GENE POLYMORPHISM OF CYP1A1/CYP2D6 AND THEIR ASSOCIATION WITH ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) IN KASHMIRI CHILDREN

Dissertation Submitted for the Award of the Degree of Master of Philosophy in Biochemistry

By

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UNDER THE JOINT SUPERVISION OF

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CERTIFICATE

Certified that the work in the dissertation entitled ***"GENE POLYMORPHISM OF CYP1A1/CYP2D6 AND THEIR ASSOCIATION WITH ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) IN KASHMIRI CHILDREN."*** is the bonafide work of Ms. Nidha Sadiq Shapoo and has been carried out under our guidance and supervision in the Department of Biochemistry, University of Kashmir. This work is suitable for the award of M.Phil. Degree in Biochemistry.

It is further certified that no work under this heading has previously been submitted to the University of Kashmir for the award of any degree or diploma, to the best of our belief.

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DECLARATION

I, Nidha Sadiq Shapoo, declare that the work embodied in this dissertation entitled "***GENE POLYMORPHISM OF CYP1A1/CYP2D6 AND THEIR ASSOCIATION WITH ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) IN KASHMIRI CHILDREN***" has been carried out by me in the Department of Biochemistry, University of Kashmir, Srinagar and is original. The work embodies the results of my observations which are advancement to the previous knowledge in the subject.

Place: Srinagar **Nidha Sadiq Shapoo**

Date:

Dedicated to my Mom...

Who cherished my dreams through sacrifice

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In the name of Allah, the most beneficent and exceedingly merciful

Though only my name appears on the cover of this dissertation, a great many people have contributed to its production. I owe my gratitude to all those people who have made this dissertation possible and because of whom my research experience has been one that I will cherish forever, and I would like to thank them all and in particular,

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Finally, I would like to thank and acknowledge all the children and parents who gave of their time to participate in this study, for sharing experiences that have helped provide a greater understanding of and deeper insight for my research on Acute Lymphoblastic Leukemia.

NIDHA SADIQ SHAPOO
Place: Srinagar,
May-2013

µg	Microgram
µM	Micromolar
µl	Microlitre
^{o}C	Degree Celsius
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myelocytic Leukemia
bp	Base Pair
BPB	Bromophenol blue
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myelocytic Leukemia
DME	Drug metabolizing enzymes
dUMP	Deoxy thymidine monophosphate
dUMP	Deoxy uridine monophosphate
DDW	Double distilled water
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribose nucleotide –triphosphate
EDTA	Ethylene diamine tetra acetate
EGIL	European Group for the immunological classification of leukemias
EMF	Electro motive force
EtBr	Ethidium bromide
FAB	French-American-British Classification
Fig.	Figure
g	Grams
Hr	Hours
Ig	Immunoglobulins
Kb	Kilo base pair
KDa	Kilo Dalton
$KHCO_3$	Potassium bicarbonate
$MgCl_2$	Magnesium chloride
m	Meter
MTHFR	Methylene tetrahydrofolate reductase
M	Molar
mg	Miligram
min	Minutes

ml	Mililitre
mM	Mili Molar
MML	Mixed Lineage Leukemia
mRNA	Messenger ribonucleic Acid
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NF1	Neurofibromatosis type I
ng	Nanogram
$NH4Cl_2$	Ammonium Chloride
O.D	Optical density
O.R	Odds ratio
ppm	Parts per million
PCR	Polymerase Chain Reaction
pmol	pico mole
rpm	Revolutions per minute
RT	Room temperature
SNA	Samples not Amplified
SDS	Sodium dodecyl Sulphate
Sec	Second
SKIMS	Sheri- Kashmir Institute of Medical Sciences
SNP	Single Nucleotide Polymorphism
STE	Sodiumchloride-Tris-Ethylenediaminetetraacetic acid
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus* DNA polymerase
Tm	Melting temperature
Tris	Tris(hydroxyl methyl amino methane)
UTR	Untranslated Region
UV	Ultra violet
WHO	World Health Organization

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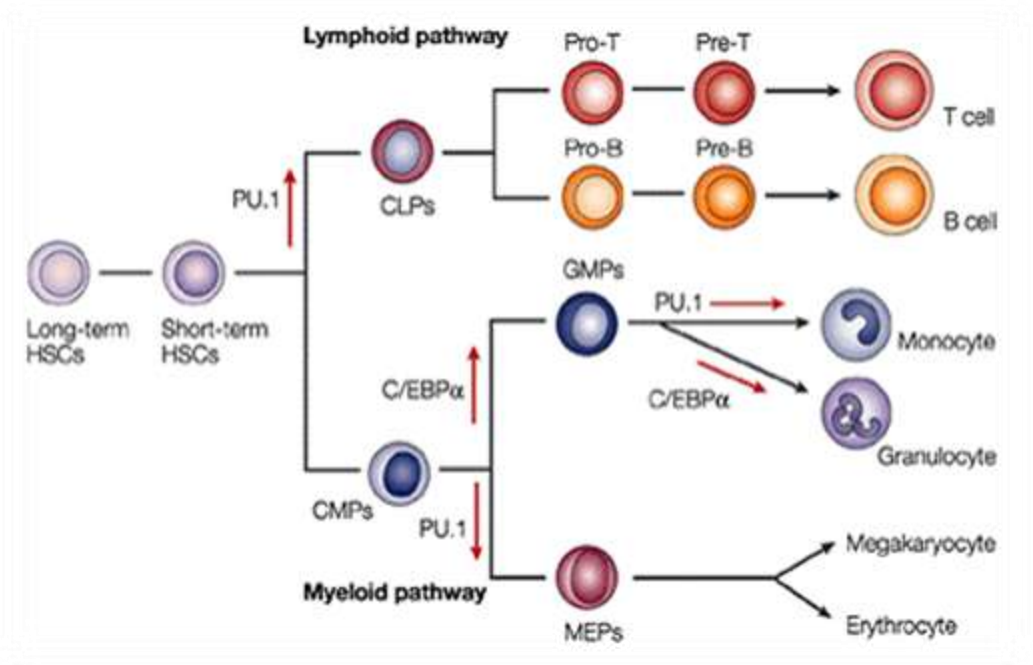
Acute lymphoblastic leukemia (ALL) is a type of cancer that affects immature lymphocytes developing in the bone marrow. Genetic susceptibility to leukemias can be related to the polymorphisms in CYP2D6 and CYP1A1 genes and consistent with this paradigm several polymorphisms have been identified in the pathogenesis of lymphoid malignancies.CYP2D6 and CYP1A1- the member of the cytochrome P_{450} mixed-function oxidase system, are the most important enzymes involved in the metabolism of various drugs in the body. To determine whether these genes played a similar role in childhood leukemogenesis in the Kashmiri children, we compared the allele frequencies of 120 childhood ALL patients and 110 controls for the CYP1A1 and CYP2D6 genes. Genotyping was done by PCR-RFLP technique and results were validated by direct sequencing of the PCR products. We observed that the CYP2D6 (G) allele frequency was 97.27% in the controls and 45% in cases and CYP2D6 (A) allele frequency was 2.72% in the controls and 55% in cases. While as in case of CYP1A1 the frequency of CYP1A1 (T) allele was 88.75% in the controls and 83.88% in cases and CYP1A1 (C) allele frequency was 11.25% in controls and 16.66% in cases. This difference in frequency was found to be statistically insignificant with a P>0.05. The CYP2D6 wild genotype frequency was found to be present in 33.33% of the cases and 96.36% of the controls, the hetero variant in 23.33% of the cases and 1.82% of controls, and the mutant genotype in 43.33% of cases and 1.82% of controls. While as in case of CYP1A1, the wild genotype was found to be present in 70.83% of cases and 85.45% in controls, the hetero genotype in 25% of the cases and 22.73% of controls and the mutant was found to be present in 4.17% of the cases and 0.9% of controls. We observed that an increased risk associated with CYP2D6 Mutant genotype 43.33% (OR = 68.90, 95% CI, 16.02-296.3; P = <0.0001) and Wild genotype 33.33% and low with hetero genotype 23.33% of CYP2D6 as compared to the controls and also increased risk is associated with CYP1A1 wild (T/T) genotype 70.83 % and hetero 25% (OR 1.33, 95% CI, 0.72- 2.43; P = 0.44) and low in case of mutant (C/C) genotype 4.17% (OR = 5.53, CI 95% 0.63-48.31, P = 0.19) as compared to the controls. Thus, our study suggests that there is an association between gene polymorphism of CYP2D6 and CYP1A1 and the development of ALL in Kashmiri children.

Chapter: 1

Introduction

The term 'leukemia' was coined in 1856 by the German pathologist **Rudolph Virchow** to describe a disease characterized by unusual quantities of white cells visible in the blood when studied under a microscope. Leukemia is no longer regarded as a single disease, but rather a collection of disorders with overlapping symptoms. Leukemia is a cancer arising from hematopoietic cell lines **(Bloomfield et al., 2001)** that produce blood cells. Leukemia is thus a hematological malignancy, in which malignant blood cells or their precursors proliferate without control and accumulate in bone marrow and blood resulting in abnormal blood cells. Leukemia appears to be related to damage to chromosomes or genes **(National Cancer Institute, 1996)**.The damage disrupts the process by which blood cells achieve their final and functional form. Leukemia is the most common cancer in children accounting for about 1 out of 3 cancers in children **(American Cancer Society, 2012)** and these are the leading cause of cancer death in children under 15 years of age and the seventh most common form of cancer death overall.



(***Courtesy: Tenin, 2003***)

Fig 1.1: Tentative scheme of Hematopoiesis

Although the cause of Leukemia is uncertain, symptoms of the disease occur due to accumulation of immature blood cells in the bone marrow which prevents the normal production of red blood cells, white blood cells and platelets. Symptoms also include bruising, pallor, fatigue, fever, bone pain, and anorexia **(Mulhern et al., 2003)**.

Leukemia is often described as being either acute (fast growing) or chronic (slow growing). Almost all childhood leukemia is acute.

- **Acute leukemias**

  There are two main types of acute leukemia:

  - **Acute lymphocytic (lymphoblastic) leukemia (ALL):** About 3 out of 4 cases of childhood leukemia are ALL. This leukemia starts from the lymphoid cells in the bone marrow.
  - **Acute myelogenous leukemia (AML):** This type of leukemia, also called acute myeloid leukemia, acute myelocytic leukemia, or acute non-lymphocytic leukemia, accounts for most of the remaining cases. AML starts from the myeloid cells that form white blood cells (other than lymphocytes), red blood cells, or platelets.

- **Hybrid or mixed lineage leukemias:** In these rare leukemias, the cells have features of both ALL and AML. In children, they are generally treated like ALL and respond to treatment like ALL.
- **Chronic leukemias**

Chronic leukemias are much more common in adults than in children. They tend to grow more slowly than acute leukemias, but they are also harder to cure. Chronic leukemias can also be divided into two types:

- **Chronic myelogenous leukemia (CML):** This leukemia rarely occur in children.
- **Chronic lymphocytic leukemia (CLL):** This leukemia is extremely rare in children.

- **Juvenile myelomonocytic leukemia (JMML)**

This rare type of leukemia is neither chronic nor acute. It begins from myeloid cells, but it doesn't grow as fast as AML or as slow as CML. It occurs most often in young children (under age four).

ALL is the single most common diagnosis in pediatric oncology. Half to two-thirds of all ALL cases occur in children. ALL comprises 75-80% of childhood acute leukemias, while 20% are acute myelocytic leukemia (AML) and the remaining cases are chronic myelocytic leukemia (CML approximately 2%) and myelodysplastic syndrome (MDS) **(Pui et al., 1999)**. Acute leukemia accounts for only 2 percent of all cancers in the Unites States **(Krajinovic et al., 2004)**. ALL can occur at any age of childhood, but is most common between the age of 2 and 3 years, with 50% of the patients <5 years of age **(Gustafsson et al., 1998 and Sather et al., 1986)**.

Although ALL is the most common type of leukemia in young children, but the disease also affects adults, especially those aged 65 years and older. Many people with ALL can be cured. However, despite the available treatments, ALL remains a serious and life-threatening disease in some patients **(European Medicines Agency, 2009)**. In patients under 15 years old, they account for over 30% of all malignant diseases **(Pui et al., 2008)**. In the majority of cases of childhood leukemia, the cause is unknown. While a number of causes and highly suspected risk factors have been identified, reviews stress that these are responsible for only a very small number of cases. The known and highly suspected causes include genetic factors (2–3% of cases are associated with Down syndrome) and exposure to ionizing radiation in utero and after birth **(Stiller et al., 2004 and Belson et al., 2007)**. Infectious diseases are likely to have a role in the etiology of childhood leukemia, especially ALL **(Belson et al., 2007 and O'Connor et al., 2007)**. Delayed exposure to infection during early infancy could result in an abnormal response, leading to development of leukemia. ALL could also be a rare response to a specific although unidentified infectious agent. Other environmental risk factors have been less clearly identified. The International Agency for Research on Cancer has concluded that extremely low-frequency electromagnetic fields are possibly carcinogenic to humans, based on consistent statistical associations of high-level residential magnetic fields with a doubling of risk of childhood leukemia (**Lyon, International Agency for Research on Cancer, 2002**). Several studies suggest that children exposed to certain hazardous chemicals have an increased risk of leukemia, with benzene being the most frequently suspected causal agent **(Belson et al., 2007; Kingsley et al., 2007 and Holme et al., 2007)**. A number of papers have shown statistical associations between the risk of childhood leukemia and exposure to pesticides during pregnancy or childhood **(Belson et al., 2007; Zahm et al., 1998;**

Ma et al., 2002; Infante-Rivard et al., 2007 and Rudant et al., 2007). The risks associated with environmental leukemogens may be modified by genetic susceptibility **(Infante-Rivard et al., 2007 and Urayama et al., 2007)**. A number of ecological studies show a positive correlation between leukemia, particularly ALL, and increasing socioeconomic status **(Poole et al., 2006 and Committee on Medical Aspects of Radiation in the Environment, 2009)**. The reasons for this are not known.

The etiology of the majority of ALL are unknown and commonly involve gene - environment interactions that may result in chromosome translocations, deletions and inversions **(Skibola et al.,1999)** but the development of ALL probably arises through the gene-environment interactions should not be fully understood and should be investigated further **(Greaves et al., 1997)**. The frequencies of the different subtypes of ALL have been related to different factors in different countries. Nevertheless, though individuals are exposed to these environmental and lifestyle risk factors. Acute leukemia develops only in a small proportion of the exposed people, indicating that the host genetic factors might play an important role in the genesis of leukemia. Several genetic variations have been evaluated as possible risk factors for leukemia by meta-analyses and various investigations have focused on the xenobiotic metabolism, DNA repair pathways, and cell-cycle checkpoint functions that might interact with environment, dietary, maternal, and external factors to affect the development of ALL. Previous evidence indicates that carcinogen-metabolizing genes may play critical role in determining individual susceptibility to malignancies **(Boffetta et al., 2010 and Klaunig et al., 2010)**. Genetic variations in these genes may change the activities of their encoded enzymes, possibly by altering their expression and function. A number of published studies support a possible role for polymorphism in genes encoding cytochrome P_{450}, NAD(P)H quinineoxidoreductase, glutathione-S-transferases, serine-hydroxymethyltransferases, methylenetetrahydrofolate reductase, cell cycle inhibitors and thymidylate synthase. Thus, different genetic polymorphisms exert different effect on acute lymphoblastic leukemia risk. Nevertheless, only a few gene polymorphisms associated with leukemia susceptibility have been identified. To explore the role of other genetic polymorphisms on the risk is required.

Although the future looks promising with increasing knowledge of cancer biology and new treatment possibilities being developed, the etiology of the disease in Kashmir is

still a matter of speculation and proposed association with various environmental factors and genetic predisposition need to be deciphered. The understanding of the contribution of genetic predisposition with the malignancy can be extended to other regions in India and rest of the world particularly cancer belt regions and may be helpful in shifting from chemotherapy to chemoprevention. The study will pave way for other studies aimed at ascertaining the biomarkers for early detection and diagnosis. Hence, we are interested in understanding the status of these suspected polymorphisms that causes the inter-individual variability in drug response, influencing the treatment of several diseases. This necessitates the need for identifying the gene polymorphisms and also it will help in the development of most effective and specific drugs in ALL patients, as it has been rightly said:

"Cancer is a word, not a sentence." - John Diamond

Chapter: 2

Review of Literature

ACUTE LYMPHOBLASTIC LEUKEMIA

Acute lymphoblastic leukemia (ALL) is a malignant proliferation of lymphoid cells blocked at an early stage of differentiation. ALL is a biologically heterogeneous disorder characterized by morphologic, immunologic, cytogenetic, biochemical, and molecular genetics. Characterizations of leukemia lymphoblast are needed to establish the diagnosis or to exclude other possible causes of bone marrow failure and, finally, to classify ALL subtypes. This heterogeneity reflects the fact that leukemia may develop at any point during the multiple stages of normal lymphoid differentiation.

2.1 Epidemiology of ALL

Most ALL cases occur in children, with an incidence of 3 to 4/100,000 in patients 0 to 14 years of age and ~1/100,000 in patients older than 15 years, in the United States **(National Cancer Institute, 2009)**. ALL is the single most common diagnosis in pediatric oncology representing nearly one third of all pediatric cancers. In children, ALL represents 75% of all acute leukemias (which in turn represent 34% of all cancers in this age group), with a peak incidence at 2 to 5 years of age **(Gurney et al., 1995)**. This percentage is much lower in adults, in whom acute myeloid leukemias (AMLs) and chronic lymphocytic leukemias are more common **(Gurney et al., 1995)(American Cancer Society, 2009)**. There is a slight male predominance in all age groups and a significant excess incidence among white children (**National Cancer Institute, 2009**). The incidence of ALL, particularly T-cell ALL, is slightly higher among boys than girls (**Kersey et al., 1973; Zahm and Devesa, 1995; Ries et al**., **1998 and Margolin et al., 2001**). Girls, however, have a higher incidence of all leukemias during the first year of life **(Gurney et al., 1995 and Ries et al., 1998).**

ALL presents primarily as de novo disease, with only rare cases occurring as secondary neoplasm (**Shivakumar et al., 2008**). A variety of genetic and environmental factors have been related to ALL. It occurs with increased frequency in patients with Down syndrome, Bloom syndrome, neurofibromatosis type I (NF1) and ataxia telangiectasia **(Spector et al., 2006)**. In addition, exposure in utero to ionizing radiation, pesticides, and solvents have also been related to an increased risk for childhood leukemia **(Spector et al., 2006)**. Leukemia-specific fusion genes or immunoglobulin (Ig) and clonal Ig gene rearrangements have been identified in

neonatal spot (Guthrie) cards of patients who later developed ALL **(Gale et al., 1997 and Taub et al., 2002)**.

Geographical differences in the incidence, age distribution and subtypes of childhood leukemia are found among countries in different parts of the world **(Gustafsson et al., 1982; Miller et al., 1995 and Swensen et al., 1997**).The reported incidence rates are highest in Costa Rica, Australia, United States (among white children) and Germany. The rates are intermediate in most European countries and lowest in India and among black children in the United States **(Parkin et al., 1988 and Gurney et al., 1995).**

2.2 CLASSIFICATION

Acute lymphoblastic leukemia (ALL) encompasses a group of lymphoid neoplasms that morphologically and immunophenotypically resemble B-lineage and T-lineage precursor cells. These neoplasms may present predominantly as a leukemic process, with extensive involvement of the bone marrow and peripheral blood or may be limited to tissue infiltration, with absent or only limited (less than 25%) bone marrow involvement. The latter cases are typically designated as lymphoblastic lymphomas (LBLs). The current World Health Organization Classification of hematopoietic neoplasms designates these disorders as B- or T-lymphoblastic leukemia/lymphoma **(Swerdlow et al., 2008**). Acute leukemia can be classified in many ways: (1) By morphology and cytochemistry supplemented by immunophenotyping, as proposed by French-American-British (FAB) group **(Bennet et al., 1976)** (2) Proposed World Health Organization Classification of Acute Leukemia **(Harris et al., 1999)** (3) By immunophenotyping alone, as proposed by the European Group for the immunological classification of leukemias **(EGIL) (Bene et al., 1995 and Hayhoe et al., 1988)**.

