Molecular Studies of Hematological Disorders in Pakistani Population

By

MADIHA SADIQ

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University Islamabad

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By

Madiha Sadiq 42-FBAS/MSBT/F12

Supervisor Dr. Nyla Jabeen

Co-Supervisor Dr. rer. nat. Rashda Abbasi

Department of Bioinformatics and Biotechnology,

Faculty of Basic and Applied sciences,

International Islamic University Islamabad

(2015)

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

Department of Bioinformatics and Biotechnology
International Islamic University, Islamabad

Dated:_____

FINAL APPROVAL

It is certificate that we have read the thesis entitled "Molecular Studies of Hematological Disorders in Pakistani Population" submitted by Ms. Madiha Sadiq and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the Master of Philosophy in Biotechnology.

COMMITTEE

External Examiner
Dr. Ghulam Murtaza
Department of Biotechnology
Comsats Institute of Information Technology
Abottabad, Pakistan

Internal Examiner
Dr. Bushra Uzair
Assistant Professor
Department of Bioinformatics and Biotechnology
International Islamic University, Islamabad

Supervisor
Dr. Nyla Jabeen
Assistant Professor
Department of Bioinformatics and Biotechnology
International Islamic University, Islamabad

Co supervisor
Dr. Rashda Abbasi
Senior Scientific Officer,
Institute of Biomedical and genetic Engineering, Islamabad

Acting Chairperson
Dr. Naveeda Riaz
Assistant Professor
Department of Bioinformatics and Biotechnology
International Islamic University, Islamabad

Dean, FBAS
Dr. Muhammad Sher
International Islamic University, Islamabad

1-4-15

A thesis submitted to Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad as partial fulfillment of requirement of degree of Masters in Biotechnology

Dedication

I dedicate this dissertation to my parents, who have been a great support for me. Without their prayers and kind gesture, I would not have been able to accomplish my work.

Declaration

I hereby declare that the work present in the following thesis is my own effort except where otherwise acknowledged and that the thesis is my own composition. NO part of the thesis has been previously presented for any other degree.

Date_____

Madiha

(Madiha Sadiq)

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All thanks to the *Almighty ALLAH*, the most Gracious, most Merciful, Whose kindness and mercy has enabled me to accomplish this research work successfully. I am greatly indebted to Him for providing me with the strength, courage, guidance and patience to complete my project. Allah says in Qur'an: ***"Therefore remember Me (by praying, glorifying). I will remember you, and be grateful to Me (for My countless favors on you) and never be ungrateful to Me" [Al-Baqarah 2:152]***

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Madiha Sadiq

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LIST OF ABBREVIATIONS

µl	Micro litre
ACD	Anticoagulant Citrate Dextrose
AFBMTC	Armed Forces Bone Marrow Transplant Centre
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
ANLL	Acute Non-Lymphoblastic Leukemia
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
Bp	Base Pair
CI	Confidence Interval
CLL	Chronic Lymphoblastic Leukemia
CML	Chronic Myeloid Leukemia
DBE	Dibromoethane
DCM	Dichloromethane
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELF-MF	Extremely Low Frequency Magnetic Field
FAB	French-American-British
GSH	Glutathione
GSTM1	Glutathione S-Tranferase Mu 1
GSTP1	Glutathione S-Tranferase Pi 1
GSTs	Glutathione S-transferases
GSTT1	Glutathione S-Tranferase Theta 1
HSC	Hematopoeitic Stem Cells
IRNUM	Institute of Radiotherapy and Nuclear Medicine
LCO	Leukemia Cell-of-Origin
LSC	Leukemia Stem Cells
MAPEG	Membrane-Associated Proteins in Eicosanoide and Glutathione Metabolism
MPO	Myeloperoxidase
MTHFR	Methylenetetrahydrofolate Reductase
NQO1	NAD(P)H quinone oxidoreductase 1

NRPB	National Radiological Protection Board
OR	Odds Ratio
PBLs	Peripheral Blood Lypmohocytes
PBSCT	Peripheral Blood Stem Cells Transplantation
PCR	Polymerase Chain Reaction
P-Value	Probability Value
RBC	Red Blood Cells
Rpm	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCT	Stem Cell Transplantation
SDS	Sodium dodecyl sulfate
SKMCH	Shaukat Khanum Memorial Cancer Hospital
STE	Saline Tris EDTA
WHO	World Health Organization

ABSTRACT

Leukemia is a cancer of white blood cells, which results in the abnormal maturation of blood cells escaping the normal mechanism of homeostatic control. The normal hematopoietic system gets disturbed by some genetic event, leading to the development of leukemia. The prevalence of leukemia is quite high in different regions of the World, where in Pakistan, among all cancer cases; about 3.1% cases of leukemia were reported in 2012. Leukemia is categorized into four major types; based on the cell type from which the leukemia is originated (Lymphoid or Myeloid) or on the speed of disease progression (Acute or Chronic). Many environmental pollutants, in addition to genetic factors, have been identified as the risk factors for causing the disease. Glutathione S-Transferases (GSTs) is an assembly of different enzymes which are involved in the Phase II of the detoxification of environmental pollutants. They do this by transforming the water insoluble xenobiotic compound to a water soluble form and hence facilitate its elimination. Among the different types of GST enzymes, GST-Theta 1 (GSTT1) and GST-Mu 1 (GSTM1) are of prime importance owing to their role in developing cancers. GSTT1 and GSTM1 genes are prone to polymorphisms, which form the basis of many diseases, including leukemia. In the current study, the association of polymorphisms of GSTT1 and GSTM1 genes was analyzed on 158 leukemia samples and 360 controls, with respect to leukemia sub-type, age, gender and ethnicity from Pakistani population. The results indicated a significant protection of disease in Pathan males with GSTT1 null. The age group above 30 years was found to be highly protected among Pathan. Deletion of both genes also showed significantly increased protection among Pathan males. Punjabi also showed significantly increased protection against leukemia in absence of both genes. It is therefore concluded that the null polymorphisms of these genes have a protective role in our population. It is recommended to include higher number of samples each from different leukemia sub-types and all ethnic groups of Pakistan in the future studies, in order to have an improved understanding of the association of the polymorphisms of these genes.

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1. HEMATOLOGICAL DISORDERS

Hematological or blood disorders include a large variety of diseases that primarily affect the red and white blood cells or the hemostatic system covering platelets and clotting factors. Red cell disorders include various types of anemia and thalassemia, while the white cell disorders involve non-neoplastic (e.g leukopenia, leukocytosis) and neoplastic diseases (leukemia, lymphoma) (Aster, 2010). Hematological disorders such as thalassemia, anaemia and leukemia, constitute a major class of genetic disorders in Pakistan (Alwan and Modell, 1997). Beta thalassemia, for instance, is found in about 5 percent of Pakistani population (Khattak and Saleem, 1992). On the other hand, the hematological malignancies like acute lymphoblastic leukemia, Non-Hodgkin's Lymphoma are among the most common cancers in Pakistan. According to the Punjab Cancer Registry Report 2, the most frequently occurring hematological malignancies among children are acute lymphoblastic leukemia (16.3%), Hodgkin's lymphoma (11.8%) and non-Hodgkin's Lymphoma (11.1%) (PCCR, 2012).

1.2. LEUKEMIA

Leukemia is the cancer of white blood cells that develops due to abnormal maturation of blood cells from the hematopoietic stem cells (Pui and Evans, 2006), escaping the normal homeostatic control mechanism. This results in the deregulation of processes like growth factor signaling, self- renewal, differentiation and apoptosis (Pui *et al.*, 2004).

1.3. NORMAL HEMATOPOIESIS SYSTEM

The process of formation of the cellular elements of the circulating blood is called hematopoiesis (Ahmed, 2002). All types of blood cells originate from bone marrow. Before birth, in the earliest phase of embryogenesis, blood cells are derived from the yolk sac mesoderm. The process occurs in the developing liver during the second trimester while in the third trimester of embryonic development, bone marrow

becomes the major organ of hematopoiesis. After birth, all blood cells arise from a single cell type in bone marrow, referred to as the pluripotential hematopoietic stem cells. In the normal hematopoiesis system, the hematopoietic stem cells proliferate and gives rise to two major cell lineages; namely the myeloid lineage and lymphoid lineage. The myeloid cell lineage forms thrombocytes, erythrocytes, granulocytes (neutrophils, basophils and eosinophils) and monocytes. This formation takes place in bone marrow. The lymphocytes arising from lymphoid lineage, on the other hand, arise partly in bone marrow and partly in lymphoid organs like lymph nodes, spleen and thymus (Fig 1.1) (Ahmed, 2002; Gyton and Hall, 2006; Mescher, 2010).

1.4. LEUKEMOGENESIS

Leukemogenesis may be defined as the process of development or production of leukemia. Leukemia arises from the abnormal growth of hematopoietic stem cells. It is found to involve multiple transformation events that block differentiation of normal cells and provide proliferation capacity. Studies conducted on acute myeloid leukemia have shown that certain genetic and epigenetic changes are required for the development of leukemia. These events lead to the formation of pre-leukemic stem cells (pre-LSC), including the initial leukemia cell-of-origin (LCO), which then differentiate into leukemia stem cells (LSC). These LSCs have the ability to grow into leukemia, hence interfering with the normal hematopoietic system, blocking the differentiation process and resulting in large number of immature cells (Fig 1.2) (Pandolfi *et al.*, 2013). Two molecular processes are thought to occur in formation of leukemia; the arrest of differentiation process (Gilliland and Tallman, 2002) and the proliferation of cells to form a bulk of leukemic cells (Hope *et al.*, 2004). Leukemia, commonly acute leukemia and chronic myeloid leukemia that is on the verge of transformation into acute leukemia, is characterized by the presence of blast cells (Fig 1.3). Blast cells are the immature precursor cells termed as myeloblasts or lymphoblasts, depending on whether they arise from myeloid or lymphoid lineage, respectively. These are comparatively bigger in size than the normal cells and possess an immature nucleus (Gallagher et al., 2012).

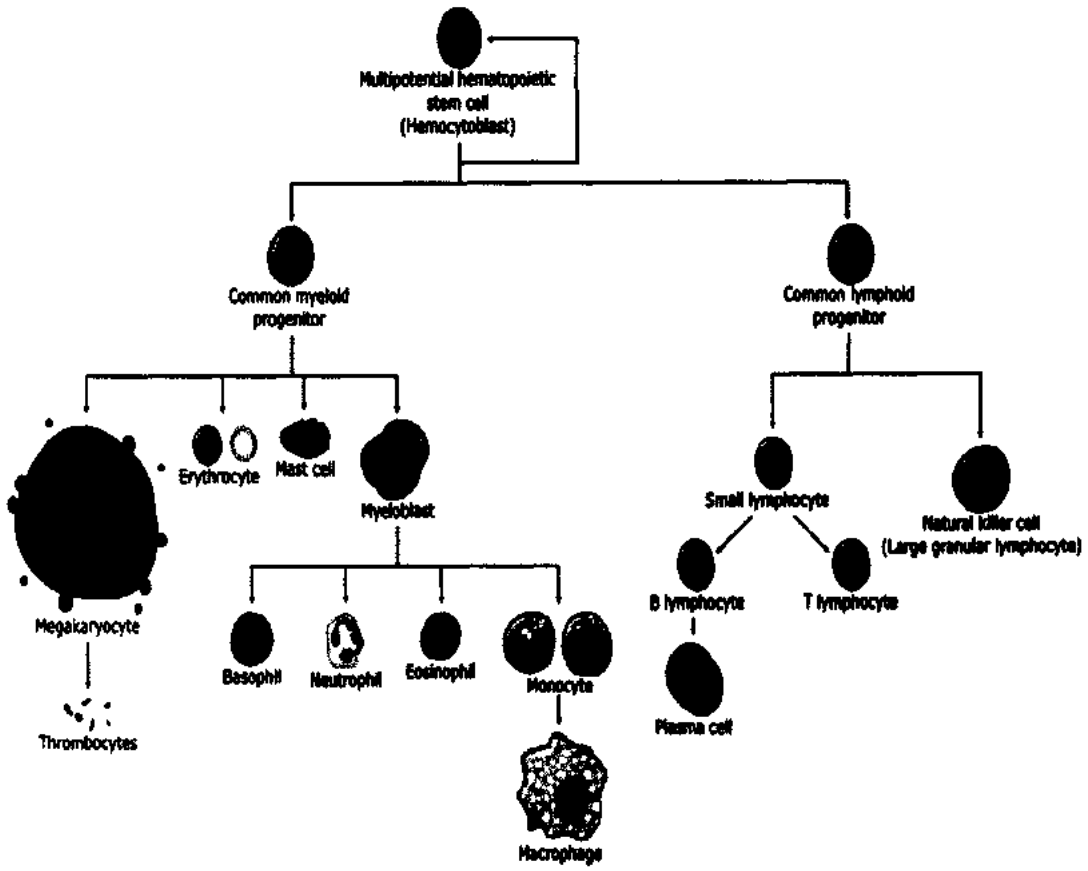

Figure 1.1: Events leading to the normal hematopoiesis in body. There are two lineages, myeloid lineage that results in the formation of erythrocytes, thrombocytes and granulocytes and the lymphoid lineage that leads to the formation of lymphocytes. (http://stemcells.nih.gov/info/scireport/2006report.html)

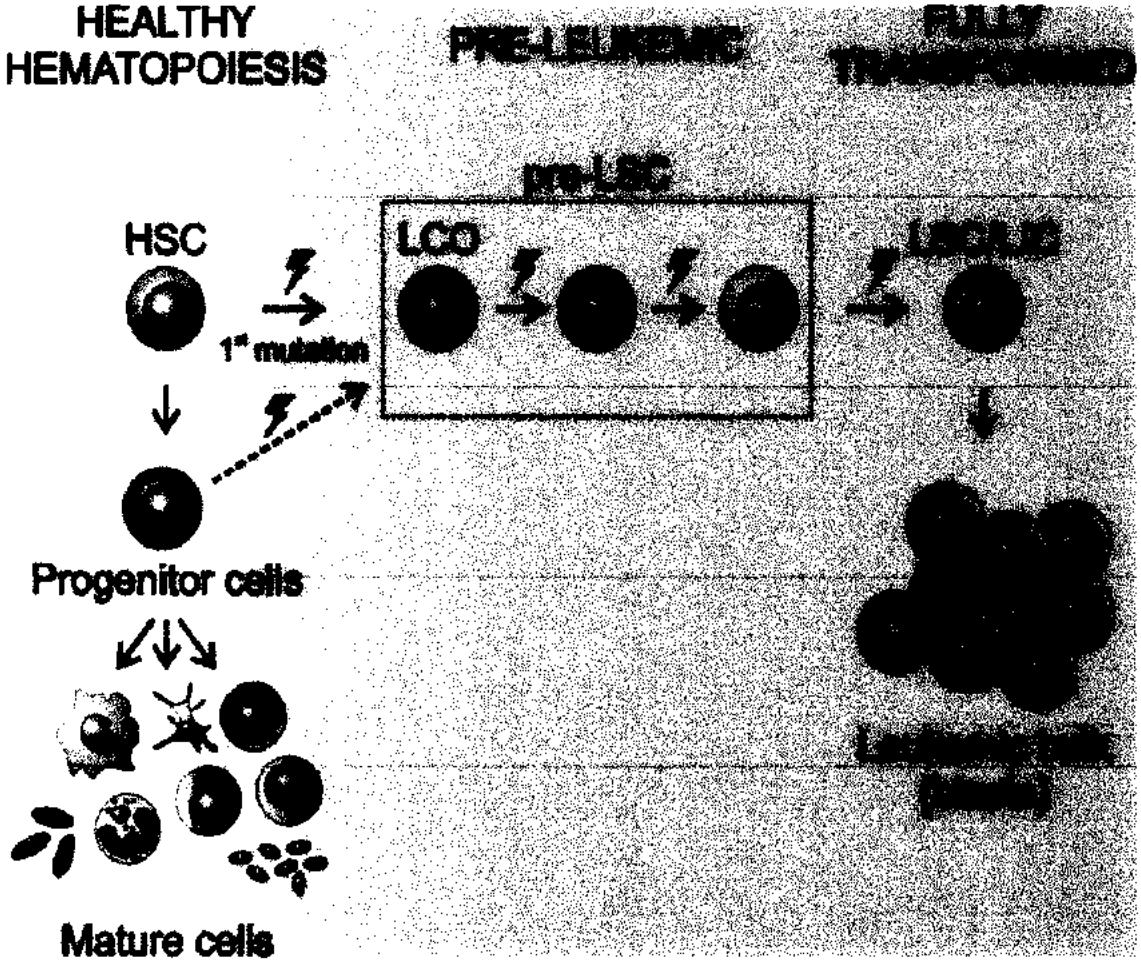

Figure 1.2: Process of healthy hematopoiesis and leukemogenesis. In normal hematopoietic system, the hematopoietic stem cells differentiate into the mature blood cells. However, any genetic or epigenetic change may result in the formation of pre-leukemic stem cells, which end in developing leukemic bulk and hence a fully transformed leukemia. (Pandolfi *et al.*, 2013).

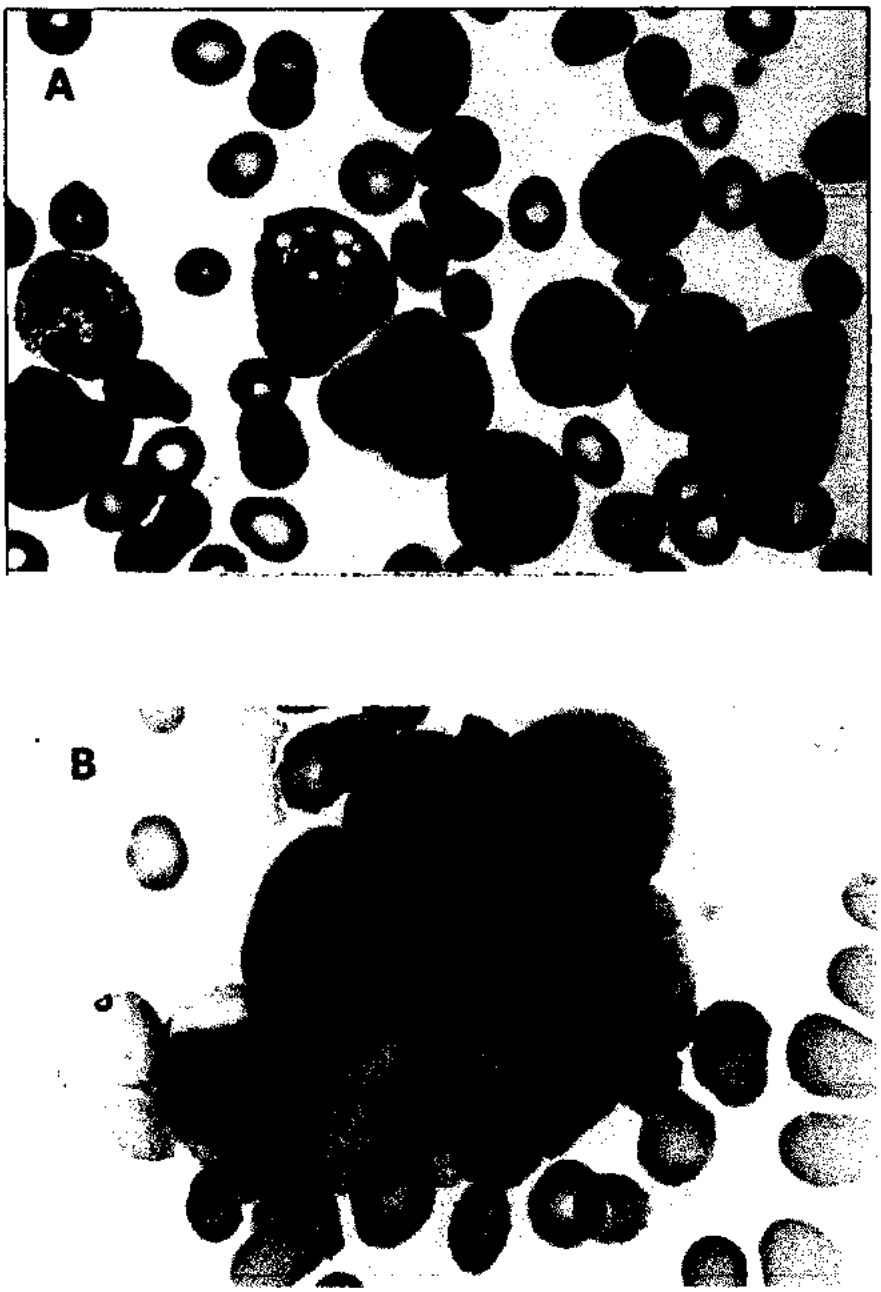

Figure 1.3: A. Morphology of the normal White Blood Cells (Neutrophils). B. Peripheral blood film showing characteristic blast cells (Gallagher et al., 2012)

1.5. SYMPTOMS OF LEUKEMIA

Leukemia is characterized often by a triad of the clinical signs due to reduction in the numbers of normal blood cells. These include anemia due to lack of red blood cells, infection by the deficiency of white blood cells (neutropenia) and bleeding caused by a decrease in blood platelets, called thrombocytopenia. Leukemia also occurs with fatigue by depression of marrow function and fever due to infection. Because of marrow expansion, the patients also suffer from bone and joint pain. Other clinical symptoms include vomiting, headache and nerve palsies due to manifestations of central nervous system (Velasco *et al.,* 2006; Gallagher et al., 2012).

1.6. EPIDEMIOLOGY OF LEUKEMIA

1.6.1. WORLD WIDE

The worldwide prevalence of leukemia is quite high with an average incidence rate adjusted for age estimated to be 12.8 per 100,000 people, and the age-adjusted mortality rate is 7.1 per 100,000 per year worldwide, reported for 2006 to 2010 (Howlader *et al.,* 2012). According to the reports by World Health Organization (WHO), the incidence and death rate of leukemia in males is estimated to be 2.7% and 3.3%, respectively. In females, however, the incidence and mortality rates of leukemia are lower with 2.3% incidence and 3.2% mortality (GLOBOCAN, 2012).

1.6.2. UNITED STATES

In the United States, about 310,046 people are predicted to be either suffering from leukemia or are in remission (Leukemia and Lymphoma Society, Facts 2013). According to the American Cancer Society, there has been an increasing trend in the age-adjusted death rates caused by leukemia in males in United States from 1930-2009. In 2013, about 52,380 new cases of leukemia are expected with 30,100 cases in males and 22,280 in females (Siegel *et al.,* 2014);

1.6.3. EUROPEAN COUNTRIES

WHO estimated about 82329 (2.4%) and 53796 (3.1%) new cases of leukemia, in 2013, in the European Region of the world. The incidence of leukemia in European males is 2.6% while the mortality rate is 3.1%. On the other hand, the incidence and

mortality rate of leukemia in European females is 2.2% and 3.2%, respectively (http://globocan.iarc.fr/).

1.6.4. ASIA

In Asia, about 167448 (2.5%) new cases of leukemia are estimated in 2013 and 138375 (3.1%) death rates are expected. In Asian men, the estimated incidence of leukemia is 2.6%, while the expected mortality rate is 3.0%. In contrast, the Asian women have an estimated incidence of 2.3% and a mortality rate of 3.2% (http://globocan.iarc.fr/).

1.6.5. PAKISTAN

Pakistan also has a considerable prevalence of leukemia. According to the WHO 2012 (http://globocan.iarc.fr), the expected occurrence and death rate of leukemia in both genders is 3.1% and 4.1%. The expected incidence of leukemia among Pakistani males and females is 4.5% and 2.1%. The mortality rate is, however, 5.4% in males and 3.0% in females (http://globocan.iarc.fr/). In 1995, the Karachi Cancer Registry was the first cancer registry organized in Pakistan. The first incidence based study was conducted on the population of Karachi South from 1995 to 1997 and focused on collecting data from 35 different hospitals, laboratories and death registry offices. According to this study, about 4,286 new cases of cancer were registered in the area of Karachi South, with 2160 cases in men and 2108 cases in women (Bhurgri *et al.*, 2000). In Pakistan, the occurrence of acute lymphoblastic leukemia is highest amongst children. According to the Punjab Cancer Registry Report 2012 of Shaukat Khanum Memorial Cancer Hospital (SKMCH), Acute Lymphoblastic Leukemia is considered to be the first among five most common cancers prevailing in children. The incidence for childhood ALL in 2011 was reported to be about 16.3% of the total 5370 cancer cases registered (PCRR, 2012). Among the top ten malignancies reported at the SKMCH from December 1994- December 2012, there were 3368 cases (6.02%) of leukemia out of the total 58,761 malignancies registered, making it the second most prevalent hematological malignancy in the country (Collective Cancer Registry Report From Dec. 1994 Uptil Dec. 2012).

1.7. TYPES OF LEUKEMIA

Leukemia can be classified by two ways. First, leukemia may be categorized on the basis of the speed of progression of the disease (acute or chronic). **Acute** leukemias progress very rapidly and if left untreated, may be fatal within days or weeks. **Chronic** leukemias are characterized by slow disease progression and patient may survive for longer periods of life (Gallagher et al., 2012). Secondly, leukemia may be divided on the basis of the location of the cells from where the disease has originated. If the abnormal clone of cells derives from the **myeloid** lineage, then it is termed as myeloid, myelobastic, myelocytic or myelogenous leukemia. On the other hand, if the abnormal cells develop from the **lymphoid** origin, then they are called lymphoblastic or lymphocytic leukemia (Gallagher et al., 2012). Leukemia is, thus, classified into four types considering these two conditions together (Kasteng *et al.,* 2007).

1. Acute Lymphoblastic Leukemia (ALL)
2. Acute Myeloid Leukemia (AML)
3. Chronic Lymphoblastic Leukemia (CLL)
4. Chronic Myeloid Leukemia (CML)

1.7.1. ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

Acute Lymphoblastic Leukemia (ALL) encompasses 80% of acute leukemias (Cheok and Evans, 2006) and involves the infiltration of the blood and bone marrow with an expanding clone of neoplastic cells (Fig 1.4 A) (Hirt *et al.,* 1997). The characteristic feature of acute lymphoblastic leukemia is the presence of more than 20% blast cells in bone marrow (Hoelzer and Gale, 1987). The most common signs and symptoms occurring in ALL include pallor, breathlessness and fatigue due to aneamia, fever and mouth ulcers due to infections, gum bleeding, bruising and petechiae caused in thrombocytopenia. ALL is also characterized with bone pain due to tissue infiltration of marrow, gums, liver/spleen, or nodes etc. (Gallagher et al, 2012). Acute Lymphoblastic Leukemia is categorized into three subtypes; L1, L2 and L3 by the FAB classification system (Viana *et al.,* 1980; Reaman *et al.,* 1999; Pui *et al.,* 2004). Most common malignancy reported in childhood is ALL. Among children below the age of 15 years, around 33.6 in every 1,000,000 children are diagnosed with ALL

(Silverberg, 1979). The peak age for the disease occurrence is 2-5 years (Miller *et al.*, 1981) and the incidence of ALL is found to be 10 per 100,000 children in this age (Pui *et al.*, 2008). The global age-adjusted death rate for ALL is, however, reported to be 0.5 per 100,000 individuals per year. These rates were based on the reports from 2006-2010 (SEER-Stat Fact Sheets - Acute lymphocytic leukemia, http://seer.cancer.gov/). In Pakistan, the prevalence of acute lymphoblastic leukemia is highest amongst children. According to the Punjab Cancer Registry Report 2012, ALL is considered to be the first among the five most common cancers prevailing in children with approximately 16.3% cases (PCRR, 2012).

1.7.2. ACUTE MYELOID LEUKEMIA (AML)

Acute Myeloid Leukemia is the hematological malignancy that interferes with the normal differentiation of myeloid cells. This causes an increase in immature progenitor cells due to their accumulation in the bone marrow and results in decrease in the number of blood cells (Fig 1.4 B) (Gallagher et al., 2012). The most common clinical signs of the disease include fever by infection, pallor due to breathlessness, petechiae, bleeding and bruising, bone pain and headache (Kumar *et al.*, 2010). AML has been classified into eight sub-types (M0-M7) according to the French-American-British (FAB) classification system, which divides them on the basis of morphology, differentiation level and lineage of leukemia blast cells (Bennett *et al.*, 1985). The FAB classification has been modified by WHO, dividing AML into four groups; (i) AML with genetic aberrations, (ii) AML with Multiple Dysplastic disorder (MDS)-like features, (iii) AML Therapy-related and (iv) AML not otherwise specified, classified according to FAB system (Vardiman *et al.*, 2002). The new cases of AML in US for the year 2014 are estimated to be 18,860 for both the genders while 10,460 deaths are estimated (Siegel *et al.*, 2014). The disease is predominant in males than in females (Kosary *et al.*, 1995; Aziz and Qureshi, 2008).

