Chapter one

Introduction and literature review

1.1 Introduction

Leukemia is a malignant progressive disease in which the bone marrow and other blood forming organs produce increased numbers of immature or abnormal leukocytes. A myeloid or lymphoid characterized by circulating neoplastic cells but also encompassing similar cases in which there are neoplastic cells in the bone marrow but not in the peripheral blood (Bain*et al.,* 2003).

These suppress the production of normal blood cells, leading to anemia and other symptoms . The main classification is into four types: acute and chronic leukaemias, which are further subdivided into lymphoid or myeloid . Acute Lymphoblastic Leukemia (All) is the most common malignancy in childhood .It accounts for one fourth of all childhood cancers and approxmately75% of all childhood leukemias(Hoffbrand*etal* .,2006).

Genetic polymorphisms of carcinogen detoxifying enzyme have been variously associated with the development of leukaemia. For example, deficiency of glutathione S transferases (GSTM1 and GSTT1), enzymes that detoxify electrophilic metabolites by catalysing their conjugation to glutathione. Glutathione S transferases (GST) are a family of enzymes involved in phase-II detoxification of endogenous and xenobiotic compounds. Polymorphisms in GST genes have been associated with susceptibility to different diseases (Aly*et al*., 2013).

1.2 Literature review

1.2.1 Acute lymphoblastic leukemia (ALL)

Is a malignant disorder that originates in a single B or T lymphocyte progenitor. Proliferation and accumulation of blast cells in the marrow result in suppression of hematopoiesis and, thereafter, anemia, thrombocytopenia, and neutropenia**(**Figure 1.1).Lymphoblasts can accumulate in various extramedullary sites, especially the meninges, gonads, thymus, liver, spleen, and lymph nodes. ALL has many subtypes and can be classified by immunologic, cytogenetic, and molecular genetics methods (Marshall*et al.*,2007). Acute lymphoblastic leukemia (ALL) is primarily a childhood disease, with the peak incidence between the ages of 2 to 3 years. It diminishes in frequency until it reaches a nadir from about the ages of 25 to 50, after which it increases to achieve a second, but minor, peak at ages older than 80, but progressively deteriorates with increasing age (Tkachuk*et al.*, 2007). Common presenting features are those of anemia and low platelets. There is a subtype, the Burkitt type ALL, that is more serious, presenting typically with prominent tissue infiltration,especially in the jaws or abdomen (Norman, 2009).

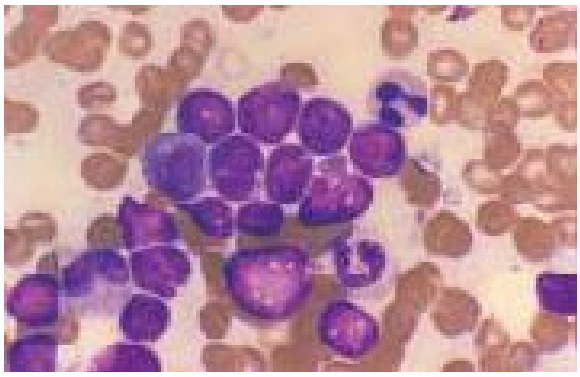


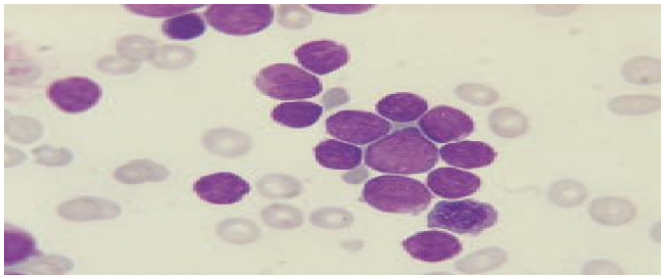
Figure (1.1):Blood film of patient with acute lymphoblastic leukaemia (Provan ,2003**) .**

1.2.1.1 Classification of Acute lymphoblastic leukemia (ALL)

1.2.1.1.1 Morphological Classification (French–American–British)

1.2.1.1.1.1L1Subtype

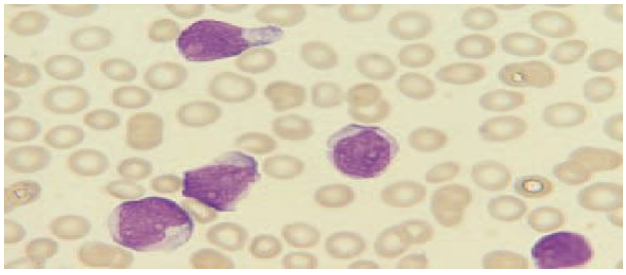
Small monomorphic type, small homogeneous blasts, single inconspicuous nucleolus, regular nuclear outline; commonest subtype(Figure1.2).



