Chattopadhyay I, Kataki AC, Sharma J, Zamoawia E, Verma Y, Kapur S, Saxena S. "Prevalence of Glutathione-S-transferase (GST) polymorphisms in tobacco-associated malignancies in high risk Northeast Indian population". International symposium on cancer Biology at National Institute of Immunology, New Delhi, November 12-14, 2007.

Brief Biography of the Supervisor

Brief Biography of the Supervisor

Name	**Dr. Sujala Kapur, M.D.,**
Designation	**Scientist E**
Address	**Institute of Pathology (ICMR)**
	Safdarjung Hospital Campus,
	New Delhi – 110029.
	Telephone 2619 8402-06
	Fax 2619 8401
	Email sujalakapur@gmail.com

Research Interests

- Gene expression, copy number variations and high thriughput sequencing using microarrays, Affymetrix and Illumina based platforms and validation by Quantitative real time RT-PCR
- Molecular biomarkers in hematopoietic lymphoid malignancies.
- Flow cytometric assays to analyse chemotherapeutic sensitivity in leukaemic cells

Fellowships/Award

- Awarded Scientific Fellowship, Kiel University, Germany, 1995 & 1998
- Awarded INSA Visiting Fellowship, 2004
- Awarded In-country WHO Fellowship, 2004

Extramural projects

- Immunogenetic profile of nasopharyngeal cancer in a high-prevalence region of Northeast India *(Collaborative, Multicentric, DBT Project)* (1).
- Genome-wide analysis of genetic alterations in patients with esophageal cancer from Northeast India using single nucleotide polymorphism arrays *(Collaborative, Multicentric, ICMR Task Force Project)* (3)
- Tobacco Use and Pesticide Exposure in causation of carcinoma oesophagus and other cancers at North East India *(Collaborative, Multicentric, ICMR Task Force Projects)* (3) (completed).
- Flow cytometric assays to evaluate prognosis and patient's response to chemotherapy in acute leukemia (*Extramural ICMR Project)* (1) (completed).

Training Programmes

- Training in Molecular Pathology, Kiel, Germany
- Training in Microarray Techniques at Department of Biochemistry, Indian Institute of Science, Bangalore and Ann Arbor, Michigan.
- Training in Laboratory Genetics at Department of Anatomy, All India Institute of Medical sciences, New Delhi**.**

Membership of Professional Societies

- Member, IAPM
- Member, IAPM, Delhi Chapter
- Member, Indian association of Cancer research
- Member, HUGO

Thesis guide for DNB/PhD/MSc students

DNB - 6
Ph.D. Thesis Completed - 1
Ongoing Ph.D. Thesis - 4
M.Sc. Students Dissertation done/completed - 8

Publications

Year	S.No.	Publications
2011	1	Study on predictive role of AR and EGFR family genes with response to neo-adjuvant chemotherpy in locally advanced breast cancer in indian women .Singh LC, Chakraborty A **Kapur S.** Saxena S *(Accepted, 2011) Medical Oncology*.
2011	2	Investigation on the Role of *p53* Codon 72 Polymorphism and Interactions with Tobacco, Betel Quid, and Alcohol in Susceptibility to Cancers in a High-Risk Population from North East India. Ihsan R, Thoudam D, Yadav DR, Mishra AK, Sharma J, Zomawia E, YVerma , Phukan R, Mahanta J, Kataki AC, **Kapur S,** Saxena S. *DNA and Cell Biology. March 2011, 30(3): 163-171.*
2011	3	Association of Glutathione *S*-Transferase, *EPHX*, and *p53* codon 72 gene polymorphisms with adult Acute myeloid leukemia; Chauhan PS, Ihsan R, Yadav DS, Mishra AK, Bhushan B, Abha Soni, Kaushal M, Thoudam RD, Sumita S, Gupta DK, Mittal V, Saxena S, **Kapur S.** *DNA and Cell Biology. January 2011, 30(1): 39-46*
2010	4	Contribution of germline *BRCA2* sequence alterations to risk of familial esophageal cancer in high-risk area of India. Kaushal M, Chattopadhyay I, Phukan RK, Purkayastha J, Mahanta J, **Kapur S**, Saxena S. *Disease of the Esophagus. 23:71-75, 2010.*
2010	5	Aberrant phenotypes in childhood and adult acute leukemia and its association with adverse prognostic factors and clinical outcome. Bhushan B, Chauhan PS, Saluja S, Verma S, Mishra AK, Siddiqui S, **Kapur S**. *Clin Exp Med. 2010 Mar;10(1):33-40.*