1.7.3. CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

Chronic Lymphocytic Leukemia is a sub-type of leukemia that involves accumulation of monoclonal B lymphocytes in blood, bone marrow, spleen and lymph node (Rozman and Montserrat, 1995). A characteristic feature of CLL is the presence of $\geq$ 5000 clonal B-lymphocytes in the peripheral blood, an important feature studied at diagnosis (Fig 1.5 A) (Hallek *et al.*, 2008). According to the current view about CLL,

it is a heterogeneous disease deriving from B-lymphocytes that vary in V gene mutations (Chiorazzi *et al.,* 2005). The patients with CLL often show no signs and symptoms and are identified by the abnormal blood counts. However, some symptoms of chronic lymphocytic leukemia may include fever, aneamia, lymphadenopathy (palpable enlargement i.e., > 1 cm, of one or more lymph nodes) and enlargement of liver and spleen, a condition called as hepatospleenomegaly (Gallagher et al., 2012). The disease presents itself with immune disturbances in patients (Rozman and Montserrat, 1995). The median age of disease occurrence is 65 years, with a median survival of about 10 years (Gallagher et al., 2012). The existence of CLL is frequent in the Western region of the world, where it accounts for about 20% of all the leukemias. However, in Asiatic zone the prevalence of disease is lesser, making up only 2.5% of all leukemia cases (Keating, 1994).

1.7.4. CHRONIC MYELOID LEUKEMIA (CML)

Chronic Myeloid Leukemia (CML) is the malignant monoclonal disease involving the hematopoietic stem cells. It results in an abnormal increase in the myeloid, erythroid, monocytic, megakaryocytic and the platelets in the peripheral blood, thereby characterized as a myeloproliferative disorder (Fig 1.5 B) (Fialkow *et al.*,1967; Fialkow *et al.,* 1977). The symptoms of Chronic Myeloid Leukemia may include weight loss, shortness of breath due to symptomatic anemia, occasional headache, abdominal discomfort caused by spleen enlargement, uncommon bruising and bleeding and fever and sweats in absence of infection. In some patients, however, CML may occur with no symptoms or signs. (Gallagher et al., 2012). There are three phases of CML, in which disease occurs; namely chronic, accelerated and blastic phases (Kantarjian *et al.,* 1993). The disease is more prevalent in older age groups, the median age being 53 years, though it may target any age (Sawyers, 1999). CML is reported to affect males more commonly than females (Aziz and Qureshi, 2008). It is characterized by slow progression, and if left untreated may get transformed to acute myeloid (75% cases) or acute lymphoblastic leukemia (25% cases) (Gallagher et al., 2012). The incidence of CML in United States has been estimated by the American Cancer Society for the year 2014. It is expected that approximately 5,980 new cases of CML will be diagnosed, with 3130 cases in males and 2850 cases in females. The estimated number of deaths will be 810, with 550 men and 260 women dying due to CML (http://www.cancer.org/).

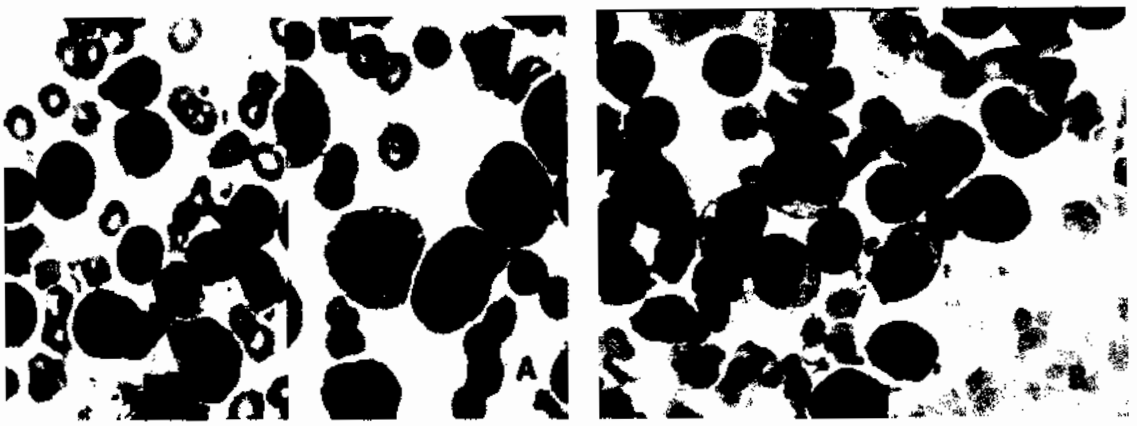

Figure 1.4: Image of cells in acute leukemias A. Image of the bone marrow aspirate from patients with acute lymphoblastic leukemia, stained with Wright-Giemsa stain. They can be seen to contain blast cells (Tsao *et al.*, 2004). B. Image of cells of acute myeloid leukemia stained with Wright-Giemsa stain (Ayala *et al.*, 2012).

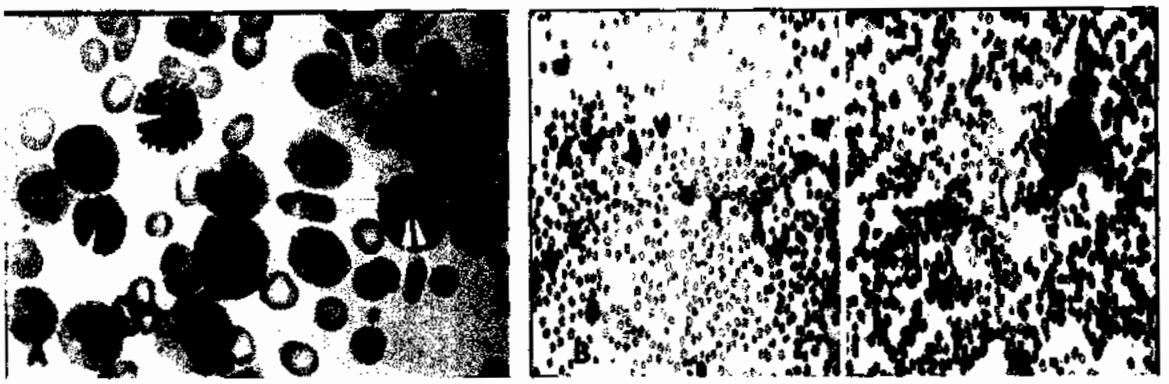

Figure 1.5: Image of cells of chronic leukemias. A. Image of the peripheral blood smear from chronic myeloid leukemia patient (Goldman, 1997). B. Image of the peripheral blood smear and bone marrow aspirate from chronic lymphocytic leukemia patients.

1.8.DIAGNOSIS AND TREATMENT OF LEUKEMIA

FAB system of classification is used for the categorization and initial diagnosis of leukemia. Diagnosis of leukemia is done by different techniques, consisting of both phenotypic and genotypic analysis. The first step in the diagnosis of leukemia is based on morphology, which involves the examination of blast cells and their cytology. This may give an immediate diagnosis of the disease and its stage on the basis of FAB classification. The acute leukemias are also diagnosed by cytochemical studies done by determination of certain enzymes, specific to AML and ALL, hence classifying them (Bene *et al.,* 1999). Phenotypic analysis is done by immunophenotyping using flow cytometry to enable correct diagnosis (Pui *et al.,* 2001). Diagnosis is also done by cytogenetic analysis of the blast cells in blood and bone marrow of the patient (Bhojwani *et al.,* 2012; McGregor *et al.,* 2012). Since leukemia mostly involves genetic rearrangements, therefore, cytogenetic analysis may be helpful in diagnosis (Heim and Mitelman, 1992). Genotypic studies are, however, done by using the highly sensitive techniques like RT-PCR, Fluorescent in situ hybridization and flow cytometry, to determine the chromosomal abnormalities resulting in the development of leukemia (Pui and Evans, 1998; Pui *et al.,* 2001).

Acute Leukemia, both acute myeloid and acute lymphoblastic leukemia, is being treated with different chemotherapy protocols in order to achieve a complete remission (80%) and a high rate of disease free survival (about 40%). In addition to chemotherapy, acute leukemia is also being treated with the Stem Cell Transplantation (SCT), from bone marrow grafts or may involve transplantation of peripheral blood stem cells (PBSCT) (Hoelzer *et al.,* 2002; Burnett *et al.,* 2011). Among chronic leukemia, tyrosine kinase inhibitors, such as Imatinib, are used for the treatment of chronic myeloid leukemia (Cohen *et al.,* 2002). However, like acute leukemia, chronic myeloid leukemia has also been treated with bone marrow transplantation (Goldman *et al.,* 1986; Or *et al.,* 2003). Many alkylating agents and purine analogues are being used to treat chronic lymphocytic leukemia, as a first line of treatment (Ferrajoli and O'Brien, 2004) whereas; it may also be treated by allogeneic bone marrow transplantation (Cheson *et al.,* 1996).

1.9. RISK FACTORS FOR LEUKEMIA

Leukemia is a multi-factorial disease, caused by interplay of various environmental and genetic factors. A number of factors like age, gender, ethnicity/race, parental occupational exposures, sensitivity to the treatment regimens (radiotherapy and chemotherapy) and genetic syndromes have been reported to have a significant association with the development of leukemia (Smith *et al.,* 1999).

1.9.1. ENVIRONMENTAL FACTORS

Ionizing radiations, occupational exposure to benzene and alkylating agents found in chemotherapy are thought to be the important risk factors for leukemia (Linet *et al.,* 2007).

Occupational Exposure of Parents

Occupational Exposure of Parents is an important risk factor for causing childhood leukemia (Reinier, 2002). It has been documented that the exposure of parents to paints, plastics, pesticides, chemicals and solvents increases the risk of childhood leukemia (Savitz and Chen 1990; Zahm and Ward 1998).

Air Pollutants

Many air pollutants like tobacco smoke, benzene, butadiene and nitrogen dioxide have been studied for their role as a significant risk factor causing leukemia (Buffler *et al.,* 2005).

Tobacco Smoke

There has been mixed views regarding association of tobacco smoke as a causative agent of leukemia. In some studies, a significant association was found between the parental smoking and childhood acute leukemia (Stewart *et al.,* 1958; Stjernfeldt *et al.,* 1986; John *et al.,* 1991; Shu *et al.,* 1996; Ji *et al.,* 1997), while some studies report no significant association (Pershagen *et al.,* 1992; Klebanoff *et al.,* 1996).

Benzene

Benzene, on the other hand, is also regarded as a risk factor for adult leukemia, mainly Acute myeloid leukemia (AML) (Buffler *et al.,* 2005). Increased risks of leukemia and elevated mortality rates were reported among exposed workers in China (Yin *et al.,* 1996). Benzene is found in occupational exposures as well as in food,

cigarette smoke and in vehicular or industrial emissions, kilns and furnace smoke and gases. Benzene, nitrogen dioxide and butadiene are included in the category of HAPs or hazardous air pollutants, that cause leukemia and other cancers (Buffler *et al.*, 2005).

Radiation

The risk of leukemia is found to be elevated with exposure to low doses of ionizing radiations like x-ray films. Among the non-ionizing radiation, it is thought that extremely low frequency magnetic field (ELF-MF) in diverse measures has a strong association with the development of childhood leukemia. It has been suggested by the National Radiological Protection Board (NRPB) in the United Kingdom that exposure of parents to a dose of more than 0.4μT of magnetic field cause leukemia risk (Ahlbom *et al.*, 2001)

Others

Household solvents including paints, petroleum products, metals and pesticides are reported to pose serious threat to children causing childhood leukemia (McBride, 1998).

1.9.2. GENETIC FACTORS

There are many genetic factors known to contribute to the development of leukemia, be it the highly penetrant mutations or the low penetrant genetic polymorphisms (Sheilds and Harris, 2000). Genes associated with the xenobiotic metabolism are one of the genetic factors that contribute to the increased risk of developing leukemia (Chen *et al.*, 1997; Krajinovic *et al.*, 1999; Infante-Rivard *et al.*, 2000). Xenobiotic metabolism occurs in two phases, Phase I controlled by Cytochrome P450 enzymes and Phase II controlled by Glutathione S-transferases (GSTs) (Guengerich *et al.*, 1995). Many genes from the Cytochrome P450 family, such as CYP1A1 and CYP2E1 polymorphisms, have been shown to be associated with childhood leukemias (Krajinovic *et al.*, 1999; Infante-Rivard *et al.*, 2000; Krajinovic *et al.*, 2002). An elevated risk of leukemia was also found to be caused by polymorphisms in genes encoding myeloperoxidase (MPO) and NAD(P)H quinone oxidoreductase 1 (NQO1) which are involved in Phase I metabolism (Smith *et al.*, 2001; Krajinovic *et al.*, 2002). Among polymorphic variants of Phase II metabolism associated genes, the

deletion of GSTT1 and GSTM1, low function variant of GSTP1 gene and the genotypes of NAT2 resulting in slow acetylation have been found to be associated with an elevated risk of infant leukemia and more specifically Acute Lymphoblastic Leukemia (Chen *et al.*, 1997; Krajinovic *et al.*, 1999; Krajinovic *et al.*, 2000; Saadat and Saadat, 2000). Furthermore, the polymorphisms in genes encoding the methylenetetrahydrofolate reductase (MTHFR) enzyme, involved in folate metabolism pathway, have been found connected with an elevated risk of adult as well as childhood leukemia (Frosst *et al.*, 1995; Duthie, 1999; Wiemels *et al.*, 2001; Franco *et al.*, 2001).

Apart from these, several chromosomal translocations have been observed that may alter the normal pathways of hematopoietic differentiation and hence may result in oncogenic activity caused by abnormal expression of a gene, transcription factor activation (Crans and Sakamoto, 2001). Major translocations associated with ALL include *BCR-ABL*, t(8, 2), t(8; 14) *AML1-ETO* and t(1; 19) (Aricò *et al.*, 2000; Ernst *et al.*, 2002). About 50-70 percent of AML patients harbor different translocations, deletions and other chromosomal abnormalities. Some cytogenetic abnormalities such as, *AML1-ETO, CBFB-MYH11*, *PML-RARA* and mutations of *FLT3* have been well-studied. (Bench *et al.*, 2005; Mrozek *et al.*, 2008; Dohner and Gaidzik, 2011). In CML, the most important and well studied translocation is *BCR-ABL*, which is found in more than 95% of the cases with CML (van Dongen *et al.*, 1999; Pui *et al.*, 2004).

1.10. DETOXIFICATION PATHWAYS

Humans are surrounded by a large range of natural (plant and fungal toxins) and man-made toxic pollutants (Guengerich, 1990; Sheehan *et al.*, 2001). These xenobiotic substances result in toxic effects upon the organisms and are considered to be one of the primary causes of cancer in humans. They include polycyclic aromatic hydrocarbons found mostly in smoke, tobacco, pesticides and various carcinogens found in air, soil and water (Guengerich, 2003; Peluso *et al.*, 2004). Cells normally possess a system for the degradation of harmful endogenous or exogenous toxins. This system consists of an enzymatic array that catalyzes the detoxification process, involving biological transformation of the carcinogenic compounds (Guengerich, 1990, Haydes and Pulford, 1995; Guengerich, 2000).

1.10.1. PHASES OF DETOXIFICATION

In conjugation reactions, the xenobiotic substance is normally thought to possess a functional active site to react with the conjugating moiety (Liska, 1998). There are certain non-reactive xenobiotic substances that have no such site and hence unable to get converted to the water-soluble form. For the bio-transformation of compounds, two phases were proposed, namely functionalization and conjugation. The first phase involves the formation of a reactive site in xenobiotic compound using oxygen and second phase adds up a water-soluble group to the reactive site (Estabrook, 1996). The detoxification system therefore is a multi-step process. It comprises three phases; I, II and III. Phase I is the name given to functionalization step and Phase II involves the conjugation step. In this system, the xenobiotic compound is being converted into a water-soluble component by Phase I and II detoxification enzymes, following its elimination by Phase III enzymatic reactions (Guengerich, 1990). Cytochrome P450 is a group of proteins, catalyzing Phase I detoxification of harmful xenobiotic compounds into less harmful and water soluble form, while the Phase II detoxification is carried out by glutathione S-transferases (Guengerich, 1990). Phase I detoxification pathway is carried out by the Cytochrome P450 (CypP450) enzymatic array (Vermeulen, 1996). This phase may, therefore, result in the formation of more harmful metabolites which, if left un-transformed by Phase II enzymes, are carcinogenic too (Ritchie *et al.,* 1980; Meyer *et al.,* 1990; Kawajiri *et al.,* 1990; Nebert *et al.,* 1991; Dalay *et al.,* 1993).

1.11. GLUTATHIONE S-TRANSFERASES

GSTs are soluble, dimeric enzymes having a molecular weight of approximately 50kDa (Tsuchida *et al.,* 1990). Glutathione S-transferases belongs to the class of enzymes carrying out Phase II metabolism, which are invovled in the detoxification of carcinogens and in removal of reactive oxygen species, like superoxide radical and Hydrogen Peroxides, from the body (Pemble *et al.,* 1994; Sheehan *et al.,* 2001). They, therefore, act on both endogenous (Reactive oxygen Species) formed as a result of body's metabolic processes as well as the foreign compounds (Nebert and Vasiliou, 2004). GSTs are located in liver where they carry out detoxification of drugs and other substances (Hayes and Strange, 2000). Glutathione S-Transferases catalyzes the conjugation of the Glutathione (GSH) into a variety of electrophillic compounds. The xenobiotic substance acts a substrate to glutathione and resulting compound, glutathione S-conjugate, is an endogenous water soluble substance leading to its elimination from the body (Fig 1.6) (Sheehan *et al.,* 2001; Townsend & Tew, 2003). GSTs, therefore, provide defense against the reactive and toxic compounds, including the reactive oxygen species like hydrogen peroxides, superoxides, mostly formed as a result of the reactions of Cytochrome P-450 enzymes (phase I detoxification).

1.11.1. MECHANISM OF DETOXIFICATION BY GSTS

Glutathione S-Transferase acts to catalyze the transformation of a harmful compound by forming a complex with Glutathione (GSH), which results in the formation of thiolate (GS^-) anion from GSH. The GS^-, hence, binds with the hydrophobic xenobiotic compound adjacent to it via a thioether bond between sulfur atom of GSH and substrate (Mannervik, 1985; Hayes and Pulford, 1995). The sites of GSH-binding and hydrophobic-substrate binding are termed as G- and H- sites (Fig 1.7) (Mannervik, 1985). There is a chance that the conjugation reaction of GSTs gets reversed, and results in the formation of compounds that are even more toxic than the original xenobiotic substrate, hence causing various diseases including cancer (Strange *et al.,* 1997; Strange and Fryer, 1999;.Cotton *et al.,* 2000).

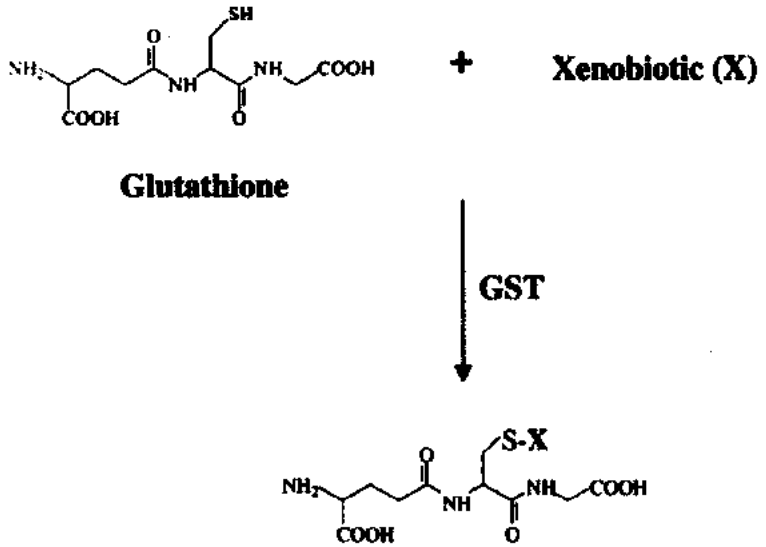

Figure 1.6: Catalysis of the Conjugation by GST (Townsend & Tew, 2003)

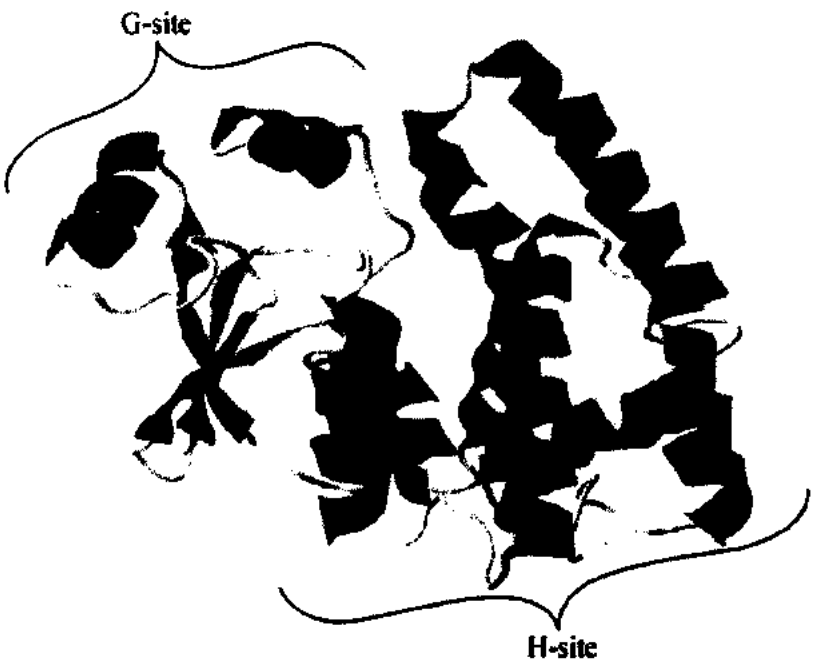

Figure 1.7: Structure of Glutathione S-Transferase showing G- and H- binding sites (Yasemin, 2011)

1.11.2. SUBSTRATES OF GLUTATHIONE S-TRANSFERASES

There are wide varieties of natural and artificial (man-made) chemical compounds that act as substrates for GSTs. They metabolize exogenous agents like insecticides, herbicides, environmental pollutants like acrolein, atrazine, lindane, inorganic arsenic, methyl parathione and DDT etc., pharmaceutical drugs, industrial intermediates and carcinogens (Hayes *et al.,* 2005; Abel *et al.,* 2004a, Abel *et al.,* 2004b). Many chemotherapeutic agents like adriamycin, chlorambucil, carmustine cis-platin busulfan etc. are also detoxified by GSTs, hence resulting in resistance against many drugs used in anti-cancer therapies (Lien *et al.,* 2002; Hamilton *et al.,* 2003). Among the most important epoxides, detoxified by GSTs, are those derived from the Phase I detoxification process by Cytochrome P-450 enzyme like polycyclic aromatic hydrocarbon epoxides, formed in liver, lungs and Gastrointestinal organs and others derived from environmental carcinogens like aflatoxin B1 (Kelly *et al.,* 2000). GSTs also function in providing protection from the by-products of oxidative stress, such as reactive oxygen species, by detoxifying them (Babusikova *et al.,* 2009). The cooked protein rich food is another source of important carcinogenic compounds, namely Heterocyclic amines, being detoxified by GSTs (Coles *et al.,* 2001).

1.11.3. CLASSIFICATION OF GLUTATHIONE S-TRANSFERASES

The Glutathione S-Transferases (GSTs) are categorized into two distinct super-families, namely microsomal and cytosolic GSTs (Hayes & McLellan, 1999). The microsomal superfamily comprises the trimeric proteins, designated as the MA-PEG (membrane-associated proteins in eicosanoide and glutathione metabolism), (Jakobsson *et al.,* 1999). The cytosolic GSTs are, on the other hand, larger super-family that includes dimeric and soluble enzymes. On the basis of their amino acid sequences, the cytosolic GSTs are further divided into eight sub-classes among humans, designated as *Alpha α, Pi (π), Theta (θ), Mu (μ), Kappa (κ), Sigma (δ) Zeta (ζ)* and *Omega (ω)* (Mannervik *et al.,* 1985; Board *et al.,* 2000). The difference in the individual's ability to respond to a number of diseases, like cancer, is influenced by the polymorphisms in these detoxification enzymes (Forsberg *et al.,* 2001).

Glutathione S-Transferase-Mu1

Glutathione S-Transferase Mu gene family consists of 5 genes; GSTM1, GSTM2, GSTM3, GSTM4 and GSTM5. These are situated on chromosome 1p13.3 in a cluster *(5'-GSTM4- GSTM2, GSTM1, GSTM5, GSTM3-3')* and are spaced 20 kilo basepairs apart (Fig 1.8) (Xu *et al.,* 1998). GST Mu1 acts on a number of compounds including 1-chloro-2,4-dinitrobenzene (CDNB), AFB1-epoxide; trans-4-phenyl-3-buten-2-one etc. GSTM1 is distributed in different organs of the body including liver, testis, lung, brain and kidney though the it is highly expressed in liver (Hayes and Strange, 2000). The null genotype (deletion) of GST-Mu is observed among 50% of the human population. It results in the individual's susceptibility to risk caused by the harmful xenobiotic compounds (Patskovsky *et al.,* 1999; To-Figueras *et al.,* 2000; Patskovsky *et al.,* 2000). In case of the gene deletion, there is no conjugation of xenobiotic substrate to GSH and hence lacks the biotransformation of harmful substrates and their removal via bile and urine (Bhattacharjee *et al.,* 2013).

Glutathione S-Transferase -Theta1

Glutathione S-Transferase Theta gene family is composed of two genes, GSTT1 and GSTT2, located on chromosome 22q11. These genes are separated by a distance of 50 bp approximately (Fig 1.9) (Coggan *et al.,* 1998). GSTT1 is expressed in liver, kidney, brain and small intestine etc. (Hayes and Strange, 2000). This gene functions in detoxifying a number of industrial chemicals, such as halogenated organic compounds (Thier *et al.,*, 1996; Hayes and Strange, 2000; Landi, 2000; Hayes *et al.,* 2005) and BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) etc. (Lien *et al.,* 2002). It also results in the GSH conjugated activation of a number of substrates like dichloromethane (DCM), chemical used in paint stripper and in synthesizing pharmaceutical drugs and plastics, dibromoethane (DBE) used as lead scavenging agent (Hayes and Strange, 2000), trichloroethene and 1,2,3,4-butadiene diepoxide (BDE) (Their *et al.,* 1996) used in rubber industry. Different allelic variations of GSTT1 have been studied, that in turn result in affecting the function of gene accordingly. The null or a deletion of GSTT1 has been linked with loss of the gene and consequently its function (Hayes and Strange, 2000) and is observed in 10-20% individuals (Pemble *et al.,* 1994; Rebbeck, 1997; Strange and Fryer, 1999). The individuals with a null condition are unable to carry out the conjugation of xenobiotic

compound with GSH and are, therefore, termed as non-conjugators (Pemble ete al., 1994; Hayes and Strange, 2000).

1.12. POLYMORPHISMS IN GSTS

Humans show differences in their capacity to detoxify harmful pollutants owing to the genetic variation in the GST enzymes among population. Most of the phase I and II enzymes among humans are polymorphic within population (Kłaassen *et al.*, 1996; Smith *et al.*, 1995; Seidegard and Ekstrom, 1997; Autrup, 2000). Normally the GSTT1 and GSTM1 genes code for the enzymes that function in detoxification. In the absence (null) of these genes, however, the function of detoxification is lost because of lack of corresponding enzymes (Landi, 2000). The null polymorphism of GSTT1 and GSTM1, therefore, are found to be associated with developing leukemia (Rollinson *et al.*, 2000; Balta *et al.*, 2003).