Figure(1.2):Peripheral blood film in L1ALL. Showing lymphoblasts and NRBC,thelymphoblasts vary in size but are relatively uniform in morphology. The smaller blast cells show some chromatin condensation (Bain, 2006).

1.2.1.1.1.2 L2Subtype

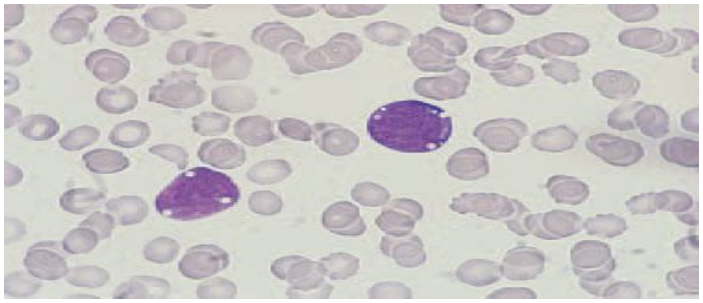
Large heterogeneous type larger blasts, more polymorphic and multinucleolate, irregular frequently clefted nuclei with conspicuous nucleol (Figure 1.3).



Figure(1.3):Peripheral blood film in L2 ALL. The blast cells are larger and more pleomorphic than in LI ALL and in this case have a more diffuse chromatin pattern; one of the blasts has a hand-mirror conformation (Bain, 2006) .

1.2.1.1.1.3 L3Subtype

Burkitt cell type—large homogeneous blasts, abundant strongly basophilic cytoplasm with vacuoles; associated with B-cell phenotype. Malignant tumour of haemopoietic precursor cells of the lymphoid lineage probably arising from the marrow in most cases (Figure1.4) (Hoffbrand*et al.*, 2005).



Figure(1.4):Peripheral blood film in L3 ALL. The blast cells are medium sized with strongly basophilic vacuolated cytoplasm. This case was shown to have a mature B-cell immunophenotype(Bain, 2006).

1.2.1.1.2 WHO Classification of ALL

The new WHO classificationdifferentiates subtypes based on phenotype to precursor B cell , precursor T cell and cytogenetic abnormalities. The FAB L1 and L2 designations are not used, and L3 is considered to be a leukemic phase of Burkitts lymphoma**(**Kern,2002).

Precursor B-cell ALL :Cytogenetic subgroups (with oncogenes involved) t(9;22)(q34;q11); BCR/ABL(the Philadelphia chromosome), t(v;11q23); myeloid-lymphoid leukemiagene MLLrearranged , t(1;19)(q23;p13); E2A/PBX1,and t(12;21)(p12;q22); TELAML1**(**Kern,2002).

1.2.1.1.3 Immunological classification of ALL

Besides morphologic analysis, the peroxidase technique is the most important basic method because lymphoblasts are always peroxidase-negative, regardless of their immunophenotype(Heilmeyer*et al*,.2005) .

1.2.1.1.2.1 B lineage

Pro B-ALL: HLA-DR+,TdT+,CD19+ (5% children; 11% adults).

Common ALL: HLA-DR+,TdT+,CD19+,CD10+ (65% children; 51% adults).

Pre B-ALL: HLA-DR+,TdT+,CD19+,CD10,cytoplasmic IgM+ (15% children; 10% adults).

B-cell ALL: HLA-DR+, CD19+,CD10,surfaceIgM+ (3% children; 4% adults)(Hoffbrand*et al.*, 2005).

1.2.1.1.2.2 T lineage

Pre-T ALL: TdT+,cytoplasmic CD3+,CD7+ (1% children; 7% adults).

T-cell ALL: TdT+,cytoplasmic CD3+, CD1a/2/3+,CD5+ (11% children; 17% adults) (Hoffbrand*et al.*, 2005).