2010	6	Distribution of Glutathione S-transferase T1 and M1 genes polymorphisms in North East Indians: A potential report. Thoudam RD, Yadav DS, Mishra AK, Kaushal M, Ihsan R, Chattopadhyay I, Chauhan P, Sarma J, Zomawia E, Verma Y, Nandkumar A, Mahanta J, Phukan RK, **Kapur S**, Saxena S. *Genet Test Mol Biomarkers. 2010 14(2) 163-169, PMID: 20073549*
2010	7	Genome-wide analysis of chromosomal alterations in patients with esophageal squamous cell carcinoma exposed to tobacco and betel quid from high-risk area in India. Chattopadhyay I, Singh A, Phukan RK, Purkayastha J, Kataki A, Mahanta J, Saxena S, **Kapur S** . *Mutat Res. - Genetic Toxicology and Environmental Mutagenesis 696 (2010) 130–138. PMID: 20083228*
2010	8	Mutation of FLT3 gene in acute myeloid leukemia with normal cytogenetics and its association with Clinical and Immunophenotypic features. Chauhan P, **Kapur S**. *Medical Oncology, 2010;10 (1):33-40.*
2010	9	Role of EPHX1 gene polymorphisms in esophageal cancer of high-risk area in India. Ihsan R, Chatterjee I, Phukan R, Mishra AK, Purkayastha J, Sharma J, Zamoawia E, Verma V, Mahanta J, Saxena S, Sujala S,**Kapur S**. *J of Gastroenterology and Hepatology 2010, 25, 1456-62.*
2010	10	Polymorphisms of glutathione-S-transferase genes and the risk of aerodigestive tract cancers in the Northeast Indian population. Yadav DS, Devi TR, Ihsan R, Mishra AK, Kaushal M, Chauhan PS, Bagadi SA, Sharma J, Zamoawia E, Verma Y, Nandkumar A, Saxena S, **Kapur S**. Genet Test Mol Biomarkers. 2010 Oct;14(5):715-23. Epub 2010 Sep 20
2010	11	Betel quid chewing as an environmental risk factor for breast cancer. Kaushal M, Mishra AK, Raju BS, Ihsan R, Chakraborty A, Sharma J, Zomawia E, Verma Y, Kataki A, **Kapur S**, Saxena S. Mutat Res. 2010 Dec 21;703(2):143-8. Epub 2010 Aug 20.
2009	12	Cytokeratin immunoexpression in esophageal squamous cell carcinoma of high-risk population in Northeast India. Singh A; **Kapur S**; l Chattopadhyay I; Purkayastha J; Sharma J; Mishra A; Hewitt S M; Saxena S. *Applied Immunohistochemistry & Molecular Morphology 17 (5), 419-524, 2009*.
2009	13	Molecular profiling to identify molecular mechanism in esophageal cancer with familial clustering. Chattopadhyay I, Phukan RK, Singh A, Vasudeva M, Purkayastha J, Hewitt S, Katak A, Mahanta J, **Kapur S**, Saxena S. *Oncology Reports, 21(5), 1135-46, 2009*.
2008	14	Subcutaneous panniculitis-like T-cell cutaneous lymphoma. Singh A, Kumar J, Kapur S, Ramesh V. *Indian J Dermatol Venereol Leprol. Mar-Apr, 74 (2): 151-3, 2008.*
2007	15	Gene expression profile of esophageal cancer in North East India by cDNA microarray analysis. Chattopadhyay I, **Kapur S**, Purkayastha J, Phukan RK, Kataki A, Mahanta J, Saxena S. *World J Gastroentero,l* 13(9): 1438-1444, 2007.
2007	16	Relation of cell viability and apoptosis with clinical remission following induction chemotherapy in ALL and AML. Bhushan B, Ahuja D, Verma S, Saluja S, Siddiqui S, **Kapur S**. *Journal of Experimental and Clinical Cancer Research.* 26 (3) September 2007.
2006	17	Gene expression profile in esophageal cancer patients of Northeast region of India. Chatterji I, **Kapur S**, Mahanta J, Phukan RK, Barooah MN, Kataki AC, Purkayastha J, Saxena S. *J Cancer Res Ther*, 2 (1), S 20, 2006.
2006	18	Limitation of FAB diagnosis in Acute leukemia. Bhushan B, Saluja S, Ahuja D, Verma S, Gupta DK, **Kapur S**. *J Cancer Res Ther*, 2 (1), S 22, 2006.