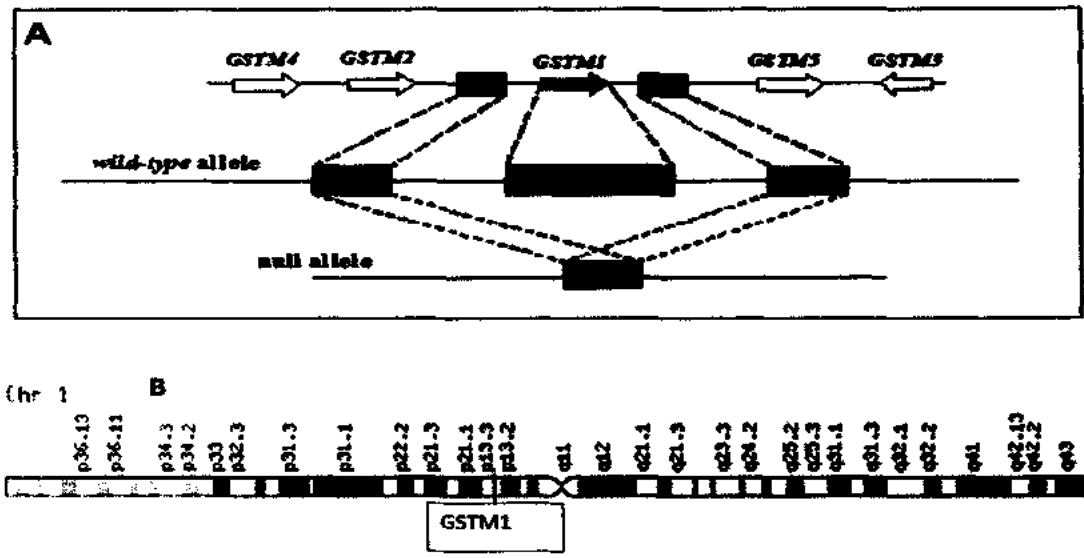

Figure 1.8: Glutathione S-transferase Mu gene family A: Localization of Glutathione S-transferase Mu gene family (100 kb region) and the event showing the deletion of GSTM1. B: The position of GSTM1 on chromosome 1 (1p13.3) (Parl, 2005).

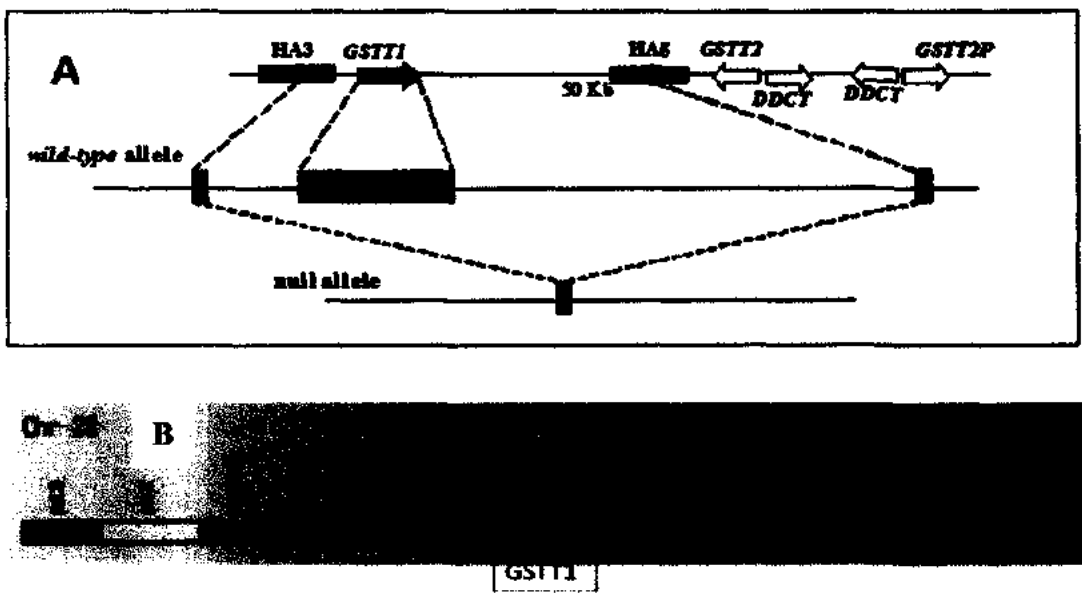

Figure 1.9: Glutathione S-tranferase Theta sub-family. A: Structural localization of Glutathione S-transferase theta genes and deletion of 50kb region of GSTT1. **B:** The position of GSTT1 on chromosome 22 (22q11.2) (Parl, 2005).

1.13. ASSOCIATION OF GSTT1 AND GSTM1 IN LEUKEMIA

One of the most important risk factors of leukemia is exposure to environmental pollution (Infante-Rivard *et al.,* 1999). The pollutants like benzene, ionizing radiation etc. have been proposed as risk factors for leukemia development, particularly acute leukemias (Perera *et al.,* 1996; Rothman *et al.,* 2001). The human body naturally possesses a mechanism that removes harmful toxins and hence provides protection against the toxicity of environmental chemicals and pharmaceutical drugs (Hayes and Pulford, 1995; Guengerich, 2000). This system is composed of an array of enzymes, called Glutathione S-Tansferases, that perform the detoxification of these environmental carcinogens. There may be individual variation, however, in the capability to metabolize the chemical toxic compounds because these enzymes exhibit polymorphisms. This may also depict the differences among individual towards leukemia risk (Taningher *et al.,* 1999).

Glutathione S-transferases are believed to express in hematopoietic stem cells, where they perform the role of detoxification of harmful compounds and hence, protect the cells from genetic damage. A defect in the respective GST genes may, however, result in failure of detoxification, exposing the cells to harmful compounds (genotoxins) and thereby, accumulating genetic changes in the process of leukemogenesis (Voso *et al.,* 2002). The data regarding expression of GST genes in specific lineages of hematopoietic system is limited. In order to analyze the function of GSTs in the development of various hematopoietic malignancies, the expression of GSTs has been studied in specific cell lineages of hematopoiesis (Wang *et al.,* 2000). Glutathione S-Transferases are found to be differentially expressed in the hematopoietic cell lines. GSTM1 is found predominantly in the lymphoid lineage while GSTT1 is observed to be greatly expressed in erythroid lines. These genes are, hence, believed to confer cytoprotective role in corresponding lineages (Wang *et al.,* 2000). Some compounds like benzene and other cytotoxic drugs have been found to cause the genetic damage and hence, mutation, by acting directly on the bone marrow cells (Rothman *et al.,* 1997; Pedersen-Biergaard *et al.,* 1987). The presence of GSTT1 null and GSTM1 null genotypes is thought to increase cells' susceptibility to genotoxins and may be considered as a major cause of hematopoietic malignancies, including leukemia (Wang *et al.,* 2000). Shao and colleagues (2007) examined the expression of cytochrome P450 as well as Glutathione S-transferase enzymes in the derived

Hematopoietic Stem Cells that were isolated from human fetal liver from donors during their second trimester. They observed the expression of both GSTT1 and GSTM1 in the fetal liver as well as HSC derived from them. Most of the fetal liver donors, however, possessed GSTM1 deletions (Shao *et al.,* 2007)

Many studies have been performed to determine whether any association occurs between the null polymorphisms of GSTT1 and GSTM1 with the possibility of developing leukemia, but no consistent risk association has been observed (Balta *et al.,* 2003). Among the different polymorphisms of GSTs, the null of GSTT1 and GSTM1 depicts a complete deletion of the respective genes and hence an absence of the functionally active enzyme (Landi, 2000). An increased risk of acute leukemias is observed to be associated with GSTT1 and GSTM1 null polymorphisms (Crump *et al.,* 2000; Saadat and Saadat, 2000; Rollinson *et al.,* 2000; Arruda *et al.,* 2001; Balta *et al.,* 2003). A case-control study was conducted to verify the role of different genes involved in xenobiotic metabolism, in susceptibility to acute leukemia. They included a total of 155 ALL patients and 94 AML patients in their study and divided them in adult and pediatric groups. They observed no association with GSTT1 null genotype in both AML (OR = 0.8, 95% CI = 0.4–1.7) and ALL patients (OR = 0.9, 95% CI = 0.5–1.6). However, they reported a significant association of GSTM1 null with increased risk of AML (OR = 2.1, P = 0.04), though the overall percentage of GSTM1 null was higher among AML and ALL patients compared to the controls. Aydin-Sayitoglu and colleagues, therefore, suggested that GSTM1 null genotype may contribute towards the development of acute leukemias (Aydin-Sayitoglu *et al.,* 2006).

Another study performed on children from Turkish population, also investigated the role of certain genes, including GSTT1 and GSTM1, in childhood leukemia development. They examined 144 and 33 patients with childhood acute lymphoblastic leukemia (ALL) and acute non-lymphoblastic leukemia (ANLL), respectively and compared them to 185 healthy individuals. The frequency distribution of GSTM1 null was equal among ALL and controls (55%), but the frequency was higher among ANLL patients (61.3%). The GSTT1 null percentage, on the other hand, was found slightly lower among the ALL patients (20.9%) than in controls (22.7%). In case of ANLL, there was a significantly lower representation of GSTT1 null (6.5%) (P- Value

= 0.05, OR = 0.24, 95% CI = 0.05–1.03). GSTT1 null genotype was believed to contribute towards a considerable protection in the development of ANLL in this population, though no significant association with the deletion of these two genes was observed to acute leukemia risk. It was also suggested that there might be a 4-fold lesser risk of ANLL development among Turkish children with GSTT1 null (Balta et al, 2003).

A study was conducted on 47 children from North Portugal with acute lymphoblastic leukemia. The polymorphisms of GSTT1 and GSTM1 were studied for their risk of developing ALL. All the three genotypes for each of the gene were studied (Y/Y, Y/N, N/N), no association was observed, except for GSTM1 null. This deletion genotype was found in higher percentage among ALL patients (68.1%) than in healthy controls (49%) and a significant association was observed (P-value = 0.035; OR = 2.2). This study, hence, suggested the correlation of the null genotype of GSTM1 with a greater risk of ALL. A combination effect was also examined by evaluating the influence of GSTT1 and GSTM1 together. No significance was, however, observed in combination (Alves *et al.*, 2002).

Furthermore, a meta-analysis of 26 case-control studies was performed. Tang and colleagues (2013) studied 3252 cases and 5024 controls. In overall analysis, they found GSTM1 null to be linked with childhood acute leukemia risk (OR = 1.30; 95%CI, 1.11-1.51). The risk of leukemia with GSTM1 null was found to be higher in Asians (OR = 1.94; 95% CI, 1.53-2.46) and Black population (OR = 1.76; 95% CI, 1.07-2.91). Among the acute leukemias, the risk of GSTM1 null polymorphism was shown to be associated with ALL (OR = 1.33; 95%CI, 1.13-1.58). GSTT1 null was, however, found to confer an increased risk of acute leukemia in Asian population. Hence, this analysis also supported the view that GSTM1 null increases the susceptibility to acute leukemia (Tang *et al.*, 2013).

A recent study was conducted on the assessment of GSTT1 and GSTM1 polymorphisms along with certain other genes, on the risk of Chronic Myeloid Leukemia (CML) (Banescu *et al.*, 2014). A total of 489 individuals, including 321 control samples and 168 unrelated adults with CML were studied. The null genotype of GSTM1 was found to be equally distributed among the patients (59.5%) and control group (59.2%), whereas, GSTT1 null was much lower in patients (20.8%) as

compared to controls (79.1%). On the other hand, the GSTM1 wild was present in patient (40.5%) and controls (40.8%) with equal percentage, but the percentage of GSTT1 was higher in patients' group (79.2%) than the controls (20.9%). No association was, however, observed with presence or deletion of both the genes in risk for developing CML. The characterization of samples into various factors, like age, gender, phase of disease as well did not show any association with GSTT1 and GSTM1 (Banescu *et al.,* 2014).

Other studies conducted on CML gave variable results. In a case-control study, carried out on CML patients from Turkish population, the polymorphisms of GSTT1 and GSTM1 were determined. In this study, GSTT1 null was found to be significantly associated with CML risk (OR 3.53, 95% CI = 2.08-6.00; P<0.0001). The combination effect of both the genes was also studied and observed that the simultaneous null of GSTT1 and GSTM1 ($GSTT1^-/GSTM1^-$) also was significantly associated (OR: 9.47; 95% CI: 3.61-24.87) with a risk of developing CML, though the frequency of the combination with the presence of both genes i.e. $GSTT1^+/GSTM1^+$ was much higher in the studied population. Therefore, this study suggested an increased risk of CML with GSTT1 null and GSTT1 null/GSTM1 null. Among the rest of combinations considered, the $GSTM1^+/GSTT1^-$ genotype was found to have a 2.5-fold increased risk of CML than $GSTT1^+/GSTM1^-$ (Ozten *et al.,* 2011). Ozten and colleagues, however, restricted their results to the Turkish population and hence, further studies on other populations were required (Ozten et al, 2011).

In another study conducted on 75 CML patients and 124 controls, from Kashmiri population, increased risk of CML was found to be significantly associated with GSTT1 null (OD = 2.12, 95% CI: 1.12– 4.02; P value = 0.0308). The percentage of GSTT1 wild and null was reported to be 64% and 36% in cases, respectively. In controls, the GSTT1 wild was observed in about 79% and GSTT1 null in 21% (Bhat *et al.,* 2012). Bhat and colleagues, therefore, suggested a positive association of GSTT1 null and CML risk, and believed that the presence of GSTT1 wild might confer protection against CML. They also studied combined effect of GSTT1 and GSTM1, and observed that the individuals with the absence of both genes were having a significantly increased risk (OD = 3.09, 95% CI: 1.122–8.528; P value = 0.0472) of developing CML (Bhat *et al.,* 2012).

1.14. AIMS OF STUDY

The major aim of the present study was to evaluate;

- The role of null polymorphisms of GSTT1 and GSTM1 in the development of acute (AML, ALL) and chronic (CML) leukemias in Pakistani Population.

- The association of these null polymorphisms in leukemia with respect to ethnic groups (Pathan, Punjabi etc.), gender (Male and Females) and age of patients.

TH-19473

CHAPTER 2
MATERIALS AND METHODS

MATERIALS AND METHODS

2.1. SAMPLING

2.1.1. Sample Collection

The study was conducted at the Institute of Biomedical and Genetic Engineering (IBGE), Islamabad from October 2013 to December 2014. A total of 158 samples from leukemia patients were collected from different hospitals. Patients suffering from any of the four types of leukemia were eligible for sampling. Bone marrow samples of a few patients were also obtained where available. The study was approved by the Ethical Committee of IBGE, Islamabad.

2.1.2. Collection Centers

The samples were collected from different hospitals in Islamabad, Peshawar, Rawalpindi and Lahore. The main hospitals targeted were Armed Forces Bone Marrow Transplant Centre (AFBMTC) Rawalpindi, Pakistan Institute of Medical Sciences (PIMS) Islamabad, Jinnah Hospital Lahore and Institute of Radiotherapy and Nuclear Medicine (IRNUM) Peshawar. A few samples were also collected from Holy Family Hospital Rawalpindi, KRL Hospital Islamabad and Benazir Bhutto Hospital Rawalpindi, depending upon the availability of patients with leukemia.

2.1.3. Data Collection

A written informed consent was obtained before sample collection from the patients and controls. Information was collected via a standardized questionnaire. The data was gathered over a wide range of variables including age, ethnic groups, gender, education class, disease status etc. Information regarding the lifestyle and eating habits was also obtained from both cases and controls.

2.1.4. Storage and Processing Of Samples

3-5 ml of samples (peripheral blood/bone marrow) were collected in BD vaccutainers (BD Franklin Lakes NJ, USA) containing "ACD solution A (Acid or Anticoagulant Citrate Dextrose Solution, Solution A)". Blood or bone marrow samples were stored at 4 °C till their processing. The samples either stored or fresh, were processed for separation of serum, Peripheral Blood Lypmohocytes (PBLs) and Red Blood Cells

(RBCs), following collection. Samples were centrifuged at 2000 rpm for 5 minutes by IEC HN-SII Centrifuge (International Equipment Company, U.S.A), till the layers got separated. Aliquots of the layers were stored at -70 °C. The remaining blood was stored at 4 °C for DNA extraction.

2.2.DNA EXTRACTION

DNA of the blood/bone marrow samples were extracted using the following methods after optimization.

1. DNA extraction by Phenol-Chloroform extraction
2. DNA extraction by Salting out Protocol
3. DNA extraction by Kit

Optimization of the protocol was done to obtain a good quality of DNA. The optical densities of DNA samples were measured using Nanodrop 2000C Spectrophotometer (ThermoScientific INC., U.S.A.). Working dilutions for samples, containing DNA concentration of more than 110 ng/µl, were prepared (100ng/µl in 50 µl).

2.2.1. DNA Extraction By Phenol-Chloroform Extraction Method

DNA was extracted following the method described by (Sambrook *et al.,* 1989). Cell lysis buffer 1 (KHCO3, NH4Cl, and 0.5M EDTA) was added twice to the blood samples, placed on ice for 30 minutes and centrifuged (Mistral 3000i Centrifuge, Fisons, UK) at 1200 rpm for 10 minutes at 4 °C. The resulting pellets were then re-suspended and 4.75 ml of STE (Saline Tris EDTA) and 250 µl of 10% SDS were added to samples drop-wise with vortex (G-560E Scientific Industries, INC., Bohemia). After that, 10 µl of Proteinase K (20mg/ml) was added to the samples and were then placed overnight in a water bath (Orbit Shaker Bath, Lab-Line, U.S.A) at 55 °C. The next day, samples were extracted with equal volumes of equilibrated phenol and chloform-isoamyl alcohol (24:1) independently, shaken for 10 minutes, kept on ice and centrifuged each time for 10 minutes at 3200 rpm at 4 °C. The aqueous layers were collected and 10 µl of RNase was added to them. The samples were then placed in water bath shaker fro incubation for 2 hours at 37 °C. Following this, approximately 250 µl of 10% SDS solution was added along with 10 µl of Proteinase K (20mg/ml) and then incubated at 55 °C. The samples were subjected to a

second round of extraction with equal phenol and chloroform-isoamyl alcohol (24:1) volumes. The DNA was then precipitated out from the finally obtained aqueous layer by adding 500 µl of 10M Ammonium Acetate and 5 ml of ice-chilled iso-propanol. The samples were then centrifuged againat 4 °C for 60 minutes at 3200 rpm. Pellet was re-suspended, washed with 70% ethanol (3200 rpm for 40 minutes at 4 °C) and then air-dried. DNA was dissolved by re-suspending the pellet in Tris-HCl (pH 8.0) and finally stored at 4 °C. The compositions of reagents used in this method are shown in Table 1.

2.2.2. DNA Extraction By Salting Out

Salting out method, described by Miller and colleagues (1988), was also used to extract DNA. Frozen blood samples (-20 °C) were washed with Tris –EDTA (T.E.) (Tris-HCl 10mM with pH 8.0 and EDTA 2mM) buffer to obtain a pellet of cells. The cells were then resuspended in 3-6ml of TNE buffer (10mM Tris-HCl, pH 8.0, 2mM EDTA and 400mM NaCl) in a 15 ml falcon tube. Around 12.5 µl of Proteinase K (20mg/ml) alongwith 100-200 µl of 10% of SDS solution were added to each sample and were left overnight at 37 °C for protein digestion. About 0.5-1 ml of saturated NaCl (5 Molar) was added to the samples. The samples were shaken vigorously for 30 seconds, kept for 5-10 minutes on ice and then centrifuged at 1500 rpm for 10-15 minutes. The supernatant containing DNA was then transferred to another 15 ml tube (Biologix Research Company, USA), and centrifuged again at the same speed. Equal volume of the ice-chilled iso-propanol was then added to the supernatant. The samples were inverted multiple times till the DNA precipitated out. DNA was then washed with chilled 70% ethanol, air-dried and dissolved in T.E. buffer The compositions of reagents used in this method are shown in Table 1.

2.2.3. DNA Extraction By Kit

DNA of some samples having volume less than 1 ml was extracted using RBC Genomic DNA Extraction Kit (Cat. No. YGB300 Genomic DNA Extraction Kit/ Lot. No. D026101; Real Biotech Corporation) as described by the manufacturer. To 200 µl of blood, 10 µl Proteinase K (20 mg/ml) was added, mixed and left for 5-10 minutes. Then 200 µl of GB buffer was then added and mixed by vortexing. Mixture was placed in a 70 °C water bath for incubation and inverted regularly. Around 200 µl ethanol (96-100%) was added to the mixture once it got clear, and was mixed for 10

seconds. Precipitate, if any, was broken up by pipetting. Sample mixture was then applied to a GD column placed in 2 ml collection tube and centrifuged for 5 minutes at 13,000 rpm. After that, the sample was washed by adding 400 µl of W1 buffer and centrifuged for 30 seconds at 13,000 rpm. The flow-through liquid was discarded and washed again by adding 600 µl of Wash Buffer and centrifuged for 30 seconds at 13,000 rpm. After discarding the flow-through liquid of this step, the sample was dried by centrifugation at 13,000 rpm for 3 minutes. Finally, DNA was dissolved in 130 µl of preheated (70 °C) Elution Buffer. Stock samples were stored at -20 °C.

Table 2.1: Composition of the different reagents used in DNA extraction done by Phenol-Chloroform method and Salting out protocol

Reagents	Composition
Cell lysis buffer	1g KHCO3, 8.29g NH4Cl, and 200 µl 0.5M EDTA
Saline Tris EDTA	3M NaCl 33.3ml (100mM), 1M Tris 50ml (50mM), 0.5mM EDTA 2.0ml (1mM)
10% SDS Solution	10g SDS in 100 ml de-ionized water
Chloroform Iso-amylalcohol	24 volumes Chloroform, 1 volume Iso-amylalcohol
10M Ammonium Acetate	77g of ammonium acetate in 100 ml de-ionized water
Tris-HCl	121.1 g Tris-Base in 1L de-ionized water
Tris –EDTA (T.E.)	Tris-HCl 10mM with pH 8.0 and EDTA 2mM
TNE buffer	10mM Tris-HCl, pH 8.0, 400mM NaCl 2mM EDTA
Saturated (5M) NaCl	146.2 g of NaCl in 500 ml de-ionized water
10X TBE	540g Trizma Base, 275g Boric Acid, 45.22g EDTA in 5L de-ionized water
0.5M EDTA	186.15g EDTA in 1L de-ionized water

2.3.POLYMERASE CHAIN REACTION

A multiplex Polymerase Chain Reaction (PCR) for amplification of the extracted DNA samples was performed and GSTT1 and GSTM1 null polymorphism was identified using a Thermal Cycler PxE 0.2 (Thermo Electron Corporation). PCR conditions were maintained as described by Sherif and colleagues (1996). The final reaction volume used was 10µl. Two primer pairs were used in the reaction, which include primers for GSTT1 and GSTM1. A third pair of an internal control primer, amplifying the CYP1A1 gene, was used. Final concentrations of reagents used in the reaction, final reaction volumes, the primer sequences and the PCR cycling conditions are shown in Tables 2.2, 2.3 and 2.4.

2.4.GEL ELECTROPHORESIS

The PCR products were subjected to agarose gel electrophoresis and analyzed on a 2% Agarose gel, prepared by mixing 2g of agarose in 100 ml of 1 X TBE Buffer using Maxicell EC 360M Electrophoretic Gel System (E-C Apparatus Corporation St. Petersburg, Florida, U.S.A). For loading, 5 µl of Orange G dye was added to the PCR Product (10 µl). GeneRuler 100 bp DNA Ladder (ThermoScientific INC., U.S.A.) was used for determining size of the product. The gel was run at 120V for an hour and was then subjected to Ethidium Bromide staining for 30 minutes. The amplified product was viewed under UV-Transilluminator for confirming the presence or absence of a 480 bp band that represents GSTT1 and a 215 bp band representing GSTM1. A third 312 bp band for the internal control was always present in amplified products (Fig. 2.1). Image was taken using gel documentation system (UVITECH, Cambridge).

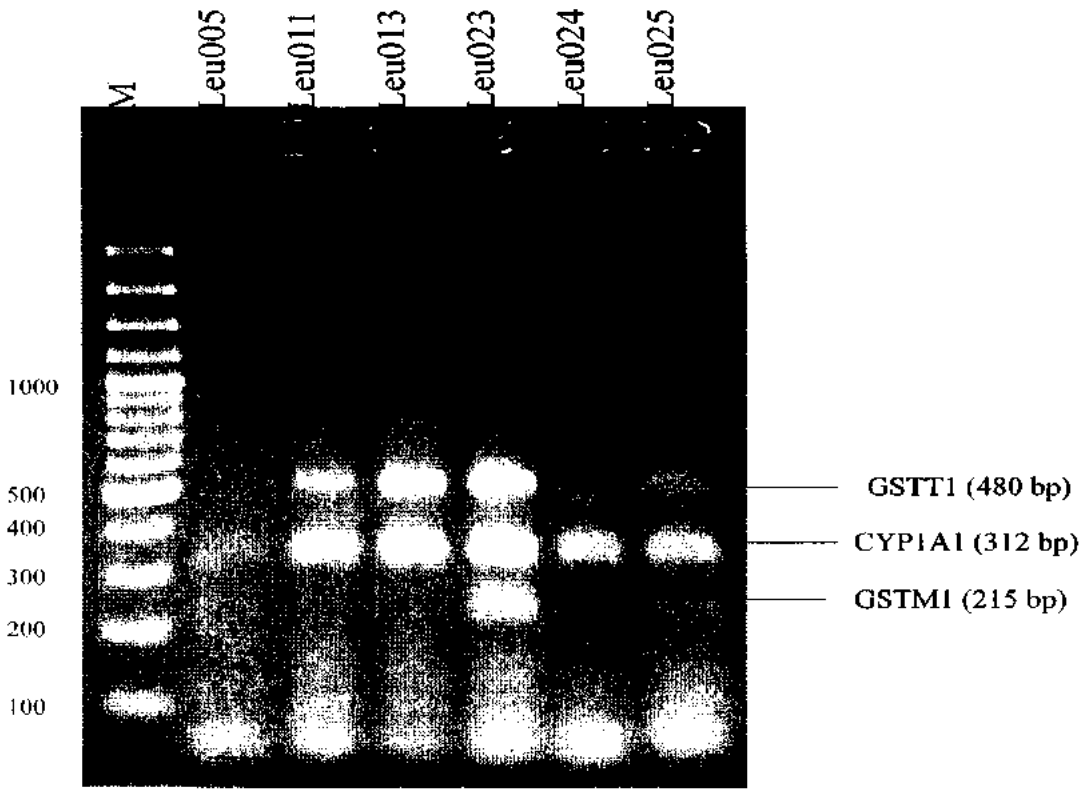

Figure 2.1: Agarose gel electrophoresis of the PCR amplicons. (From left to right) Lane 1-M (Ladder), Lane 2-Leu005, Lane 3-Leu011, Lane 4-Leu013, Lane 5-Leu023, Lane 6-Leu024, Lane 7-Leu025. The band of 480 bp represents GSTT1, band at 215 bp confirms GSTM1 and 312 bp band shows CYP1A1 Internal Control and are shown on the right. The band sizes of ladder are depicted on the left.

Table 2.2: Stock and final concentrations of the reagents used in the Polymerase Chain Reation

no.	Reagents	Stock Conc.	Final Conc.	Volume (µl) for 10 µl reaction	Volume (µl) for 50 µl reaction (Sherif *et al.*, 1997)
1	PCR Buffer (10X)	10 X	1X	1	5
2	dNTPs (mM)	2.5	0.2	0.8	4
3	GSTT1 F (µM)	10	0.5	0.5	2.5
4	GSTT1 R (µM)	10	0.5	0.5	2.5
5	GSTM1 F (µM)	10	0.5	0.5	2.5
6	GSTM1 R (µM)	10	0.5	0.5	2.5
7	Internal control F (µM)	10	0.5	0.5	2.5
8	Internal control R (µM)	10	0.5	0.5	2.5
9	MgCl2 (mM)	25	1.5	0.6	3
10	Taq DNA Polymerase(U)	5	2	0.2	0.4
11	DNA sample (ng/µl)	100	50	1	5
12	De-ionized Water			3.2	17.6

Table 2. 3: Sequences and melting temperatures of the primers used (GSTT1, GSTM1and CYP1A1 as internal primer)

No.	Primers	Sequence	Melting Temperature *
1	**GSTM1 F**	5'-GAA CTC CCT GAA AAG CTA AAG C	58.7 ºC
2	**GSTM1 R**	5' -GTT GGG CTC AAA TAT ACG GTG G	62.6 ºC
3	**GSTT1 F**	5' –TTC CTT ACT GGT CCT CAC ATC TC	59.6 ºC
4	**GSTT1 R**	5' –TCA CCG GAT CAT GGC CAG CA	70.5 ºC
5	**CYP1A1 F**	5' –GAA CTG CCA CTT CAG CTG TCT	59.7 ºC
6	**CYP1A1 R**	5'- CAG CTG CAT TTG GAA GTC CTC	62.2 ºC

*https://genome.ucsc.edu/cgi-bin/hgPcr?hgsid=384987381_l9vinEuHqknwa8Vmqxg3Jr5CW3jv

Table 2.4: Cycling conditions of polymerase chain reaction, showing , temperature required, the time of each cycle and total number of cycles per step

No.	Step	Temperature	Time	No. of Cycles
1	Initial Melting	94 °C	5 minutes	1 cycle
2	Melting	94 °C	2 minutes	35 cycles
	Annealing	59 °C	1 minute	
	Extension	72 °C	1 minute	
3	Final Extension	72 °C	10 minutes	1 cycle

2.5.STATISTICAL ANALYSIS

Allele and genotype frequencies were tested to determine the deviation of samples from Hardy-Weinberg equilibrium. For this analysis, Chi Square test for the goodness of fit with one degree of freedom was performed. The statistical software package SAS (version 9.2) was used to perform statistical analysis of the data. Logistic regression analysis was conducted on the data to compute Odds Ratio (OR) along with 95% confidence intervals (CI). The analysis was conditioned for variables like age, gender and ethnic group, which were found to be differently distributed among cases and controls.