2.2.1 French-American-British Classification.

In the 1970s, a group of French- American and British (FAB) leukemia experts divided ALL into 3 subtypes (L1, L2 and L3), based on the way the leukemia cells looked under the microscope after routine staining. Acute lymphoblastic leukemia (ALL) is divided in FAB L1 (children), L2 (older children and adult), and L3 (patients with leukemia secondary to Burkitt's lymphoma. These types are defined according to two criteria (1) the occurrence of individual cytological features and (2) the degree of heterogeneity among the leukemic cells. The features considered are cell size,

chromatin, nuclear shape, nucleoli, degree of basophilia in the cytoplasm and the presence of cytoplasmic vacuolation **(Bennett et al., 1976)**.

- **ALL-L1: Homogenous cells (Small cell):** One population of cells within the case. Small cells predominant, nuclear shape is regular with occasional cleft. Nuclear contents are rarely visible. Cytoplasm is moderately basophilic. L1 accounts 70% of patients. The L1 type is the acute leukemia that is common in childhood, with 74% of these cases occurring in children 15 years of age or younger.
- **ALL-L2: Heterogeneous cells:** Large cells with an irregular nuclear shape, cleft in the nucleus are common. One or more large nucleoli are visible. Cytoplasm varies in colour and nuclear membrane irregularities. L2 accounts 27% of ALL patients. The FAB-L2 blast may be confused with the blasts of acute myeloid leukemia. Approximately 66% of these cases of ALL in patients older than 15 years are of type 2.
- **ALL-L3: Burkitt's lymphoma type:** Cells are large and homogenous in size, nuclear shape is round or oval. One to three prominent nucleoli and sometimes to 5 nucleoli are visible. Cytoplasm is deeply basophilic with vacuoles often prominent. Intense cytoplasmic basophilia is present in every cell, with prominent vacuolation in most. A high mitotic index is characteristic with presence of varying degrees of macrophage activity. Mature B-Lymphoid markers are expressed by most cases.

The World Health Organization (WHO) classification has changed the grouping of ALL to reflect increased understanding of the biology and molecular pathogenesis of the diseases. In addition to discarding the L1-L3 terms, the new classification characterizes these heterogenous diseases based upon immunophenotype into 3 basic categories: precursor B-cell ALL, precursor T-cell ALL and mature B-cell ALL (Burkitt lymphoma/leukemia) **(Jaffe et al., 2001)**.

2.2.2 European Group for the Immunological classification of Leukemias (EGIL)

Immunologic surface and cytoplasmic marker studies are of great significance for classification of acute Lymphoblastic leukemias. The European Group for the Immunological Classification of Leukemias (**EGIL**) (**Bene et al., 1995 and Hoelzer et al., 2002**) has proposed that ALL be classified on the basis of immunophenotype alone. Initial attempts to classify ALL stemmed from the observation that

lymphoblasts and normal lymphoid precursors express common antigens. Based on the expression of these antigens, the consensus considers a 20% minimum threshold to define a positive reaction of blast cells to a given monoclonal antibody.

The earliest B-lineage markers are CD19, CD22 (membrane and cytoplasm) and CD79a (**Campana et al., 1988**). A positive reaction for any two of these three markers, without further differentiation markers, identifies pro-B ALL. The presence of CD10 antigen (CALLA) defines the "common" ALL subgroup. Cases with additional identification of cytoplasmatic IgM constitute the pre-B group, whereas the presence of surface immunglobulin light chains defines mature B-ALL.

T-cell ALL constitute approximately 25% of all adult cases of ALL. T-cell markers are CD1a, CD2, CD3 (membrane and cytoplasm), CD4, CD5, CD7, CD8, CD2, CD5 and CD7 antigens are the most immature T-cell markers, but none of them is absolutely lineage specific, so that the unequivocal diagnosis of T-ALL rests on the demonstration of surface/cytoplasmic CD3.

ALL of B or T lineage can additionally express stem-cell antigen CD34. It has little diagnostic relevance but can be prognostically important **(De Waele et al., 2001)**.The scoring system recently proposed by the EGIL group addressed the characterization of the acute lymphoblastic leukemia as B or T lineage ALL by including the most specific markers for the lymphoid lineages among those of earlier stages of cell differentiation and some non-specific but stem-cell markers. The system introduced a modified terminology specific to each 'maturation' step within the B- or T-cell lineage **(EGIL, 1995)** and was confirmed as adequate for both diagnosis and sub classification of ALL **(Thalhammer-Scherrer et al., 2002)**.

2.3 CYTOGENETICS IN ALL

ALL is a group of cytogenetically distinct diseases related to clinical characteristics. The cytogenetic grouping of ALL facilitates understanding of the differences in etiology and epidemiology of different disease subtypes. The main reasons for performing cytogenetic analysis in ALL include obtaining information on prognosis and monitoring the disease and MRD status at levels beyond the sensitivity of cytomorphologic methods. Recurrent genetic abnormalities are hallmark of acute leukemia and provide insights into the molecular mechanisms of leukemogenesis **(Armstrong et al., 2005)**. The most frequent targets of genetic alterations involved in

hematological disorders are genes controlling transcription and tyrosine kinases **(Mitelman et al., 2004)**. The large variety of genetic alterations includes point mutations and deletions, but the main genetic characteristics of acute leukemia are translocations and numerical chromosome imbalances resulting in hyper or hypodiploidy. These chromosomally defined subtypes also show distinctive patterns of global gene expression in microarray analysis **(Greaves and Wiemels et al., 2003)**.

2.3.1 CHROMOSOMAL TRANSLOCATIONS

Cytogenetic analyses of childhood ALL have been found to be of both biological and clinical importance, and banded karyotyping is today part of the routine diagnostic procedure. Chromosomal aberrations in ALL can be divided into two groups, which can be present simultaneously: Abnormalities in the number of chromosomes (ploidy) and structural aberrations, such as translocations, deletions, partial duplications, inversions, and dendritic chromosomes. During the last three decades, numerous primary and secondary, chromosomal abnormalities, both numerical and structural, have been described in ALL and also shown to correlate to clinical parameters and outcome **(Mitelman et al., 1997 and Mitelman et al., 2002)**.

Two of the most constant findings are that hypodiploidy (less than 46 chromosomes per cell) and presence of Philadelphia chromosome are risk factors for treatment failure. Massive hyperdiploidy in ALL (greater than 50 chromosomes per cell), a typical childhood feature in this disease (1-10 years of age), has shown different degrees of impact on the prognosis with differences in treatment intensity, although in most studies it has been shown to be an indicator of a lower risk for treatment failure.

Chromosome translocations either result in inappropriate expression of an oncogene by juxtaposition of the entire coding sequence under constitutive activated regulatory elements of a partner gene or more commonly in leukemia in the formation of a chimeric fusion gene with novel properties. An increasing number of promiscuous genes (e.g. *MLL*, *ETV6* or *NUP98*) that recombine with numerous different partner genes have been identified and, thus, the number of fusion genes exceeds the number of affected genes.

In childhood BCP-ALL the most common genetic rearrangement is the t(12;21) (p13;q22), which fuses *ETV6* to *RUNX1* and is present in about 25% of BCP-ALL

cases.Fig.2.1**(Armstrong et al., 2005)**. Other commonly found chromosomal aberrations in childhood ALL are the t (1; 19) (q23; p13)/ *E2A-PBX1 (TCF3-PBX1),* the t(9;22)(q34;q11)/*BCR-ABL1*, and hyperdiploidy (presence of greater than 46 chromosomes), which is often associated with a *FLT3* mutation (Fig. 5) **(Armstrong et al., 2005)**. Rearrangements of the *MLL* gene occur in up to 80% of infant ALL and are associated with a pro-B ALL phenotype **(Attarbaschi et al., 2006; O'Neil and Look et al., 2007 and Pieters et al., 2007)**.

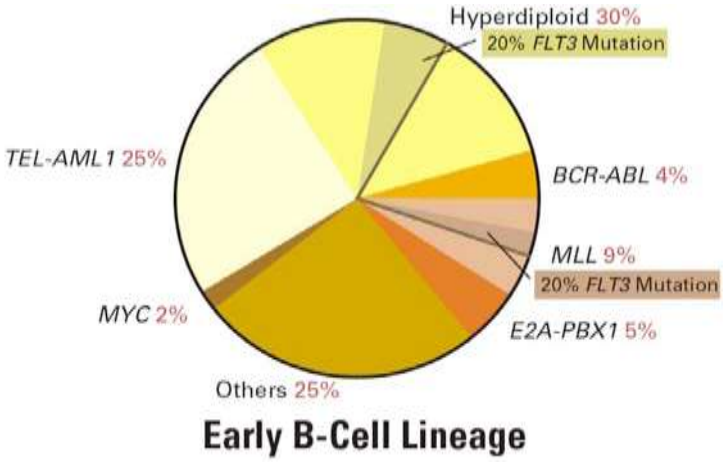


Figure 2.1: Pie diagram showing relative frequencies of chromosomal aberrations found in childhood B-ALL (Armstrong et al., 2005).

Recently, pediatric ALL was further characterized by genome-wide analyses using high resolution SNP arrays and DNA sequencing, which uncovered that in about 40% of BCP-ALL genes implicated in B-cell development and differentiation are targets of mutations, deletions or structural rearrangements **(Mullighan et al., 2007)**. These genes comprised *IKZF1* (*Ikaros*), *IKZF3* (*Aiolos*), *LEF1*, *EBF1*, *TCF3* (*E2A)*, and *PAX5* **(Kuiper et al., 2007 and Mullighan et al., 2007)**. In addition to micro deletions in transcription factors involved in B-lineage development, recurrent deletion of *BTG1* a negative effector of B-cell proliferation was observed. Moreover, other genes frequently affected by copy number losses were those controlling G1/S cell cycle progression (e.g. *CDKN2A, CDKN2B,* and *RB1*), and such deletions were detected in 54% of BCP-ALL and 86% of T-ALL, respectively **(Kuiper et al., 2007)**.

BCP-ALL, analysis of neonatal blood spots for leukemia specific rearrangements showed that most T-ALL cases are more likely initiated postnatally **(Fischer et al., 2007**). Interestingly, the *ETV6-RUNX1* and the *RUNX1-RUNX1T1* (*AML1-ETO*) fusion genes could be detected 100 times more often in blood samples from healthy

newborns as the risk of the corresponding leukemia **(Mori et al., 2002)**. The *ETV6-RUNX1* positive leukemic blasts of the twin diagnosed with full-blown BCP-ALL showed a deletion of the second *ETV6* allele, whereas the *ETV6-RUNX1* positive cells of the healthy twin harbored one intact copy of *ETV6.* These data strongly support the notion that inactivation of the second unrearranged *ETV6* allele indeed represents a crucial cooperating mutation **(Hong et al., 2008)**.

2.3.2 COOPERATIVE MUTATIONS

Although fusion oncogenes encoded by chromosomal translocations are a hallmark of pathogenesis of ALL. It seems likely that other genetic lesions are also needed to induce overt leukemia **(Knudson et al., 1971)**. A well-characterized example is the deletion or epigenetic silencing of the cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*) located in 9p21.3.This gene encodes the tumor suppressors p16INK4A and p14ARF **(Lukas et al., 1995 and Stott et al., 1998)**. Inactivation of this gene neutralizes both the TP53 and retinoblastoma pathways, which control the transition of cell cycle from G1 phase to S phase, thus serving as tumor suppressor proteins. Deletions of *CDKN2A* are present in about 70% of T-ALL and 30% of B-cell precursor ALL **(Bertin et al., 2003)**.

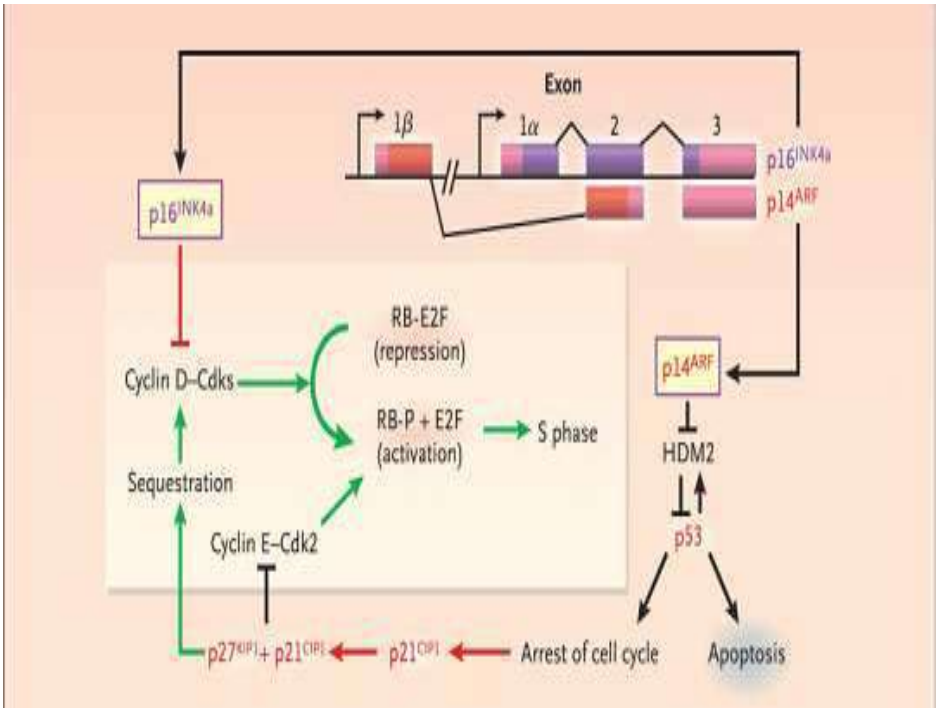


Fig 2.2: The Retinoblastoma pathway and p53 Tumor suppressor cross talk (Pui et al., 2004).

NOTCH1 has been identified as a partner gene in t (7; 9), found in less than 1% of T-ALL cases **(Ellisen et al., 1991)**. It encodes a transmembrane receptor that regulates normal T-cell development **(Maillard et al., 2005)**. Despite the rare involvement of *NOTCH1* in translocations, recent studies have shown its importance in T-ALL through activating mutations. Such mutations involving NOTCH1 are present in more than 50% of T-ALL patients **(Weng et al., 2004**; **Grabher et al., 2006 and Marks et al., 2009)**. The mechanisms by which aberrant NOTCH signaling causes T-ALL remain unclear. Expression of oncogenes, such as *MYC*, probably plays an important role. Evidence suggests that the MYC oncoprotein is an important downstream mediator of the pro-growth effects of NOTCH1 signaling in the developing thymocytes **(Weng et al., 2004)**. Activating mutations in *NOTCH1* can induce T-ALL in experimental models and could be the initiatory event in most human T-cell leukemias (**Grabher et al., 2006**).

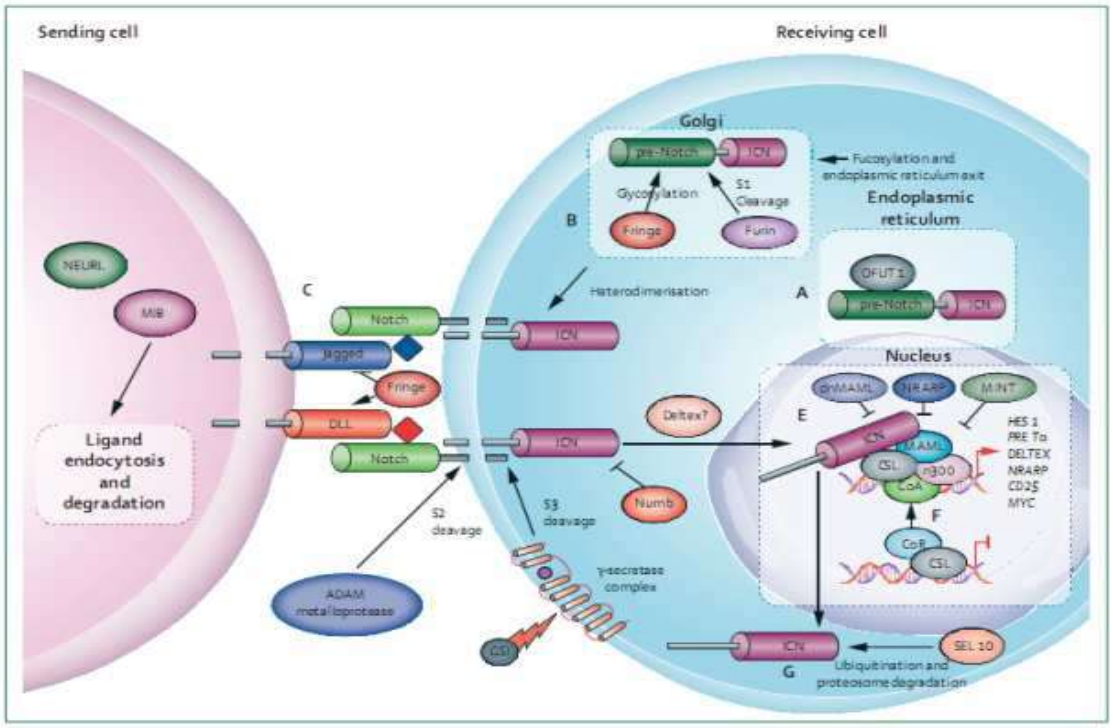


Fig 2.3: Notch signaling pathway in normal thymocytes (Pui et al., 2008)

2.4 ETIOLOGY OF LEUKEMIA

ALL is a heterogeneous grouping of biological subtype of leukemias and smaller studies of the past may have lacked sufficient statistical power to examine potential risk factors. Thus, one emerging theme concerning the etiology of childhood ALL is the need to separately study different biological groups of ALL. Epidemiologic studies of acute lymphoblastic leukemia in children have examined several internal and external risk factors for childhood ALL. These factors included a number of possible risk factors e.g., environmental, dietary, socioeconomic, genetic or immunological factors in an effort to determine the etiology of the disease. Knowledge of these particular risk factors can be used to support measures to reduce potentially harmful exposures and decrease the risk of disease.

2.4.1 ENVIRONMENTAL FACTORS

2.4.1.1 Ionizing radiations

Large epidemiological studies have documented that ionizing radiation under certain conditions of exposure induces human cancers including Acute lymphoblastic leukemia **(Jablon et al., 1970 and Black et al., 1987)**. Ionizing radiation is one of the few exposures for which the causal relationship with childhood leukemia, particularly AML, has been established **(Ron et al., 1998; Sali et al., 1996**; **United Nations Scientific Committee on the Effects of Atomic Radiation, 1994 and Mahoney et al., 2004)**. Currently, in utero low-dose ionizing radiation exposure is recognized as an established risk factor for childhood cancers under the assumption that the fetus may be more susceptible to the leukemogenic effects of radiation than the child. The magnitude of the risk depends on the dose of radiation, the duration of exposure, and the age of the individual at the time of exposure. Studies have demonstrated the relationship between the degree of irradiation and occurrence of leukemia **(Moloney et al., 1955 and Miller et al., 1967)**. The potential effect of ionizing radiation exposure on children may occur during preconception, pregnancy, or the postnatal period.