2006	19	Reliability of detecting rRNA sequences of Chlamydia trachomatis with fluorescence in situ hybridization without amplification. **Kapur S**, Margoob A, Singh V, Guido K, Salhan S, Mittal A. *Acta Cytol*, 50(3): 277-83, 2006.
2005	20	The role of p53 and anaplastic lymphoma kinase genes in the progression n of cutaneous CD30+ lymphoproliferative diseases. **Kapur S**, Tiemann M, Menke AOH, Schubert C, Parwaresh R. *Ind J Med Res,* 121, 46-54, 2005.
2005	21	Apoptotic and proliferative related gene expression in non-Hodgkin's lymphoma. S Saluja S, Ghai R, Sharma M, Bhasin S, Gupta DK, Kataria S, **Kapur S**. *Ind J Med Res,* 121, Suppl, 95, 2005.
2005	22	An Unusual Presentation of primary lymphoma of the thyroid in a young male patient. Ghai R, Bharat R, Sunita S, **Kapur S**. *Ind J Pathol Microbiol,* 48 (3), 385-87, 2005.
2005	23	Idiopathic Myelofibrosis with generalized Peristitits in a 4 year old girl.J of Paed. Hematology: Mandeep W ,Mehta R, Paul P, Pr Saluja, S, Sharma, M , **Kapur S,**2005,27-5:278-282
2005	24	Extra-medullary lymphoid blast crisis in a patient of chronic myelogenous leukemia. Khunger JM, Arora S, Sharma U, **Kapur S**. *Indian J. Hemat & Blood Transf.*; 23 (4): 39 – 40, 2005.
2005	25	Congenital leukaemia: A case report. Khunger JM, Srivastava SK, **Kapur S,** Gupata DK. *Indian J. Hemat & Blood Transf.*; 23 (4): 39 – 40, 2005.
2003	26	Cytochemical, immunocytochemical and flow cytometric analysis for the diagnosis of acute leukemia. S Saluja, **S Kapur**, M Sharma, DK Gupta, M Narang. *Ind J Med Paediatric Oncol*. 24 (3), 59-60, 2003.
2002	27	Polymerase chain reaction for detection of endocervical Chlamydia trachomatis infection in women attending a gynecology outpatient department in India .Singh V.,Rastogi S.,Gaarg **S,Kapur** S,Kumar A.,Salhan S and mittal A Acta Cytologica 46(3):540-544 2002.
2001	28	Early mycosis fungoides: Molecular analysis for its diagnosis and the absence of p53 gene mutations in cases with progression J Dermatol Sci, 26 (1), 36-45. 2001. **Kapur S,** Menke MAOH, Tiemann M, Schubert C, Parwaresh R.
1999	29	Chlamydia trachomatis infection in pregnancy: risk factor for an adverse pregnancy outcome. *Br J Biomed Sci*, 56, 94-98, 1999. S.Rastogi, **S.Kapur**, S.Salhan, A.Mittal.
1998	30	Incidence of HIV infection and its predictors in blood donors in Delhi. *Indian J Med Res*, 108, 45-50,1998. S. Kapur, A. Mittal.
1997	31	Coexisting pathogens in cervicovaginal infection. *Ind J Sex Trans Dis.* 18, 1997. **S.Kapur**, S.Gupta, A.Mittal.
1996	32	Host immune response in chlamydial cervicitis. *Br J Biomed Sci*. 53(3) 214-220 1996. A. Mittal, **S. Kapur**, S. Gupta.
1995	33	Clinical trial with Praneem polyherabal cream in patients with abnormal vaginal discharge due to microbial infection. *Australian & New Zealand J of Obst Gynaecol*. 1995, 35(2), 190. A. Mittal, S. **Kapur, S**.Garg, S. N. Upadhyaya, S. Suri, S.K. Das, S. Gupta, G. P. Talwar.
1995	34	Infertility due to Chlamydia trachomatis infection: what is the appropriate site for obtaining samples? Genitourin Med. 71, 267-269, 1995. A. Mittal, **S. Kapur**, S. Gupta.
1995	35	Rising trend of HIV positive individuals among blood donors in Delhi. *Ind. J Sex Transm Dis*. 16; 15-17, 1995. R.Singh**, S. Kapur**, A. Mittal.
1994	36	Prevalence of Chlamydia trachomatis infection in female genital tract. *Indian J Sex Transm* Dis. 15; 15-18, 1994. A. Mittal, **S. Kapur**, & S.Gupta.