CHAPTER 3

RESULTS

RESULTS

One hundred and fifty eight samples from leukemia patients were screened in the present study. These samples comprised 110 males and 46 females; with the male percentage higher as compared to females (gender of 2 patients was unknown). A total of 360 randomly selected healthy population was included in the current study. Of these, informed consents and data was obtained from142 healthy individuals, having no history of leukemia in their family. The data was collected via questionnaire and information regarding their health, education, lifestyle, ethnicity, age and eating habits were collected. About 100 Pathan control samples were provided by Khyber Medical College. Forty six Punjabi samples were kindly given by Dr. Nafees Ahmed. The remaining 177 control samples were provided by Dr. Sadia Rehman. The genotypes of all cases and control samples were entered accordingly.

3.1.DISTRIBUTION OF LEUKEMIA SAMPLES IN SUB-TYPES

Samples from the four major sub-types of leukemia were included. Among these 76 (48.10%) samples were of Acute Lymphoblastic leukemia (ALL), 49 (31.01%) samples of Acute Myeloid Leukemia (AML), 31 (19.62%) samples of Chronic Myeloid Leukemia (CML) and 2 (1.27%) samples of Chronic Lymphocytic Leukemia (CLL) (Fig 3.1).

3.2.DISTRIBUTION OF LEUKEMIA SAMPLES IN ETHNIC GROUPS

The samples belonged to different areas and hence were divided on the basis of ethnicity. Among these, 50% (79) of the patients were "Pathan". In rest of pateints, 29 (18.4%) were "Punjabi" and 10 (6.33%) samples were from "Northern Areas". The information for 40 (25.3%) patients regarding their ethnicity was unavailable (Fig 3.2).

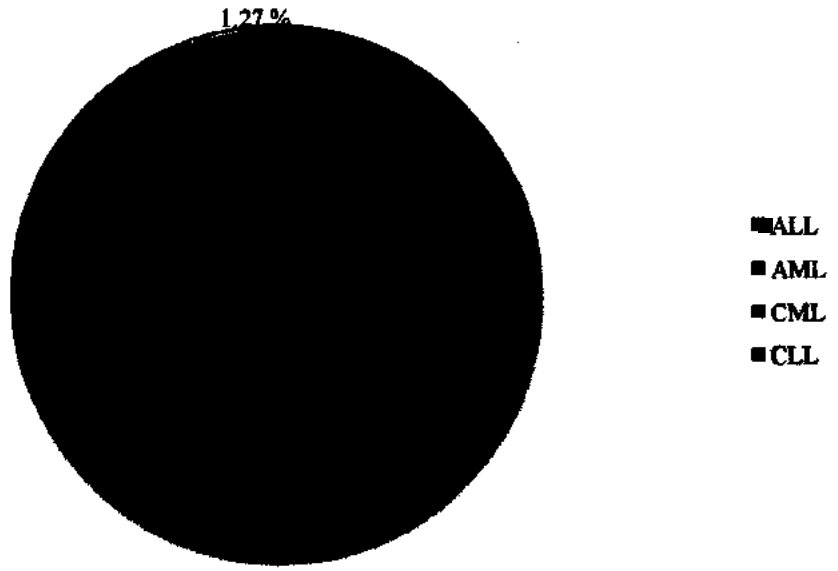

Figure 3.1: Percentage distribution of patients samples in sub-types of leukemia

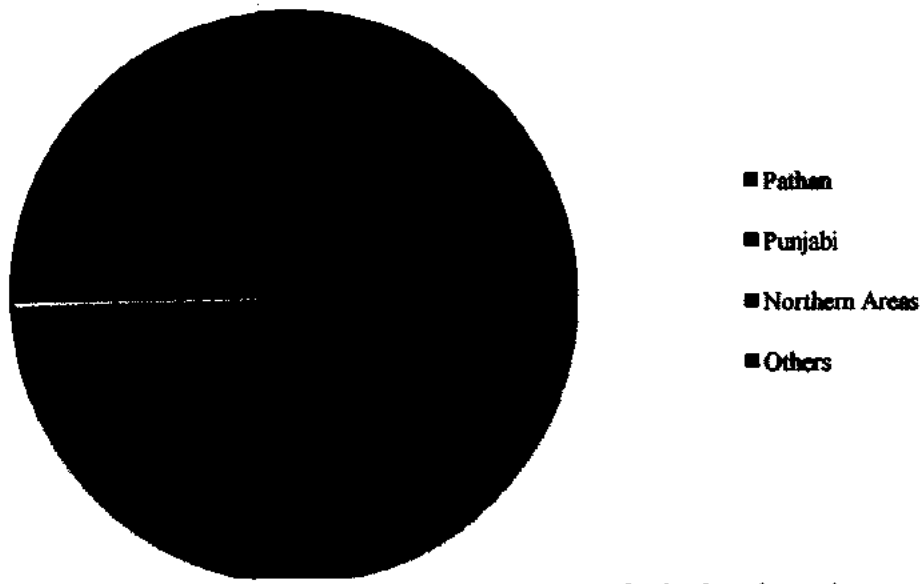

Figure 3.2: Percentage of different ethnic groups among the leukemia patients

3.3. DISTRIBUTION OF LEUKEMIA SAMPLES IN MALES AND FEMALES

The frequency of male patients was higher as compared to females. Among the 158 patients, 110 (69.6%) were males while 46 (29.11%) were females. Two (1.27%) samples were included with unconfirmed gender information (Fig 3.3).

3.4. GENDER BASED DISTRIBUTION OF LEUKEMIA SAMPLES OF DIFFERENT ETHNIC GROUPS

The distribution of males and females in ethnic groups was such that among "Pathan" samples, 63.29% (50) were males and 36.71% (29) females (Fig 3.4). There were 24 (83.33%) males and 5 females (16.67%) in "Punjabi" group (Fig 3.5), and 7 (70%) males and 3 (30%) females in "Northern areas" category (Fig 3.6). In the group with unknown ethnic background, there were 74.36% (29) males and 25.64% (10) females (Fig 3.7).

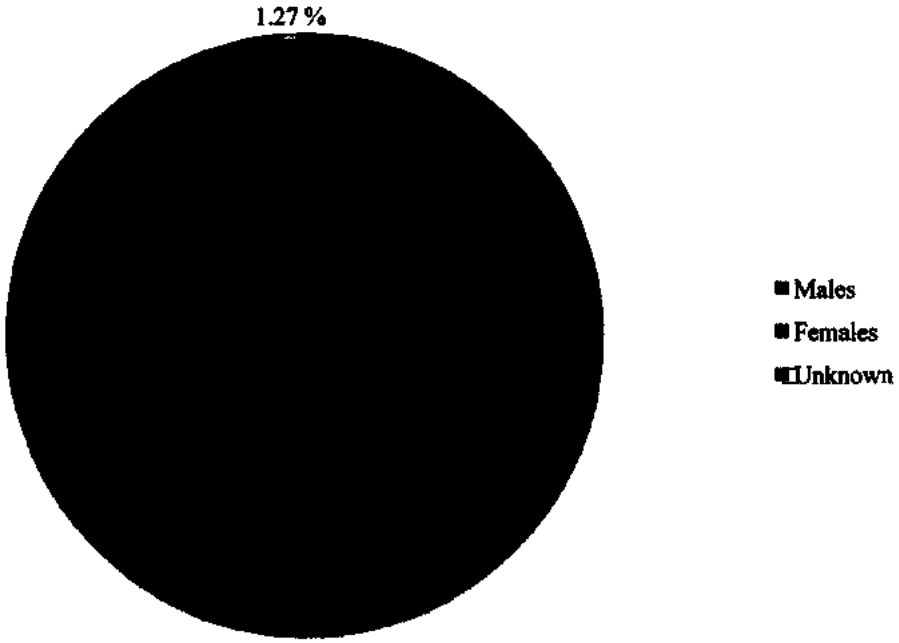

Figure 3 3: Males and females percentage in leukemia samples

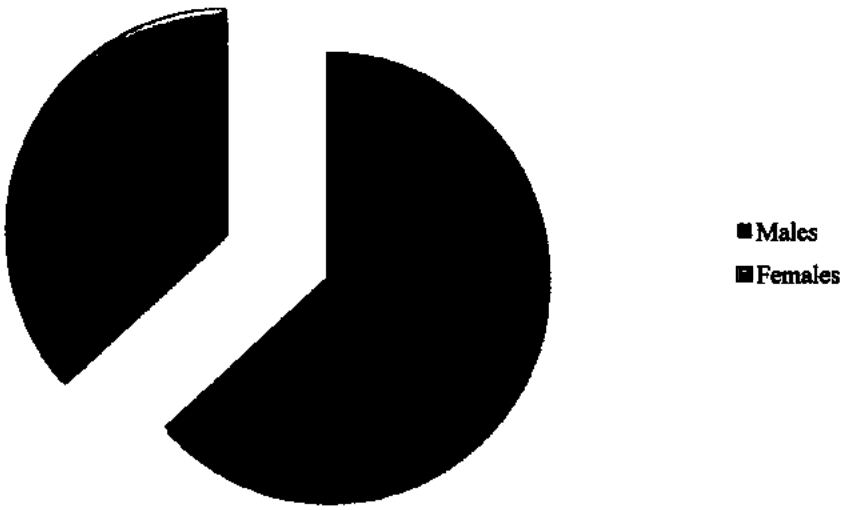

Figure 3.4: Males and females percentage in "Pathan" Leukemia Samples

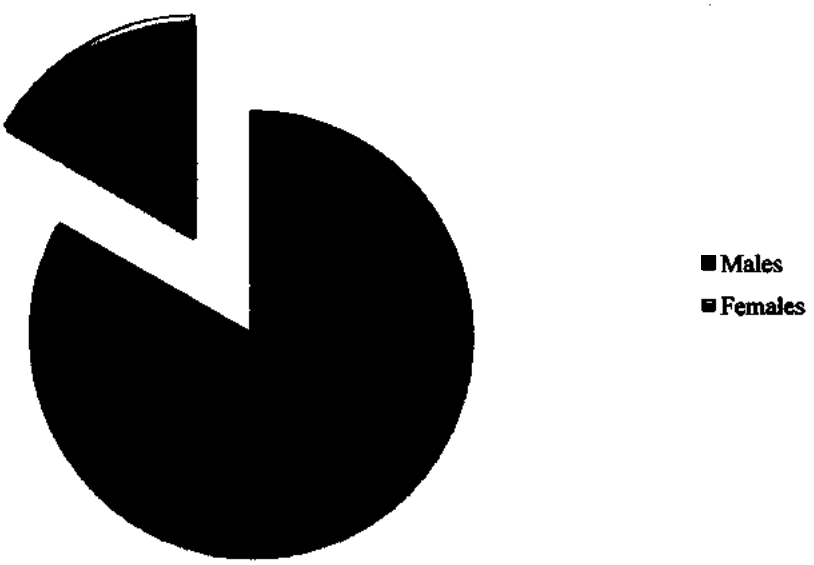

Figure 3.5: Males and females percentage in"Punjabi" Leukemia Samples

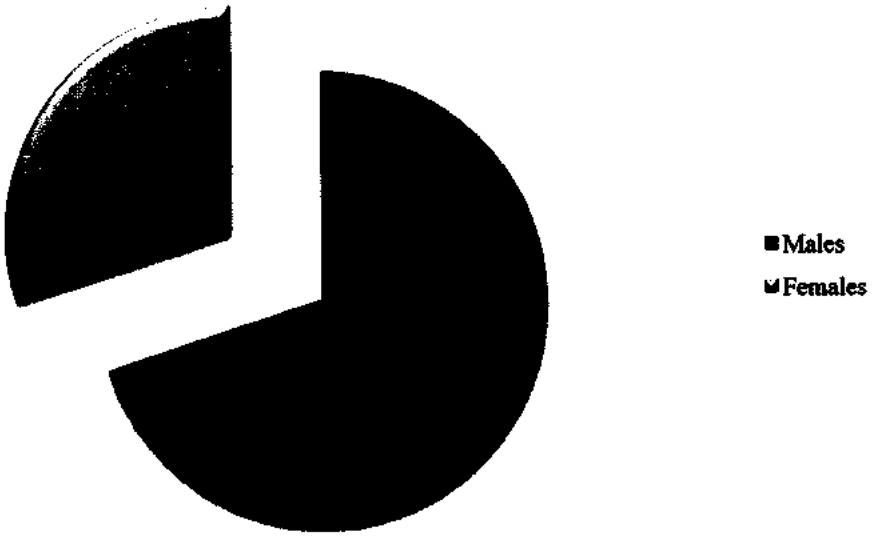

Figure 3.6: Males and females percentage in "Northern Areas" Leukemia Samples

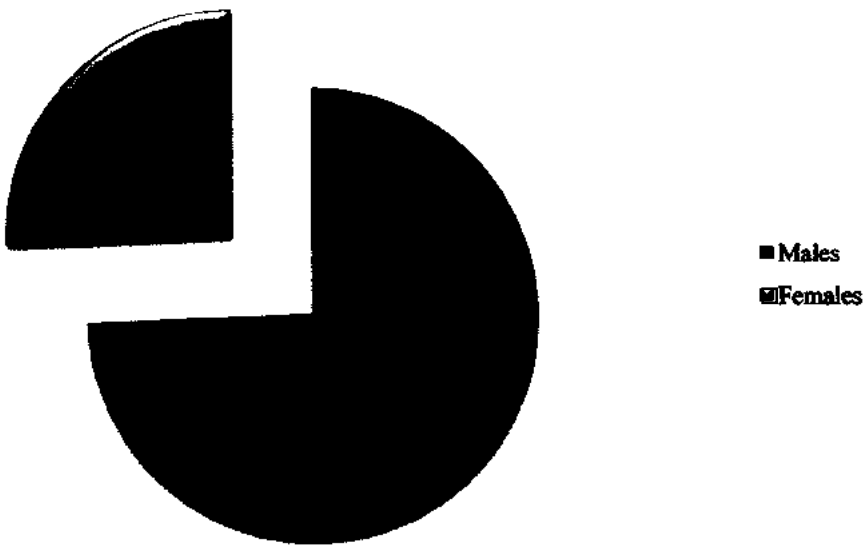

Figure 3.7: Males and Females percentage in Leukemia Samples with unknown ethnic group

3.5. DISTRIBUTION OF LEUKEMIA SAMPLES IN DIFFERENT AGE GROUPS

The ages of included patients ranged from a few months to more than 70 years old. They were, therefore, divided into three major groups; "Group A" included patients with ages from 0 – 15 years, "Group B" consisted of ages ranging from 16 – 30 years and "Group C" contained patients aged Above 30 years. An extra group namely "Group N" was made, containing the patients whose age was unknown. According to this distribution of age, there were 48 (30.38%) patients with ages between 0 – 15 years, 51 (32.28%) patients between 16 – 30 years and 48 (30.38%) patients above 30 years old. The ages of 11 (6.96%) patients were unknown and included in Group N (Fig 3.8).

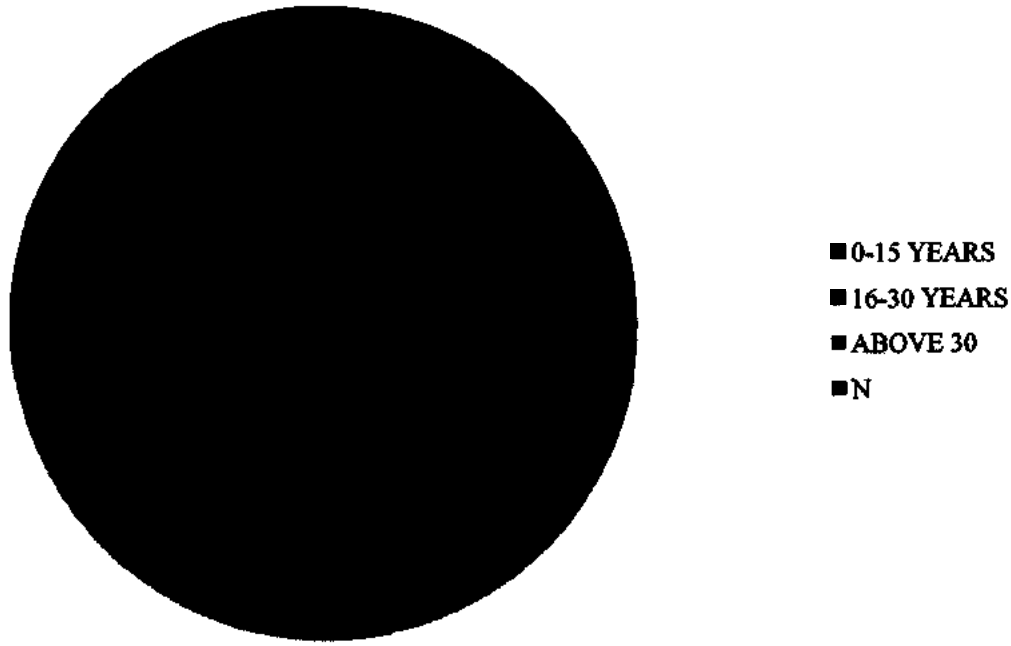

Figure 3.8: Percentage distribution of leukemia patient samples with respect to different age groups

3.6.GENOTYPING OF LEUKEMIA SAMPLES

The 158 included leukemia samples were processed for PCR optimized with conditions for genotyping. DNA extracted from all the collected leukemia samples were genotyped using the multiplex PCR protocol followed by Sherif *et al.*, 1996. Samples were screened for all possible genotypes. The presence of GSTT1 and GSTM1 were confirmed by 480 bp and 215 bp band on agarose gel, respectively. A 315 bp band showing the internal control was always visible, confirming the amplification (Fig 3.9).

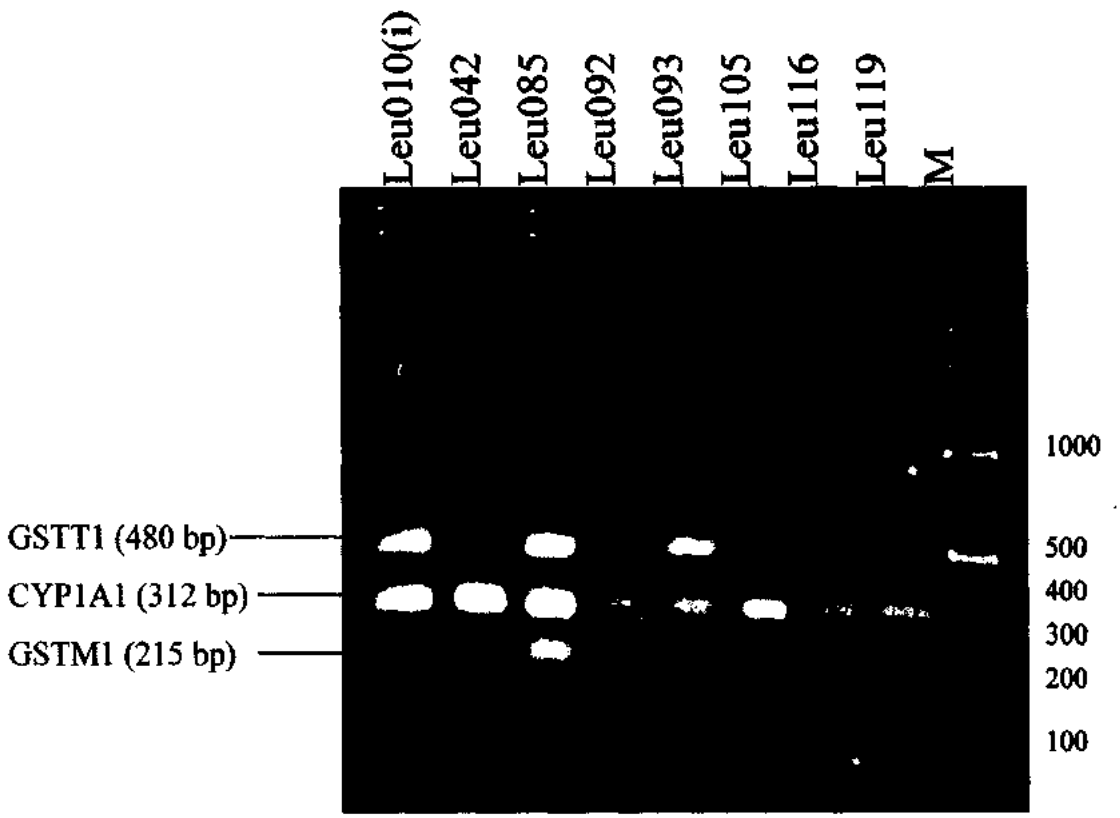

Figure 3.9: Agarose gel electrophoresis of the PCR amplicons. (From left to right) Lane 1-Leu010, Lane 2-Leu042, Lane 3-Leu085, Lane 4-Leu092, Lane 5-Leu093, Lane 6-leo105, Lane 7-116, Lane 8-Leu119, Lane 9-M (Ladder). The bands of 480 bp representing GSTT1, 215 bp confirming GSTM1 and 312 bp for CYP1A1 Internal Control are shown on the left. The band sizes of ladder are depicted on the right.

3.7.ASSOCIATION OF GSTT1 WITH LEUKEMIA

3.7.1. Percentage of GSTT1 in Leukemia Samples

Out of the 158 leukemia samples being studied, 116 (74.8%) cases harbored GSTT1 wildwhile 39 (25.1%) cases had GSTT1 null. A total of 360 controls were also included in the study and screened for the wild and null GSTT1. Among them, 265 (73.6%) controls contained GSTT1 wild while 95 (26.4%) controls showed GSTT1 null (Fig 3.10).

3.7.2. Percentage of GSTT1 in Sub-Types of Leukemia

On the basis of leukemia sub-types, 52 (68.42%) patients with Acute Lymphoblastic Leukemia (ALL) had GSTT1 wild and 24 (31.58%) ALL cases had GSTT1 null. Among Acute Myeloid Leukemia (AML) patients, 36 (76.60%) possessed the GSTT1 wild while 11 (23.40%) got the GSTT1 null. Twenty six (86.67%) patients with Chronic Myeloid Leukemia (CML) patients contained GSTT1 wild while 4 (13.3%) had GSTT1 null. There were only 2 samples of Chronic Lymphocytic Leukemia (CLL), both of which possessed GSTT1 wild. They were omitted from final analysis owing to the small number of samples (Fig 3.11).

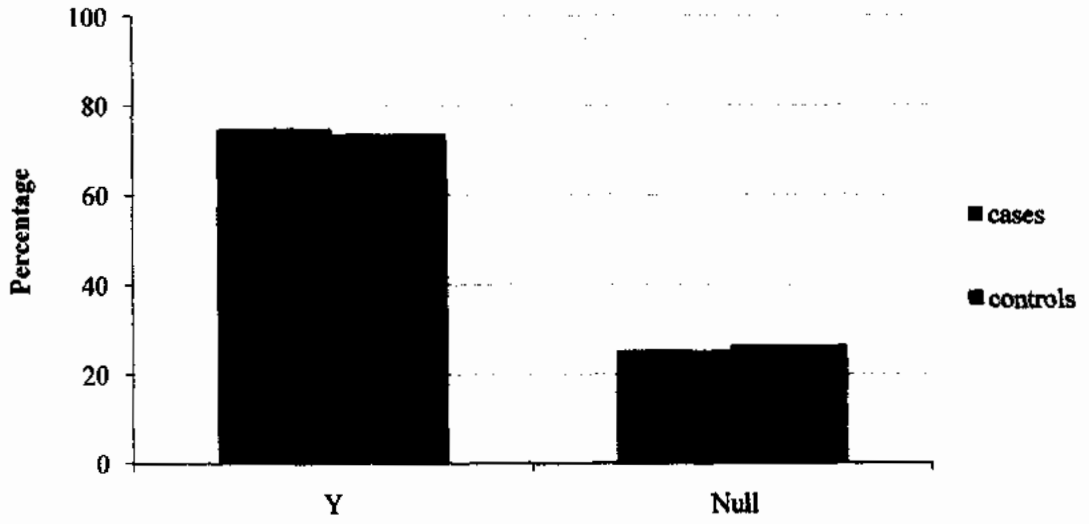

Figure 3.10: Percentage of presence and absence of GSTT1 in cases and control samples

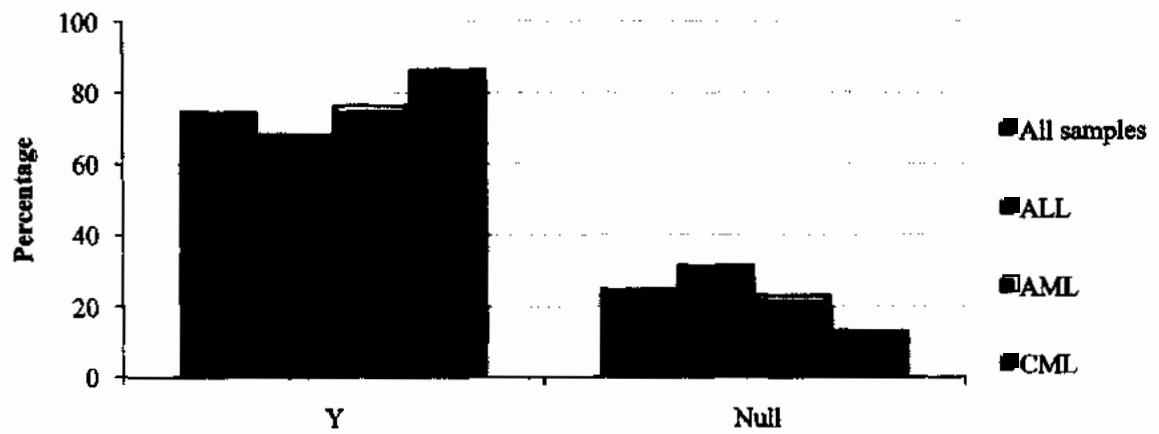

Figure 3.11: Percentage of the presence and absence of GSTT1 in all leukemia samples and the four sub-types of leukemia

3.7.3. Percentage of GSTT1 in Leukemia Samples and Controls with Different Ethnic Groups

The leukemia samples were also divided on the basis of the ethnic origin of individuals. Among the 158 leukemia patient samples, 62 (79.49%) "Pathan" cases had GSTT1 wild and 16 (20.51%) had GSTT1 null. A total of 22 (75.86%) "Punjabi" samples contained GSTT1 wild while 7 (24.14%) had GSTT1 null. Among "Northern areas" samples, 8 (80%) cases possessed GSTT1 wild, whereas GSTT1 null was found in 2 (20%) cases. Amongst the patients with unknown ethnic origin, 64.10% (25) individuals possessed GSTT1 wild while 35.90% (14) lacked the gene (Fig 3.12).

A total of 360 control samples were included and sorted according to their ethnic background. Of these, 147 (73.13%) "Pathan", 116 (77.33%) "Punjabi" and 3 (100%) "Northern Areas" control samples had GSTT1 wild. On contrary, 54 (26.87%) "Pathan" control samples and 34 (22.67%) "Punjabi" samples had GSTT1 null. None of the samples from "Northern areas" had GSTT1 absent in them. Only 5 samples were included with unconfirmed ethnic background and among them GSTT1 wild was present in 2 samples, while GSTT1 null was found in 3 individuals (Fig 3.13).