2.4.1.2 Non- Ionizing Radiations

Numerous epidemiologic studies have reported associations between measures of power-line electric or magnetic fields (EMFs) and childhood leukemia. The basis for

such association remains unexplained some studies have found a small association, **(Savitz et al., 1990**; **Hatch et al., 1998; Ahlbom et al., 2000**; **Greenland et al., 2000; Rivard et al., 2003)** while others have not **(Myers et al., 1990; Linet et al., 1997 and Kleinerman et al., 2000)**. The inconsistent results of the EMF and childhood leukemia studies may be due in part to differing methods for assessing residential magnetic field exposures and unmeasured EMF characteristics **(Hardell et al., 1995; Bowman et al., 2001; Brain et al., 2003)**. Furthermore, investigations of animals with exposure to much higher levels of EMFs than humans have not shown increased risk for hematopoietic neoplasia **(Brain et al., 2003)**.

2.4.1.3 Chemicals

Parental occupational exposure to chlorinated solvents, paints or thinners and plastic materials could cause leukemia in children. Parental occupational exposure to some certain chemicals like benzene can trigger the development of acute lymphoblastic leukemia in their offspring **(Greaves et al., 2002)**. A recent occupational study **(Glass et al., 2003)** found excess risk for leukemia associated with cumulative benzene exposures and benzene exposure intensities at lower levels (less than 60 ppm-years) than had been previously reported (as high as 220 ppm-years) **(Belson et al., 2007)**. Some studies have investigated the possible etiologic factors for childhood leukaemia related to parents occupational and home exposures **(Lowengart et al., 1987 and Shu et al., 1999)**.The results indicated an increased risk of leukemia for children whose father had an occupational exposure after the birth of the child to chlorinated solvents, spray paint, dyes or pigments, methyl ethyl ketone, and cutting oil.

2.4.1.4 Pesticides

There is growing evidence in support of the association between pesticide exposure and childhood leukemia. Most of the studies evaluating exposure to household pesticides and risk of childhood leukemia suggest that an increased risk is associated with in utero and postnatal pesticide exposures **(Ma et al., 2002)**. This suggests that newborns and children may be particularly sensitive to the carcinogenic effects of pesticides **(National Research Council, 1993 and Zahm et al., 1998)**. Most children exposure to pesticides is from home, lawn and garden use **(Grossman et al., 1995**). Other sources of exposure can include local agricultural applications, contaminated food, parental occupation, and pet products. Some studies have reported increased risk

with mothers, who had frequent prenatal pesticide exposure in the garden **(Infante-Rivard et al., 1999)** or during Pregnancy **(Lowengart et al., 1987)** and increased risk during childhood with garden insecticides and garden fungicides **(Menegaux et al., 2006).**

2.4.1.5 Alcohol cigarette and illicit drug use

The passive smoking literature is inconsistent regarding an association of maternal or paternal smoking on the risk of childhood ALL. It is unclear whether maternal or paternal cigarette smoking before or during pregnancy is a risk factor for developing childhood leukemia **(Shu et al., 1996 and Brondum et al., 1999)**. Some studies have reported that parental smoking, either maternal, paternal, or both, had a significant effect on childhood acute leukemia **(Ji et al., 1997)** while other studies found no association between parental smoking and childhood leukemia **(Pang et al., 2003)**. Two studies found that the frequency, amount and duration of paternal smoking before conception were related to significantly elevated risk, after adjusting for the effect of maternal smoking **(Sorahan et al., 1995; Ji et al., 1997 and Brondum et al., 1999)** however, found no association between the disease and paternal smoking at any time.

2.4.2 DIETARY FACTORS

There has been little systematic research on maternal dietary factors in childhood ALL. Most studies of maternal diet have focused on specific food groups such as vitamins A and D, **(Shu et al., 1988)**, cured meats, **(Blot et al., 1999)**, supplementation with folate **(Thompson et al., 2001)** or foods containing topoisomerase II inhibitors and their relationship to childhood acute lymphoblastic leukemia. Cured meats, which contain N-nitroso precursors that can be converted to carcinogenic N-nitroso compounds in an acidic environment, have been hypothesized to increase the risk of childhood leukemia either through maternal consumption during pregnancy or child consumption early in life **(Blot et al., 1999)**. Another study have shown that there is a significant positive association of childhood ALL with increasing consumption of DNA topoisomerase II inhibitor-containing foods such as beans, fresh vegetables, canned vegetables, fruit, soy, regular coffee, black tea, cocoa and wine **(Ross et al., 1996).**

2.4.3 IMMUNOLOGIC FACTORS AND POPULATION MIXING THEORY

Several studies contribute to the theory that a transmissible agent is potentially involved in the oncogenic process of childhood acute lymphoblastic leukemia. Some studies showed that the peak incidence of childhood ALL and that of common childhood infections both occur among children 2–5 years of age, as this age group possess sophisticated immune systems **(Greaves et al., 2002)**.While some showed viral etiology associated with some human cancers (e.g., Epstein-Barr virus for Burkitt lymphoma) **(Greaves and Alexander et al., 1993) (Kinlen et al., 1995)**.Susceptible individuals who live in rural areas in which the population suddenly increases, or for which the composition changes regularly, may be exposed to infectious agents that are brought into the area by new residents, possibly triggering a cluster of cases of ALL. The theory implies that the differences in rates of leukemia in rural areas are due to differences in herd, or population, immunity. Results from other studies support this theory **(Kinlen et al., 1995; Alexander et al., 1997; Gilman et al, 1998; Koushik et al., 2001 and Boutou et al., 2002)**.

Kinlen (1995) hypothesized that increased rates of leukemias are seen following periods of population mixing, which can introduce new infectious agents into a community of individuals who were previously unexposed or who may have susceptibility to the infectious agent. Similarly, *Greaves (1988 and 1997)* hypothesized that the development of leukemia is related to inadequate development of the infant's immune response or to a lack of exposure to infections in early childhood, resulting in an abnormal immune response. When these children are later exposed to common infections (the "delayed infection" hypothesis). Down's syndrome has also been associated with an increased risk of pediatric leukemia **(Taub et al., 2001 and Reynold et al., 2002)**.

2.4.4 SOCIO-ECONOMIC STATUS

In the vast majority of the epidemiologic studies focusing on childhood ALL, the role of socioeconomic status (SES) in the causal pathway is controversial. Early ecologic and descriptive studies from the United States suggested that higher SES was a possible risk factor for childhood leukemia while early United Kingdom studies reported mixed results **(Reinier et al., 2002)**. *Pelin (2002)* in his study observed that the socioeconomic status of the patients and the control group did not show a

significant difference, with approximately 75% belonging to lower socio-economic status.

2.4.5 GENETIC FACTORS

Genetic factors ranging from predisposing highly penetrant mutations to low penetrant genetic polymorphisms have been shown to significantly influence the interindividual variation in cancer incidence **(Shields et al., 2000)**. Therefore several internal and external risk factors contribute to root cause of cancer development i.e activation of oncogenes and inactivation of defense genes **(Nishihira et al., 1993)**. Certain gene polymorphisms have been shown to alter the risk of development of leukemia and these variations can interact with diet, other environmental exposures, and individual immune function to be major determinants of susceptibility. However, clonal evolution, characterized by complex karyotypic changes and the modest concordance rate for ALL in identical twins strongly suggest that additional genetic events, occurring mainly in the postnatal period, are required for progression to full malignancy **(Greaves et al., 1999).** Consistent with this paradigm, several genetic alterations, due to incorrect DNA synthesis or altered methylation status of oncogenes and/or tumor suppressor genes, have been identified in the pathogenesis of lymphoid malignancies **(Zing et al., 1997; Kuppers et al., 1999 and Vanesse et al., 1999).**

2.4.6 FOLATE-METABOLIZING ENZYMES

The folate cycle is important in DNA synthesis, DNA repair and DNA methylation. Methylenetetrahydrofolate reductase (MTHFR) has a major impact on the regulation of the folic acid pathway due to the conversion of 5, 10 methylenetetrahydrofolate (methylene-THF) to 5-methyl-THF. The two common polymorphisms of the MTHFR gene are 677 C→T and 1298 A→C, which are known to reduce the enzyme activity, leading to a decreased pool of methyl-THF particularly in the folate-deficient states. The polymorphic forms of MTHFR increase the level of methylene -THF leading to a subsequent reduction of uracil in DNA, protecting the DNA from double stranded breaks and consequently from chromosomal alterations **(Blount et al., 1997)**. MTHFR variants are known to influence disease processes and several studies in literature have reported on reduced MTHFR activity and susceptibility to lymphoid malignancies. MTHFR, either heterozygous and / or homozygous for 677T and 1298C polymorphisms have been shown to reduce the risk of ALL **(Chatzidakis et**

al., 2006 and Kamel et al., 2007), while other studies have found no association (**Chiusolo et al., 2004; Schnakenberg et al., 2005 and Thirumaran et al., 2005)**. Some studies found a protective role of T677 and C1298 variants in a subset of childhood leukemias **(Skibola et al., 1999; Smith et al., 1999 and Wiemels et al., 2001)** whereas, some observed the protective effect only for the MTHFR 677 homozygous (TT) genotype **(Franco et al., 2001)**. On the other hand, studies from India and Philippines have reported an increased risk of ALL among children for both polymorphisms **(Reddy et al., 2006 and Alcasabas et al., 2008)**. There have been several published studies with contradicting results of association between MTHFR polymorphisms and acute lymphoblastic leukemia. Multiple factors could be the reason for the difference between the observations in this study and results reported by others. Those include differences in ethnicity, gene-gene interaction, and gene environment interaction but it may be associated with increasing the risk for ALL. These results need to be confirmed by a larger sample size.

2.4.7 XENOBIOTIC METABOLIZING ENZYMES

Genetic susceptibility studies of genes that encode enzymes with critical roles in xenobiotic metabolism and membrane transport have shown associations with an increased risk of childhood acute lymphoblastic leukemia **(Chen et al., 1997; Krajinovic et al., 1999; Wiemels et al., 1999; Infante-Rivard et al., 2000; Krajinovic et al., 2000; Saadat et al., 2000; Franco et al., 2001; Krajinovic et al., 2002; Smith et al., 2002; Canalle et al., 2004 and Jamroziak et al., 2004)**. The complete metabolism of xenobiotic compounds is divided into two phases, each utilizing different sets of metabolic enzymes. The metabolic activation of the xenobiotics performed by the phase I enzymes are usually necessary in order for the phase II enzymes to convert this activated intermediate into a detoxified water soluble compound that can be easily eliminated from the cell **(Lang et al., 1999)**. Genetic polymorphisms that disrupt the equilibrium between these two phases compromise the host's ability to respond appropriately to several xenobiotics which may potentially increase the host's susceptibility to develop cancer.

The cytochrome P_{450} is a member of superfamily heme-thioloate monooxygenase enzymes (EC 1.14.14.1) that is involved in the oxidative metabolism of a number of endogenous and exogenous compounds like steroids, drugs, carcinogens and mutagens. In humans, 57 functional CYP_{450} genes and 58 pseudogenes have been

described **(Nelson et al., 2004 and Guengerich et al., 2008)**.The liver expressing CYP_{450} variants including many non-drug and drug metabolizing enzymes (Figure 2.4 A). Of the drug metabolizing CYP_{450} variants, the clinically important ones include phase I drug metabolizing enzymes (DMEs) like CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1, CYP3A4, CYP3A5 and CYP2D6 (Figure 2. 4 B).

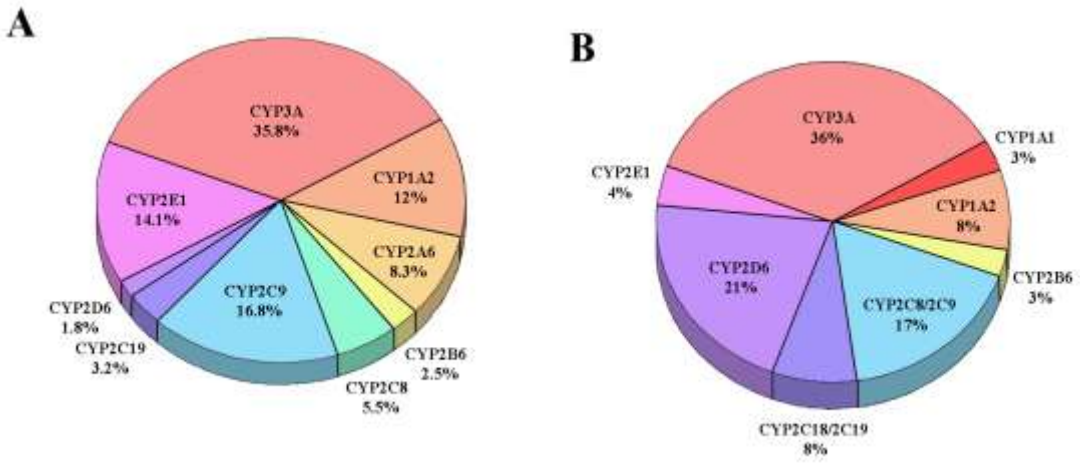


Fig 2.4*: *Pie diagram showing the relative abundance of human P_{450} enzymes in the liver (A) and the percentage of pharmaceuticals metabolized by respective P_{450} enzymes (B).

The drug metabolizing CYP_{450s} are responsible for the metabolism of 40-45% of all marketed drugs and 70-80% of all phase I dependent drug metabolism **(Ingelman-Sundberg and Rodriguez-Antona et al., 2005 and Guengerich et al., 2008)**.The activity of these CYP_{450} enzymes determine: (1) The rate of biotransformation of a parent drug into active and/or inactive metabolites. (2) The concentration of the drug and/or its metabolites that will be achieved in the body and (3) the rate of elimination of the drug and its metabolites.

Children may be particularly vulnerable to environmental toxins because of their greater relative exposure, immature metabolism and higher rate of cell division and growth. In this context, functional polymorphisms in xenobiotic metabolizing enzymes have been postulated to be of relevance in determining susceptibility to ALL **(Perera et al., 1997)**.These enzymes interact with environmental, dietary, maternal and other external and internal factors that affect the development of ALL. For example, inactivating polymorphisms of detoxifying enzymes (e.g., glutathione S-

transferase, reduced nicotinamide adenine dinucleotide phosphate: quinineoxido-reductase) have been variously associated with the development of ALL. However, these findings need to be confirmed by larger studies with careful attention to ethnic and geographic diversity in the frequency of polymorphisms **(Pui et al., 2004)**.

2.5 HUMAN CYTOCHROME P_{450}2D6 GENE

Cytochrome P_{450}, family 2, subfamily D, polypeptide 6 (CYP2D6) is the first identified enzyme controlled by single gene located on the long arm of chromosome 22q13, encoding protein of 497 amino acids **(Zhou et al., 2009).** It is the most polymorphic gene of metabolic enzymes so far, metabolizing nearly 20%~25% drugs in clinically with large individual differences **(Kimura et al., 1989; Wilkinson et al., 2005 and Sistonen et al., 2007).** It accounts for about 2% of all hepatic P_{450s}, but its role in drug metabolism is extensively higher than its relative content, metabolizing about 20-30% of all drugs on the market. In other words, it can be called a high-affinity low capacity enzyme. The polymorphism of CYP2D6 was independently discovered in three different labs **(Mahgoub et al., 1977; Tucker et al., 1977 and Eichelbaum et al., 1979)**.

The CYP2D6 function in any particular subject may be described as one of the following:

- **Poor metaboliser**–these subjects have little or no CYP2D6 function.
- **Intermediate metabolizers**–these subjects metabolize drugs at a rate somewhere between the poor and extensive metabolizers.
- **Extensive metaboliser**–these subjects have normal CYP2D6 function.
- **Ultrarapid metaboliser**–these subjects have multiple copies of the CYP2D6 gene expressed, and therefore greater-than-normal CYP2D6 function.

The type of CYP2D6 function of an individual may influence the person's response to different doses of drugs that CYP2D6 metabolizes. The nature of the effect on the drug response depends not only on the type of CYP2D6 function, but also on the extent to which processing of the drug by CYP2D6 results in a chemical that has an effect that is similar, stronger, or weaker than the original drug, or no effect at all. For example, if CYP2D6 converts a drug that has a strong effect into a substance that has a weaker effect, then poor metabolizers (weak CYP2D6 function) will have an

exaggerated response to the drug and stronger side-effects; conversely, if CYP2D6 converts a different drug into a substance that has a greater effect than its parent chemical, then extensive metabolizers (strong CYP2D6 function) will have an exaggerated response to the drug and stronger side-effects **(Lynch et al., 2007)**.

CYP2D6 GENE STRUCTURE

CYP2D6 belongs to the CYP2D gene family. The CYP2D gene cluster which is located on chromosome 22q13.1 **(Kimura et al., 1989)** consists of three genes: (1) CYP2D6, a functional gene (2) CYP2D8, a pseudogene that arose by gene conversion, and (3) CYP2D7, a nonfunctional gene that arose by gene duplication (Figure 2.5). In the CYP2D family, CYP2D6 is the only gene that encodes a functional enzyme **(Gaedigk et al., 2005)**. The wild type CYP2D6 gene is ~4.4 kb long. It is composed of 9 exons and a 1491 bp long open reading frame (ORF) that translates into a protein with 497 amino acids **(Kimura et al., 1989)**. Many alternative spliced transcript variants that encode different isoforms have also been reported. The CYP2D6 gene is highly polymorphic; these polymorphisms affect the pharmacokinetics, metabolism, safety and efficacy of drugs that are metabolized by it **(Ingelman-Sundberg et al., 2005).**

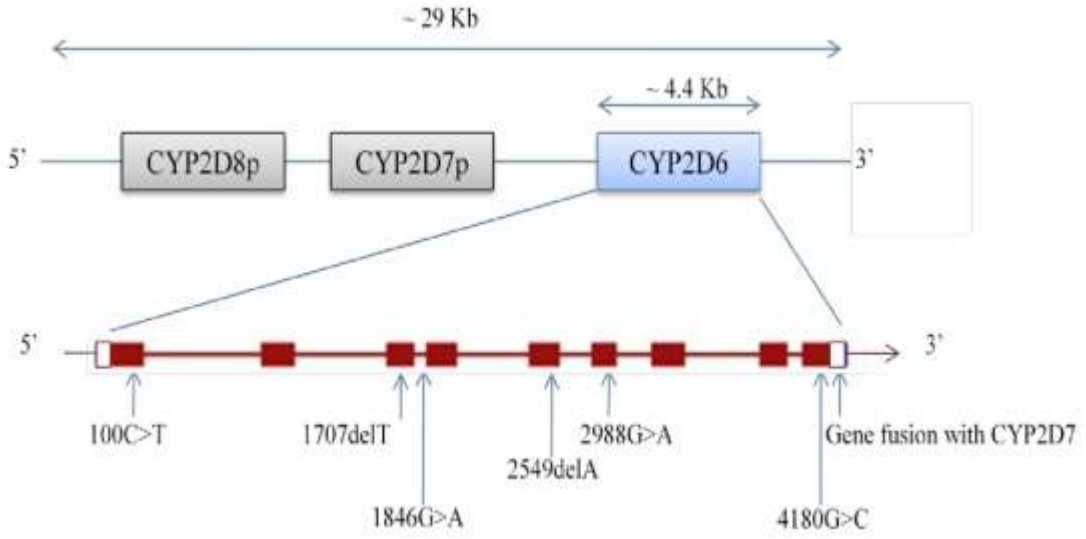


Fig 2.5: Representative picture of CYP2D6 gene, the brown boxes represent the exons and the white boxes represent the untranslated regions (UTRs). Some of the CYP2D6 representative variants are also shown; del denotes deletion; > denotes conversion.

The CYP2D6 gene is not highly conserved across species. The rat and mouse orthologs equivalent to human CYP2D6 are cyp2d1 and cyp2d22, respectively.