1.2.1.2 Etiology

Unknown, some factors seem to be implicated:

Radiation: Exposure to moderate to high doses of ionizing radiation increases the incidence of leukemia. Fetuses and young children exposed to smaller doses of ionizing radiation also have an increased incidence of acute leukemia. Leukemia that develops after radiation exposure differs from de-novo AML in that a preleukemic phase is often present, chromosomal aberrations are frequent, treatment is less successful, and survival time is short(Schumacher*et al.*, 2000).

Chemicals:Several chemicals and drugs are leukemogenic. The incidence of leukemia is increased in Exposure to high doses of benzene causes patients treated with alkylating agents (e.gmelphalan, chlorambucil), epipodophyllotoxins (e.getoposide), anthracyclines, and actinomycin D, Most patients developing leukemia after exposutroe these agents develop AML. Similar to radiation-induced leukemia, chemical-induced leukemia

usually has a preleukemic phase, chromosomal abnormalities, poor response to treatment, and short survival(Schumacher*et al.*, 2000).

Chromosomal and Genetic Factors:Asmallnumber of childhood leukemias are associated with chromosomal disorders, such as Down syndrome and minefelter's syndrome. Several inherited disorders also predispose to leukemia, including Bloom' ssyndrome, Fanconi's anemia, and Li-Fraumeni syndrome. Patients with structural abnormalities of the retinoblastoma gene on chromosome 13 are also at increased risk of developing leukemia (Schumacher*et al.*, 2000).

Viruses: Viruses have been found to cause leukemia in animals. Viruses are also implicated in human leukemogenesis, especially in the chronic leukemias and lymphomas. With T.ALL HTLV (humanTcelllymphotrophic virus) in T cell ALL and hairy cell leukemia (Schumacher*et al.*, 2000).

Immunological Factors:Patient with acquired or congenital immune deficiency syndrome or subjected to prolonged immunosuppressive treatment has high incidence of leukemia (Schumacher*et al.*, 2000).

1.2.1.3 Incidence and pathogenesis:-

ALL is the most common form of leukaemia in children; its incidence is highest at 3-7 years, falling off by 10 years with a secondary rise after the age of 40 years. The common (CDI0+) precursor B type which is most usual in children has an equal sex incidence; there is a male predominance for T-cell ALL (T-ALL). The pathogenesis is varied. In a proportion of cases the first event occurs in the fetus *in utero,* with a secondary event possibly precipitated by infection in childhood. This is discussed further onp. 130. In other cases, the disease seems to arise as a posh1atal mutation in an early lymphoid progenitor cell. Commonest malignancy in

childhood with the majority of cases in the 2–10 age group (median 3.5 years). Five times more frequent in childhood than AML. Rare leukaemia in adults, 0.7 to 1.8/100,000 annually. In adults, there is a peak at 15–24 years and a further peak in old age (2.3/100,000 >80 years) (Hoffbrand*etal.,* 2005).

1.2.1.4 Clinical features

Clinical features are a result of the following.

Bone marrow failure Anaemia (pallor, lethargy and dyspnoea); neutropenia (fever, malaise, features of mouth, tlu'oat , respiratory, perianal or other infections; and thrombocytopenia (spontaneous bruises,purpura , bleeding gums and menorrhagia, epistaxis, rectal, and retina.

Organ infiltration Tender bones, lymphadenopathy (55%) , moderate splenomegaly(49%), hepatomegaly(45%), orchidomegaly and meningeal syndrome (headache, nausea and vomiting, blmring of vision and diplopia). Fundamental examination may reveal papilloedema and sometimes haemorrhage. Many patients have a fever which usually resolves after starting chemotherapy. Less common manifestations include testicular swelling or signs of mediastinal compression in T-ALL (Hoffbrand*etal*., 2006).

Infection: particularly chest, mouth, perianal, skin (Staphylococcus, Pseudomonas, HSV, Candida). Fever, malaise, sweats.

Signs of leucostasis e.g. hypoxia, retinal haemorrhage, confusion or diffuse pulmonary shadowing.Mediastinal involvement occurs in 15% and may cause SVC obstruction. CNS involvement occurs in 6% at presentation and may cause cranial nerve palsies especially of facial VII nerve, sensory disturbances and meningism(Themle*t al*., 2004).