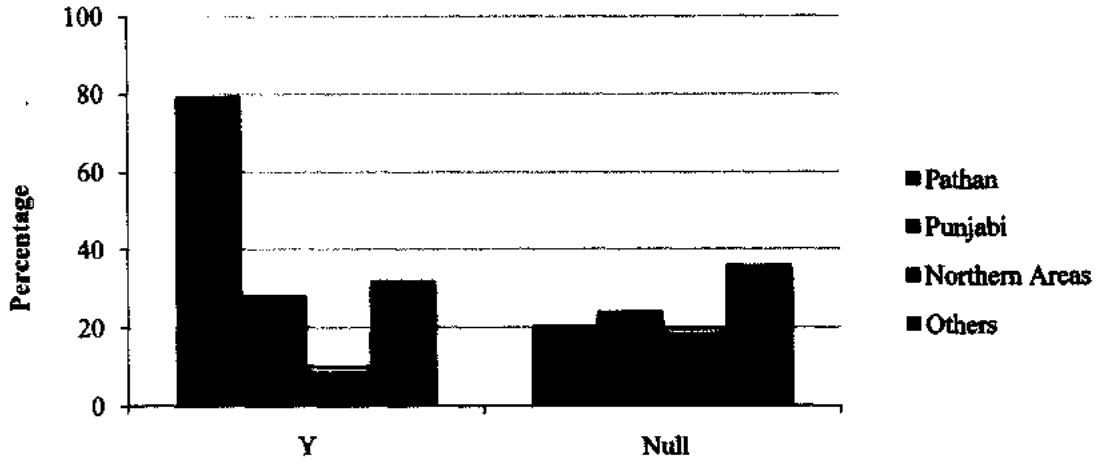

Figure 3.112: Percentage of the presence and absence of GSTT1 in patients with different ethnic backgrounds

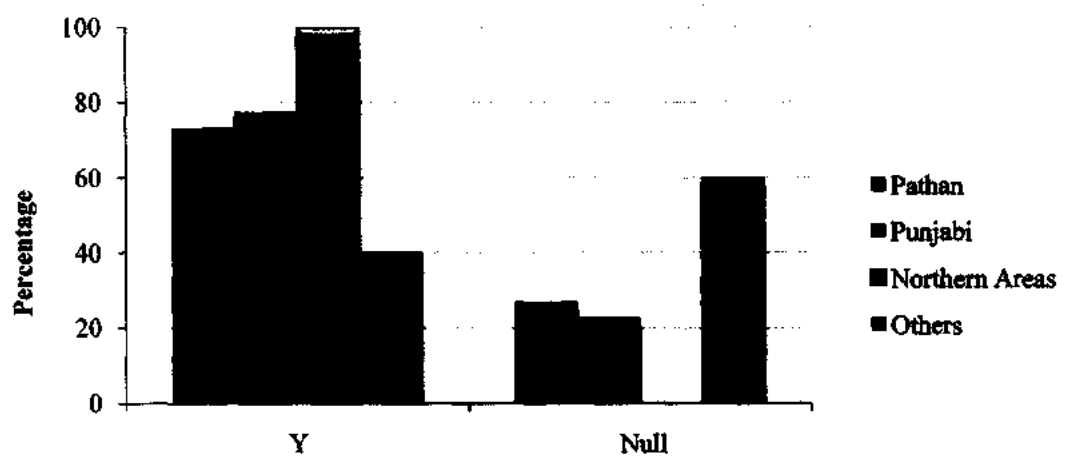

Figure 3.13: Percentage of the presence and absence of GSTT1 in controls with different ethnic backgrounds

3.8. CHI SQUARE ANALYSIS FOR GSTT1 IN LEUKEMIA SAMPLES AND ITS SUB-TYPES

In order to test the samples for Hardy-Weinberg equilibrium (HWE), the Chi-Square analysis was performed for goodness of fit with a degree of freedom of 1. The T-critical value for Chi-Square was 3.84. Results were considered significant with a probability value (p-value) of ≤ 0.05 The Chi Square analysis for GSTT1 in all leukemia samples showed the values below the critical value of 3.84 and a higher p value was observed. The genotype distribution in this case was consistent with Hardy-Weinberg Equilibrium and did not show any deviation (Fig 3.14).

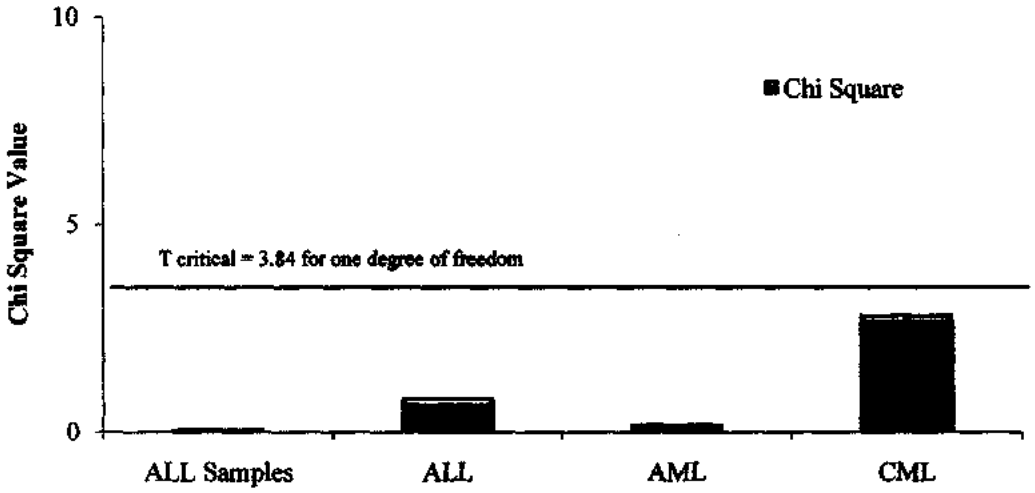

Figure 3.14: Analysis of the Chi-Square values for GSTT1 in all leukemia samples and the sub-types of leukemia. The Chi-Square values were below the T-critical of 3.84, and did not show deviation from HWE.

3.9.ASSOCIATION OF GSTM1 WITH LEUKEMIA

3.9.1. Percentage of GSTM1 in Leukemia Samples

The difference between the percentage of GSTM1 wild and null was much reduced. Among the 158 samples of leukemia studied, 46.45% (72) cases harbored GSTM1 wild while about 53.55% (83) cases had GSTM1 null. Three hundred and sixty healthy individuals were studied and screened for the GSTM1 gene. A similar trend was found among the control samples. Of the 360 control samples, 48.61% (175) controls contained GSTM1 wild while 51.39% (185) controls showed the deletion or null of GSTM1 (Fig 3.15).

3.9.2. Percentage of GSTM1 in Subtypes of Leukemia

The leukemia samples were characterized on the basis of sub-types of leukemia (ALL, AML, CML). According to this division, 37 (48.68%) patients with Acute Lymphoblastic Leukemia (ALL) had GSTM1 wild and 39 (51.32%) cases of ALL had GSTM1 null. Twenty (42.55%) patients with Acute Myeloid Leukemia (AML) possessed the GSTM1 wild and 27 (57.45%) had the GSTM1 null. Among the Chronic Myeloid Leukemia (CML) patients 14 (46.67%) individuals contained GSTM1 wild while 16 (53.33%) had GSTM1 null. There were only 2 samples of Chronic Lymphocytic Leukemia (CLL), of which one patient possessed GSTM1 wild and the other one contained GSTM1 null. CLL samples were omitted from final analysis owing to their small number (Fig 3.16).

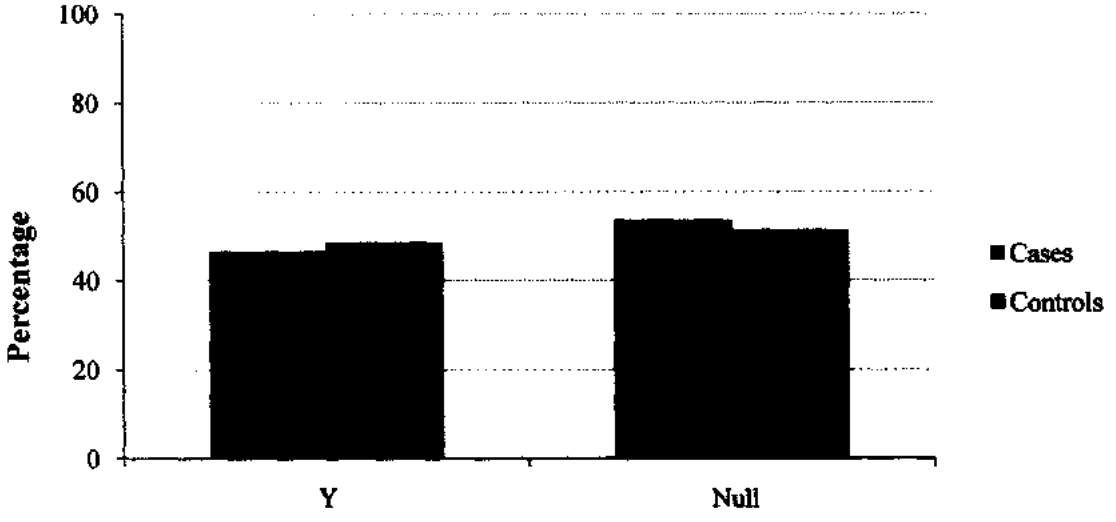

Figure 3.15: Percentage of presence and absence of GSTM1 in cases and control samples

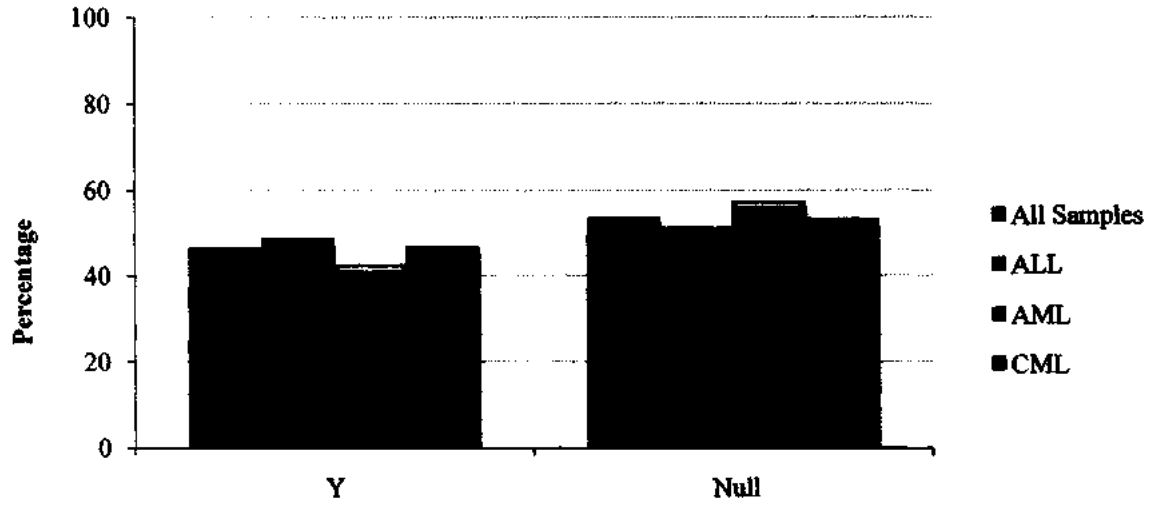

Figure 3.16: Percentage of the presence and absence of GSTM1 in all leukemia samples and the four sub-types of leukemia

3.10. PERCENTAGE OF GSTM1 IN LEUKEMIA SAMPLES AND CONTROLS WITH DIFFERENT ETHNIC GROUPS

The patients belonged to different ethnic backgrounds and were distributed accordingly. Out of the screened leukemia patient samples, 39 (50%) "Pathan" cases had GSTM1 wild and 39 (50%) had GSTM1 null. Among "Punjabi" samples, 10 (34.48%) individuals contained GSTM1 wild, while 19 (65.52%) patients showed GSTM1 null. Three (30%) patients from "Northern Area" cases possessed the GSTM1 wild genotype, whereas GSTM1 null was found in 7 (70%) cases. Amongst the patients with unknown ethnic origin, 51.28% (20) individuals possessed GSTM1 wild while 48.72% (19) had GSTM1 null (Fig 3.17).

A total of 360 control samples were included and sorted according to their ethnic background. The GSTM1 wild was present in 98 (48.76%) "Pathan", 70 (51.24%) "Punjabi" and 2 (66.67%) "Northern area" samples. A total of 103 (51.24%) "Pathan" control individuals, 80 (53.33%) Punjabi and 1 (33.33%) northern areas samples showed the GSTM1 null. Five samples with no information regarding their ethnic background were included. Two out of five patients (40 %) possessed GSTM1 wild while the rest of 3 individuals (60%) showed GSTM1 null (Fig 3.18).

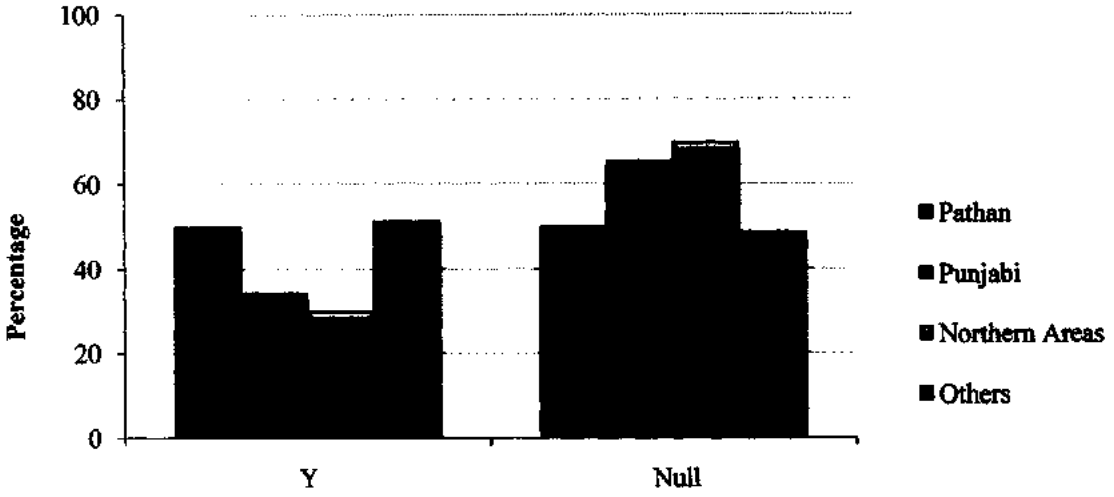

Figure 3.17: Percentage of the presence and absence of GSTM1 in cases with different ethnic backgrounds (Pathan, Punjabi, Northern Areas and others)

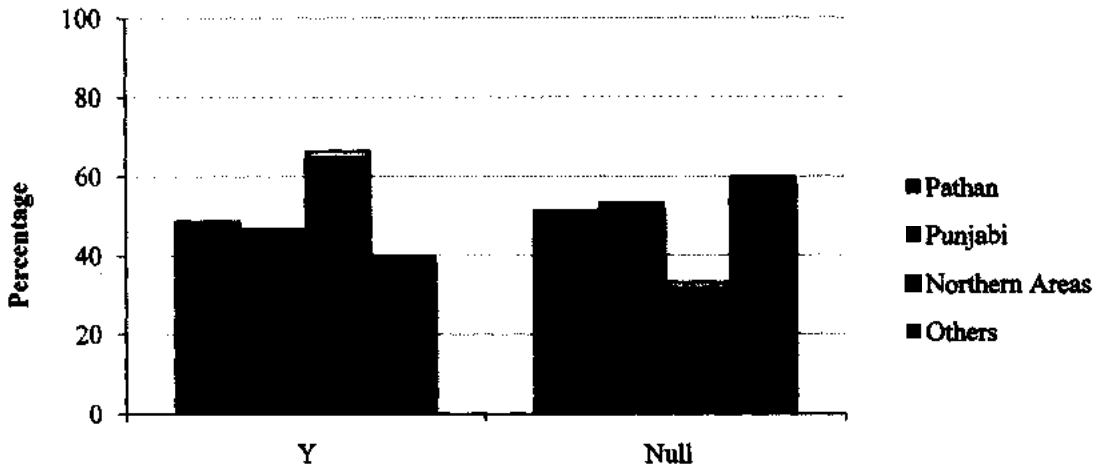

Figure 3.18: Percentage of the presence and absence of GSTM1 in controls with different ethnic backgrounds (Pathan, Punjabi, Northern Areas and others)

3.11. CHI SQUARE ANALYSIS FOR GSTM1 IN LEUKEMIA SAMPLES

The Chi-Square analysis was performed for goodness of fit with one degree of freedom, to test the samples for deviation from Hardy-Weinberg equilibrium. In case of GSTM1, the Chi Square values for all leukemia samples were lower than the T-critical value of 3.84 and showed higher probability values. For GSTM1, Acute Myeloid Leukemia (AML) showed a comparatively higher Chi-Square value. Therefore, the distribution of genotypes showed no deviation from Hardy-Weinberg Equilibrium (Fig 3.19).

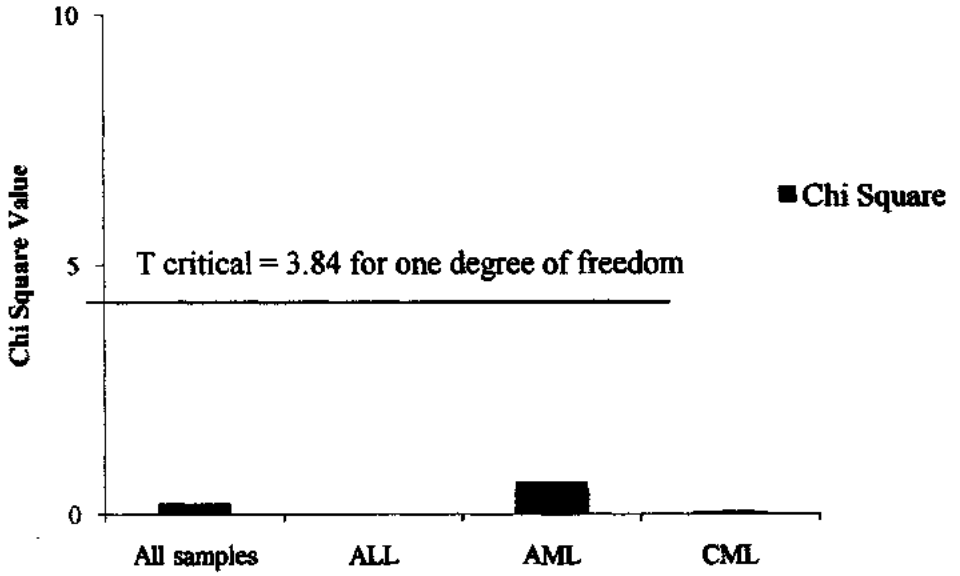

Figure 3.19: Analysis of the Chi-Square values for GSTM1 in all Leukemia samples and its subtypes. The Chi-Square values were below the T-critical value of 3.84 showing no deviation from HWE.

3.12. ASSOCIATION OF GSTT1 POLYMORPHISMS WITH LEUKEMIA

3.12.1. Association of GSTT1 with all leukemia samples and its sub-types

In order to study the association of GSTT1 with leukemia, logistic regression was performed. The relative risk was calculated by Odds Ratio along with 95% Confidence Interval (CI). The analysis was done by considering the variables like gender, ethnic group and leukemia sub-type. The Odds Ratio calculated for all leukemia samples and its subtypes showed no significance in case of GSTT1 null. The presence of GSTT1 wild was taken as reference equal to 1. The Odds Ratio for Chronic Myeloid Leukemia (CML) was highest (OR = 2.33; 95% CI = 0.79-6.85) among the other sub-types of leukemia, showing the highest risk linked with GSTT1 null. A considerably increased risk was associated with GSTT1 null, when observed in all leukemia samples (OR = 1.07; 95% CI = 0.69-1.64), Acute Myeloid Leukemia (AML) samples (OR = 1.17; 95% CI = 0.57-2.39) and Acute Lymphoblastic Leukemia (ALL) samples (OR = 1.15; 95% CI = 0.81-1.65). Due to the small sample size of Chronic Lymphocytic Leukemia (CLL), they were not included in further statistical analysis (Fig 3.20).

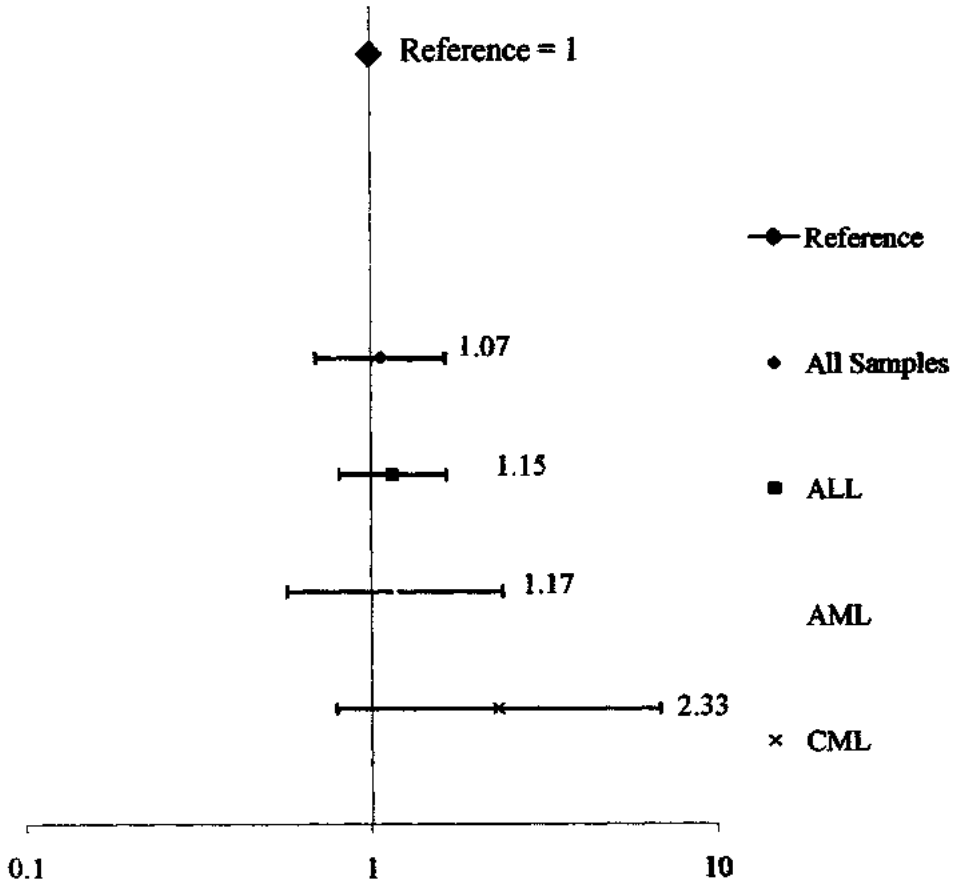

Figure 3.20: Association of GSTT1 with all Leukemia samples and its sub-types; ALL, AML, CML. The presence of GSTT1 was taken as reference.

3.12.2. Association of GSTT1 with Leukemia Patients from Different Ethnic Backgrounds

The odds ratio was calculated for GSTT1 in samples with different ethnic backgrounds. There was no significance observed in these ethnic groups. The GSTT1 wild was taken as a reference equal to 1. In "Pathan" ethnic group, a considerable amount of risk was found to be associated with GSTT1 null (OR = 1.42; 95% CI = 0.76-2.68). In "Punjabi" samples, however, a reduced risk was observed and showed protection (OR = 0.92; 95% CI = 0.36-2.34) with GSTT1 null. The sample size of "Northern Areas" was negligible, so it was removed from the final analysis. The group containing samples with unknown ethnic backgrounds showed the highest odds ratio (OR = 2.68; 95% CI = 0.39-18) and a higher risk associated with GSTT1 null (Fig 3.21).

3.12.3. Association of GSTT1 with Gender

The Odds Ratio calculation was also performed for "Pathan" and "Punjabi" males and females, because of the higher number of samples in these groups. The other groups were ignored, since there were small sample sizes and analysis could not be done. The presence of GSTT1 (GSTT1 wild) was taken as reference equal to 1. In case of "Pathan" samples, significance was found among the males and a high protection (OR = 0.543; 95% CI = 0.319-0.926; P-value = 0.024) was observed to be associated with GSTT1 null. "Pathan" females, however depicted a considerable non-significant protection with GSTT1 null. Among the "Punjabi" samples, there was no significance observed with GSTT1 null. However, in "Punjabi" males, there was a elevated risk (OR = 1.35; 95% CI = 0.45-4.08) associated with GSTT1 null, whereas, a significant protection (OR = 0.35; 95% CI = 0.05-2.34) was observed in "Punjabi" Females (Fig 3.22).

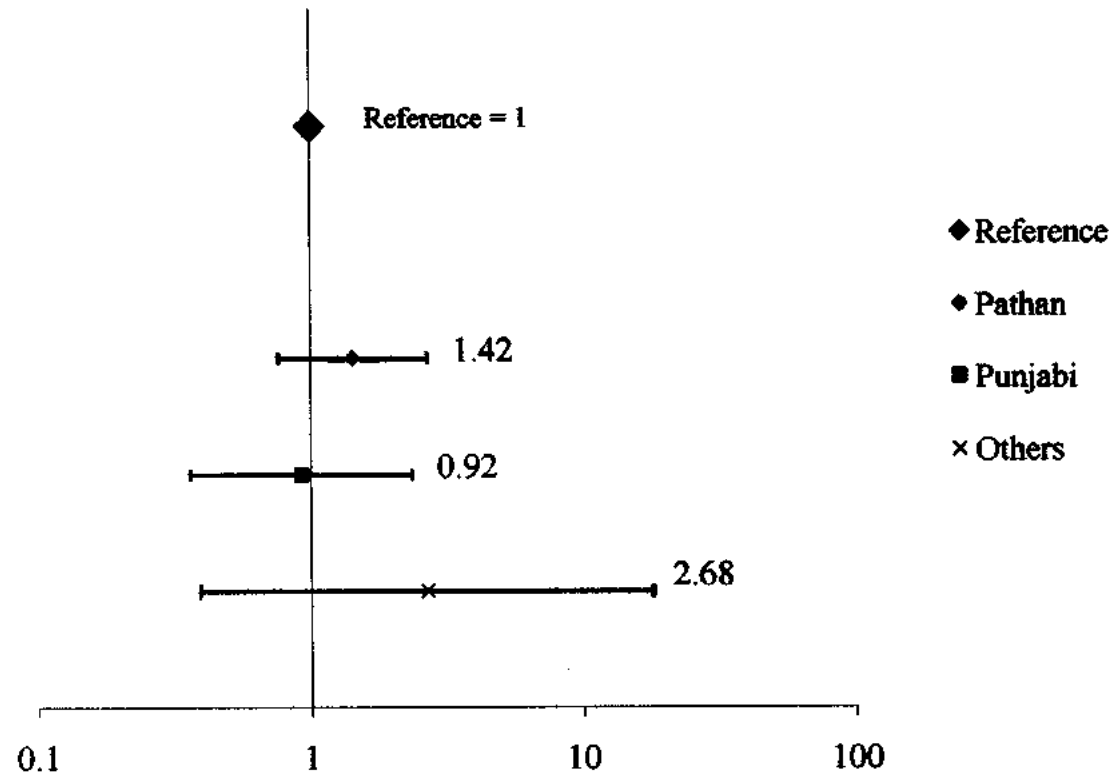

Figure 3.21: Association of GSTT1 with different ethnic groups including Pathan, Punjabi and Others. The presence of GSTT1 was taken as reference equal to 1.