Among these three species, the drug metabolism profile is very different. They exhibit variability in substrate specificity and also enzyme inhibition **(Bogaards et al., 2000)**. Among rats and mice, there is also considerable inter strain difference in the metabolic profile (**Corchero et al., 2001**). For example, debrisoquine, a CYP2D6 substrate drug, is metabolized to 4-hydroxy debrisoquine by humans and Sprague-Dawley rats, but not by Dark Agouti rats, or by C57BL/6, DBA/2 and ddY strains of mice **(Al-Dabbagh et al., 1981 and Masubuchi et al., 1997)**. Hence, model organisms are generally considered to be inadequate for preclinical studies of CYP2D6 substrate drugs. However, a humanized CYP2D6 transgenic mouse model is now available and has been shown to be a good experimental model resembling the human extensive metabolizer phenotype for some drugs, including debrisoquine **(Corchero et al., 2001)**.

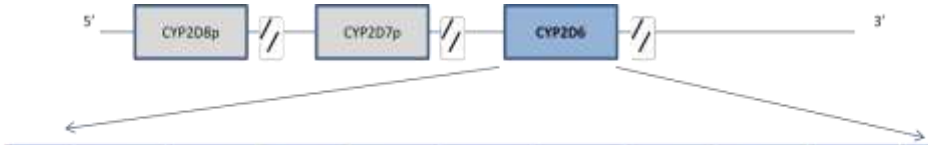
CYP2D6 GENE POLYMORPHISMS

CYP2D6 is a highly polymorphic gene. So far, more than 78 alleles and allelic subvariants have been identified **(Human Cytochrome P_{450} Allele Nomenclature Committee: www.cypalleles.ki.se/cyp2d6.htm)**.

These alleles can be classified as:

- Functional alleles, with normal enzyme activity (e.g., CYP2D6*1, *2, *35, etc.)
- Reduced functional alleles, with decreased enzyme activity (e.g., CYP2D6*9, *10, *17, *29, *37, *41, etc.) and
- Nonfunctional alleles, with negligible enzyme activity (e.g., CYP2D6* 3-*8, *11- *16, *18-*20, *36, *38, *40, *42 etc.).

A CYP2D6 star-allele represents either a single genetic variant or a haplotype that results in amino acid substitution, post-transcriptional modification, post-translational modification, and alteration in transcription, splicing, or translation **(Robarge et al., 2007)**. CYP2D6*1 is the wildtype reference sequence to which all the variants are compared. As new nucleotide variants (either a single variant or a combination of variants) are identified, a unique number is assigned (e.g., CYP2D6*2, *4, *10, etc) Fig.2.6



Allele	-1584C>G	100C>T	1661G>C	1707delT	1846G>A	2549delA	2850C>T	2988G>A	4180G>C	Gene conversion
*1	Wildtype									
*2	x		x				x		x	
*3						X				
*4		x	x		X				x	
*5	Deletion									
*6				X						
*10		X	x						x	
*36		X	x							X (ex 9)
*41			x				x	X	x	

Fig 2.6: X represents single nucleotide polymorphisms (SNP) that are used for genotyping; X represents some of the representative SNPs present in the given CYP2D6 star-allele haplotype

These polymorphisms can affect CYP2D6 enzyme expression and activity. For example, individuals with CYP2D6*1/*10 and CYP2D6*10/*10 genotypes had 3-fold lower CYP2D6 protein in their liver microsomes when compared to CYP2D6*1/*1 individuals **(Shimada et al., 2001)**. Consequently, these polymorphisms affect pharmacokinetics, metabolism, safety and efficacy of CYP2D6 substrate drugs. Based on the CYP2D6 allelic make up, an individual can be classified into different categories such as ultra rapid (UM), extensive (EM), intermediate (IM) or poor (PM) metabolizer.

There are considerable differences in the frequencies of CYP2D6 gene polymorphisms in different ethnic populations. For example, CYP2D6*4, CYP2D6*3, CYP2D6*5 and CYP2D6*6 are present at a combined frequency of 20-30% in Caucasians **(Bradford et al., 2002 and Sistonen et al., 2007)** and are the primary cause of poor metabolism in this population. However, in Asians and Africans, CYP2D6*3-*6 alleles are present in less than 7% frequency (**Bradford et al., 2002**). In Asians, the primary cause for reduced enzyme activity is CYP2D6*10, an intermediate metabolizer allele that is present at a frequency of 40-60% **(Veiga et al., 2009)**. However, in both Caucasians and Africans, CYP2D6*10 is present at a frequency of less than 5% **(Bradford et al., 2002)**. In Africans, the primary cause of

reduced enzyme activity are CYP2D6*17 and CYP2D6*29. Both these alleles are present in less than 3% frequency in both Caucasian and African populations **(Bradford et al., 2002 and Sistonen et al., 2007)**.

The role of CYP2D6 in cancer susceptibility was explored in earlier works, and there are controversial findings for the poor metabolizer (PM) allele frequency and the risk of acute lymphoblastic leukemia **(Lemos et al., 1999 and Krajinovic et al., 1999)**. Some of them support the assertion that the PM and/or High efficient metabolizer (HEM) allele carriers are at increased risk for ALL because of insufficient detoxification **(Roddam et al., 2000)**.

The CYP2D6 gene polymorphism, due to single nucleotide polymorphisms, gene duplication and deletion are relatively well known. At least 60 CYP2D6 alleles are responsible for the about 200-fold variability in the metabolism. CYP2D6*3 and CYP2D6*4 are the most common nonfunctional CYP2D6 alleles in Caucasians population. The mutation in CYP2D6*3 consist of a single base-pair deletion in exon 5 and in the case of CYP2D6*4 in G to A transition in 1934 position which causes an altered reading frame shift and a premature stop codon. The carriers of deficient (CYP2D6*3/*4) or nonfunctional (CYP2D6*4/*4) alleles are poor metabolizers (PM). The homozygote for wild-type CYP2D6 alleles (CYP2D6*1/*1) belong to extensive metabolizers (EM), while heterozygous persons to intermediate metabolizers (IM). Another relatively common phenomenon in humans is the duplication or amplification of CYP2D6. The mechanism involved in the gene duplication process is likely to be due to an unequal crossover between the two sister chromatides which causes deletion of the CYP2D6 gene (CYP2D6*5) in one chromatid and duplication of the gene in the other one (CYP2D6*X2). The presence of multiduplicated genes is attributable to additional unequal crossover events affecting chromatids that already contain duplicated genes. The carriers of three or more active alleles are ultra rapid metabolizers (UM). The clinical relevance of genetic polymorphism can be seen in the wide inter individual variability of therapeutic efficacy and in the spectrum of drug adverse effects **(Agundez et al., 2001; Daly et al., 1996 and Sachse et al., 1997)**.

2.6 HUMAN CYTOCHROME P_{450}1A1

Cytochrome P_{450}, family 1, subfamily A, polypeptide 1 is a protein **(Kawajiri et al., 1999)** that in humans is encoded by the CYP1A1 gene **(Nelson et al., 2004)**.The protein is a member of the cytochrome P_{450} superfamily of enzymes **(Smith et al., 1998)**. CYP1A1 is involved in the activation of PAHs into reactive epoxide metabolites and it also hydroxylates the two major estrogens, E2 and E1, predominantly at the C-2 position **(Shimada et al., 2004 and Spink et al., 1992)**.

The CYP1A1 (P1-450) gene, located at 15q22-q24, comprises seven exons 1and six introns and spans 5810 base pairs (**Jaiswal et al., 1985 and Kawajiri et al., 1986)**. Human CYP1A1 (EC: 1.14.14.1) is mainly present in the skin, lungs, placenta, and lymphocytes and plays an important role in the metabolic activation of chemical carcinogens. Basal CYP1A1 protein expression in all tissues is thought to be low **(Nebert et al. 2004)**, but varying levels of CYP1A1 mRNA have been detected following induction by polycyclic aromatic hydrocarbons (PAH's). However, in breast tissue, the expression level of CYP1A1 mRNA has been shown to be much weaker than that of CYP1B1 mRNA **(Hellmold et al., 1998 and Iscan et al., 2001)**. Induction of CYP1A1 gene is regulated by the aromatic (aryl) hydrocarbon receptors (AhR) and the AhR nuclear translocator **(Whitlock et al., 1999)**. CYP1A1 is capable of oxidizing benzo[a]pyrene and other PAH's to carcinogenic species (**Chun et al., 1996; Chua et al., 2000; Liu et al., 2003 and Sparfel et al., 2004**). This enzyme is strongly induced by cigarette smoke and potentially associated with lung cancer **(Patterson et al., 2002 and Spivack et al., 2003)**. Like many other P_{450s}, CYP1A1 is polymorphic and genetic variation is thought to play a role in determining cancer susceptibility (**Miyoshi et al., 2002; Han et al., 2003 and Balasubramanian et al., 2004)**.

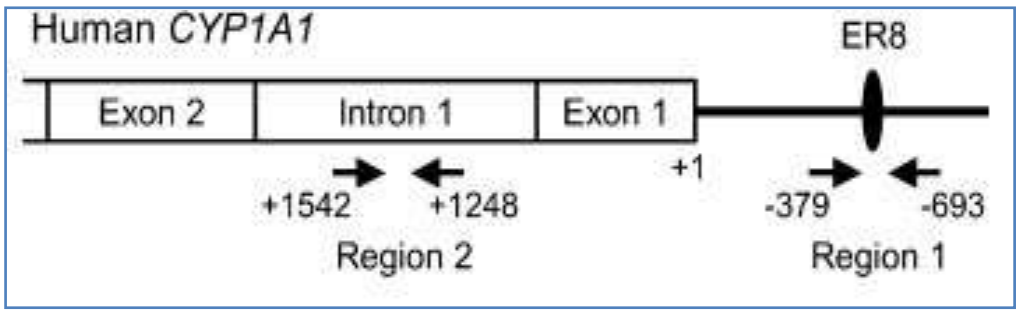


Fig: 2.7: Representative structure of CYP1A1 gene

CYP1A1 GENE POLYMORPHISMS

Several polymorphisms have been identified in CYP1A1 gene. Some of which lead to more highly inducible Aryl hydrocarbon hydroxylase (AHH) activity.CYP1A1 polymorphisms include:

- **M1**, T→C substitution at nucleotide 3801 in the 3'- non-coding region
- **M2**, A→G substitution at nucleotide 2455 leading to an amino acid change of isoleucine to valine at codon 462
- **M3**, T→C substitution at nucleotide 3205 in the 3'- non-coding region
- **M4**, C→A substitution at nucleotide 2453 leading to an amino acid change of threonine to asparagine at codon 461 **(Petersen et al., 1991; Cosma et al., 1993 and Crofts et al., 1996)**

Several polymorphic sites for the CYP1A1 have been reported. The first reported CYP1A1 polymorphism (T3801C) was detected by the MspI restriction enzyme **(Spurr et al., 1987**). This polymorphic site is located in the 3' non coding region of CYP1A1 and results in CYP1A1*2A allele. In African and Americans, an additional MspI restriction site exists in the 3' non-coding region (T3205C) resulting in CYP1A1*3 allele, but this has not been reported among Caucasians or Asians **(Crofts et al., 1993)**. Two polymorphisms in exon 7 result in amino acid changes at codons 461 (Thr→Asn) and 462 (Ile→Val), which are situated near the catalytic region of the CYP1A1 **(Crofts et al., 1994 and Cascorbi et al., 1996)**. These alleles are named CYP1A1*4 and CYP1A1*2C, respectively. Even though they are situated in adjacent codons, they are not linked with each other **(Cascorbi et al., 1996)**. Instead, strict linkage between the T3801C and Ile 462 Val polymorphisms has been observed among Caucasians **(Cascorbi et al., 1996; Hirvonen et al., 1992 and Fontana et al., 1998)**.

Polymorphism of CYP1A1 and exposure to chemicals such as polyaromatic hydrocarbons (PAHs) in relation to cancer may be given as an example for xenobiotic metabolizing enyzmes polymorphisms, chemical exposure and cancer susceptibility. Studies were carried out to demonstrate the association between CYP1A1 polymorphisms and smoking related cancers. CYP1A1 polymorphisms were found to be associated with lung **(Coles et al., 1990 and McClellan et al., 1996)**, head and

neck **(Doll et al., 1998)**, prostate **(Murata et al., 2001)** and colorectal **(Sivaraman et al., 1994)** cancers. Benzo(a)pyrene (BaP) found in the tobacco smoke, is one of the substrate of CYP1A1. Within body, BaP is converted to BaP 7, 8 diydrodiol- 9, 10-epoxide (BPDE) by several reactions of CYP family. BPDE is one of the most carcinogenic compound ever known by CYP1A1, which can bind to DNA and forms DNA-adducts **(Parke et al., 1991).** Interestingly, CYP1A1 is induced by BaP itself, which causes higher rate of CYP1A1 expression and so higher activity of CYP1A1 enzyme and increased rate of accumulation of carcinogenic compound **(Stegeman et al., 1995 and Arınç et al., 2000)**.

The polymorphism of CYP1A1 and its association with childhood ALL were studied by several groups. Previous association studies have shown that variants within cytochrome P_{450}1A1 (*CYP1A1*), a member of the CYP1 gene family of constitutive and inducible enzymes with a major role in the oxidative activation and/or deactivation of a wide range of xenobiotics, are associated with increased risk in ALL **(Krajinovic et al., 1999; Sinnett et al., 2000; Krajinovic et al., 2001 and Canalle et al., 2004)**. In particular, two common polymorphisms in the *CYP1A1* gene, a T6235C change within the 3′ noncoding region of the gene (*2A, also known as the MspI restriction fragment length polymorphism) and an A4889G change in the heme-binding domain of exon 7 (*2C), have been previously described to influence enzymatic activity **(Hayashi et al., 1991)**. An elevation in CYP1A1 enzyme activity has been found with the A4889 polymorphism in exon 7 alone and when combined with the T6235C polymorphism to form the CYP1A1*2B genotype **(Crofts et al., 1994)**. CYP1A1 polymorphisms have been found to be significantly associated with both an increased genetic susceptibility **(Krajinovic et al., 1999)** and its worse prognosis **(Krajinovic et al., 2002)** for childhood ALL among French–Canadians. Another study found that CYP1A1 polymorphisms greatly increased susceptibility of ALL among Indian children with the homozygous CYP1A1*2A conferring a 6-fold risk and the CYP1A1*2C conferring a 4-fold risk **(Joseph et al., 2004)**. However, a possible role of CYP1A1 polymorphisms in ALL was not confirmed in Turkish **(Balta et al., 2003; Aydin-Sayitoglu et al., 2006)**, Brazilian **(Canalle et al., 2004)** or Chinese **(Chen et al., 2008**) study groups.

Although research has elucidated tumorigenic characteristics of CYP1A1 polymorphisms, these studies have not examined the impact of ethnicity on these

variants and ALL risk. No study has investigated the importance of CYP1A1 polymorphisms and ethnic status by comparing Caucasian, Hispanic, and African-American children. Furthermore, no studies have explained the increased incidence of ALL in the U.S. Hispanic population.

Chapter: 3

Materials & Methods

3.1 MATERIAL

3.1.1 STUDY POPULATION

Subjects were recruited at Sher-i- Kashmir Institute of Medical Science (SKIMS), Srinagar Kashmir. The recruitment process was initiated following approval from the ethical committee of SKIMS. The diagnosis of Acute lymphoblastic leukemia (ALL) was based on the standard histopathological criteria. Controls were taken from healthy individuals of Kashmir valley from Department of Hematology, SKIMS, Soura, following the referral pattern of sex and age matched patients. None of the controls had a personal history of malignancy.

3.1.1.1 SUBJECTS

Criteria adopted for selecting the cases and controls

The criteria for including or excluding a subject in the study were formulated prior to the commencement of the study.

Cases

All the documented confirmed ALL patients were included irrespective of cancer stage but were age and gender matched.

Exclusion criteria

Under the following conditions the patients were not recruited in the study;

- Patients suffering from any disease that affected their life style.
- Patients who had received prior chemo or radiotherapy.
- Patients not belonging to Kashmir valley.
- Who did not agree to participate

Inclusion Criteria

- Documented proven ALL patients.
- Patients who were willing to take part in the study
- Patients below the age of 20 years were included.
- Patients belonging to Kashmir valley

Controls

Controls were matched with the cases by age and gender.

Exclusion criteria

- Patients who ever suffered from any kind of malignancy.
- Patients who did not agree to participate.

Inclusion criteria

- Residents of the Kashmir valley.
- Matched male and female ratio with the cases.

3.1.2 COLLECTION OF BLOOD SAMPLES

Individuals who gave consent to participate in this case-control study were included in the study. Blood samples were collected from both cases and the controls. Around three milliliter (3 ml) of peripheral blood was collected from each subject in sterilized plastic vials containing EDTA (0.5M; pH-8.0) and stored at -20^{o}C for further analysis.

3.2 METHODS

3.2.1 DNA EXTRACTION FROM WHOLE BLOOD

Blood samples of about 2 ml were obtained from the patients in the EDTA coated plastic vials and genomic DNA was isolated by Phenol-chloroform method as per the given procedure **(Sambrook and Rusell, 2001)**.

1. Cells were first lysed with 4ml of freshly prepared Lysis buffer (155mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) with gentle mixing and kept at - 20^{o}C for 15 minutes.
2. The tubes were then centrifuged at 8000 rpm for 10 minute and then supernatant was discarded.
3. The pellets were resuspended in 2 ml of SE, 100µg/ml Proteinase k (100µg/ml) and 200 µl of 10% SDS. The solution were then incubated at 37^{o}C overnight.
4. On the next day, equal volumes of Tris-saturated-phenol (pH 8) were added to the samples and mixed thoroughly by inverting the tube for 15-20 minutes.

5. The tubes were then centrifuged at 6000 rpm for 10 minutes. The aqueous supernatants were transferred to fresh tubes and to it, an equal volume of phenol-CIA (chloroform-isoamyl alcohol) were added.
6. The tubes were shaken gently for 15 minutes and then centrifuged at 7000 rpm for 10 minutes.
7. The supernatant formed were transferred to fresh tubes and an equal volume of CIA was added to them.
8. The tubes were again shaken gently for 15-20 minutes and then centrifuged at 8000 rpm for 10 minutes.
9. To the supernatant, an equal volume of chilled ethanol and 0.1 volume of sodium acetate was added to precipitate the DNA immediately.
10. After retrieving, the DNA was washed thrice with 70% ethanol and subsequently dissolved in 5 ml of DNA storage buffer and stored at 4oC for future use.

3.2.2 DETERMINATION OF CONCENTRATION, PURITY AND QUALITY OF GENOMIC DNA

3.2.2. Qualitative Analysis

The quality of the genomic DNA was assessed on 0.8% gel electrophoresis. 2µl of each DNA sample was mixed with 1µl of 1X DNA loading dye (1X loading dye consisted of 4.16 mg bromophenol blue, 4.16 mg xylene cyanol and 0.66g sucrose in 1ml water) and was loaded in the gel. The voltage was kept constant at 20V until DNA left the wells and moved into the gel,for rest of the run voltage was raised to 50V. Run was stopped when the dye had travelled nearly 2/3rd of the gel. Gel was visualized by a Gel doc system (Alphaimager^{TM} 2200, Alpha InfoTech Corporation) under UV light and picture was captured by using CCD camera system.

3.2.2.2 Quantitative Analysis

The quantity of the DNA was estimated by making appropriate dilutions to determine the optical density (OD) at 260 nm and 280 nm by double beam spectrophotometer (Spectron 2206) and the concentration was determined using equation:

$$\text{DNA } (\mu g/ml) = A_{260} \times 50 \times \text{dilution factor}$$

The ratio of A_{260}/A_{280} was calculated and the DNA samples for which the ratio was 1.7-1.9 were considered for the future use. The DNA was stored at 4^oC for a short time but the stock was kept at -20^oC for further use.