1.2.1.5 Investigations and diagnosis

1.2.1.5.1 Full blood countand blood film

Total WBC usually high with blast cells on film but may be low (previously known as aleukaemicleukaemia).

Hb, neutrophils and platelets often low and clotting may be deranged (Kern, 2002).

1.2.1.5.2 Bone marrow aspirate biopsy

The bone marrow in ALL usually shows a monomorphic population of blasts, with marked decrease in normal hematopoietic precursors of all types. “Block” PAS reactivity may be present, although this occurs in less than half of ALL cases. Reactivity for myeloperoxidase, Sudan black B, and specific and nonspecific esterases are absent (Kern, 2002).

1.2.1.5.3 Immunophenotyping of blood or marrow blasts

A panel of monoclonal antibodies is used to differentiate ALL from AML. A further panel of B-and T-lineage markers and lymphocyte maturation markers subclassify ALL.Bone marrow heavily infiltrated with blasts (≥20%)(Theml*etal.,* 2004).

1.2.1.5.4Molecular studies

Fluorescence in situ hybridization (FISH) analysis as example for detection of all patients with acute lymphoblastic leukaemia (ALL) with hyperdiploidy or ETV6–RUNX1 fusion, detection of BCR–ABL1 fusion in adults with ALL and detection of other mutations of specific oncogenes (Theml*et al.*, 2004).

1.2.1.5.5Bone marrow cytogenetics

Cytogenetic analysis has become critical for prediction of outcome and selection of therapy in ALL. Chromosomal alterations are present in

≥75% of cases. There may be abnormalities in chromosome number or structural alterations in chromosomes, usually reciprocal translocations. Provides important prognostic information in both childhood and adult ALL. Abnormalities are detected in up to 85%. The major abnormalities are clonal translocations: t(9;22), t(4;11), t(8;14), t(1;19) or t(10;14) and other structural abnormalities (9p, 6q or 12p). If no structural abnormalities are present, the abnormalities can be classified by the modal chromosome number: <46 (hypodiploid); 46 with other structural abnormalities (pseudodiploid); 47–50 (hyperdiploid); >50 (hyper-hyperdiploid). With the exception of t (9;22) each has an incidence in the order of 5–10% or less,t(9;22)(q34;q11) produces the Philadelphia chromosome found in 5% of children and 25% of adults with ALL and is a very strong adverse prognostic factor in both; the resultant BCR-ABL hybrid product is the same 210 kDa protein detected in CML in 33% but is a smaller 180 kDa protein in 66%; it can be used for minimal residual disease detection, t (8;14) is associated with B-cell ALL (L3 morphology) and occurs in 5% of cases (dysregulates the mycproto-oncogene), t(1;19) is associated with B-cell precursor ALL; t(4;11) occurs in 80% of infants with ALL and 6% of adults and fuses the MLL gene from 11q23 to the AF4 genefrom 4q21 which can be detected by PCR; all these abnormalities are associated with refractory disease and early relapse hyper-hyperdiploidy (>50 chromosomes) confers a favourable prognosis; combined +4, +10 confers a favourable outcome in B-cell precursor ALL; patients with hypoploidy (<46 chromosomes) and pseudodiploidy fare less well(Table 1.1)(Kern, 2002).

Table (1.1): Frequencies of Common Genetic Aberrations in Childhood and Adult ALL (Marshall*etal*., 2007).

Abnormality	Children	Adults
Hyperdiploidy(>50chromosomes)	23–26%	6–7%
Hypodiploidy(<45chromosomes)	1%	2%
t(1;19)(q23;p13.3)	3% in white, 11% in black	2–3%
t(9;22)(q34;q11.2)	3%	25-30%
t(4;11)(q21;q23)	2%	3–7%
t(8;14)(q23;q32.3)	2%	4%
TEL-AML1 fusion	20–25%	0–3%
HOX11L2 over expression	2–3%	1%
LYL1 over expression	1.5%	2.5%
TAL1 over expression	3–7%	12%
HOX11 over expression	0.7%	8%
MLL-ENL fusion	0.3%	0.5%
Abnormal 9p	7–11%	6–30%
Abnormal 12p	7–9%	4–6%
del(7p)/del(7q)/monosomy 7	4%	6–11%
+8	2%	10-12%

1.2.1.5.6 Chest xray and Computed tomography CXR and CT

scan CXR and CT scan needed if ALL has B-cell or T-cell phenotype for abdominal or mediastinal lymphadenopathy respectively. Lumbar puncture mandatory to detect occult CNS involvement but may be postponed until treatment reduces high peripheral blast count to prevent

seeding .Bone marrow show lymphoblasts in ALL L1 (Themle*t al*., 2004).