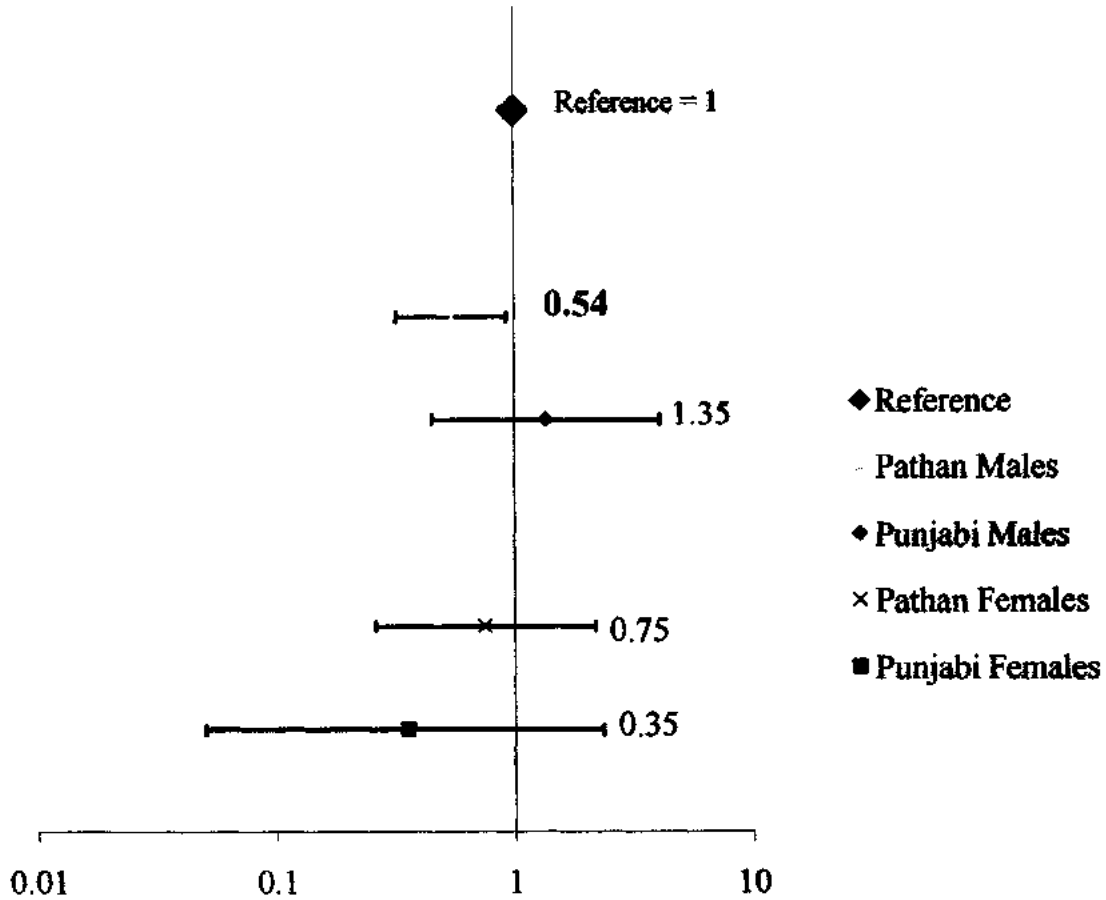

Figure 3.22: Association of GSTT1 in Pathan and Punjabi males and females. Presence of GSTT1 was taken as reference. Significant p-value is shown in red.

3.13. ASSOCIATION OF GSTM1 WITH LEUKEMIA

3.13.1. Association of GSTM1 with all leukemia samples and its sub-types

The association of GSTM1 null with leukemia was also studied by performing logistic regression. The Odds ratios were determined along with 95 % Confidence Interval (CI). The GSTM1 wild was taken as reference equal to 1 In this case, no significance was found with GSTM1 null in all leukemia samples and the three sub-types; ALL, AML, CML. The analysis for CLL wasn't done since there was a small sample size. The figure below depicts the trends for Odds Ratio and shows a considerable amount of protection with GSTM1 null (Fig 3.23).

3.13.2. Association of GSTM1 with leukemia patients from different ethnic backgrounds

The Odds Ratios were calculated to determine the association of GSTM1 with respect to different ethnic groups. The GSTM1 wild was taken as reference equal to 1. Among these, the "Pathan" samples showed significance and a considerable amount of protection (OR = 0.37; 95% CI = 0.21-0.63; P-value = 0.0003) was conferred with GSTM1 null. Punjabi individuals also depicted protection (OR = 0.60; 95% CI = 0.26-1.38) with GSTM1 null, although no significance was found. The Northern Areas individuals showed protection (OR = 0.21; 95% CI = 0.01-3.37) with GSTM1 null while persons with no information regarding their ethnic backgrounds showed a considerable risk (OR = 1.58; 95% CI = 0.42-10.52) associated with GSTM1 null (Fig 3.24).

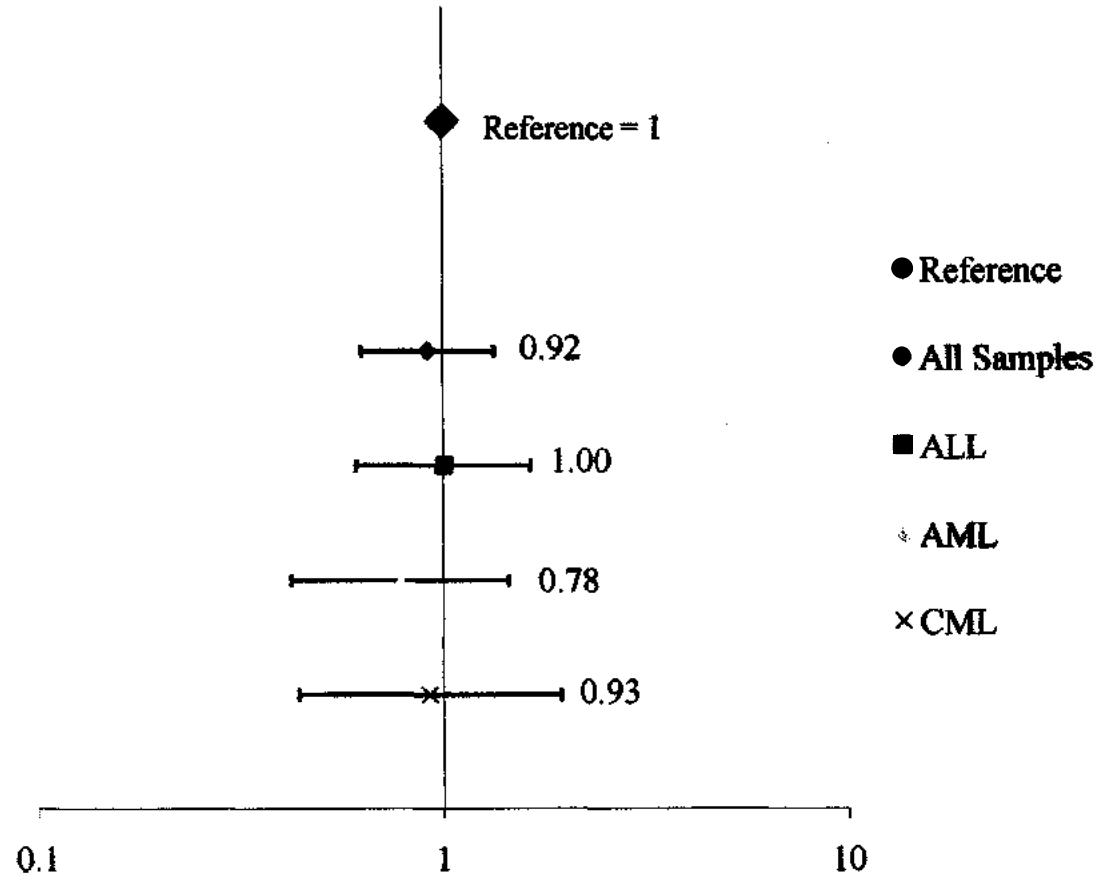

Figure 3.23: Association of GSTM1 with all Leukemia samples and its sub-types; ALL, AML, CML. The presence of GSTM1 was taken as reference equal to 1.

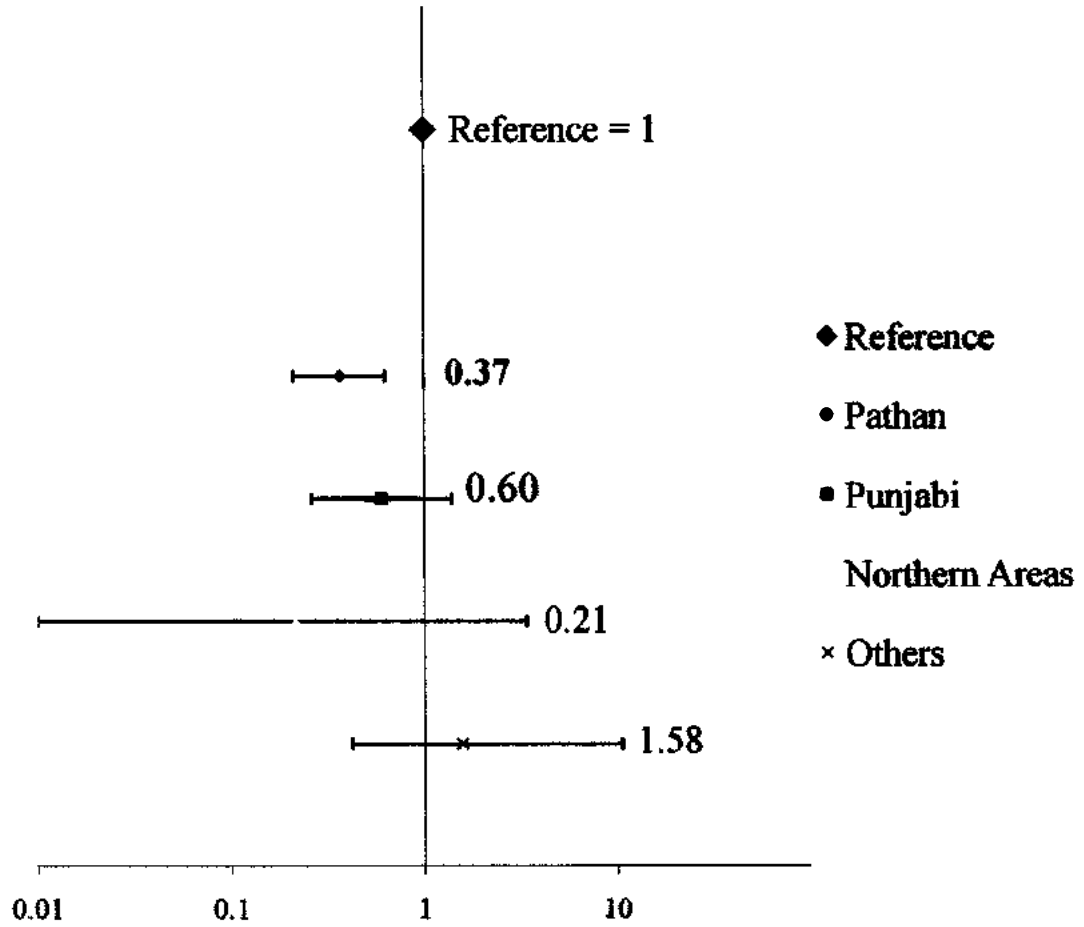

Figure 3.24: Association of GSTM1 in different ethnic groups including, Pathan, Punjabi, Northern Areas and Others. The presence of GSTM1 was taken as reference equal to 1.

3.13.3. Association of GSTM1 with Gender

Among the different ethnic groups, "Pathan" and "Punjabi" were selected for gender based analysis. The odds ratio was calculated for males and females of these two ethnic groups. The GSTM1 wild was taken as reference equal to 1. In case of "Pathan" samples, the males showed risk (OR = 1.27; 95% CI = 0.62-2.61) with GSTM1 null, while the females depicted to be protected with GSTM1 null (OR = 0.70; 95% CI = 0.26-1.92), but association was not significant. "Punjabi" males (OR = 0.73; 95% CI = 0.29-1.87) and females (OR = 0.25; 95% CI = 0.03-2.35) also showed no significance but there was protection with GSTM1 null (Fig 3.25).

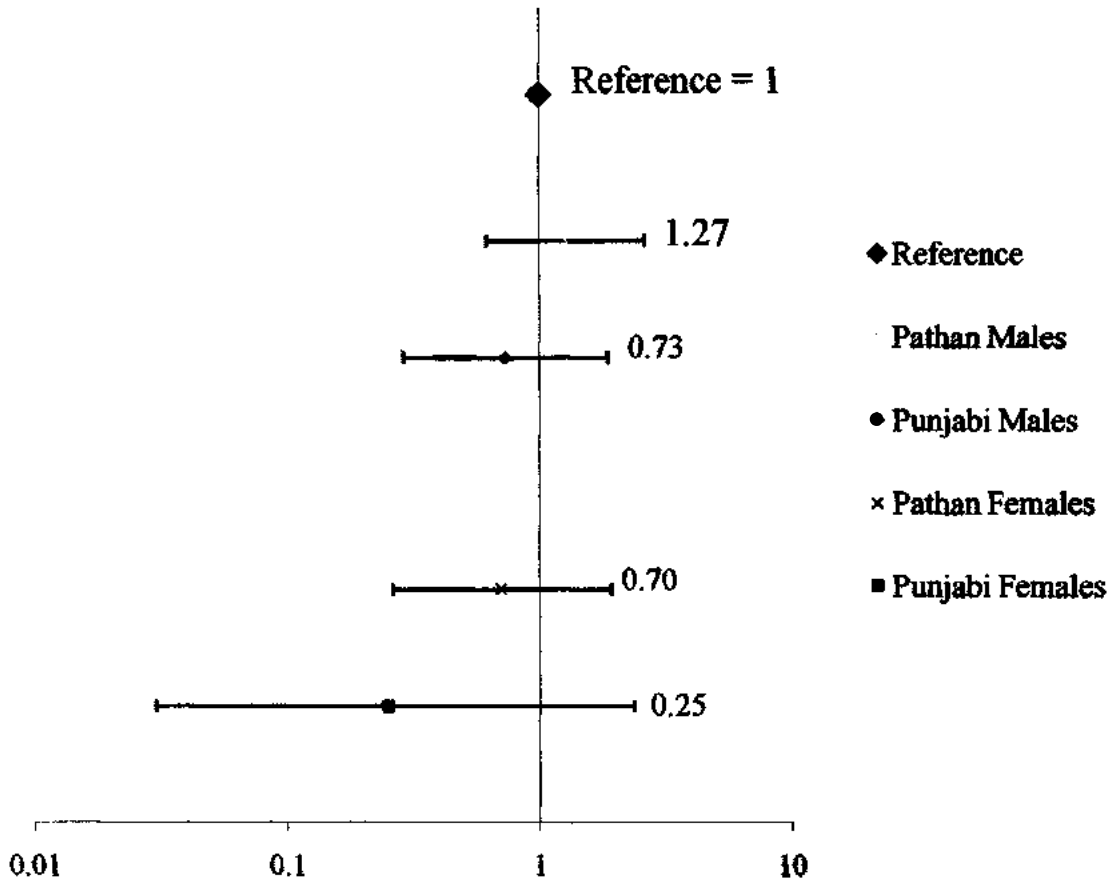

Figure 3.25: Association of GSTM1 with Pathan and Punjabi males and females.
The presence of GSTM1 was taken as reference = 1.

3.14. ASSOCIATION OF GSTT1-GSTM1 COMBINATION WITH LEUKEMIA

The individuals were characterized as those with null alleles for both genes (GSTT1 N/N, GSTM1 N/N), individuals possessing one gene and lacking other (GSTT1 Y/Y, GSTM1 N/N or GSTT1 N/N, GSTM1 Y/Y) or individuals possessing both genes (GSTT1 Y/Y, GSTM1 Y/Y). Among 158 screened leukemia samples, 59 (39.06%) individuals possessed both the genes (Y/Y). About 70 patients had heterozygous (Y/N+N/Y) condition lacking one of the two genes and containing other, with 57 (36.77%) patients Y/N and 13 (8.38%) N/Y. Among the studied samples, 26 patients (16.77%) were having a null genotype or N/N. A total of three samples gave no results (Fig 3.26).

Among 158 leukemia samples, the combined effect of both GSTT1 and GSTM1 genes was analyzed statistically and Odds ratio was calculated with 95% Confidence Interval. In this case, no significance was shown in overall samples. The individuals containing both genes (homozygous YY) were taken as the reference point of 1. The individuals showing a heterozygous condition (Y/N and N/Y), with one gene present and lacking the other, depicted a considerable increased risk associated with it. The samples with a null genotype, however, showed significant protection (Fig 3.27).

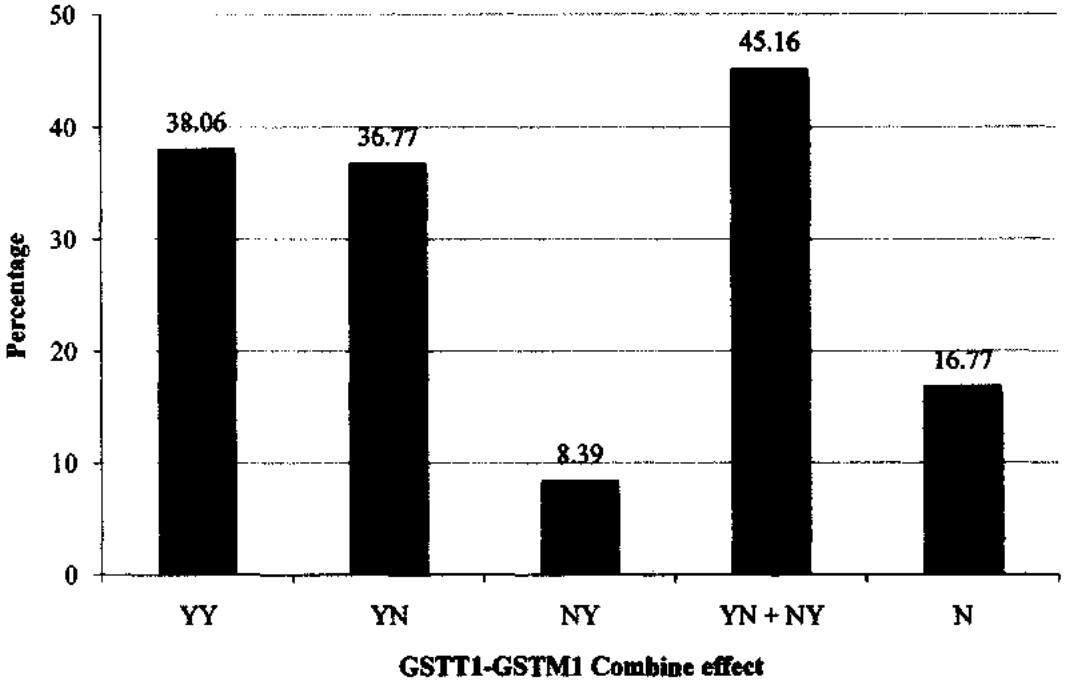

Figure 3.26: Percentage of combined effect of GSTT1-GSTM1 genotypes in all leukemia patient samples

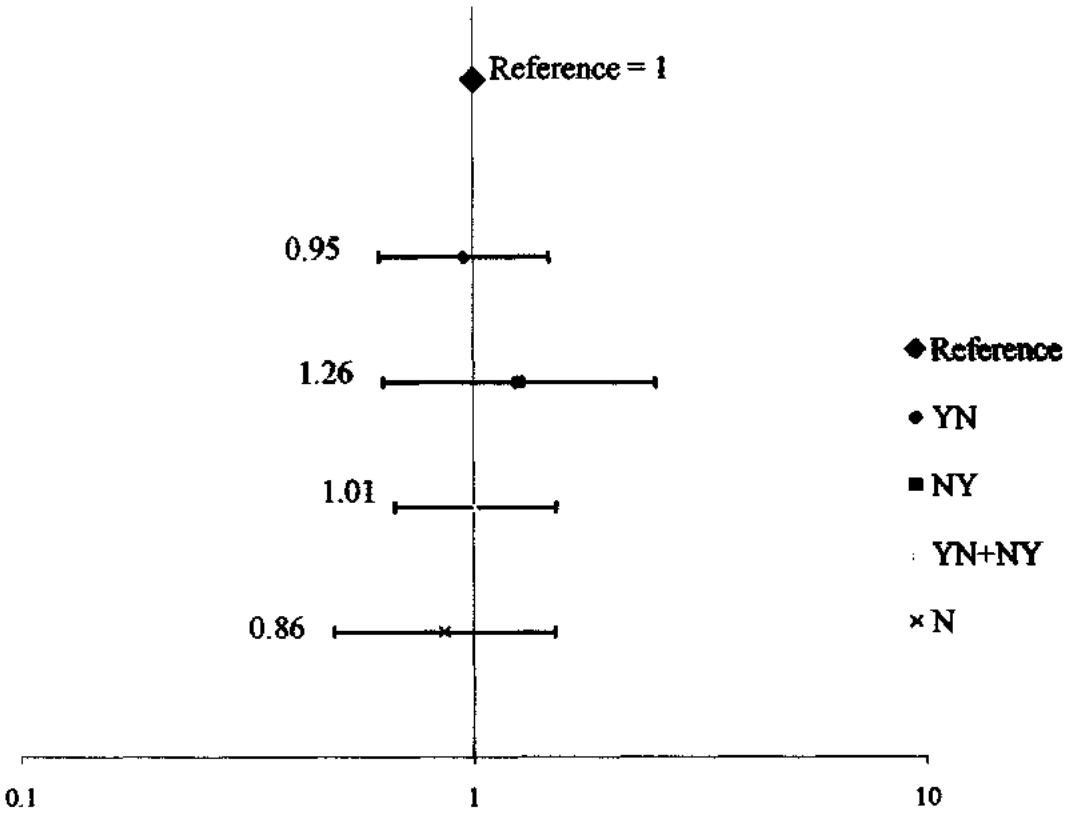

Figure 3.27: Association of combination (GSTT1-GSTM1) in all leukemia samples, considering the different genotypes. The presence of both GSTT1 and GSTM1 was taken as reference.

3.15. TRENDS OF GSTT1-GSTM1 COMBINATION EFFECT IN SUB-TYPES OF LEUKEMIA

The combination effect of GSTT1-GSTM1 was also calculated in different sub-types of leukemia namely ALL, AML and CML and the trends were observed in them separately. None of these results showed any significance statistically, as they had higher probability values and a Chi-Square value below the critical limit of 3.84. The Odds ratio of Acute Lymphoblastic Leukemia (ALL) showed a considerably increased protection (Fig 3.28). In Acute Myeloid Leukemia (AML), however, there was comparatively increase in risk of AML associated with the simultaneous deletion of both GSTT1 and GSTM1 (GSTT1-GSTM1 null). The maximum risk (OR = 2.64; 95% CI = 0.59-11.82) was associated with individuals possessing GSTT1 wild and harboring GSTM1 null (Fig 3.29). In case of Chronic Myeloid Leukemia (CML), there was a significantly higher risk. Among these, the individuals harboring a heterozygous condition (Y/N and N/Y) showed the highest risk associated with the combination effect of GSTT1 and GSTM1 (Fig 3.30).

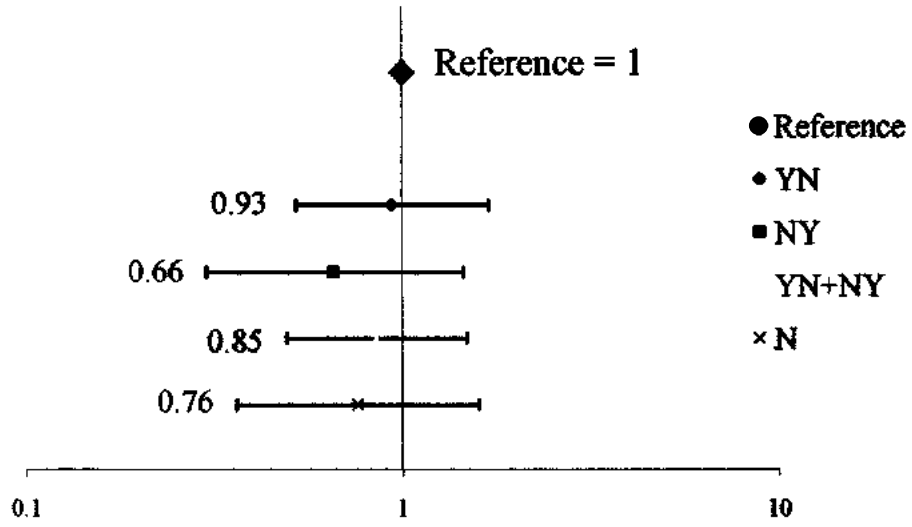

Figure 3.28: Association of combination (GSTT1-GSTM1) in Acute Lymphoblastic Leukemia (ALL). The presence of GSTT1-GSTM1 wild was taken as reference

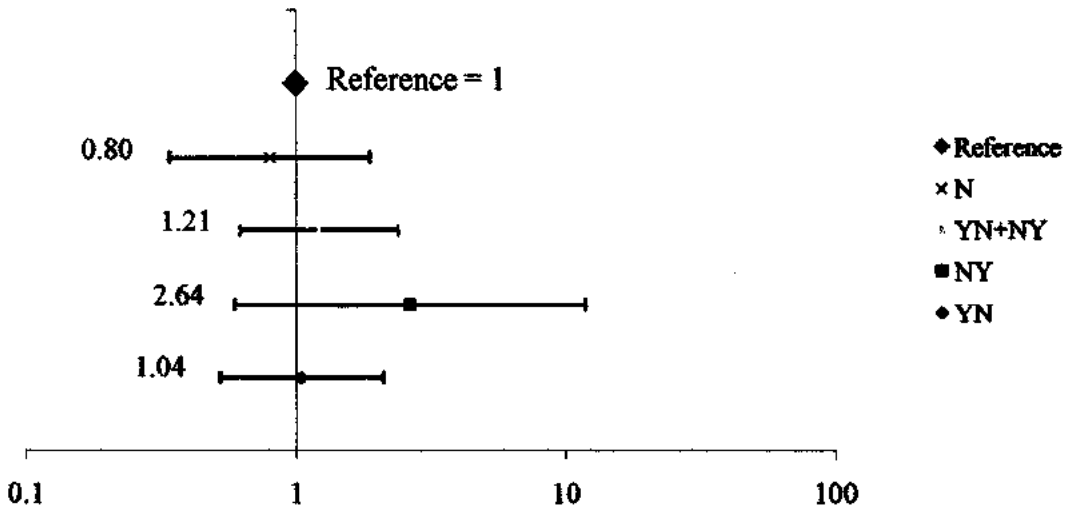

Figure 3.29: Association of combination effect (GSTT1-GSTM1) in Acute Myeloid Leukemia (AML). The presence of GSTT1-GSTM1 wild was taken as reference.

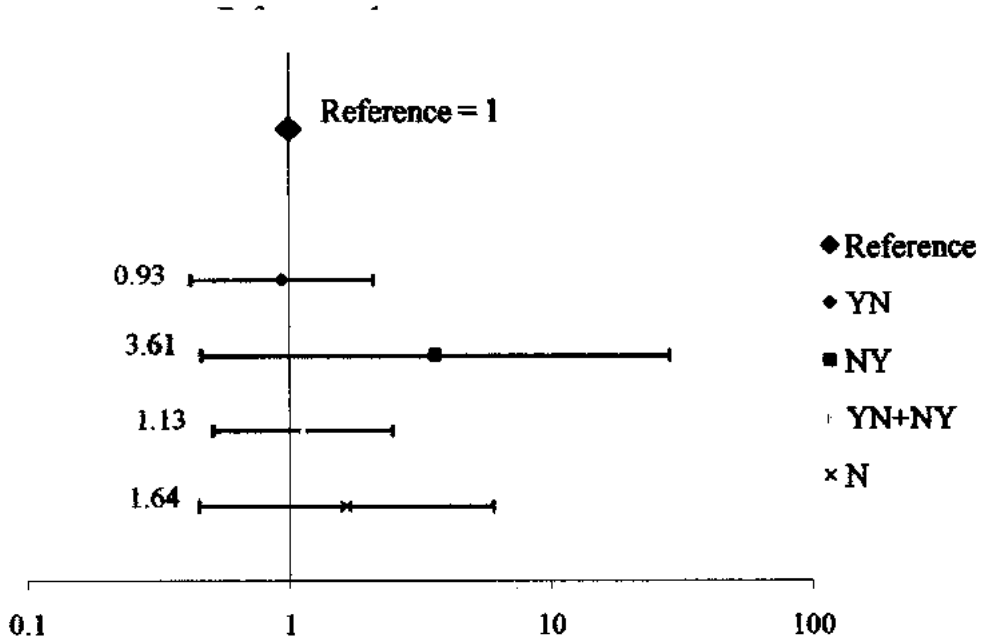

Figure 3.30: Association of combination (GSTT1-GSTM1) in Chronic Myeloid Leukemia (CML). The presence of GSTT1 GSTM1 was taken as reference.

3.16. ASSOCIATION OF GSTT1-GSTM1 COMBINATION IN DIFFERENT ETHNIC GROUPS

GSTT1-GSTM1 combined effect was also studied for the different ethnic groups and odds ratio along with 95% Confidence Interval was determined. Among these, all the possible genotypes were analyzed for "Pathan" and "Punjabi" samples. The presence of both genes GSTT1 and GSTM1 was taken as reference equal to 1. In "Pathan" Group, an increased risk was found to be associated with GSTT1-GSTM1 in combination, whereas for "Punjabi" samples, a significant protection (OR = 0.10; 95% CI = 0.04-0.24; P-value = 0.000) was observed. In case of "Northern Areas" samples, two combinations could be analyzed because of the smaller number of samples. In "Northern Areas" samples, there was protection with GSTT1-GSTM1 (Fig 3.31).