3.2.3 AGAROSE GEL ELECTROPHORESIS

- The edges of clean, dry glass plates were sealed with tape to form a mold, and were set on a horizontal section of the bench.
- Sufficient electrophoresis buffer (1xTAE) was prepared to cast the gel and to fill the electrophoresis tank.
- 0.8g of agarose was dissolved in 100 ml of 1xTAE buffer and heated in an oven until a clear transparent solution formed.
- After removing from the oven the solution was allowed to cool to 50-60^{o}C and then few (5µl) of ethidium bromide was added as visualizing agent.
- The warm agarose was poured into the mold. An appropriate comb was positioned for forming the sample slots in the gel when the agarose was added to the mold.
- The gel was allowed to set completely (30-45 min), then a small amount of TAE buffer was poured on top of the gel and comb was carefully removed.TAE buffer was poured, after removing tape also, and gel was mounted in the tank.
- Sample was mixed with few (2-3 µl) of 6X gel loading buffer.
- Sample was loaded slowly and carefully into the slot of submerged gel using disposable loading tips.
- Lid of gel tank was closed and electric leads connected. DNA migrated toward positive anode. Voltage of 1-5 V/cm was applied (distance measured from cathode to anode). The gel was allowed to run until the bromophenol blue and xylene cyanol migrated an appropriate distance through the gel.

  Pattern of separation was visualized by a Gel doc system (Alphaimager^{TM} 2200, Alpha InfoTech Corporation) under UV light and picture was captured by using CCD camera system. Samples containing high molecular weight DNA with no fragmentation/ shearing and without any apparent contamination or streaking were selected for further analysis.

3.2.4 GENOTYPING OF CYP2D6 AND CYP1A1 GENES

Once it was confirmed that the genomic DNA is present, the concentration and purity is also desirable, the two xenobiotic metabolizing enzyme genes: CYP2D6 and CYP1A1 were genotyped for their most common single nucleotide polymorphisms.

***CYP2D6* (G1934A)** and ***CYP1A1* (T6235C)** polymorphisms were identified by PCR amplification of SNP regions. The standard protocol for PCR was used, however the technique was standardized and optimized for available laboratory conditions. After standardizing all the parameters of the technique like varying annealing temperature from 56^{o}C to 64^{o}C (CYP2D6 gene) and 55^{o}C to 65^{o}C (CYP1A1 gene), dNTPs, primer and template concentration, the 5' UTR of these genes were amplified. PCR was performed in a total volume of 25µl for both genes. For these two genes, the PCR reactions composed of 50-150 ng genomic DNA, 0.2mM dNTPs, 0.4pmoles/µl of each primer and 1.5 U of Taq polymerase in 1X PCR buffer were used. Lyophilized primer stocks were diluted first to 100pmol/µl concentration and then to 20pmol/µl using mili Q water. PCR buffer contained Tris-Cl, KCl, $(NH_4)_2SO_4$ and 15mM $MgCl_2$ (pH 8.7). Amplification and specificity of amplicon obtained in the PCR reaction was analyzed by agarose gel electrophoresis on 2.5% gel. After the electrophoresis, the gel was visualized using UV-illuminator and photographed in a Gel Doc (Alphaimager^{TM} 2200, Alpha InfoTech Corporation).

3.2.4.1 *Genotyping of CYP2D6 G1934A Polymorphism*:

In this study, CYP2D6 gene was genotyped by PCR for the detection of CYP2D6 **(G1934A)** (Arg281His) polymorphism. G1934A mutation is located between the junction of intron 3 and exon 4 region of the gene. A 334bp fragment covering G1934A point mutation was amplified. The primer pair used for amplification was:

CYP2D6

Forward primer: 5'- GCCTTCGCCAACCACTCCG-3'

Reverse primer: 5'- AAATCCTGCTCTTCCGAGGC-3'

Table 3.1: Volume of different reagents used in 25µl of PCR of CYP2D6 gene

Reagent	Volume used
10X PCR reaction buffer	2.5 µl
$MgCl_2$	2.1 µl
dNTP mix	0.7 µl
Forward Primer	0.45 µl
Reverse Primer	0.45 µl
Genomic DNA	1.5 µl
Taq DNA polymerase	0.3 µl
Milli Q water	17 µl
Total Volume	**25.0 µl**

PCR Amplification

Reaction mixture in PCR tubes was gently mixed and placed in a twenty-five well automated thermal cycler (Eppendorf). The different temperatures were set as given in the Table-3.2. During this procedure precautions were taken to keep the Taq polymerase active by not only preparing the reaction under 4^{o}C temperature but also adding the various reaction components as quickly as possible. The PCR conditions were selected after extensively standardizing all the PCR parameters.

Table 3.2: PCR cycling parameters for CYP2D6 Gene

Steps	Temperature ^{o}C	Time	
Initial Denaturation	95	5 minutes	
Denaturation	95	59 seconds	33 Cycles
Annealing	59.7	59 seconds	
Extension	72	59 seconds	
Final extension	72	10 minutes	

3.2.4.2 Genotyping of CYP1A1 T6235C polymorphism:

CYP1A1 gene was genotyped by PCR method for the detection of *CYP1A1* **T6235C** polymorphism located 264bp downstream from the 3'-flanking region, forming an Msp1 restriction site (*CYP1A1m1*). A 899bp fragment covering T6235C point mutation was amplified. The primer pair used for amplification was;

CYP1A1
- Forward primer: 5'- GGCTGAGCAATCTGACCCTA-3'
- Reverse primer**: 5'-** TAGGAGTCTTGTCTCATGCCT-3'

Table 3.3: Volume of different reagents used in 25µl of PCR of CYP1A1 gene

Reagent	Volume used
10X PCR reaction buffer	2.5 µl
$MgCl_2$	1.8 µl
dNTP mix	0.7 µl
Forward Primer	0.6 µl
Reverse Primer	0.6 µl
Genomic DNA	2.0 µl
Taq DNA polymerase	0.3 µl
Milli Q water	16.5 µl
Total Volume	**25.0 µl**

Table 3.4: PCR cycling parameters for CYP1A1 Gene

Steps	Temperature ^{o}C	Time	
Initial Denaturation	95	5 minutes	
Denaturation	95	59 seconds	33 Cycles
Annealing	63	50 seconds	
Extension	72	59 seconds	
Final extension	72	10 minutes	

3.2.5 Purification and DNA Sequencing

The purification and sequencing was done commercially by using the services of Sci-Genomics, Kerala. For purification and sequencing, 50 µl of unpurified PCR product samples along with 50µl of 20µM Forward & Reverse primers were used. DNA sequences of the amplicons were obtained in pdf formats. The pdf file of each DNA sequence was used for visual inspection of the sequencing chromatograph using Acrobat Reader 5.0.

3.2.6. Statistical Analysis

The allele frequency for each allele was determined by gene counting. The allele and genotype frequencies of patients were given together with the 95% confidence interval (CI). Statistical analyses were conducted using software Graph Pad Prism version 5.0. The genotype distributions of polymorphisms were compared by $\chi 2$ – test (Pearson). A p value <0.05 was considered to be statistically significant throughout the population comparison. Comparisons of genotypes in control and ALL patient groups were done by calculation odds ratios (OR).

Chapter: 4

Results

4.1 GENERAL CHARACTERISTICS OF STUDY POPULATION

In the present study 120 blood samples from ALL patients and 110 blood samples from healthy controls were analyzed. General characteristics of the ALL patients and controls are given in table 4.1.

Table 4.1: General characteristics of the study population

Characteristics	Cases n=120 (%)	Controls n=110 (%)	P value*
Mean Age (±SD)			
>10	55 (45.83)	47(42.72)	0.73
<10	65(54.16)	63(57.27)	
Gender			
Male	70(58.33)	62(56.36)	0.86
Female	50(41.66)	48(43.63)	
Dwelling			
Rural	110 (91.67)	96 (87.27)	0.38
Urban	10 (8.33)	14 (12.73)	
Occupation			
Un-employed	95 (79.17)	80 (72.73)	0.32
Employed	25 (20.83)	30 (27.27)	

n =No. of individuals
Values represent ±S
*p using χ^2 Test ($p<0.05$, Data statistically significant)

4.2 EVALUATION OF DNA CONCENTRATION AND PURITY

The genomic DNA was extracted from the whole blood samples from the ALL patients and controls of Kashmir. The integrity, concentration and purification of the genomic DNA was checked not only by UV-spectrophotometer but also by agarose gel electrophoresis, by analyzing 3-4 µl of genomic DNA on the 0.8 % agarose gel (Fig.4.1), which reflected the intactness of genomic DNA, because the genomic DNA was restricted to the wells with no smear in the gel suggesting that DNA was not degraded. The results showed that DNA concentration of the samples ranged from 300ng/µl to 1200 ng/µl which was sufficient enough to carry out the other phases of the study. The purity of DNA was determined by A260 / A280 ratio and it was found that the ratio in all DNA preparations was in the range of 1.60-1.94.Agarose gel electrophoresis also demonstrated that DNA samples had high molecular weight without degradation. Fig. 4.1.

Lane:

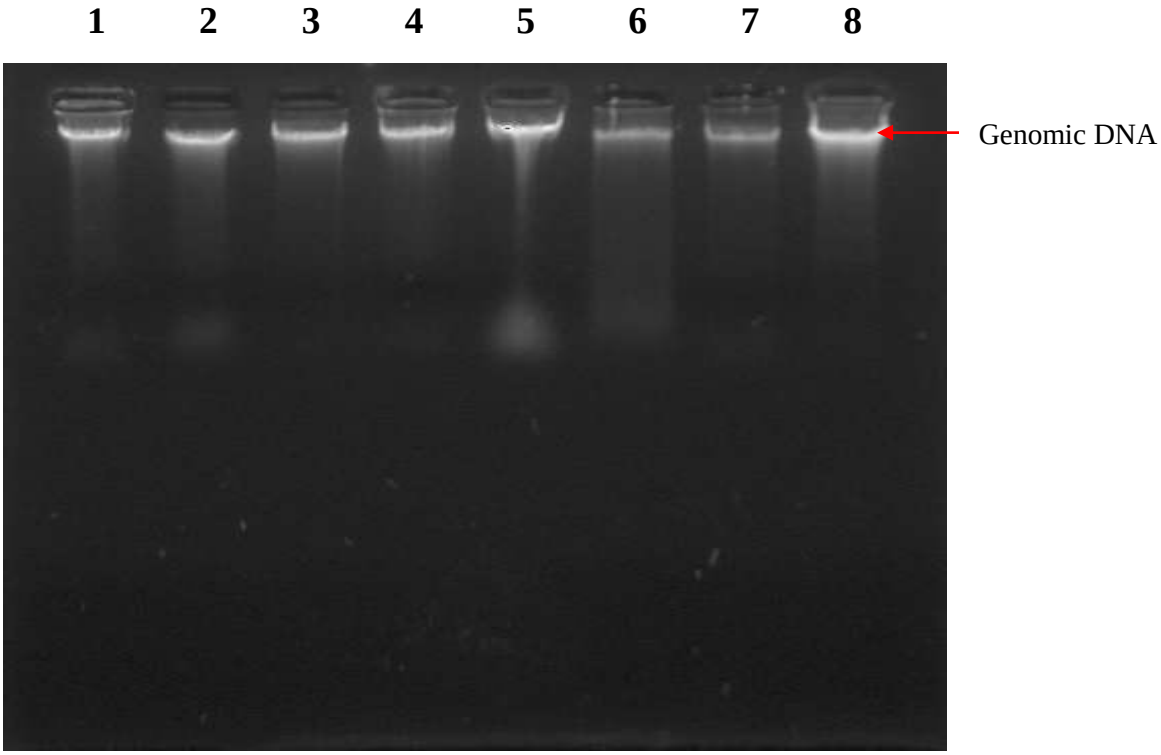


Fig 4.1: Representative gel picture showing the integrity of genomic DNA on 0.8% agarose gel. Lane 1-8 represents intact genomic DNA extracted from some cases.

Lane:

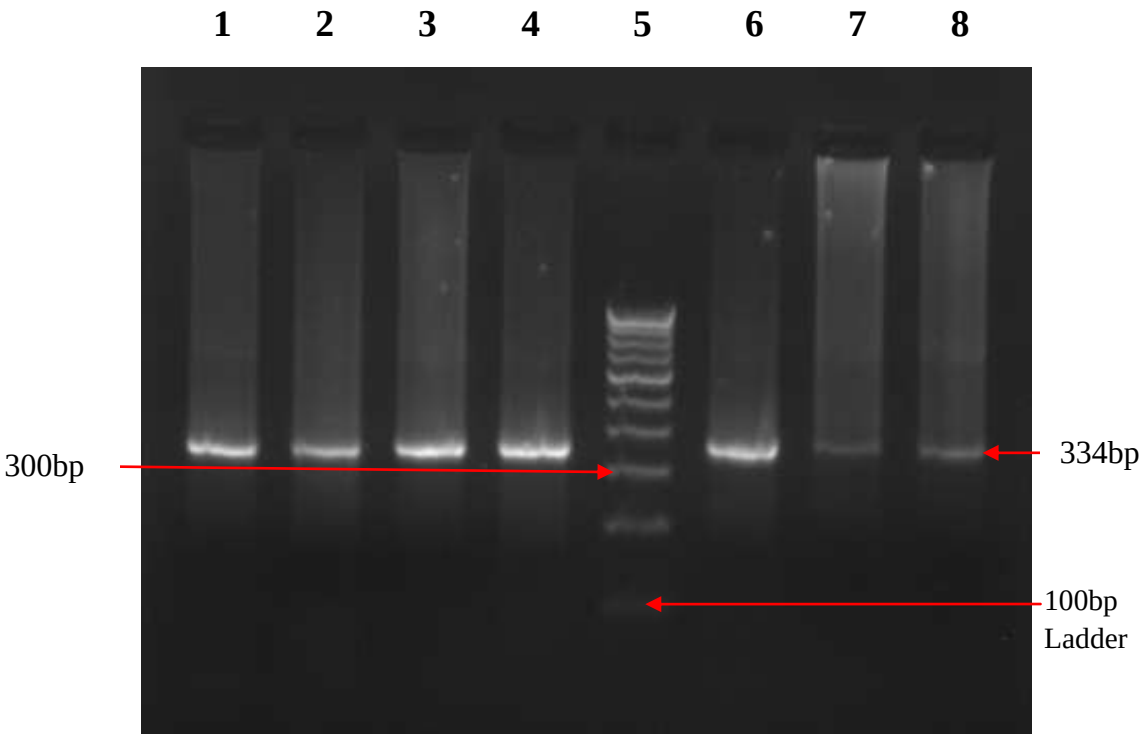


Fig 4.2: Representative gel picture showing amplification of exon 4 of CYP2D6 gene on 2.5% Agarose gel of some ALL samples. Lane no.5 represents 100bp ladder, lane no. 1, 2, 3, 4, 6, 7 and 8 represents 334bp fragment of CYP2D6 amplified PCR product

4.3 ANALYSIS OF CYP2D6*4 (G1934A) POLYMORPHISM

CYP2D6*4 (G→A transition at position 1934) polymorphism was analyzed through RFLP method, already described in the methodology section. Subsequently after amplification, PCR products were subjected to restriction digestion using BstN1 enzyme (New England Biolabs, USA). The samples were genotyped on 3% agarose gel. The normal allele i.e homozygous individuals G/G, of CYP2D6*4 polymorphism produces two fragments of size 230bp and 104bp after digestion with BstN1 restriction enzyme. Heterozygotes (G/A) produced three fragments of 334bp, 230bp and 104bp. G to A transition at position 1934 abolishes the restriction site and a fragment of 334 bp was observed in mutant allele (i.e A/A) as shown in Fig 4.3.

Lane:

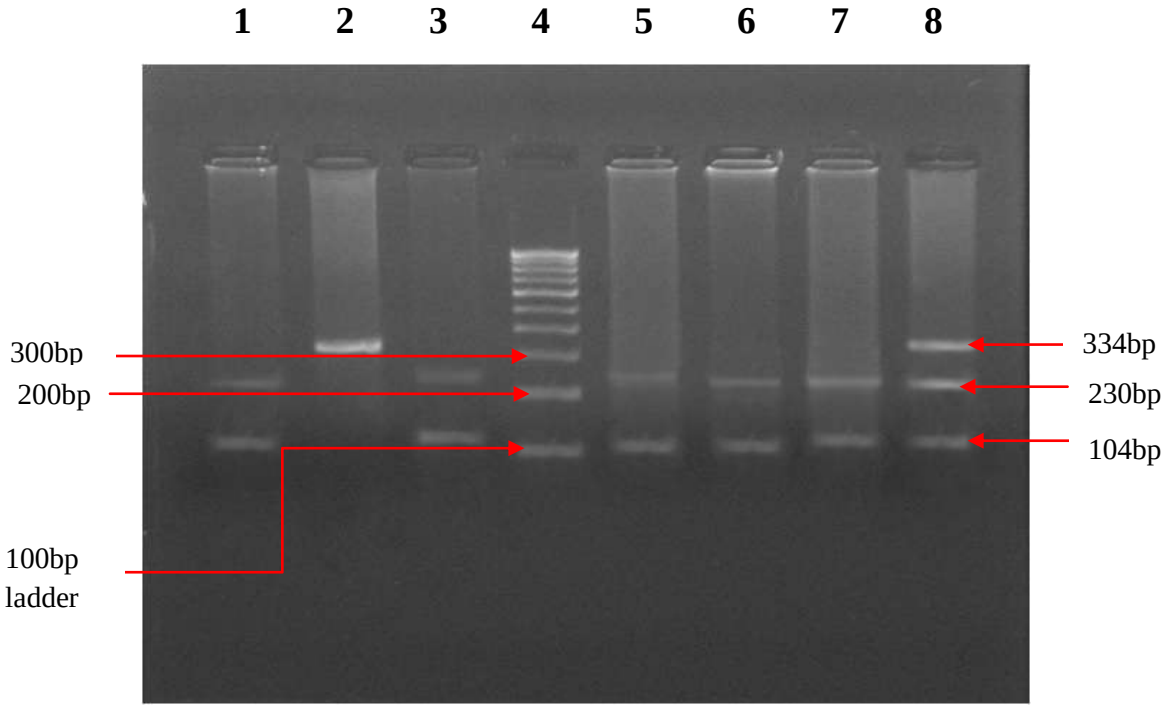


Fig 4.3: Representative gel picture showing restriction digestion pattern of amplified exon 4 of CYP2D6 gene on 3% Agarose gel electrophoresis of some ALL cases. Lane 4 represents DNA marker; lane 1, 3, 5, 6 & 7 represents the homozygous condition (G/G); lane 8 represents the heterozygous condition (G/A); whereas lane 2 represents the homozygous mutant condition (A/A).