1.2.1.6 Prognosis

The prognosis of childhood ALL has improved dramatically. Over 95% of children achieve a complete response, and over 80% of children have longterm disease-free survival and are presumed cured. The prognosis in adults is less optimistic; less than 40% of adults are cured. Efforts have been made to stratify treatment based on prognosis: patients with favorable prognostic factors can be treated less aggressively, whereas patients with adverse prognostic factors may be treated more aggressively from the time of diagnosis. Nearly all adults with ALL are considered high risk. The initial risk stratification in children is based on age and WBC count, and then readjusted after cytogenetic results are available. An anthracycline is added to the induction regimen in children considered at high risk, and the intensity of therapy during the consolidation phase is increased. Patients at particularly high risk (such as those with a Philadelphia chromosome) may be considered for allogeneic bone marrow transplant (BMT) in first CR (Kern, 2002).

1.2.1.7 Treatment

This may be conveniently divided into supportive and specific treatments. General supportive therapyincludes the insertion of a central venous cannula, blood product supportand prevention of tumor lysis syndrome. Any episode of fever must be treated promptly. Specific therapy of ALL is with chemotherapy and sometimes radiotherapy. These are used in various phases in a treatment course which usually has four components. The protocols differ in infants, children and adults and are also riskadjustedto reduce the treatment given to patients with good prognosis.

The rare B-ALL is treated by a different protocol involving short and intensive courses of drugs. Other treatments are allogeneic stem cell transplantation is indicated for patients with Ph (BCR-ABL) positive ALL, for primary refractory disease or for early relapsed disease, imatinibmesylate is included into treatment protocols for patients with BCR-ABL positive ALL. Glutathione S-transferases (EC2 .5.1.18) comprise a family of isozymes that can catalyze the covalent addition of the tripeptide glutathione (7-Glu-Cys-Gly) to a structurally diverse set of physiological and xenobiotic electrophiles (Hoffbrand*etal.*, 2006) .

1.2.2 Detoxification system

The detoxification systems are highly complex, show a great amount of individual variability, and are extremely responsive to an individual's environment, lifestyle, and genetic uniqueness. This review of the detoxification systems is meant to whet the appetite for a more in-depth look at detoxification and, as such, it may raise more questions than it answers. The detoxification system in humans is extensive, highly complex, and influenced by myriad regulatory mechanisms. This complexity of the detoxification systems and the individual uniqueness shown in ability to detoxify substances suggests one test or type of assay to fully assess detoxification status will not be possible. For some enzyme activities, such as the Cyp2D6, which are primarily influenced by genetics, determination of slow or fast metabolizers is possible with one gene analysis. However, to understand the detoxification profile of an individual, other approaches are necessary. The first analyses to identify detoxification pathways were challenge tests, in which a known amount of a substance was ingested and the amount of metabolite found in urine over a specified time period was collected and quantified. This type of test

takes into account myriad factors influencing detoxification activities and remains the standard today. The search for appropriate challenge substances and better methods to interpret the influence of the variety of detoxification activities on overall health and well-being is thechallenge we face (Deann*et al.*, 1998).

1.2.2.1 Enzyme Systems Involved in Detoxification

1.2.2.1.1 The Phase I System

The Phase I detoxification system, composed mainly of the cytochrome P450 supergene family of enzymes, is generally the first enzymatic defense against foreign compounds. Most pharmaceuticals are metabolized through Phase I biotransformation. In a typical Phase I reaction, a cytochrome P450 enzyme (CypP450) uses oxygen and, as a cofactor, NADH, to add a reactive group, such as a hydroxyl radical. As a consequence of this step in detoxification,reactive molecules, which may be moretoxic than the parent molecule, are produced. If these reactive molecules are not further metabolized by Phase II conjugation, they may cause damage to proteins, RNA, and DNA within the cell. Several studies have shown evidence of associations between induced Phase I and/or decreased Phase II activities and an increased risk of disease, such as cancer, systemic lupus erythematosus, and Parkinson's disease. Compromised Phase I and/or Phase II activity has also been implicated in adverse drug responses (Deann*et al.*, 1998).