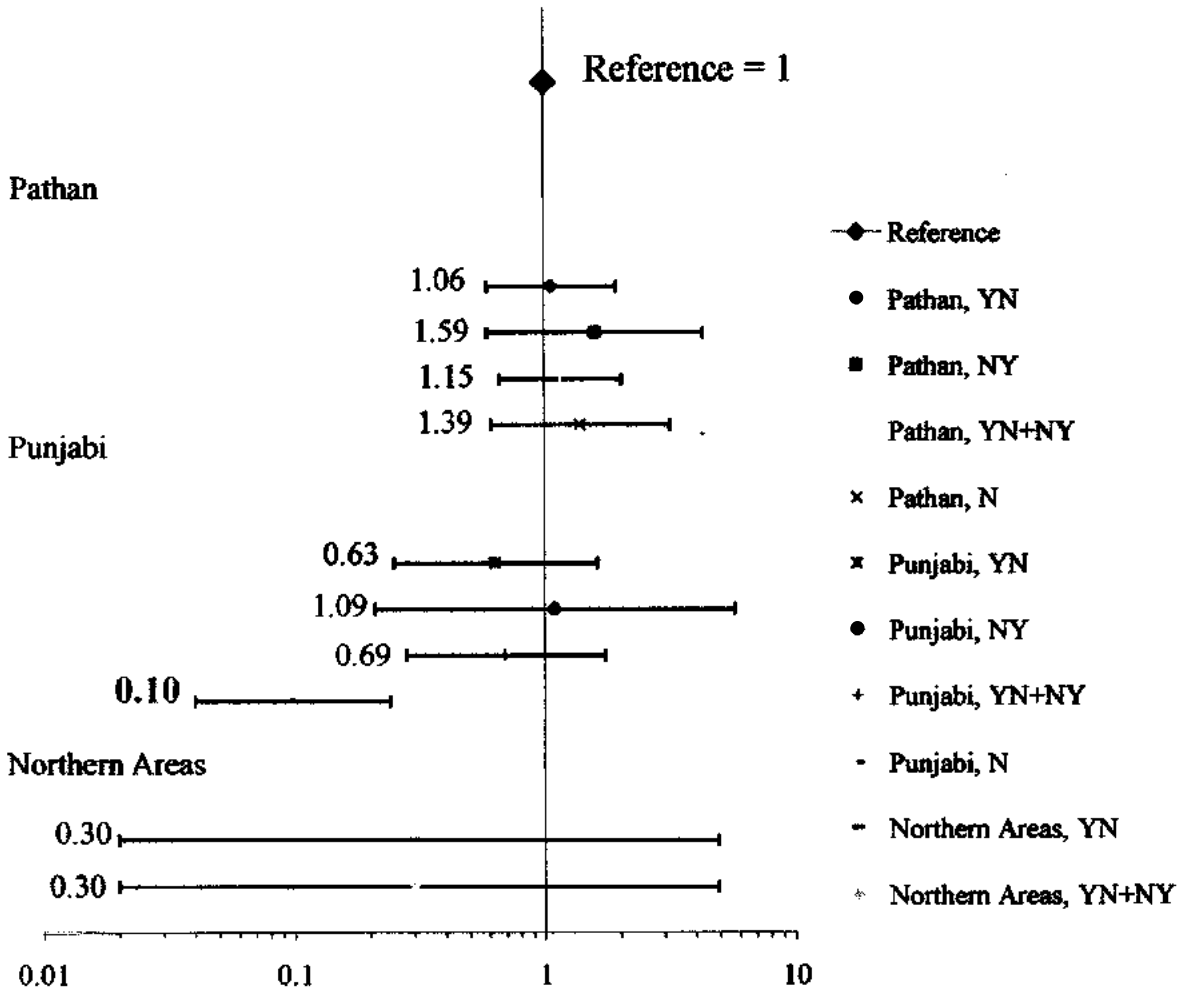

Figure 3.31: Association of combination (GSTT1-GSTM1) in different Ethnic Groups. The presence of GSTT1-GSTM1 was taken as reference equal to 1. Significant association was depicted in red.

3.17. ASSOCIATION OF GSTT1 AND GSTM1 WITH RESPECT TO COMBINATIONS OF AGE-GROUPS, GENDER, ETHNIC GROUP AND LEUKEMIA SUB-TYPES

3.17.1. Age Group and Gender

Logistic Regression was performed in leukemia samples after distributing them in different gender groups according to their ages and odds ratio was calculated along with 95% Confidence Interval. The presence of wild genotype of GSTT1 and GSTM1 genes was taken as reference equal to 1. Among all ages, males were found to have a considerable protection (OR = 1.766; 95% CI = 0.98-3.17) associated with GSTT1 null, with a borderline significance. Males from other age-groups and females of all age groups showed no association (Fig 3.32).

For GSTM1 null, no significant association was observed among males of all ages. However, the females of ages above 30 yrs showed close to significant association (p-value 0.06) with a considerable protection (OR = 0.148; 95% CI = 0.02-1.08). No association was observed with other age groups or gender (Fig 3.33).

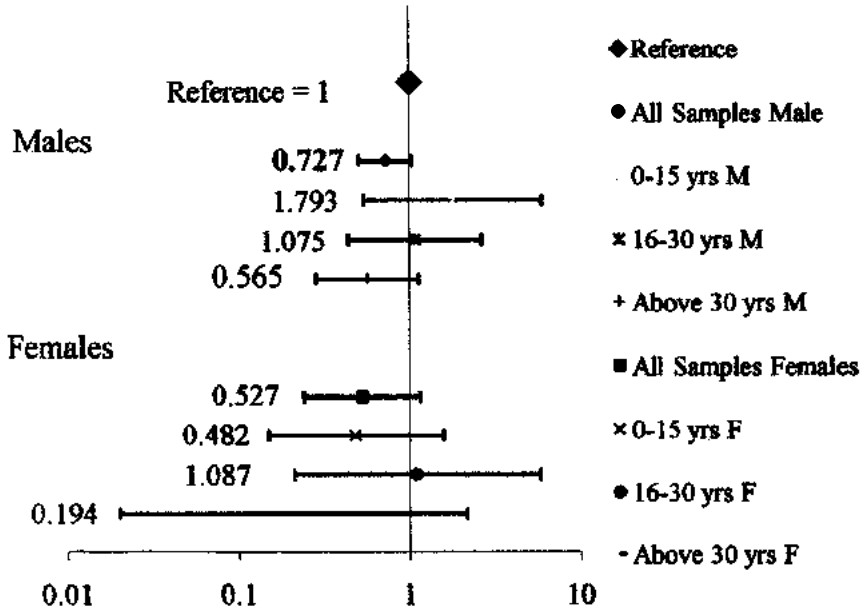

Figure 3.32: Association of GSTT1 with leukemia samples divided into age groups and gender. The presence of wild genotype of GSTT1 and GSTM1 genes was taken as reference. Values in red depict significance.

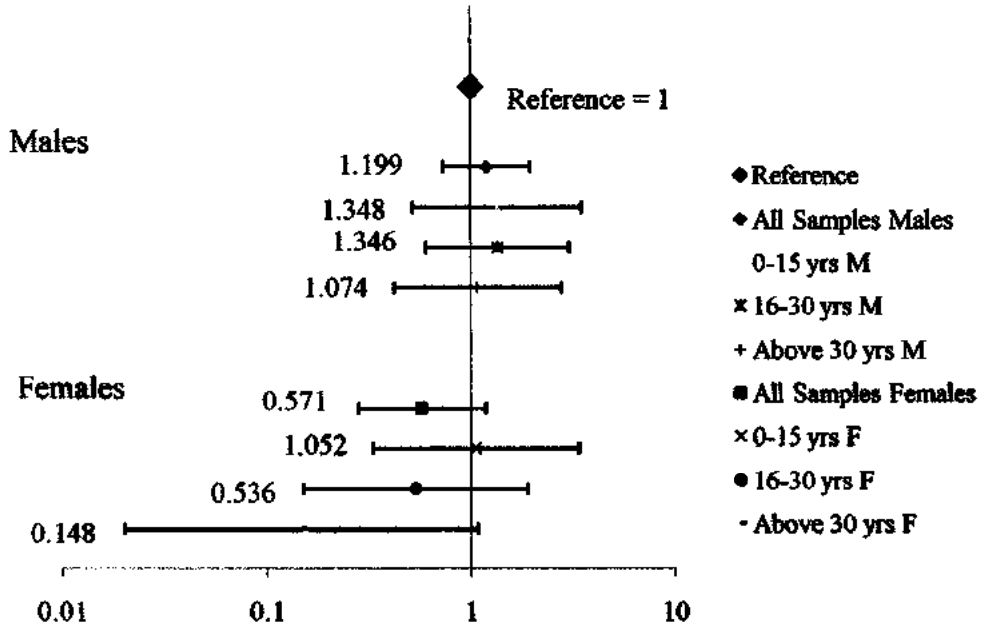

Figure 3.33: Association of GSTM1 with leukemia samples divided into age groups and gender. The presence of wild genotype of GSTT1 and GSTM1 was taken as reference. Values in red depict significance.

3.17.2. Ethnic Group and Gender

Leukemia samples were categorized into males and females with respect to their ethnic background and their odds ratio was calculated. The presence of GSTT1 and GSTM1 was taken as reference equal to 1. A significantly high protection (OR = 0.189; 95% CI = 0.049-0.730), with a probability value of 0.0108, was observed with GSTT1 null among Pathan males (Fig 3.34). No significance was observed among males and females belonging to the rest of ethnic groups. In case of GSTM1 null, there was no significant association with any of the ethnic group.

3.17.3. Ethnic Group and Age

Leukemia samples were characterized on the basis of age and ethnicity. The presence of GSTT1 and GSTM1 was taken as reference equal to 1. Among these groups, no significance was found with GSTT1 null in any of the age and ethnic group. In case of GSTM1 null a significant association was, however, observed among the patients above 30 yrs of age. A considerable protection (OR = 0.1875; 95% CI = 0.04-0.94) was depicted in this group with GSTM1 null, having a probability value of 0.041 (Fig 3.35).

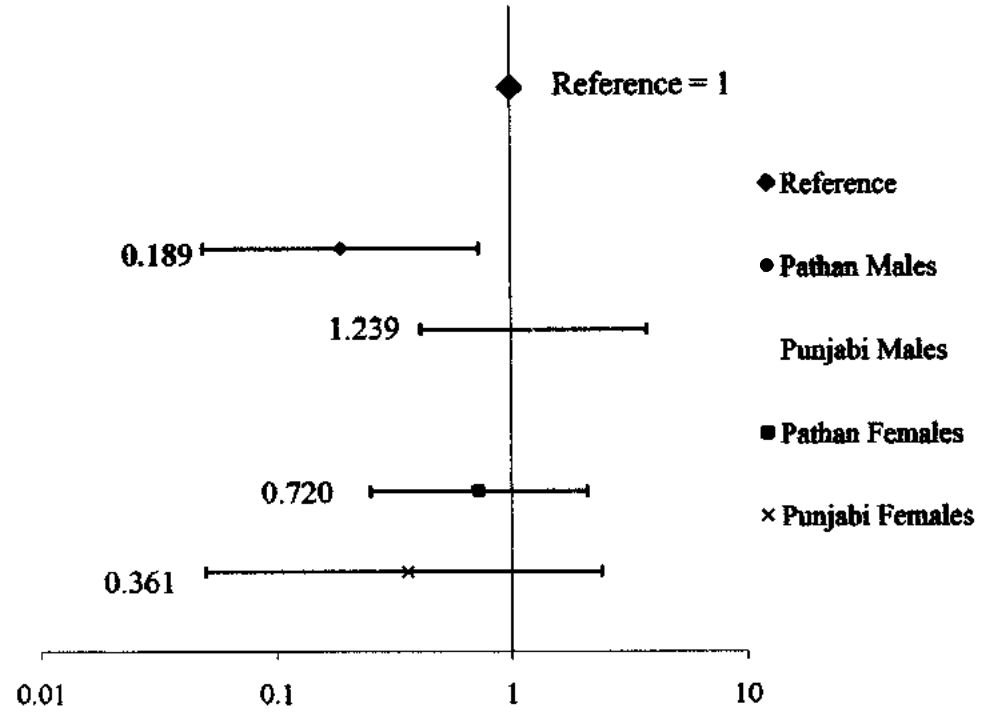

Figure 3.34: Association of GSTT1 in leukemia samples divided into gender and Ethnicity. The presence of GSTT1 was taken as reference. Significant result is shown in red.

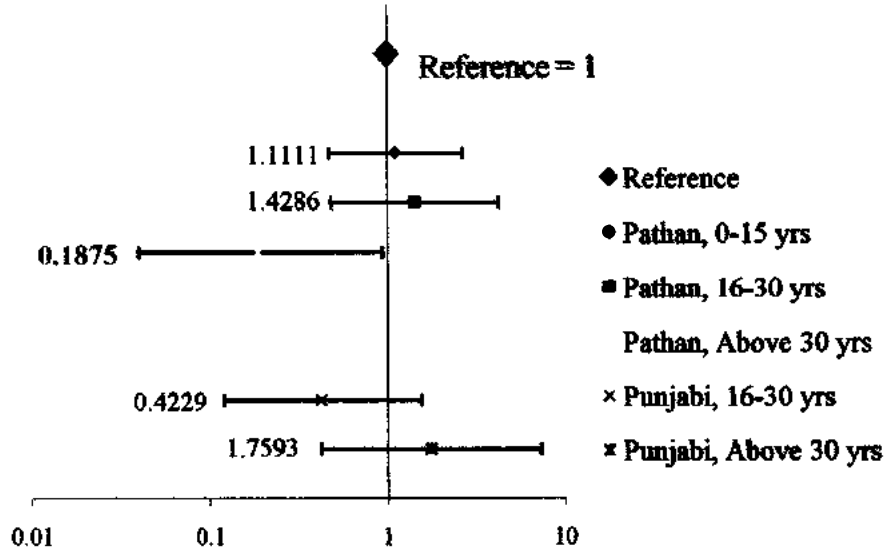

Figure 3.35: Association of GSTT1 in leukemia samples divided into age and Ethnicity. The presence of GSTT1 was taken as reference. Significant result is shown in red

3.17.4. Leukemia Sub-types and Age

Leukemia samples were also divided into four sub-types (ALL, AML, CML, CLL), on the basis of their ages. Presence of GSTT1 and GSTM1 wild genotype was taken as reference equal to 1. Odds ratios were calculated and GSTT1 null showed no significance in any of leukemia sub-type belonging to any age group. In case of GSTM1 null, though, higher protection was observed among patients with Chronic Myeloid Leukemia (CML) with age above 30 years. The result was not significant (p-value 0.061) that may be due to the small number of samples in this group.

3.17.5. Leukemia Sub-type and Gender

The individuals were categorized into males and females along with sub-type of leukemia being diagnosed. Presence of GSTT1 and GSTM1 was considered as reference equal to 1. Considering all sub-types together, the males possessed a considerably higher protection with GSTT1 null (OR = 0.727; 95% CI = 0.507-1.041). Association was, however, on the borderline of significance. Increasing the specificity, males suffering with Acute Myeloid Leukemia (AML) were found to have the increased protection (OR = 0.514; 95%CI=0.272-0.970) associated with GSTT1 null having a probability value of 0.037 (Fig 3.36). Among males with CML, an increased risk (OR = 2.318; 95% CI = 0.76-7.11) was observed with GSTT1 null, although association was not statistically significant (p-value = 0.142). No significance was observed among females with GSTT1 null, suffering from any of the leukemia sub-types. A significantly elevated risk (OR = 2.512; 95% CI = 1.244-5.072; p-value = 0.00871) was found with GSTM1 null among females diagnosed with AML (Fig 3.37).

3.17.6. Leukemia Sub-type and Ethnic Groups

Leukemia samples were also divided into sub-types with respect to their ethnic background. Presence of GSTT1 and GSTM1 was considered as reference equal to 1. In this group, there was no significant association observed with GSTT1 null and GSTM1 null.

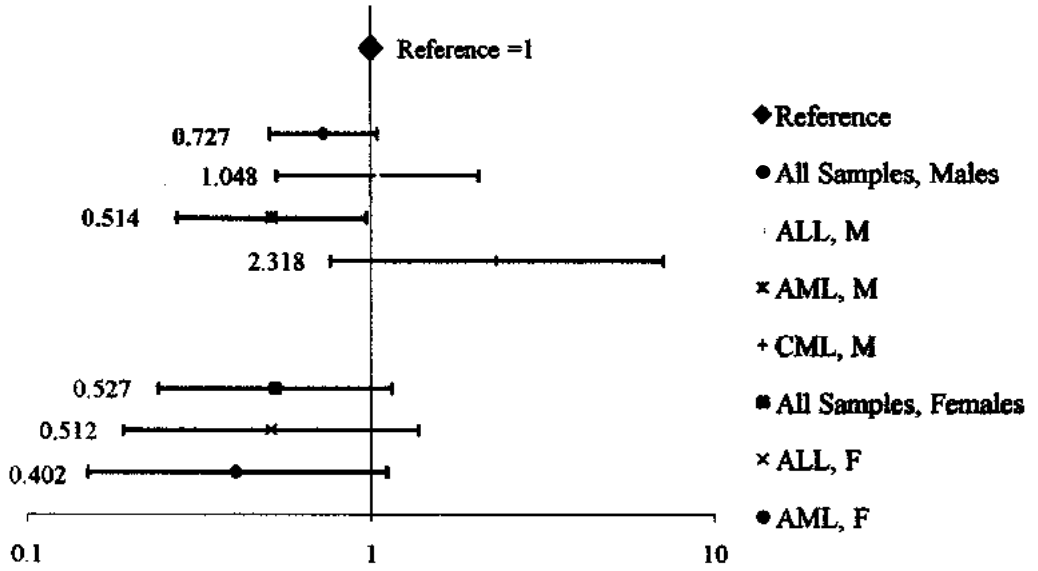

Figure 3.36: Association of GSTT1 with leukemia subtypes and gender. Presence of GSTT1 and GSTM1 was considered as reference equal to 1. Values in red show significant results.

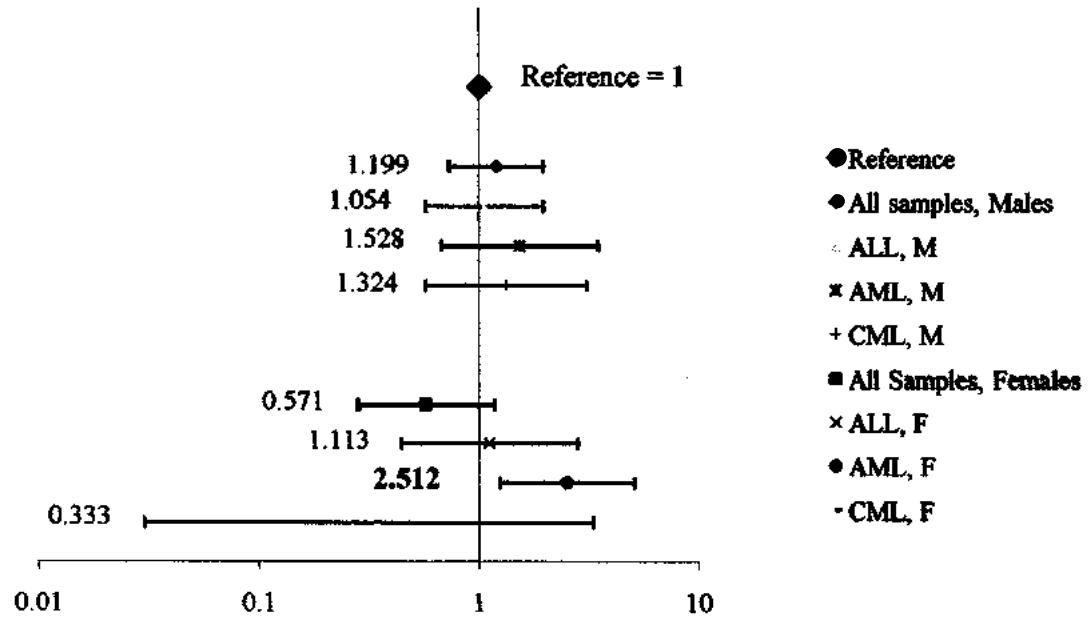

Figure 3.37: Association of GSTM1 with leukemia subtypes and gender. Presence of GSTT1 and GSTM1 was considered as reference equal to 1. Values in red show significant results.

3.18. ASSOCIATION OF GSTT1-GSTM1 (COMBINED) WITH LEUKEMIA WITH RESPECT TO COMBINATIONS OF AGE-GROUPS, GENDER, ETHNIC GROUP AND LEUKEMIA SUB-TYPES

In order to determine the combined effect of GSTT1-GSTM1 in different combinations of age, gender, ethnicity and leukemia sub-types logistic regression was done to calculate the Odds Ratio.

3.18.1. Gender and Age

Leukemia patients were divided in gender and age groups. The odds ratio was calculated along with 95% Confidence Interval for the resulting groups. The presence of GSTT1 and GSTM1 was taken as reference equal to 1. There was no significant association among males of any age. In females, of all age groups taken together, there was a significant association (p-value = 0.009) with the GSTT1-GSTM1 null (deletion of both GSTT1-GSTM1). A considerably high level of risk (OR = 0.22; 95% CI =1.462-13.675; p-value = 0.0064) was observed in this case. An elevated risk (OR = 2.00; 95%CI = 0.40-9.91) was, however, found to be associated with GSTT1-GSTM1 null in females of age between 0-15 years, although no significance was observed here (p-value = 0.396) (Figure 3.38).

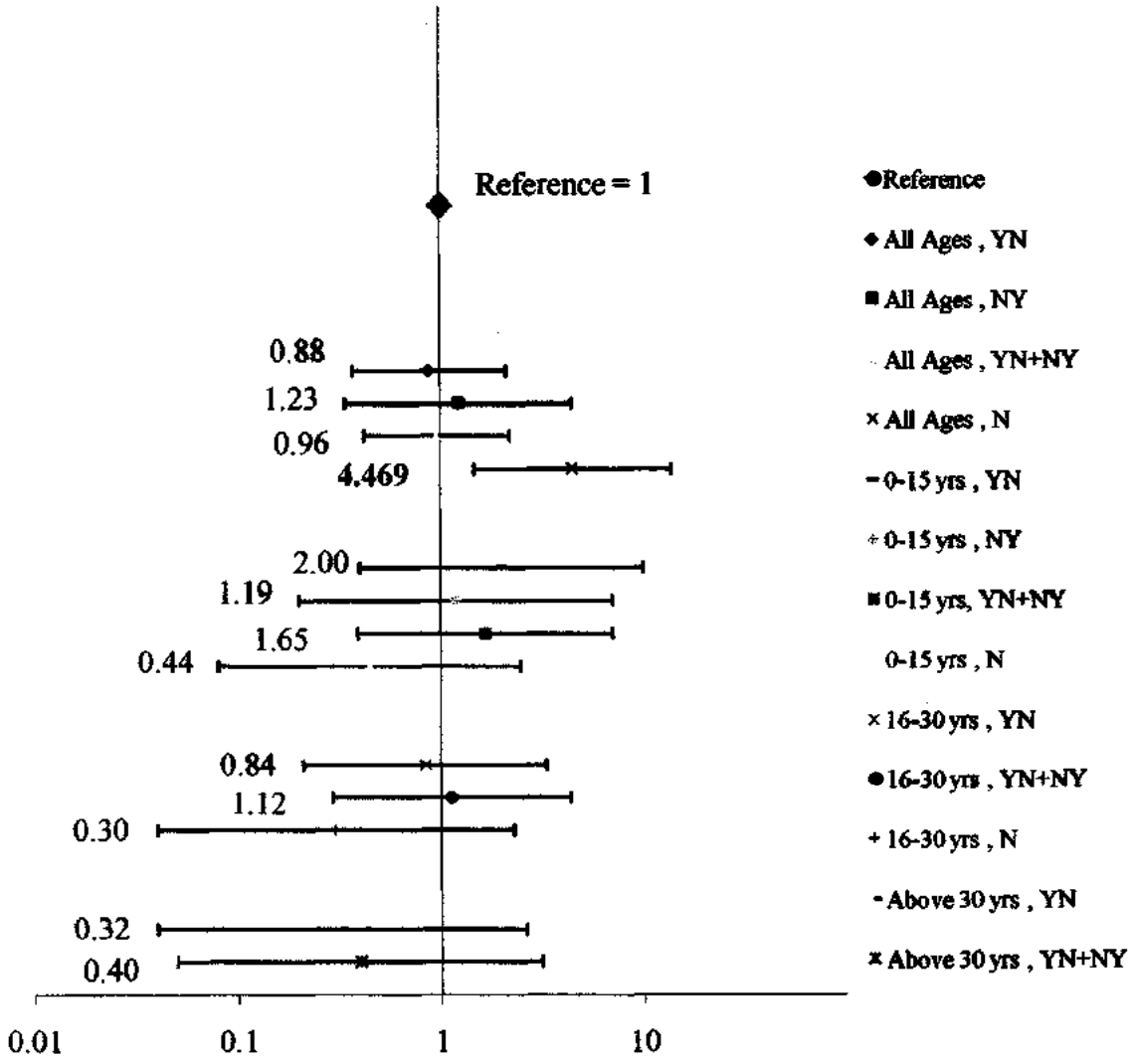

Figure 3.38: Association of GSTT1-GSTM1 (combined) in females of different age groups. The genotype combinations (YN, NY, YN+NY, N) for all ages are depicted here. A reference (YY/presence of both genes) of 1 is taken. Significant result is shown in red.

3.18.2. Ethnic Group and Gender

On the basis of ethnicity, the males and females were divided and odds ratios were calculated accordingly. The presence of both genes was taken as reference equal to 1. In this group, "Pathan" males showed significant association (p-value = 0.0157) and a greater protection (OR = 0.189; 95% CI = 0.049-0.730; p-value = 0.010) was observed with a GSTT1 null and GSTM1 null, simultaneously. A visibly increased risk was also observed among "Pathan" males associated with heterozygous NY, possessing GSTM1 wild but GSTT1 null (OR = 2.35; 95% CI = 0.55-9.96) (Fig 3.39). The association was, however, not significant (p-value = 0.247). Considering the results in females, no significance was observed in any of the ethnic group. In the present analysis, "Pathan" and "Punjabi" individuals were included only, because of the small number of samples in other combinations.

3.18.3. Ethnic Group and Age

A combination of individuals was made on the basis of their ages and ethnic background. The odds ratio calculated for this group showed no significant association in any combination.

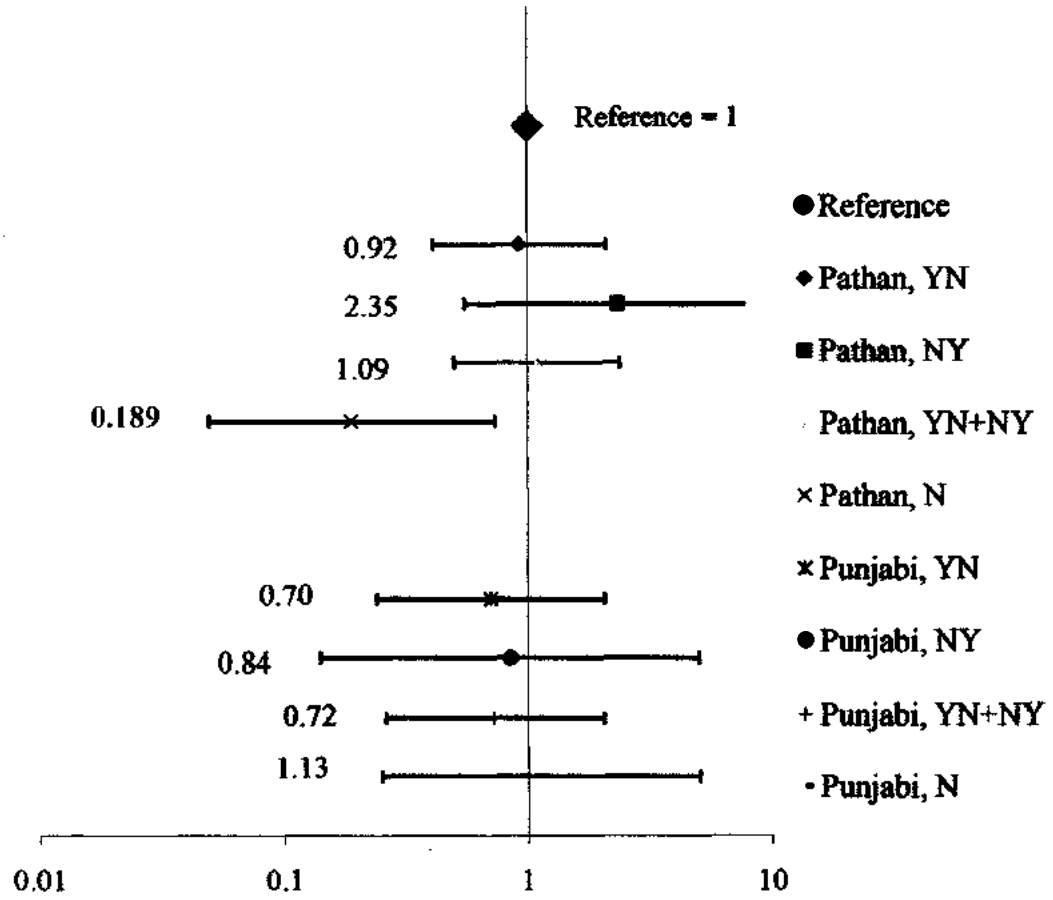

Figure 3.39: Association of GSTT1-GSTM1 (combined) in males of different ethnic groups. The genotype combinations (YN,NY, YN+NY, N) for Pathan and Punjabi males are depicted here. A reference (YY) of 1 is taken. The significant result is shown in red.