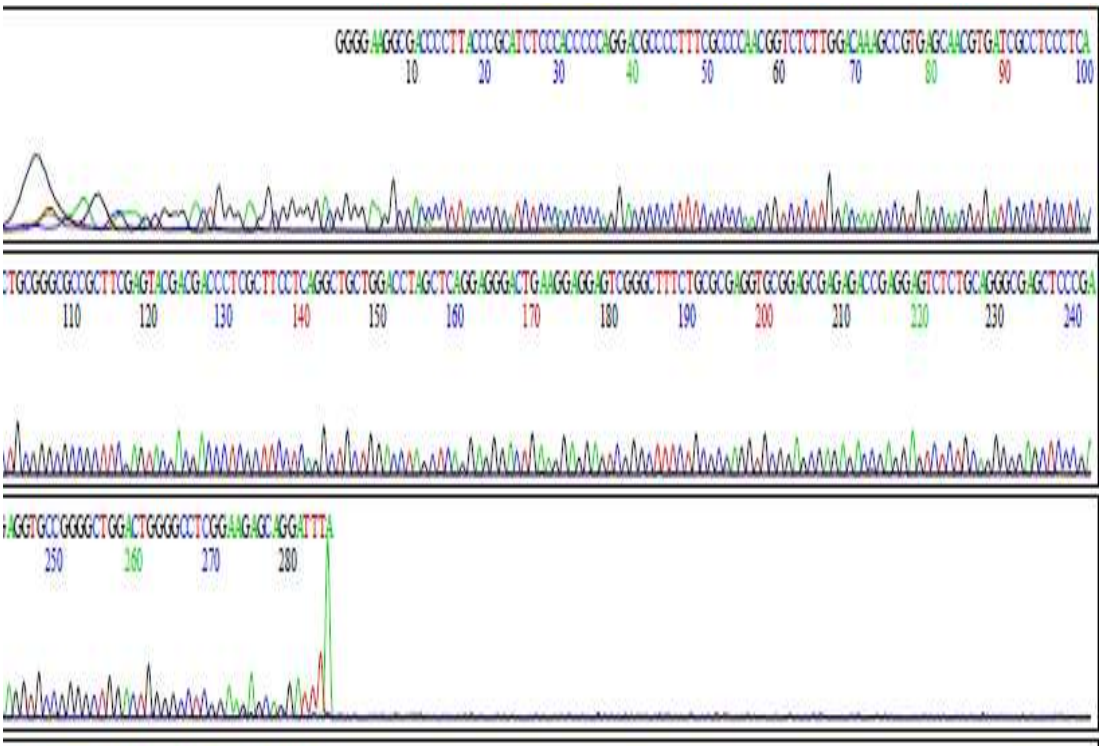


Fig 4.4: Representative chromatogram of direct sequencing for CYP2D6 gene in controls.

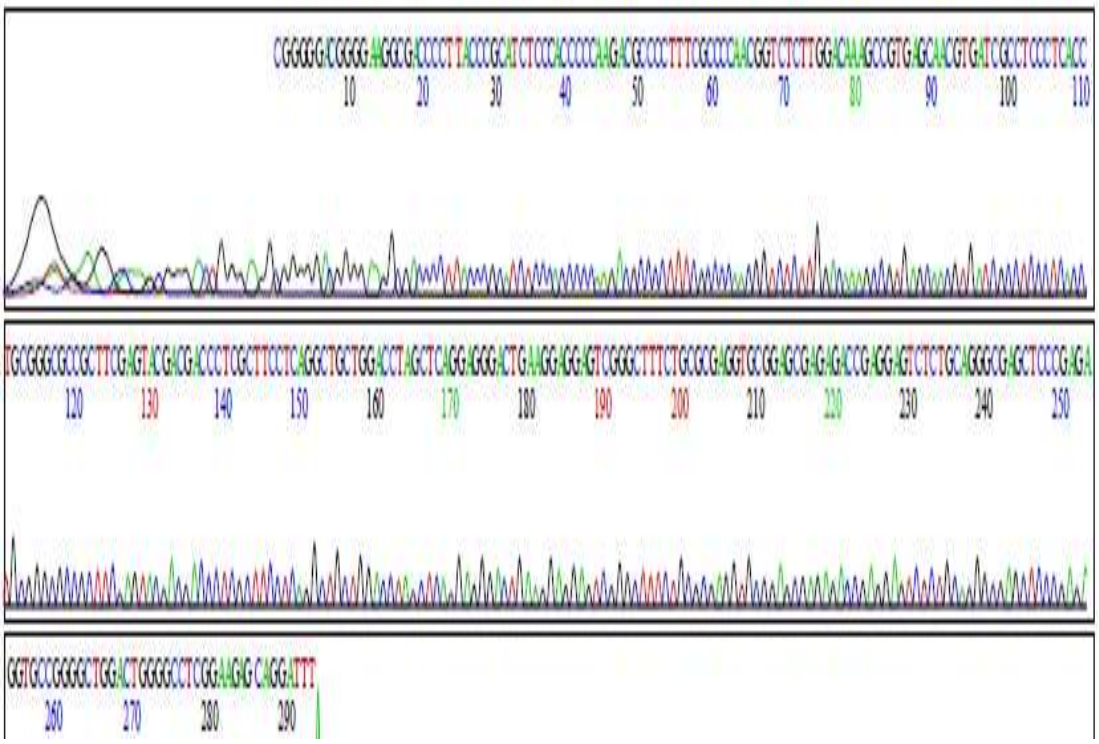


Fig 4.5: Representative chromatogram of direct sequencing for CYP2D6 gene in ALL patients

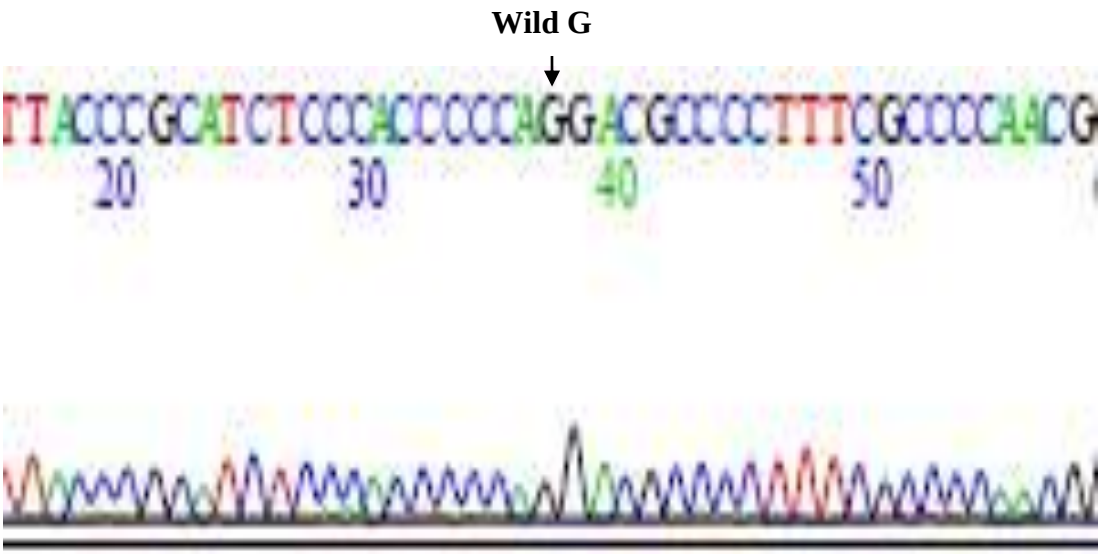


Fig 4.6: Representative chromatogram of direct sequencing for CYP2D6 unaffected individuals. The CYP2D6 of unaffected individuals contains a "G nucleotide base at 1934 position

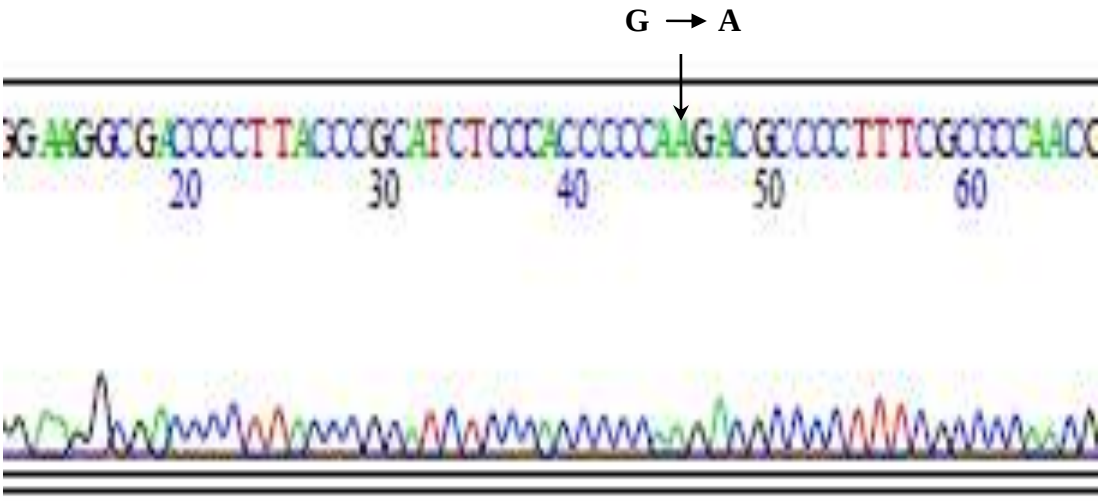


Fig 4.7: Representative chromatogram of direct sequencing for CYP2D6 affected individuals. The CYP2D6 of affected individuals contains a G → A transition at 1934 position.

Table 4.2: Genotypic Frequencies of *CYP2D6* in ALL patients and controls

Gene	Genotypes	Cases n = 120 (%)	Control n = 110 (%)	OR	(95% CI)	*P* value*
CYP2D6	Wild (G/G)	40 (33.33)	106 (96.36)	1	-	Referent
	Hetero(G/A)	28 (23.33)	2 (1.82)	37.10	8.44 – 163	< 0.0001
	Mutant(A/A)	52 (43.33)	2 (1.82)	68.90	16.02 - 296.3	< 0.0001

*P using χ^2 test
p<0.05 (Data statistically significant)

Table 4.3: Allelic frequency of *CYP2D6* in ALL patients and controls

Polymorphism	Alleles	Cases n^s (%) 240	Control n^s (%) 220	OR	(95% CI)	*P* Value*
CYP2D6*4 1934G>A	G	108 (45.0)	214 (97.27)	1	-	Referent
	A	132 (55.0)	6 (2.72)	43.59	18.62 - 102.0	< 0.0001

n^s=No. of Alleles

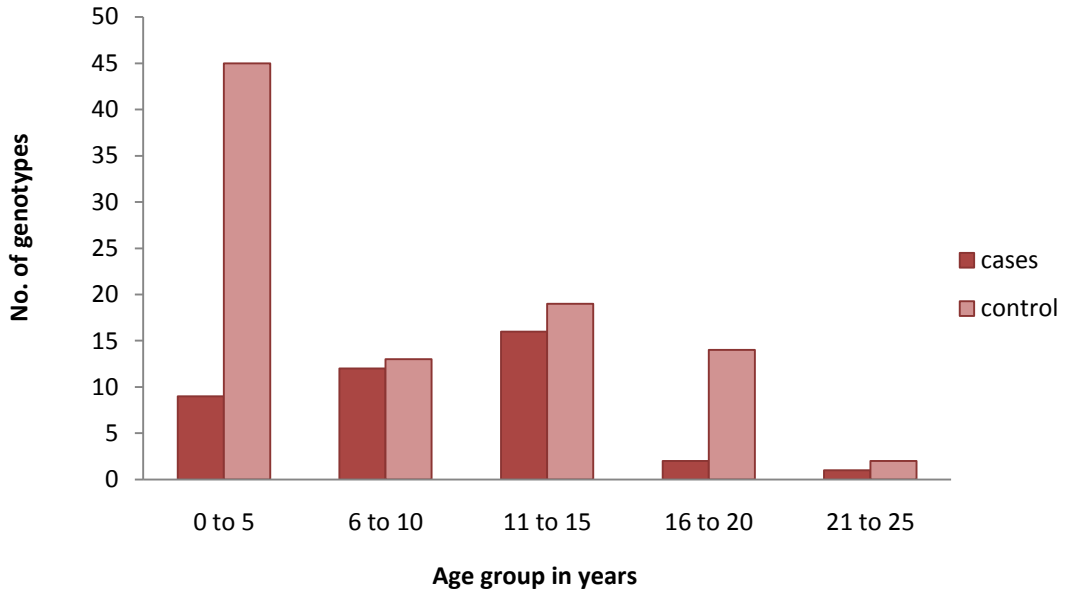


Fig 4.8: Histogram showing the genotype distribution in ALL patient and controls of Wild genotype (G/G)

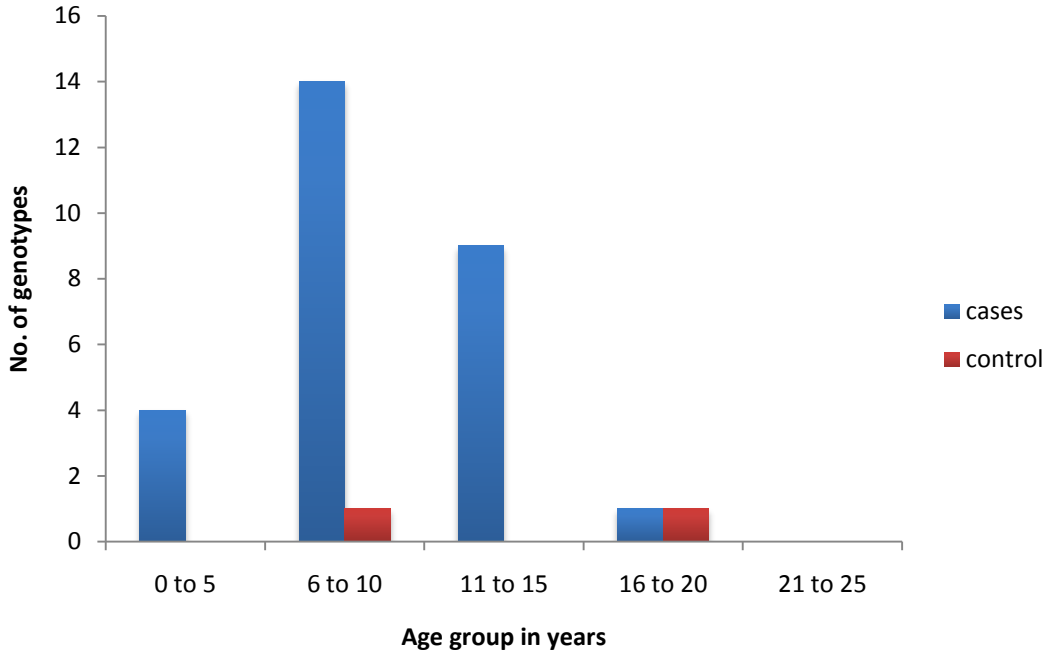


Fig 4.9: Histogram showing the genotype distribution in ALL patients and controls of Hetero genotype (G/A).

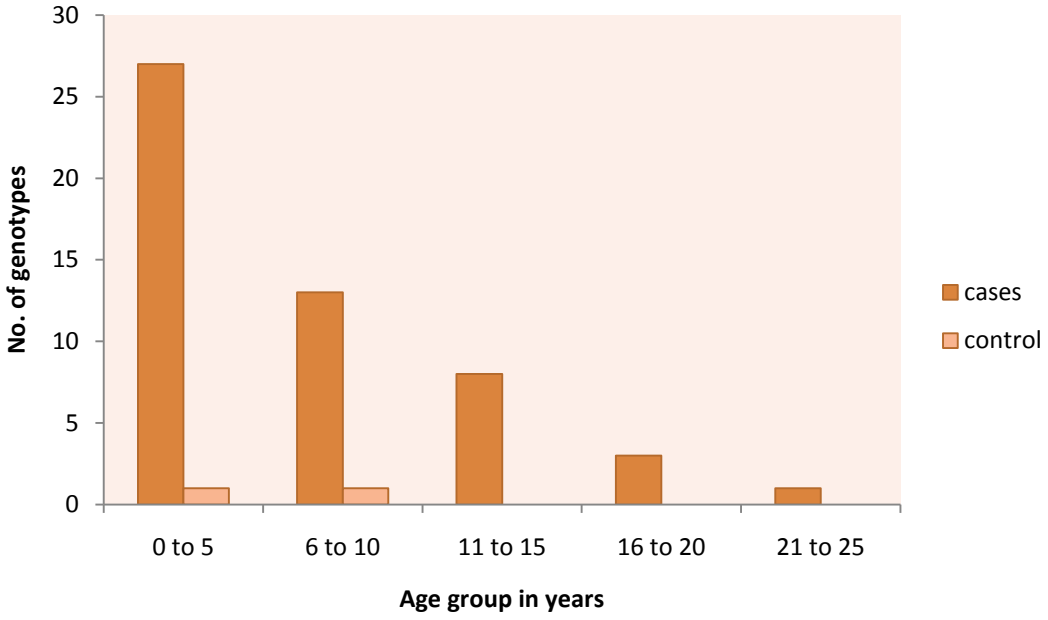


Fig 4.10: Histogram showing the genotype distribution in ALL patients and controls of Mutant genotype (A/A).

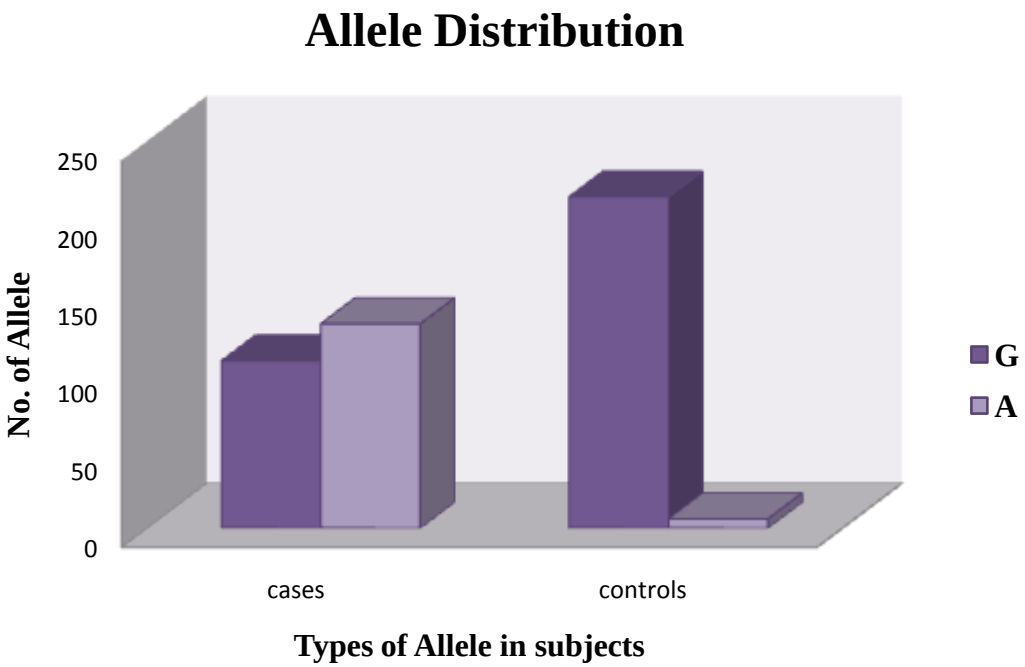


Fig 4.11: Histogram showing the allele distribution of CYP2D6 in ALL patients and controls.