1.2.2.1.2 The Phase II System

Phase II conjugation reactions generally follow Phase I activation, resulting in a xenobiotic that has been transformed into a water-soluble compound that can be excreted through urine or bile. Several types of conjugation reactions are present in the body, including glucuronidation,

sulfation, and glutathione and amino acid conjugation. These reactions require cofactors which must be replenished through dietary sources (Deann*et al.*, 1998).

1.2.2.1.3 The Phase III System

Recently, antiporter activity (p-glycoprotein or multidrug resistance) has been defined as the Phase III detoxification system.Antiporter activity is an important factor in the first pass metabolism of pharmaceuticals and other xenobiotics. The antiporter is an energy-dependent efflux pump, which pumps xenobiotics out of a cell, thereby decreasing the intracellular concentration of xenobiotics. Antiporter activity in the intestine appears to be co-regulated with intestinal PhaseI Cyp3A4 enzyme.16 This observation suggests the antiporter may support and promote detoxification. Possibly, its function of pumping nonmetabolizedxenobiotics out of the cell and back into the intestinal lumen may allow more opportunities for Phase I activity to metabolize the xenobiotic before it is taken into circulation ((Deann*et al.*, 1998).

1.2.3 Glutathione S-transferases (GSTs)

1.2.3 1Definition

Glutathione S-transferases (GST), a large group of dimeric enzymes play a critical role in the defence against oxidative stress products and various electrophilic compounds. GST, as phase II metabolic enzymes, catalyse the conjugation of reduced glutathione with potential genotoxic substances, especially those from tobacco smoke. (Reszka, 2001)

1.2.3.2 Classification of Glutathione S-transferases

Glutathione S-transferases (GSTs) constitute multifunctional enzymes that are coded by at least eight distinct loci: α (GSTA); μ (GSTM); θ (GSTT); π (GSTP); σ (GSTS); κ (GSTK); ω (GSTO); and ζ(GSTZ), each one

composed of one or more homodimeric or heterodimeric isoforms. These enzymes are involved in the conjugation reactions between glutathione (GSH) and a variety of potentially toxic and carcinogenic compounds. Additionally, GSTs display peroxidase activity and this can protect against oxidative damage. The deficiency in the activity of this enzyme can be derived from the inherited GSTs polymorphisms; GSTT1 (22q11.23), GSTM1 (1q13.3) and GSTP1 (11q13) (Aly, 2013) .

1.2.3.3 Glutathione S-transferase mu 1 (GSTM1)

The GSTM1 gene is highly polymorphic and is located on chromosome 1p13.3. A wide range of variation in *GSTM1* homozygous deletion polymorphism (approximately 20–67%) has been observed globally with regard to various ethnicities. It is often hypothesised that, due to the lack of functional GSTT1 and/or *GSTM1*, the null phenotype is unable to efficiently perform the conjugation reaction (biotransformation) and the subsequent elimination of toxic products via urine and bile. The null variant of *GSTM1* is of particular interest, as a plethora of studies have demonstrated the difference in susceptibility, exposure to environmental toxicants, resistance to chemotherapy treatment, variability in drug response, manifestation of several diseases, and, most importantly, cancerous outcomes.The four other members of the GSTμ subfamily, *i.e.*, *GSTM2*, *GSTM3*, *GSTM4*, and *GSTM5*, exhibit high levels of sequence homology and substrate specificity with *GSTM1*. Among these genes, *GSTM1* has largely been studied due to its null genotype. Although a large number of studies have attempted to associate the *GSTM1*-null genotype with cancer risk, the results are inconclusive. Several studies have attempted to identify the association of *GSTM1* null with cancer risk through meta-analysis using the existing literature; however, these

analyses failed to show a significant association of *GSTM1* with cancer. These observations prompted us to search for the functional relevance of this “well known gene” with other family members that are relatively less studied. A possible explanation of the apparently inconsistent results could be that other members of the GST family compensate for the absence of a functional GSTM1 enzyme (Bhattacharjee*et al.*, 2013).