1993	37	Chlamydial cervicitis: Role of culture, enzyme immunoassay and Giemsa cytology in diagnosis. *Acta Pathol Microbiol Immunol Scand (APMIS)*, 101, 37-40, 1993. A. Mittal, **S. Kapur**, & S.Gupta
1993	38	Screening for genital chlamydial infection in symptomatic women. *Indian J Med Res*. 98, 119-123, 1993. A. Mittal, **S. Kapur**, & S.Gupta.
1993	39	Cytologic detection of chlamydial inclusions in cervical smears: Papanicolaou versus Giemsa stain. *ActaCytol* 37(1), 108-109, 1993. S. Kapur, A.Mittal.
1992	40	Viral particles in persistent generalised lymphadenopathy. *Indian J Pathol Microbiol*. 35(3) 201-208, 1992. **S.Kapur**, N. Jain, A.K. Jain & M. Chandra.
1991	41	Persistent lymphadenopathy - a cause for concern. *Ann. National Acad. Med. Sci (India)*. 27 (3&4): 171-176, 1991**. S. Kapur**, N. Jain, R.L. Gupta, Y.N. Singh & M.Chandra.
1991	42	HIV infection in the blood donors of New Delhi, India 1½ years experience. *J Acquir immune Defic Syndr*. 4, 1008-9, 1991. Y.N. Singh, M.Chandra, A. Nanu, R.Bhasin, **S.Kapur**, N. Jain, A.K. Jain, M.Chandra
1991	43	Epidemiological patern of HIV infection in India. *JAMA (India)* 10 (27) 171-173,1991.A.N. Malaviya, Y.N. Singh, M. Chandra **& S.Kapur.**

International Proceeding

1. Genetic profile of patients with esophageal cancer in a high-incidence region of Northeast India and its association with tobacco consumption. **Kapur S**, Chatterjee I, Noor A, Mahanta J, Phukan RK, Barooah MN, Saikia B, Kataki AC, Joydeep Purkayastha J, Saxena S. In: Medimond S.r.l. (Pub) Sede Legale: Via Maserati, Bologna, Italy 2006.

2. Genomic alterations in breast cancer patients from Northeast India using 10K SNP arrays. Saxena S, Kaushal M, Sharma J, Zomawia E, **Kapur S**. *Genome Biology* 2010, **11** (Suppl 1) :P34 doi:10.1186/gb-2010-11-s1-p34.

3. Molecular Profile of Esophageal Cancer in High-risk Region of India. I. Chattopadhyay, A. Singh, S. Kapur, and S. Saxena.Abstracts for 10th World Congress of OESO, Boston, Massachusetts. *J Clin Gastroenterol* Volume 45, Number 2, February 2011.

Chapters in Books

1. Neem Research and Development. New insights into the mechanism of action of neem extracts on induction of local cell mediated immunity in the genital tract : Its implications and application. In: Neem Research and Development. Randhawa N.S. and Parmar B.S. (Eds). Society of pesticide Science, India (Pub), 1993, 227-234. G.P. Talwar, S. Upadhyay, S. Garg, R. Kaur, S. Dhawan, A. Mittal, **S. Kapur,** S.Gupta.

2. HIV/AIDS Research in India - Correlation of HIV infection with Hepatitis B and syphilis. In: O.P. Aggarwal, A.K. Sharma, A. Indrayan (eds). National Aids Control Organisation, Min of Health & Family Welfare, Govt. of India, New Delhi (Pub) 1997, 465-468. **S. Kapur**, R. Singh, A. Mittal.