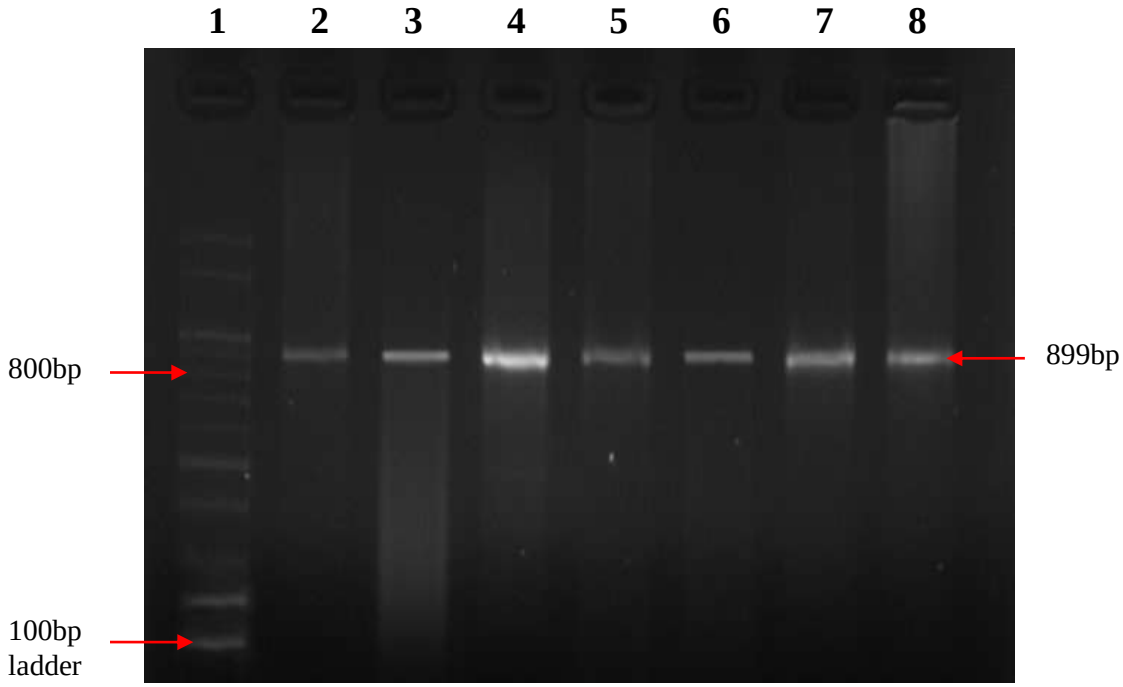


Fig 4.12: Representative gel picture showing amplification of 899bp of 3' non coding region of CYP1A1 gene on 1.5% Agarose gel of some ALL cases. Lane no.1 represents 100bp ladder, lane no. 2, 3, 4, 5, 6, 7 and 8 represents amplified PCR product.

4.4 ANALYSIS OF CYP1A1*2A T6235C (m1) POLYMORPHISM

3'UTR of CYP1A1*2A mutation of T6235C transition (m1, T→C transition at position 6235) were characterized by the PCR-RFLP approach as already described in the methodology section. PCR products so obtained were subjected for restriction digestion using Msp1 (New England Biolabs, Schwalbach, Germany). The samples were genotyped on 2% agarose gel. It was observed that the homozygous individuals (T/T) produced one band corresponding to 899 bp. Heterozygotes (T/C) produced three fragments corresponding to 899, 693 and 206 bp and homozygotes mutants (C/C) produced two bands which correspond to 693 and 206 bp fragments after digestion, as m1 T→C transition at position 6235 in CYP1A1 gene create the restriction site which results in smaller fragments of 693 and 206bp. Fig. 4.13

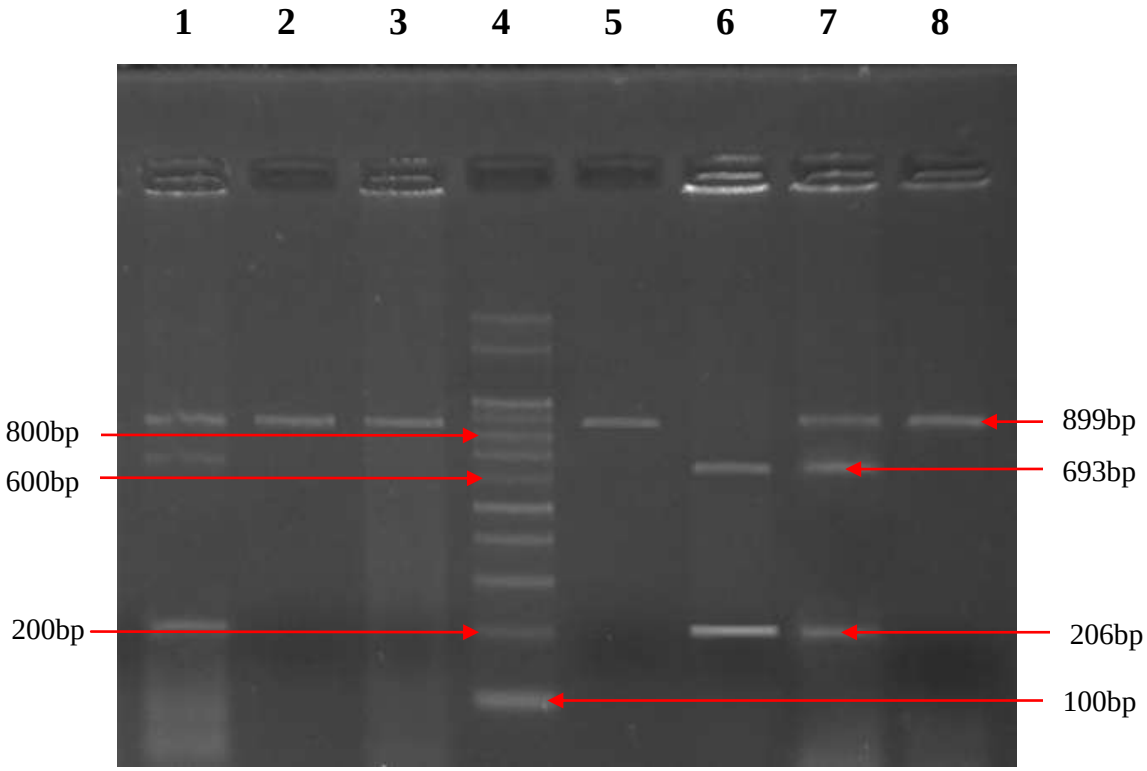


Fig 4.13: Representative gel picture showing restriction digestion pattern of the amplified product of 3' non coding region of CYP1A1 gene on 2% Agarose gel of some ALL cases. Lane 4 represents DNA marker; lane 2, 3, 5 & 8 represents the homozygous condition (T/T); lane 1 & 7 represents the heterozygous condition (T/C); whereas lane 6 represents the homozygous mutant condition (C/C).

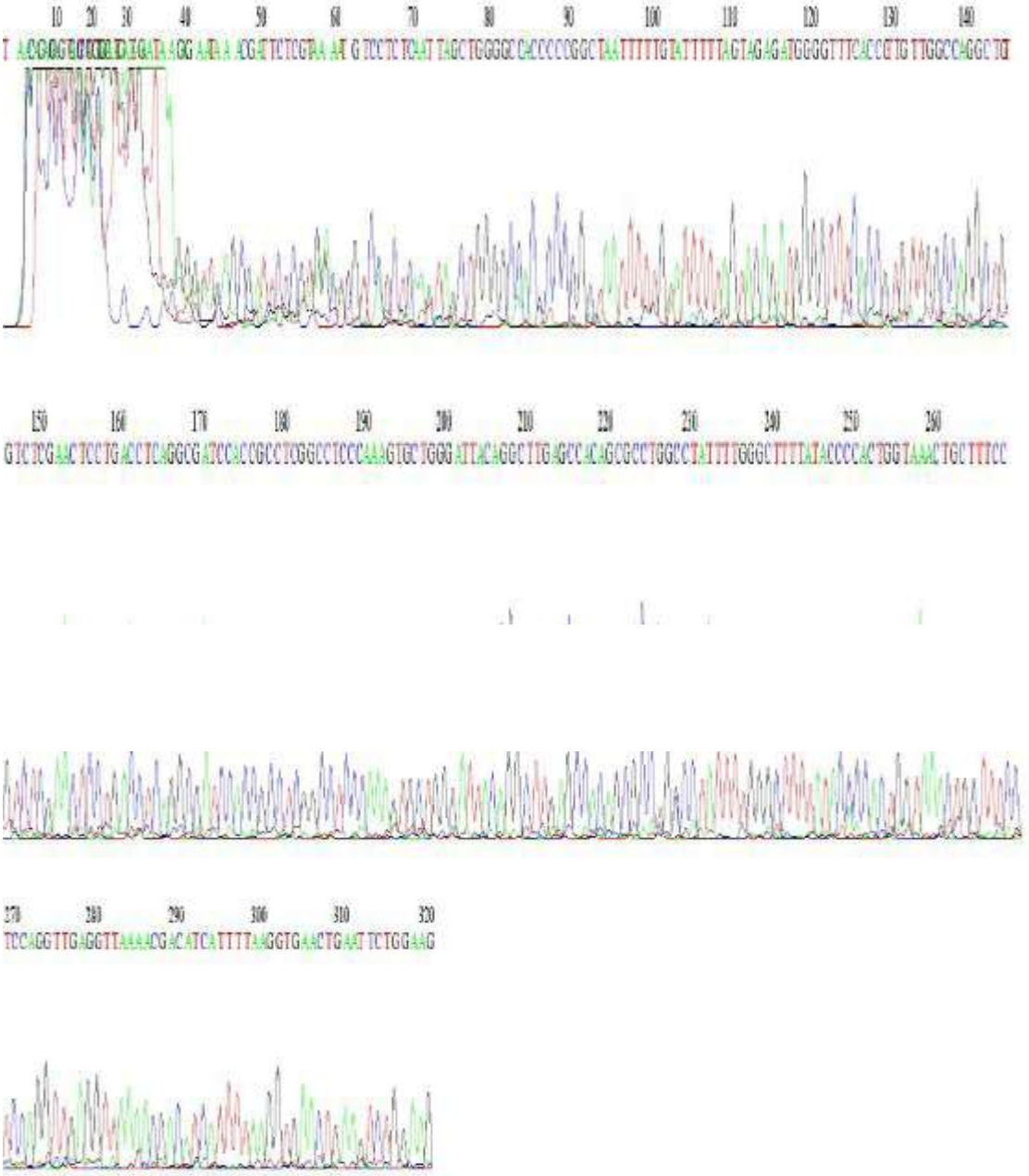
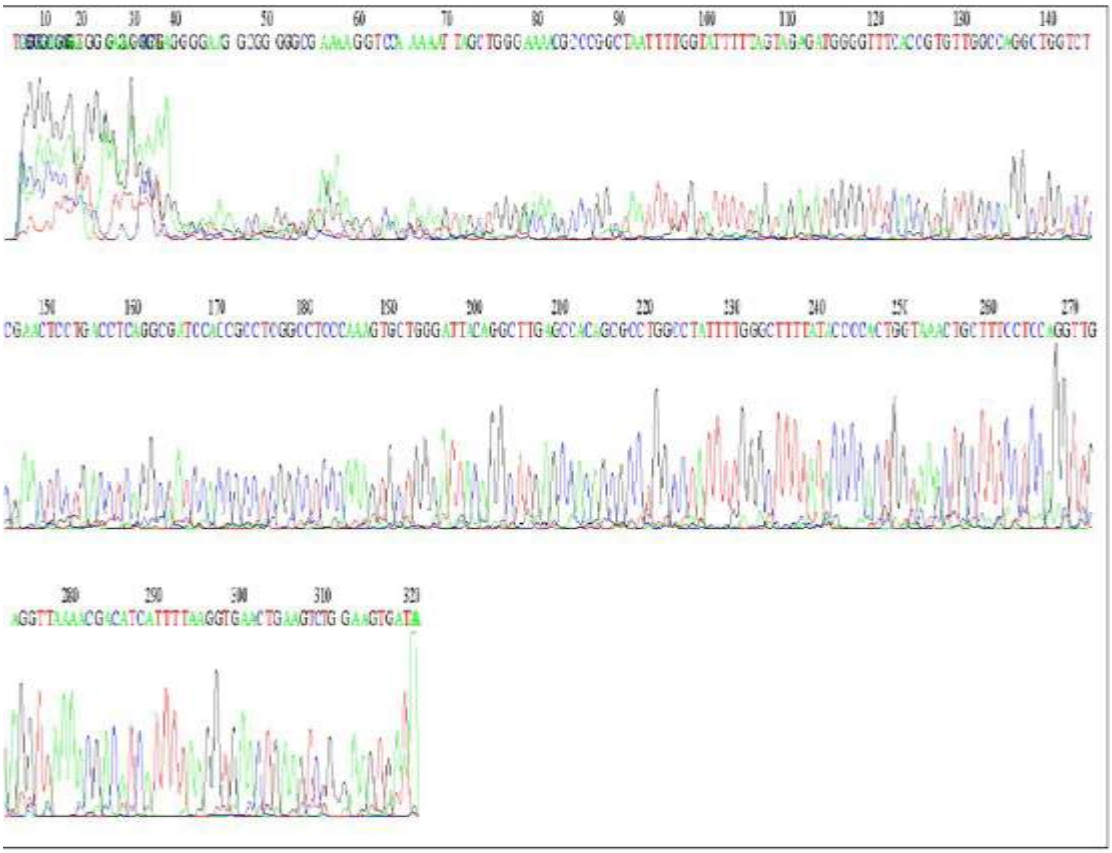


Fig 4.14*: *Representative chromatogram of direct sequencing for CYP1A1 gene in controls.



4.15: Representative chromatogram of direct sequencing for CYP1A1 gene in ALL patients.

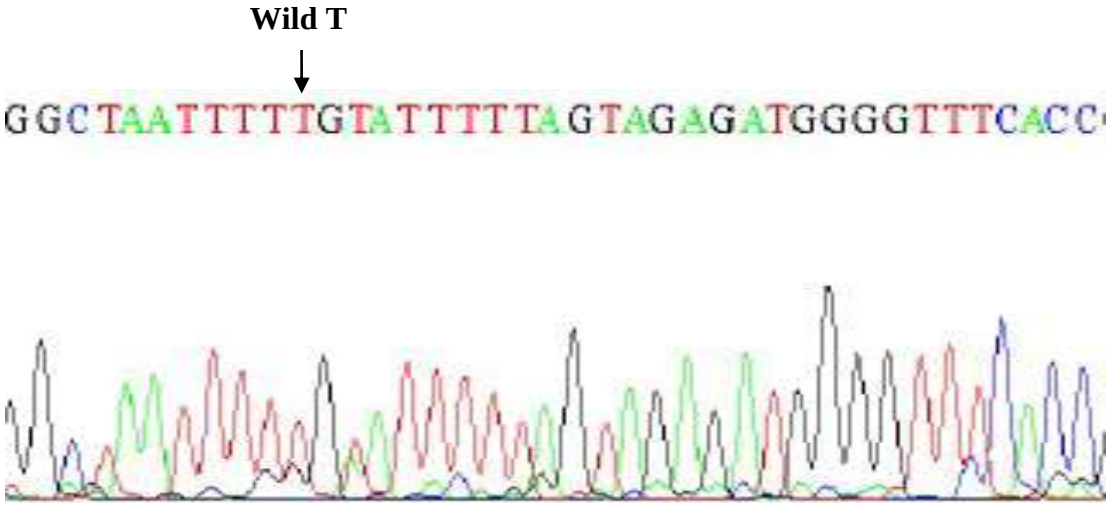


Fig 4.16: Representative chromatogram of Direct sequencing for CYP1A1 unaffected individuals. The CYP1A1 of unaffected individuals contains a "T" nucleotide base at 6234 position.

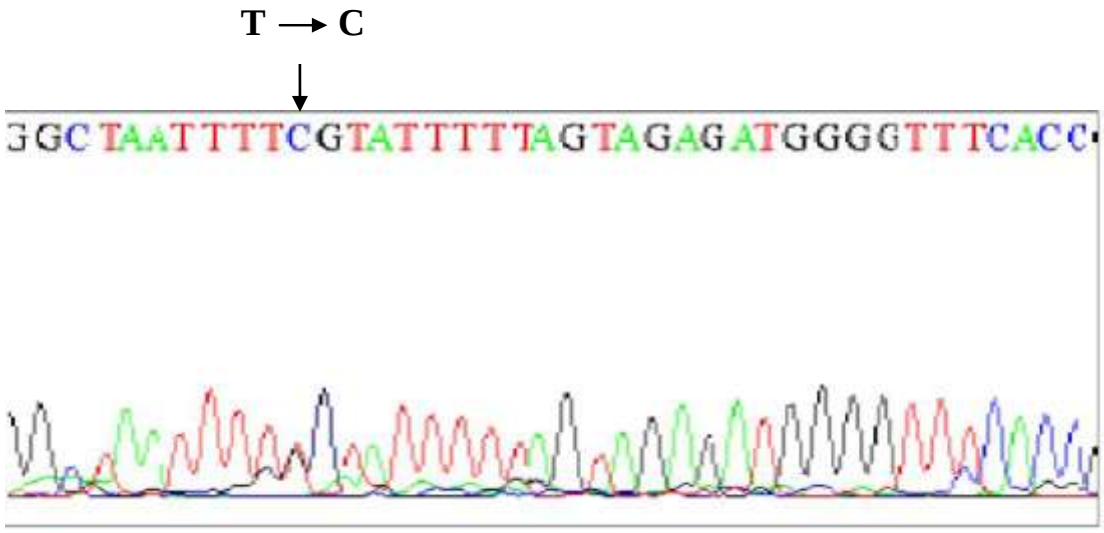


Fig 4.17: Representative chromatogram of direct sequencing for CYP1A1 affected individuals. The CYP1A1 of affected individuals contains T→ C transition at 6234 position.

Table 4.4: Genotypic Frequencies of *CYP1A1* in ALL patients and controls

Gene	Genotypes	Cases n(%) 120	Control n(%) 110	OR	(95%CI)	*P* value*
CYP1A1	Wild (T/T)	85 (70.83)	94 (85.45)	1	-	Referent
	Hetero(T/C)	30 (25)	25 (22.73)	1.33	0.72 - 2.43	0.44
	Mutant(C/C)	5 (4.17)	1 (0.91)	5.53	0.63 - 48.31	0.19

*P using χ^2 test
p>0.05 (Data statistically insignificant)

Table 4.5 The distribution of *CYP1A1* alleles in ALL patients and control

Polymorphism	Alleles	Cases n^s (%) 240	Control n^s (%) 240	OR	(95% CI)	*P* Value*
CYP1A1*2A 6235T>C	T	200 (83.33)	213 (88.75)	1	-	Referent
	C	40 (16.66)	27 (11.25)	1.57	0.93 - 2.66	0.11

n^s = No. of Alleles

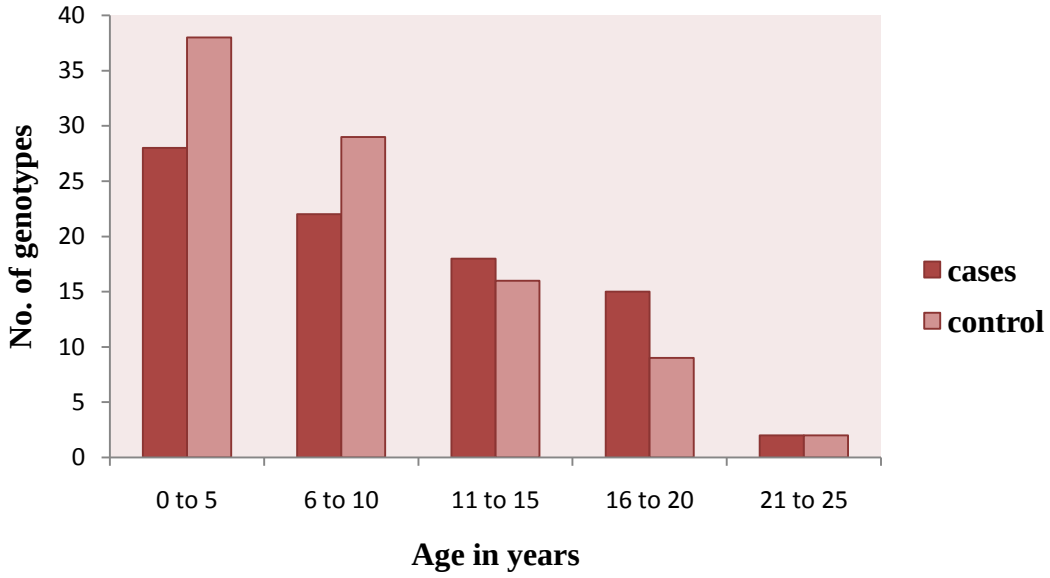


Fig 4.18: Histogram showing the genotype distribution in ALL patients and controls of Wild genotype (T/T).