3.18.4. Sub-type and Gender

The patients were categorized in males and females on the basis of the sub-types of leukemia. Logistic Regression performed on this data showed that there was no association among males with any of the leukemia sub-type diagnosed. Females, on the other hand, had a significant association (p-value = 0.009) with null condition for both GSTT1 and GSTM1. There was a considerable protection (OR = 0.22; 95% CI = 0.073-0.68) being observed among all females. The presence of both genes was considered as reference equal to 1. When divided in sub-types, the females diagnosed with Acute Myeloid Leukemia were shown to have a significant association (p-value = 0.003) with a null of GSTT1 and GSTM1. There was a higher protection (OR = 0.13; 95% CI = 0.033-0.495) associated in these females (Fig 3.40).

3.18.5. Sub-type and Ethnic Groups

The odds ratios were calculated for a combination of individuals grouped according to their ethnicity and the sub-type of leukemia they were suffering from. The presence of both genes was considered as reference equal to 1. There was no significant association observed in these groups.

3.18.6. Sub-type and Age

The patients with different sub-types of leukemia were divided on the basis of their ages. The wild genotype of both genes was considered as reference equal to 1. The odds ratio along with 95% Confidence Interval calculated showed no significance in this combination. There was, however, near significance (p-value = 0.059) observed in AML patients above 30 years of age. A considerably higher risk (OR = 3.14; 95%CI = 0.959-10.302) was found with a heterozygous condition (Y/N+N/Y) in this group.

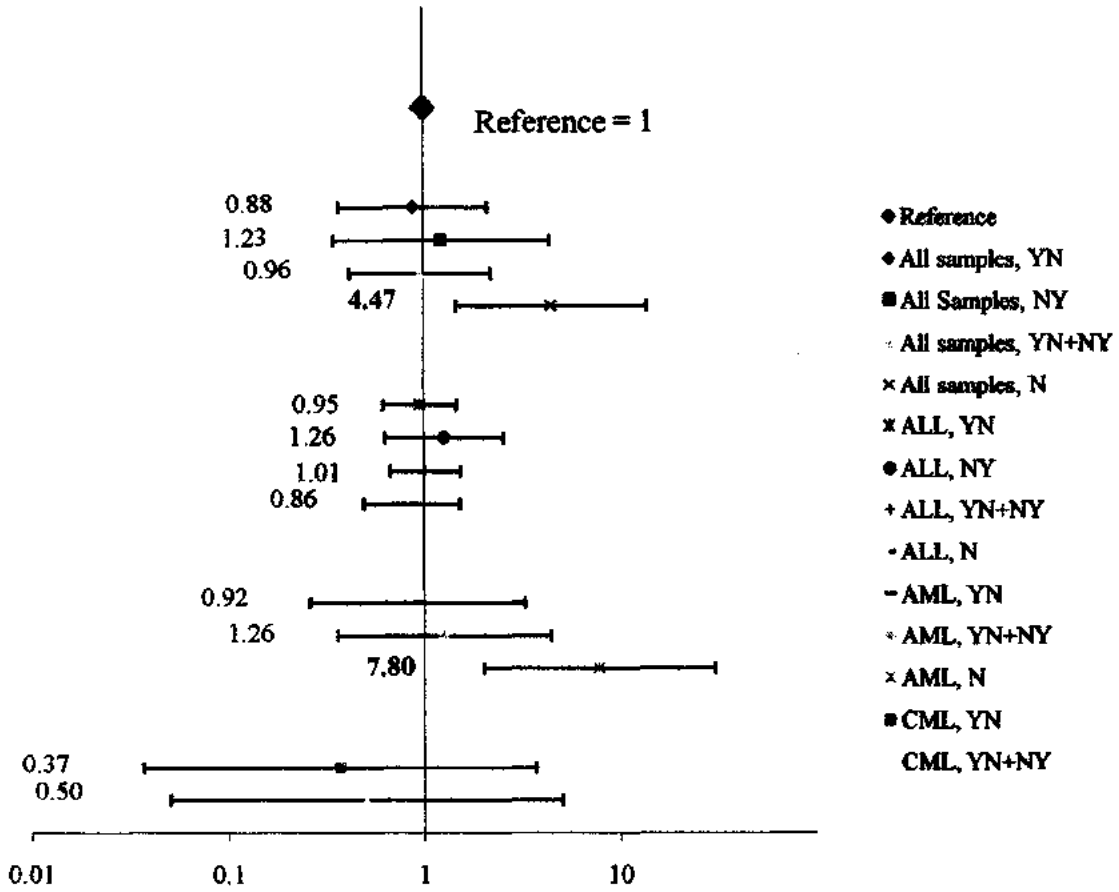

Figure 3.40: Association of GSTT1-GSTM1 (combined) in Females with different sub-types of leukemia. The genotype combinations (YN, NY, YN+NY, N) for females with ALL, AML and CML are depicted here. The presence of both genes was considered as reference equal to 1.

CHAPTER 4

DISCUSSION

DISCUSSION

Glutathione S-transferases (GSTs) are a group of enzymes that function in the detoxification of harmful xenobiotics, both endogenous (reactive oxygen species, oxygen radicals) as well as foreign (environmental pollutants, chemical carcinogens and cytotoxic drugs etc.). Individuals, however, differ in their ability to detoxify the xenobiotics and hence towards cancer, owing to the differences in the genotypes of a number of involved enzymes. The polymorphisms among these enzymes have been found to be associated to a number of diseases including cancer. In order to assess the associated risk of disease, studying only a single gene may not sufficient. (Guengerich, 1990; Sherif *et al.,* 1996). In the present study, a total of 158 leukemia samples were included, which consisted 110 males and 46 females (gender for two patients was unknown). Patients were ranging from few months old to more than 70 years old. About 76 patients were diagnosed with acute lymphoblastic leukemia, 49 with acute myeloid leukemia, 31 with chronic myeloid leukemia and only 2 patients with chronic lymphocytic leukemia were screened. In this study, the null polymorphisms of GSTT1 and GSTM1 were analyzed for their association with leukemia risk. Null polymorphism depicts the deletion of both the genes,

4.1. PERCENTAGE OF GSTT1, GSTM1 POLYMORPHISMS

The percentage of GSTT1, GSTM1 genes independently and their combined genotypes in 158 leukemia samples and 360 controls matched for ethnic group and gender were evaluated. The percentage of the presence of GSTT1(Y) and GSTT1 null were approximately equal in cases and controls. The GSTT1 null was found to be 25.1% of cases had GSTT1 null while 26.4% of controls possessed GSTT1 null. These percentages of GSTT1 null observed in current study were compared to those previously reported in other studies. The percentage of null genotypes of GSTT1 observed in the present study was in concordance to an earlier study conducted on healthy population of Southern Punjab, Pakistan (Shaikh *et al.,* 2010). They evaluated the percentage of the GSTT1 null and GSTM1 null separately and then their combined null genotype among 111 healthy individuals. They reported GSTT1 null in about 23% individuals (Shaikh *et al.,* 2010). Masood and colleagues (2010) studied

the association of GSTT1 and GSTM1 null with risk of head and neck cancer. They reported 18.6% GSTT1 null in their 150 normal control population (Masood *et al.,* 2010). Sohail and co-workers (2013) determined the frequency of GSTT1 gene in 100 healthy controls from Multan, Layyah and Rajanpur areas of Punjab, Pakistan while studying the effect of GST gene polymorphism in breast cancer risk. They reported the deletion of GSTT1 gene in 27% of their (100) healthy individuals (Sohail *et al.,* 2013). However, a study conducted on 100 healthy individuals from Faisalabad area of Punjab, Pakistan depicted a 71% of GSTT1 deletion (null), much higher than the current study and contradictory to other studies discussed (Rafiq *et al.,* 2013). The GSTT1 null percentage, assessed in the current study, was comparable to those observed in studies conducted on other populations. Singh and colleagues (2009) determined the frequencies of null polymorphisms of GSTT1 and GSTM1 in Delhi population, North India. They also reported 27.4% deletion of GSTT1 gene (Singh *et al.,* 2009). Similar percentage was observed by Rossini *et al.,* (2002) in 591 individuals of Brazilian population. The volunteers were treated for diverse pathologies, but had no history of cancer. They divided the individuals into Brazilian Whites (319) and Brazilian non-White groups (272), and observed about 25% null genotype of GSTT1 in both the groups (Rossini *et al.,* 2002). Fujihara and co-workers also reported a GSTT1 null percentage of about 25.6% among Mongolian population (Fujihara *et al.,* 2009). Other studies depicted contrasting results. In the Asian countries, the GSTT1 null was reported to be 18.4% in North India (Mishra *et al.,* 2004) and 16.8% in South India lesser than the percentages observed in this study. (Naveen *et al.,* 2004). In some studies, a higher percentage of GSTT1 null was reported such as 46% in 477 Chinese individuals (Setiawan *et al.,* 2000) and 54% among 150 Japanese individuals (Naoe *et al.,* 2000). Among the African-American population from North Carolina, 17% individuals showed a null genotype of the gene. In the White individuals from same population, the GSTT1 was deleted in 16% individuals (Millikan *et al.,* 2000). Stavropoulou *et al.,* (2006) evaluated the GSTT1 and GSTM1 polymorphisms in 165 healthy Greek individuals and reported 18.8% of individuals with GSTT1 null genotype (Stravropoulou *et al.,* 2006). A study conducted on Italian population consisting of 273 healthy controls, depicted 19% GSTT1 null genotype among them (D'Alo *et al.,* 2004). About 16.3% of GSTT1 null was reported in a Polish study conducted on 233 healthy controls (Kargas *et al.,* 2003).

In the current study, the percentage of GSTM1 null was observed to be approximately equal among cases and control population. The null genotype of GSTM1 was about 53.55% in cases while 51.39% in controls. The percentage of GSTM1 null analyzed in this study was higher as compared with those observed by Shaikh *et al.,* (2010). They reported 45% of deletion of GSTM1 in a total of 111 healthy individuals from Southern Punjab, Pakistan (Shaikh *et al.,* 2010). Masood and co-workers reported only 8% of GSTM1 null genotype among the 150 healthy individuals studied to evaluate the association of GST polymorphisms with risk of developing cancers of neck and head (Masood *et al.,* 2010). However, results from a study conducted on 100 individuals from Punjabi population of Multan, Layyah and Rajanpur showed the GSTM1 deletion in 43% individuals (Sohail *et al.,* 2013). This difference in the percentage of GSTM1 null might be attributed to the difference in the locality from where the samples were collected; Shaikh et al., (2010) and Sohail et al., (2013) studied individuals from Southern Punjabi, whereas the samples in the current study were from Northern areas. The percentage deletion of GSTM1 observed in the current study was nearly in concordance with those observed in English, Turkish, Chinese, Japanese and White (USA) populations in different studies. Among 178 individuals studied from New Castle England, 50.8% showed the GSTM1 null (Welfare *et al.,* 1999). Ada and colleagues (2004) evaluated the frequency of GSTM1 null polymorphism in 133 healthy Turkish individuals and reported approximately 51.9% of GSTM1 null genotype (Ada *et al.,* 2004). Among the Asian countries, the results were comparable to those from Chinese and Japanese populations. In a case-control study, Setiawan and co-workers (2000) determined the percentage of GSTT1 and GSTM1 null polymorphisms in 477 healthy control Chinese individuals, having no cancer history. They reported a GSTM1 null in 51% of individuals (Setiawan *et al.,* 2000). Similar percentage was found by Naoe *et al.,* (2000), who observed 51.3% GSTM1 null genotype among 150 Japanese individuals evaluated for GST polymorphisms (Naoe *et al.,* 2000). The GSTM1 null percentage was also in agreement with those reported in the White population of USA, showing 52% of GSTM1 null genotype (Millikan *et al.,* 2000). The percentages observed in the present study were, however, in contrast to those observed in North and South Indian populations with 33% and 30.4% GSTM1 null genotypes, respectively (Mishra *et al.,* 2004; Naveen *et al.,* 2004). Other studies depicted different percentages of GSTM1 null as 46.9% in Italian population (D'Alo *et al.,* 2004), 47.6% among Polish

population (Kargas *et al.*, 2003), 48.9 % in Brazilian Whites (Rossini *et al.*, 2002), 46.6% in Mongolians (Fujihara *et al.*, 2009), however a substantial difference in percentages was observed with the individuals of Brazilian non-Whites having 34.2% GSTM1 null (Rossini *et al.*, 2002), African-Americans with 28% (Millikan *et al.*, 2000) and Greek with 38.8% deletions of GSTM1 (Stavropoulou *et al.*, 2007).

The combination effect of polymorphisms of both the GSTT1 and GSTM1 genes was also studied and the percentages of the resulting genotypes were calculated among leukemia cases and healthy control population. The percentage of the GSTT1 wild/GSTM1 wild was found to be 38.06%. The GSTT1 wild and GSTM1 null was reported to be 36.77%, whereas, the percentage of GSTT1 null and GSTM1 wild was 8.39%. The null genotype of both genes, however, was 16.77%. The percentage of deletion of both GSTT1and GSTM1 genes (GSTT1/GSTM1 null) was, however, reported to be 5%, much lower than the percentage observed in current study (Shaikh *et al.*, 2010).

The variations in the GSTT1 null and GSTM1 null percentages in different studies may be accredited to the difference in the vicinity of the sample population. There may also be differences regarding the age group and gender of the studied individuals.

4.2. ASSOCIATION OF GSTT1 AND GSTM1 POLYMORPHISMS WITH RISK OF LEUKEMIA

The association of null polymorphisms GSTT1 and GSTM1 have been estimated in leukemia (AML, ALL, CML) patients from Pakistani population. Logistic regression was done to calculate odds ratio, to assess the relative risk of these polymorphisms. No significant association of GSTT1 and GSTM1 null was depicted with overall leukemia risk development. The combined effect of GSTT1 and GSTM1 null polymorphisms with risk of leukemia also did not show any significant association in any of the possible genotype combinations. The assessment of GSTT1 null polymorphism in all leukemia samples of with respect to gender, however, depicted a significant association among males. Male patients were believed to have an increased protection with GSTT1 null and showed borderline significant association. The percentage GSTT1 null was lesser in cases than controls, hence, the GSTT1 null may

be predicted to have a protective role in our population. On the other hand, there was a small difference found in GSTM1 null percentages in cases and control population and no association was observed with GSTM1 null in any of the cases. The association of GSTT1 and GSTM1 polymorphisms was evaluated in combination in all leukemia cases, with respect to their age and gender. Females were found to have an increased risk of developing leukemia with the deletion of both genes simultaneously (GSTT1 null/GSTM1 null).

4.2.1. Acute Lymphoblastic Leukemia

The association of GSTT1 and GSTM1 null polymorphisms was studied among acute lymphoblastic leukemia cases. ALL cases had higher percentages (31.58%) of GSTT1 null than controls (26.39%). A considerable risk was observed with GSTT1 null, though, the association was not significant (OR = 1.15; 95% CI = 0.81-1.65; p-value = 0.43). Similarly, the association with GSTM1 null was not significant in risk of developing ALL (OR = 1.00; 95% CI = 0.61-1.64; p-value = 0.99). The association of polymorphisms of both GSTT1 and GSTM1 combined was also studied in ALL patients. No significant association was observed with any of the genotype combination. Association of GSTT1 and GSTM1 polymorphisms was examined in ALL patients with respect to their gender and age and observed same results. No significance was observed in any genotype combination. However, the individuals with a heterozygous condition (GSTT1/GSTM1 = Y/N or N/Y) showed considerably increased risk of developing ALL, but there was no significant association (OR = 2.36; 95% CI = 0.349-15.927; p-value = 0.379).

Chan et al., (2011) also observed similar results, who studied 185 children with ALL from Javanese population and 177 healthy controls. In their study, none of the polymorphisms showed any association with ALL. Significant association was, however, depicted with GSTT1 null genotype among females, unlike our results (Chan *et al.*, 2011). Our results were also comparable to those observed in a study conducted on 144 children with ALL fom Turkish population. They reported no significance with the GSTT1 and GSTM1 null in risk of developing ALL, although GSTT1 null was believed to confer a protective role against ALL among this population (Balta *et al.*, 2003). In another study, GSTM1 null showed no significant association in risk of causing ALL, similar to our results. A significant association

was, although, observed with GSTT1 null in risk of developing ALL (Rollinson *et al.*, 2000). Pakakasama *et al.*, (2005) observed GSTT1 null to have no significant association, similar to our results. Conversely, they reported GSTM1 null and GSTT1/GSTM1 null genotypes to be associated with an increased risk of ALL (Pakakasama *et al.*, 2005). However, many studies depicted the association of these polymorphisms in risk of developing ALL. Another study conducted on 100 ALL cases and 300 healthy individuals form north Indian population, depicted GSTT1 null genotype to be associated with a 2 folds increase the risk of pediatric ALL (OR = 2.54; 95% CI = 1.50–4.32), but GSTM1 null showed no any risk. The analysis of combined effect of both genes showed that the deletion of both genes may increase the risk of developing ALL while the presence of GSTT1 wild and GSTM1 wild were shown to impart a significant protection against ALL (Moulik *et al.*, 2014). A meta-analysis was done for assessing the association of GSTT1 and GSTM1 polymorphisms with risk of ALL, the null of both the genes was believed to increase risk of developing ALL among Asian children, but no association was observed for Whites or Blacks (Moulik *et al.*, 2014). In contrast to our study, Bolufer *et al.*, (2007) also reported the deletion of GSTT1 (GSTT1 null) to be associated significantly with the ALL risk. They conducted a study on 141 ALL patients and observed a higher risk among males with GSTT1 null (Xu and Cao, 2014). Krajinovic *et al.*, (1999) also showed the significant association of GSTM1 null with the increased risk of ALL in 177 patients. However, in agreement to our study, they also reported no association with GSTT1 in ALL patients (Krajinovic *et al.*, 1999). Suneetha *et al.*, (2008) reported an increased risk of GSTM1 null with ALL among 92 Indian patients (Suneetha *et al.*, 2008). Therefore, in the current study, the GSTT1 null percentage being higher in patients might be believed to be associated with ALL risk.

4.2.2. Acute Myeloid Leukemia

The association of GSTT1 and GSTM1 null polymorphisms with Acute Myeloid Leukemia showed no significant association with GSTT1 null but a relatively increased protection might be expected with GSTT1 null. Similarly, no significance was depicted with GSTM1. The association of polymorphisms of both the genes was observed in patients with AML, and a statistically non-significant, higher risk was observed among cases with GSTT1 null/ GSTM1 wild. Upon assessing the GSTT1 null and GSTM1 null in the risk of developing AML, with respect to ages and gender,

a significant association was observed with GSTT1 null in males, showing an increased protection. Among females, on the other hand, a significantly increased risk was found to be associated with null polymorphisms of GSTM1. The analysis of polymorphisms of GSTT1 and GSTM1 combined with respect to age and gender was observed. Among males, though no significant association was found but an extremely increased risk was believed to be associated in patients with GSTT1 null/ GSTM1 wild and GSTT1 null/ GSTM1 null combinations. Among females, however, the deletion of both genes depicted to confer a significant increased risk. Evaluating the association of polymorphic variants of GSTT1 and GSTM1 together on the basis of different age groups, the GSTT1 presence and GSTM1 null genotype combination was believed to confer an increased risk among individuals of ages between 0 – 15 years and 16 – 30 years, respectively. In these groups the heterozygous condition (GSTT1/GSTM1 = Wild/Null and Null/Wild) of two genes also was thought to result in a high risk of causing AML. But in any of the case, no significance was observed. Thus in our study, null genotype of GSTT1 was believed to confer a protection from AML, especially among males. On the other hand, GSTM1 null, either separately or combined with GSTT1 null, might have a risk of developing AML, particularly in females.

Many previous studies have observed the association of null polymorphisms of GSTT1 and GSTM1 genes with AML risk. Rollinson *et al.,* (2000) observed a weak association of GSTT1 null and GSTM1 null in 479 AML patients, from Caucasian population, being studied. The association was, though, non-significant However, genotypic analysis of both genes simultaneously showed a significantly increased risk of AML in individuals possessing a wild of GSTT1 and Null of GSTM1 or vice versa (Rollinson *et al.,* 2000). Our results were in agreement to these observations, though the association was not significant owing to a small number of samples included in the study. Similar to our results, in a meta-analysis Ma et al., (2014) reported the GSTM1 null to be associated with an increased risk of AML among Chinese population (Ma *et al.,* 2014). Another recent study depicted the association of GSTT1 and GSTM1 variants with risk of developing acute myeloid leukemia among 243 cases of AML and 231 controls. They found a significant association of a null GSTM1 genotype to cause a considerably higher risk of AML. Individuals with GSTT1 null also were found to have 1.78 folds elevated risk of AML than the

individuals showing GSTT1 wild genotype and significant association was observed (Zi *et al.,* 2014). Association of GSTT1 and GSTM1 polymorphisms was determined in acute leukemia cases and showed GSTM1 null to have a significant risk with AML similar to our results. No association was, however, observed with GSTT1 null (Aydin-Sayitoglu *et al.,* 2006). In contrast to our reports, D'Alo *et al.,* (2004) reported the GSTT1 null to have significant association with AML conferring a 1.7 fold increased risk; however, GSTM1 null was not significantly associated with risk of AML. They also studied the GSTT1 and GSTM1 null polymorphisms with respect to gender, but found no significant association, in contrast to our results (D'Alo *et al.,* 2004). A met-analysis was conducted by Tang *et al.,* (2014), who reported the GSTT1 and GSTM1 null to pose an increased risk of acute leukemias, including AML, so these may be a risk factor for developing the disease (Tang *et al.,* 2014). A study conducted on a sub-type of AML, Acute Promyelocytic Leukemia, also showed the increased risk of disease to be associated with GSTT1 null and the GSTT1null and GSTM1 null together (GSTT1/GSTM1 null). The GSTM1 null was, however, not associated with risk of AML development (Mandegary *et al.,* 2011).

4.2.3. Chronic Myeloid Leukemia

In the overall leukemia samples, the higher risk with GSTT1 null was associated to Chronic Myeloid Leukemia, although the association was not significant statistically. This could be due to the small number of samples of CML included in the study. The association of null polymorphism of GSTM1 was also studied. Among all the leukemia samples and sub-types studied, no significant association was found with GSTM1 null. The association of null polymorphism of GSTT1 and GSTM1 independently was also examined with respect to gender and age of patients with CML. A considerably high risk was linked with GSTM1 null in individuals with ages between 16 – 30 years, whereas, individuals with ages above 30 years reported an increased risk associated with GSTT1 null polymorphisms. On comparing the association among males and females with the disease, it was found that males possessed an increasing risk of developing chronic myeloid leukemia. In any of these cases, however, there was no statistically significant association due to small sample number of chronic myeloid leukemia samples collected. The combination effect of both the genes GSTT1 and GSTM1 was studied to assess their association with risk of developing leukemia. In CML, the combination effect of the variants of these genes

was observed and showed an extremely higher risk with the GSTT1 null/ GSTM1 wild combination. The association was, however, not significant, that may be ascribed to the small number of CML samples included in the study. The combination effect was also determined in CML with respect to gender and age of patients. Among males and females with CML, males were found to have an elevated risk with GSTT1 null / GSTM1 wild genotype, however, the association did not reach the significance value. Males, having both genes deleted (GSTT1 null/ GSTM1 null), showed a considerably higher risk of CML, but no significance was observed. Conversely, no significant association was observed among females. Among age groups, the highest risk of CML was found with individuals between 16 – 30 years of age. The individuals belonging to this age group also showed a higher risk with a null genotype of both genes.

These results were in agreement to the study conducted by Banescu and colleagues (2014), who conducted a case-control study on 168 CML patients and 321 healthy controls and reported no significant association of GSTT1 and GSTM1 null with CML (Banescu *et al.*, 2014). He and co-workers (2014) carried out a meta-analysis including sixteen studies and determined the effect of polymorphisms of GST on the risk of developing CML. They reported no association of GSTM1 null genotype with risk of developing CML, similar to our study. However, in contrast to our results, the GSTT1 null was significantly associated (OR = 1.57; 95% CI = 1.15-2.14; P-value = 0.004) with risk of CML (He *et al.*, 2014). Another study was conducted on 107 CML patients and 135 healthy controls from Turkish population. They also showed no significance with GSTM1 null. The GSTT1 null, however, was related to risk of CML (OR = 2.82; 95% CI = 1.58-5.05; P-value < 0.001) and the CML cases had higher frequency of GSTT1 null as compared to controls, so the presence of GSTT1 was believed to have a protective role in CML (Taspinar *et al.*, 2008). Al-Achkar *et al.*, 2014 observed a null genotype of GSTT1 and GSTM1 to be significantly associated with a higher risk of CML. Upon analyzing the genotypes of both genes simultaneously, they reported the deletion of both genes to have a higher risk of developing CML. The GSTT1 null/ GSTM1 wild combination was found to have an higher risk but the association was not significant (Al-Achkar *et al.*, 2014). This finding was in agreement with our results, where the genotypic combination of GSTT1 null/ GSTM1 wild was found to have an elevated risk of CML, but was not

significant. Similar results were reported by another study, showing 4.5 folds of significant increase in the risk of CML among individuals possessing GSTT null/GSTM1 wild genotypic combination. They also reported the deletion of both genes to pose a 3.58 times higher risk of developing CML (Ovsepyan *et al.,* 2014).

4.3. ASSOCIATION OF GSTT1 AND GSTM1 POLYMORPHISMS WITH RESPECT TO ETHNIC GROUPS, GENDER AND AGE

The present study is the first to report the association of null polymorphisms of GSTT1 and GSTM1 genes in specific ethnic groups of Pakistani population, considering the variables like gender, age groups and sub-types of leukemia. A significantly increased protection was observed with GSTT1 among males from Pathan ethnic origin, irrespective of the leukemia sub-type and age of patients. Furthermore, patients from Pathan ethnic group were also observed to have a significantly increased protection with GSTM1 null. According to the age group division, this significantly increased protection was observed among Pathan patients above the age of 30 years. In addition to this, the genotypic analysis of both the genes simultaneously showed that the GSTT1 null and GSTM1 null together confer a significantly elevated protection against leukemia in Pathan males. On the other hand, the simultaneous genotype analysis of GSTT1 and GSTM1 among Punjabi patients (Males and Females) of all age-groups showed that the deletion of both GSTT1 and GSTM1 imparted significantly increased protection against leukemia development. However, the Punjabi females showed a considerably increased protection with deletion of both genes, but the association was not significant due to small number of samples. The difference of results among males and females might be related to the sex-specific hormones, which vary among both genders and hence affect their susceptibility towards disease (Chan *et al.,* 2011).

CONCLUSION

In the current study, the GSTT1 null and GSTM1 null, independently or simultaneous deletion of both the genes conferred a considerable amount of protection among our population. However, the samples included in the current study were much lesser in number as compared to those in the mentioned studies. Secondly, the difference in the ethnicity of the samples might also have an effect on the results, since none of the study on association of GSTT1 and GSTM1 null polymorphisms in leukemia (ALL, AML, CML) from Pakistani population could be found and hence results were compared with studies on other populations. The association of the null polymorphisms of both genes with leukemia was not consistent among different studies, which might be attributed to the difference in the nature of environmental and chemical compounds to which the patients are exposed and due to the differences among the patients' characteristics considered in different studies, like age, gender, treatment status of patient, ethnic background etc. It is, therefore recommended that the study must be conducted on a larger cohort of samples from Pakistani population living in different areas of the country. A larger number of samples must be included each for different sub-types of leukemia, different ethnic groups and other characteristics of patients like age, gender, locality, disease status etc.

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