1.3Previous Studies

Aprevious Study done to examine thehigher frequency of glutathione S transferase deletions in black children with acute lymphoblastic leukemia (Chen*et al*., 1996), and they found that the GSTM1 null polymorphism was detectedin (41.2%) black patients, frequencies not significantly different (*Pvalue*=0.19)(Chen *et al*., 1996).

Study done by(Saadat*et al*., 2002) to study The glutathione ***S***-transferase mu polymorphism and susceptibility to acute lymphocytic leukemia, showed that The null genotype of GSTM1 was significantly more common among leukemic patients compared with the normal control group (55.3 vs. 32.0%; *P*<0.025). The absence of the GSTμ enzyme may increase the risk of the development of leukemia. (Saadat*et al*., 2002)

Study done by (Moulik*et al*.,2014) to examine a case–control study and meta-analysis for Glutathione-S-transferase polymorphism and acute lymphoblastic leukemia (ALL) in north Indian childrenwhich show GSTT1 null mutation (odds ratio (OR) 2.54, 95% confidence interval (CI) 1.50–4.32) and GSTP1 homozygous mutation (OR 3.13, 95%CI 1.48–6.59) were found to increase the risk of childhood ALL, while GSTM1 did not alter the risk. (Moulik*et al*.,2014) .

Study done by (Tang *et al*., 2013)performed an extensive meta-analysis on 26 published case-control studies to assess the strength of association between childhood acute leukemia risk and polymorphisms of GSTM1 and GSTT1, a stratification analysis showed that the risk of GSTM1polymorphism are associated with childhood acute leukemia in group of Asians (OR = 1.94; 95%CI), Blacks (OR = 1.76; 95%CI,) With respect to GSTT1polymorphism, significant association with childhood

acute leukemia risk was only found in subgroup of Asian(Tang*et al*., 2013).

Chapter Two

Rationale and Objectives

2.1 Rationale

Based on National Cancer Institute records; acute lymphoblastic leukemia has increasing prevalence in Sudanthere is many studies revealed association of acute lymphoblastic leukemia with certain genetic abnormalities.

The frequencies of GSTs polymorphic alleles, especially GSTM1 and GSTT1 have been reported in ALL and there are several studies have been published on the relationship between GSTM1 null polymorphism and ALL. To the best of our knowledge, there are no published reports about the association between GSTM1 null polymorphism and ALLfor patients attendedNational Cancer Institute, so this study may fill the gab regarding this polymorphism and its association with Sudanese ALL patients attended toNational Cancer Institute.

2.2 Objectives

2.2.1 General Objective

To study the association between GSTM1 null polymorphism and childhood acute lymphoblastic leukemia attended toNational Cancer Institute in Gezira State, Sudan.

2.2.2 Specific Objectives

- To determine the frequency of GSTM1 null polymorphism among Sudanese patients with childhood acute lymphoblastic leukemia.
- To examine the role of GSTM1 null polymorphism as risk factor for childhood acute lymphoblastic leukemia.
- To correlate the presence of this polymorphism with patient's hematological parameter, age, gender, family history of ALL or other malignancy.

Chapter Three

Materials and Methods

3.1 Study design /area/ and duration

This is a case control study conducted at National Cancer Institute, Gezira State in the period from February to April 2016.

3.2 Study population and sample size

Forty ALL patients referring to National Cancer Institute as well as thirty apparently healthy volunteers as a control groupwere enrolledin this study.

3.3 Inclusions criteria

Patients with ALL and age range up to 15 years.

3.4 Exclusion criteria

No exclusion criteria.

3.5 Ethical considerations

The consent of the selected individuals to the study was taken from parents of patients after being informed with all detailed objectives of the study and it is health benefit in future.

3.6 Data collection

A questionnaire was filled for each of the patients (see appendixes).

3.7 Data presentation

The data were presented in tables and figure.

3.8 Sampling

Non-probability sampling method was used (only who accepted study tests / volunteers) were involved.

3.9 Sample collection

Three milliliter of venous blood collected in a plastic container containing EDTA anticoagulant and preserved at -20°C for DNA extraction.

3.10 Methodology

3.10.1 Complete Blood Count (CBC) and blast percentage

The results of CBC and blast percentage were taken from patients filesatNational Cancer Institute.