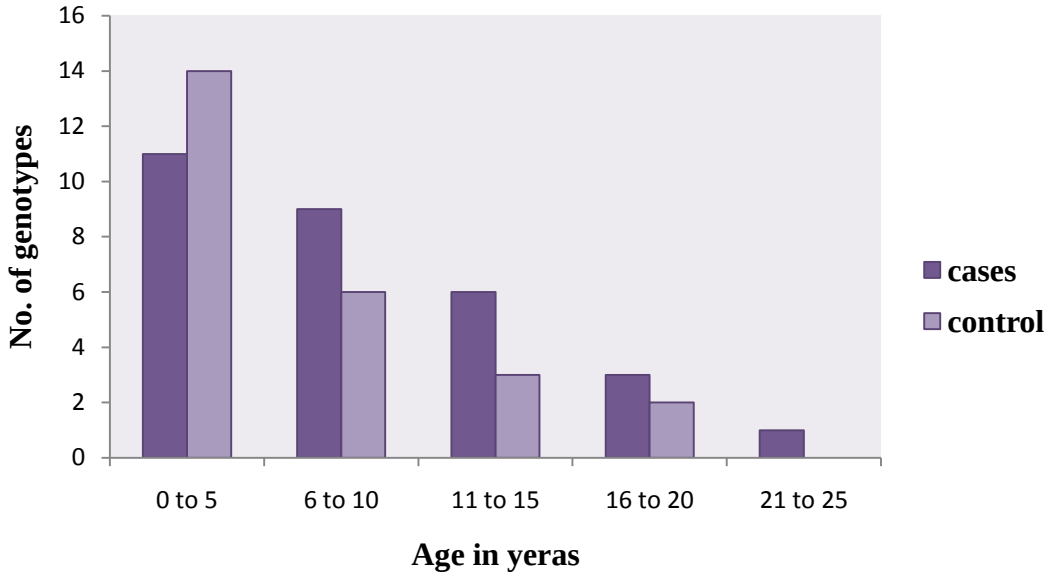


Fig 4.19: Histogram showing the genotype distribution in ALL patients and controls of Wild genotype (T/C).

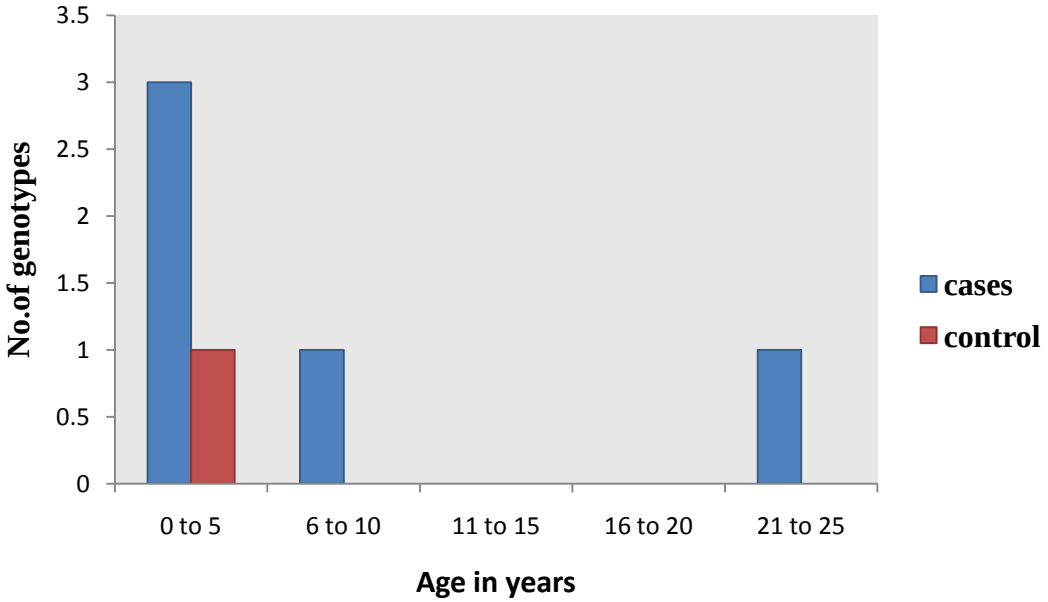


Fig 4.20: Histogram showing the genotype distribution in ALL patients and controls of** Mutant **genotype (C/C).

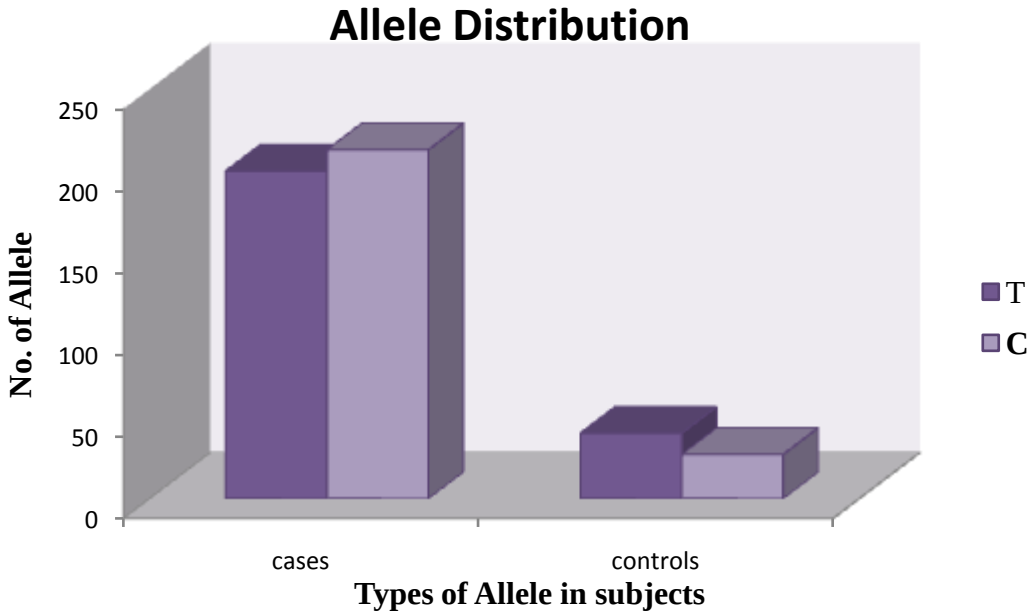


Fig 4.21: Histogram showing the allele distribution of CYP1A1 in ALL patients and controls.

Chapter: 5

Discussion

Acute lymphoblastic leukemia (ALL) accounts for approximately 74% of the leukemia cases among children in between the age group of 0 to 19 years old and an estimated 5,300 cases were diagnosed in 2010 in the United States. Despite, 80% of overall 5-year survival, ALL remains a major cause of childhood mortality **(SEER, 2011; American Cancer Society, 2011 and McNeil et al., 2002)**. There is significant ethnic variation in incidence rates with Hispanics having the highest rate (44/million), followed by Whites (36/million) and African Americans with the lowest rate (20/million) **(SEER, 2011)**. The etiology of childhood leukemia continue to be incompletely explained but have been linked to environmental exposures and multiple low penetrance of genetic factors (**McNeil et al., 2002 and Bolufer et al., 2006)**. Studies of childhood leukemia have suggested that genetic variants within xenobiotic metabolizing enzymes (XMEs) significantly affect susceptibility to childhood ALL (**Sinnett et al., 2000; McNeil et al., 2002; Canalle et al., 2004 and Buffler et al., 2005)** and that XME polymorphisms may be significant predictors of chemotherapy response and survival for children suffering from leukemia **(Krajinovic et al., 1999; McNeil et al., 2002; Canalle et al., 2004 and Buffler et al., 2005)**. Genetic susceptibility to leukemias can be related to the polymorphisms in CYP2D6 and CYP1A1 genes and consistent with this paradigm several polymorphisms have been identified in the pathogenesis of lymphoid malignancies. Cytochrome P_{450} 2D6 (CYP2D6) and Cytochrome P_{450}1A1 (CYP1A1), the member of the cytochrome P_{450} mixed-function oxidase system, are the most important enzymes involved in the metabolism of various drugs in the body. Also, many drugs are activated by CYP2D6 and CYP1A1 to form their active compounds. While CYP2D6 is involved in the oxidation of a wide range of substrates. There is considerable variability in its expression in the liver, while as CYP1A1 is involved in the metabolic activation of aromatic hydrocarbons (polycyclic aromatic hydrocarbons). These genes are highly polymorphic in the population; certain alleles result in the poor metabolizer phenotype, characterized by a decreased ability to metabolize the enzyme's substrates and certain result in intermediate metabolizer, characterized by intermediate capacity to metabolize the substrate **(Krajinovic et al., 2002).** However, actual phenotype depends on the nature of drug substrate and non-genetic factors, particularly co-medications. Alternative spliced transcript variants encoding different isoforms have

been found for these genes. Taking this information into consideration, it seems plausible that inter- individual differences in CYP2D6 activity can produce adverse effects or lack of therapeutic effect with an altered risk for cancers **(Nageswararao et al., 2010)**. The most common inactivating mutation is the transition G1934A at the splice site resulting in truncated protein with no enzymatic activity (**Smith et al., 1995)**. Also, studies have shown that variants within cytochrome P_{450} 1A1 (CYP1A1), a member of the CYP1 gene family of constitutive and inducible enzymes are associated with increased risk in ALL (**Gough et al., 1990 and Canalle et al., 2004)**. Thus, CYP2D6 and CYP1A1 encodes crucial enzyme in drug metabolism and therefore, are good candidates for studying the effect of polymorphisms on drug metabolism and their role in development of malignancies. The present study was conducted on Kashmiri children to investigate gene polymorphism of CYP2D6 and CYP1A1 and their association with ALL. The study was carried out with 120 ALL cases 110 controls (age group, sex, region, occupation) by RFLP-PCR analysis. In the transition of CYP2D6*4 1934G>A, it was observed that the CYP2D6 (G) allele frequency was 97.27% in the controls and 45% (OR=1, CI 95%) in cases and CYP2D6 (A) allele frequency was 2.72% in the controls and 55% (OR=43.59, 18.62-102) in cases. This difference in frequency was found to be statistically significant with a $P = 0.0001$ ($p< 0.05$). While as in the transition of CYP1A1*2A 6235T>C, the frequency of CYP1A1 (T) allele was 88.75% in the controls and 83.88% (OR=1, CI 95%) in cases and CYP1A1 (C) allele frequency was 11.25% in controls and 16.66% (OR=1.57, CI 95%, 0.93-2.66) in cases. This difference in frequency was found to be statistically insignificant with a $P= 0.11$ ($p> 0.05$). The CYP2D6 wild (G/G) genotype (Extensive metabolizer) frequency was found to be present in 33.33% of the cases and 96.36% of the controls, the hetero (G/A) variant (Intermediate metabolizer) in 23.33% of the cases and 1.82% of controls, and the mutant (A/A) genotype (Poor metabolizer) in 43.33% of cases and 1.82% of controls. Similarly in case of CYP1A1, the wild (T/T) was found to be present in 70.83% of cases and 85.45% in controls, the hetero (T/C) genotype in 25% of the cases and 22.73% of controls and the mutant (C/C) was found to be present in 4.17% of the cases and 0.9% of controls. We observed that an increased risk associated with CYP2D6 Mutant(A/A) genotype 43.33% (OR = 68.90, 95% CI, 16.02-296.3; $P = <0.0001$) and Wild (G/G) genotype 33.33% (OR = 1; 95%CI) and low with hetero (G/A) genotype 23.33% of CYP2D6 as compared to the controls and also increased risk is associated with

CYP1A1 wild (T/T) genotype 70.83 % (OR 1, CI 95%) and hetero 25% (OR 1.33, 95% CI, 0.72- 2.43; *P* = 0.44) and low in case of mutant (C/C) genotype 4.17% (OR = 5.53, CI 95% 0.63-48.31, *P* = 0.19) as compared to the controls. We observed that in CYP2D6, the incidence of ALL is higher in the children of age group in between 0 to 5 years in cases and least in the age group of 21 to 25 in cases, when compared to the controls (Fig 4.10). Similarly, in case of CYP1A1, the incidence of ALL was found to be higher in the children of age group 0 to 5 years in cases as compared to controls (Fig. 4.20).

We also observed that in CYP2D6, although the proportion of patients who were homozygous mutant (A/A) was higher in cases than in controls, the difference was not statistically significant when using wild (G/G) genotype as a reference (OR= 68.9; 95% CI, 16.02-296.3; *P* = < 0.0001). Similarly, we observed the frequency of the heterozygous genotype (G/A) when compared with A/A genotype was not much different between the cases and controls hence, statistically insignificant (OR=37.1; 95% CI, 8.44- 163; *P* = < 0.0001). Similarly, in CYP1A1 we observed that the proportion of patients who were homozygous mutant (C/C) was lower in cases and in controls, the difference was statistically significant when using wild (T/T) genotype as a reference (OR= 5.53; 95% CI, 0.63-48.31; *P* = 0.19). Similarly, we observed the frequency of the heterozygous genotype (T/C) when compared with T/T genotype was again lower in cases and controls hence, statistically significant (OR=1.33; 95% CI, 0.72-2.43 ; *P* = 0.44). This finding is in line with the findings of **(Krajinovic et al., 1999 and Nageswararao et al., 2010)** and other studies. Therefore, the polymorphism CYP2D6 needs to be evaluated in detail to conclude its role on the development of acute lymphoblastic leukemia in Kashmiri population. On the basis of results, our study suggests that there is an association between gene polymorphism of CYP2D6 and CYP1A1 and the development of ALL in Kashmiri children. However these findings need to be substantiated with larger sample size to clarify the real contribution of these genes in the susceptibility to ALL in different world populations.

The reasons for contrary results obtained from several studies remain ambiguous and might be attributed to differences in ethnic backgrounds and the selection of the population studied, differences in sample sizes, and gene-environment interactions, such as diet, exposure to chemicals and nutritional intake. Furthermore, inherited biases accompanied with hospital based case –control studies may also be attributed

to spurious findings or false positive results. Due to limited studies reporting the influence of gene polymorphism involved in drug metabolism on ALL, more investigations are necessary to find a clear relationship of these genes to susceptibility to ALL.

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Appendix

Chemicals and other miscellaneous items

The details of chemicals and other miscellaneous items used in the study

Chemicals/Enzymes	Source
Proteinase k	Sigma
Chloroform	Qualigens
Isoamyl alcohol	Qualigens
EDTA-disodium	CDH
SDS	Sigma
Tris-HCl	Himedia
Magnesium chloride	Sisco Research Lab
Ethanol	Jiangsu Huaxi International Co.Ltd
Phenol	Qualigens
Sodium acetate	S.D. fine chem.pvt ltd
Ethidium bromide	Himedia
Bromophenol blue	Himedia
Tris – base	Himedia
Acetic acid	Qualigens
Sucrose	Sisco Research Lab
Sodium hydroxide	RANKIN
Sodium chloride	Sisco Research Lab.Pvt.Ltd
Agarose low EEO	Sisco Research Lab.Pvt.Ltd
Isopropanol	Qualigens
Ammonium chloride	D.D. Fine Chem.Pvt.Ltd

Taq DNA polymerase	GENETAQ™
dNTPs	Sigma Aldrich
100bp Ladder	Sigma Aldrich
Oligonucleotides	Sigma Aldrich

REAGENTS

REAGENTS FOR DNA EXTRACTION

Chloroform isoamyl alcohol (CIA)

Chloroform	24.0 ml
Isoamyl alcohol	1.0 ml

Chloroform: isoamyl alcohol, in the ratio 24:1 was prepared by mixing 24 ml of Chloroform and 1 ml of isoamyl alcohol. The solution was stored at 4˚C in dark bottle.

DNA storage buffer:

0.5 M EDTA	0.01 ml
1 M tris	0.5 ml

Final volume was made 50 ml with sterile distilled water.

Lysis buffer

1 M tris	2.0 ml
0.5M EDTA	400 µl
10% SDS	30.0 ml

Final volume of the solution was made 100ml with sterile distilled water.

Lysis solution

1 M Ammonium chloride	15.5 ml

1M Potassium bicarbonate	1.0 ml
0.5 M EDTA	200 µl

Final volume was made 100 ml with sterile distilled water.

Proteinase k

Proteinase k	10 mg

Proteinase k was dissolved in 1 ml of deionized water and stored at -20˚C in aliquots of

1 ml each.

SE Solution

5 M Sodium chloride	3 ml
0.5 M EDTA	8 ml

Final volume was made 200ml with sterile distilled water. pH was adjusted to 8.

SDS (10%)

SDS	10 g

SDS was dissolved in 100 ml sterile distilled water.

Sodium acetate (3M)

Sodium acetate	40.83 g

Sodium acetate was dissolved in 100 ml sterile distilled water; pH of the solution was adjusted to 5.0 using acetic acid.

Saturated phenol

0.2% β- mercaptoethanol

8-hydroxy quinoline

0.1 M Tris HCl buffer	800 ml

0.5 M Tris HCl buffer	1000 ml
Phenol	1000 ml

The mixture obtained by adding equal volume of 0.5M Tris-Cl buffer and melted phenol was stirred for 15 min on magnetic stirrer. Two phases were allowed to separate. Upper aqueous phase was removed and equal volume of 0.1 M Tris-chloride buffer was added. The saturation with 0.1 M Tris-Cl buffer was repeated till phenol>7.8 pH was obtained. A pinch of β mercaptoethanol and 8-hydroxy quinoline was added. Phenol was stored at 4˚C in a dark bottle.

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

Agarose (1%)

Agarose	1.0 g
Buffer	100 ml
EtBr	5.0 µl

Agarose was dissolved in a buffer and heated till a clear solution is formed. EtBr was then added to the solution.

Bromophenol blue

Bromophenol blue	0.4 g
Sucrose	20.0 g

Bromophenol blue was dissolved in 100 ml of distilled water.

From the above stock solution 31.25 ml was taken and sucrose was added. Final volume was made to 50 ml with distilled water.

Ethidium bromide

Ethidium bromide 10 mg

Ethidium Bromide was dissolved in 1ml of distilled water. The solution was stored in a dark bottle at 4˚C.

50X TAE (pH 8.0)

Tris base	242 g
0.5MEDTA	100 ml
Glacial acetic acid	57.1 ml

Final volume was made 1000 ml with distilled water.

1X TAE (pH 8.0)

50 X TAE 20 ml

Final volume was made 1000 ml with distilled water.

REAGENTS FOR PCR

Stock

Deoxyribose nucleotide triphosphate (dNTP), 10mM each dATP, dGTP, dCTP, dTTP and Taq polymerase (5U/ml) 10X Taq buffer Primers: 100 mM in sterile demonized water (Sigma) 100bp DNA ladder (100µg/ml)

EQUIPMENTS

The details of equipments used during the course of study

Equipments	**Source**
Deep freezer (-20°C)	Nirmal Instruments
Centrifuge	Avanti™
Spectrophotometer	ELICO
Vortex mixer	Remi
Hot plate	SHIV
Magnetic stirrer	SHIV
Thermal cycler	Eppendrof

Electrophoresis apparatus	BangloreGeni
Power supply	Genei
Uv-Tran Illuminator	Hoeser
Incubator	Techno
Autoclave	Techno
Refrigerator	Godrej
Ph meter	HANNA
Gel documentation unit	Alpha Innotech Corporation
Stabilizer	Philips