3.10.2 DNA extraction by salting out method

3.10.2.1 Principle

RBCs werehemolysed by alkaline solution (Red Cells lysis buffer), Then the membranes were digested by solution containing detergent and proteases (White Cells Lysis Buffer) then protein was precipitated out by saturated NaCl and centrifugation finally DNA was precipitated by absolute ethanol then washed by 70% ethanol and eluted in TE buffer.

3.10.2.2 Procedure

300 µL of blood sample was placed in 1.5 ependorf tube, 1000 µL RCLB was added to the tube, mixed well, centrifuged at 2500 rpm for 10 minutes, supernatant was discarded and the pellet (WBCs) washed again with 1000 µL RCLB(repeated until clear pellet was optained). 300 µL WCLB, and 10 µL of 10% SDS were added to the clear white pellets. The mixure was incubated for 1 hour at room temperature.100 µL of 6ml Nacl was added to precipitate the protein and mixed well by vortex. 200 µL f ice cold chloroform were added to tube and centrifuged at 1200 rpm for 6 minate. The aqueous phase as transferred carefully to clean ependorf tube, and to which double volume of cold absolute ethanol was added to precipitate the DNA. The tube was centrifuged at 12000 rpm for 5 minutes. The supernatant was poured off without disturbing the

precipitate and then washed with 600 µL 70% ethanol. The tube content was centrifuged at 7000 rpm for 5 minutes, the ethanol was discarded and the tube was left to air dry. The pellets were resuspended in 50 µL TE buffer and leaved to dissolve overnight.

3.10.3 Determination of DNA quality and purity

Part of the DNA solution was mixed with loading dye (1 in 5) and DNA quality and purity was determined using gel electrophoresis.

3.10.4 DNA storage

DNA was transferred into 1 ml eppindroff tube and preserved at -20 ^{o}C until PCR is performed.

3.10.5 Molecular analysis

3.10.5.1 Detection of GSTM1null genotype

The primers sequence used are shown in table(3-1)

Table (3-1) Oligonucleotideds sequences for GSTM1

Primer	Primer direction	Sequence	Product size (bp)
GSTM1	Forward primer	5’GAA CTC CCC TGA AAA GCT AAA GC3’	219
	Reverse primer	5’GTT GGG CTC AAA TAT ACG GTG G3’	
Beta globin	Forward primer	5'GAAGAGCCAAGGACAGGTAC-3'	500
	Reverse primer	5'-CAACTTCATCCACGTTCACC-3'	

All patients were screened for the presence of GSTM1 null genotype using allele specific PCR.

PCR mixture of 20 µL was prepared using premix master mix (Maxime^{TM}PCR premix kit (i-Tag)) for each sample, with positive and negative controls in sterile0.5 ml eppindroff tube as follow:

Table (3-2) PCR mixture

Reagents	Volume
Double D.W	14 µL
Forward primer	1 µL
Reverse primer	1 µL
Template DNA	4 µL
Total reaction volume	20 µL

Optimized cycling protocol for PCR analysis of GSTM1 on the TECHN (TC-312) as follow:

Table (3.3) PCR protocol

Profile	Temperature	Timeduration	Number of cycles
Initial denaturation	94^{o}C	10 minutes	1
Denaturation	95^{o}C	1 minutes	35
Annealing	62^{o}C	1 minutes	
Extension	72^{o}C	1 minutes	
Extension final	72^{o}C	10 minutes	1

3.10.5.2 Demonstration of PCR product

Five µL of the PCR product (ready to load) was electrophoresed on 2% agarose gel and was stained with ethedium bromide, 1X TBE buffer was used as a running buffer. The voltage applied to the gel was 100 volt with time duration of 10 minutes . 50bp DNA ladder was used as molecular weight marker with each patch of samples.finally, PCR product was demonstrated by gel documentation system ”SYNGENE”.

3.11 Interpretation of result

The GSTM1 normal genotype will give band (219 bp) while GSTM1 null give no band. Beta globin primer was used as positive control for all negative GSTM1null genotype and give band (500 bp)band.

3.12 Data Analysis

Data of this research was analyzed using the statistical package for the social sciences (SPSS) version 11.5 (independent t-test was usedto compare patient's age and hematological parameter in null and normal GSTM1 genotype, chi square test wasused to investigate the correlation between GSTM1 null polymorphism with gender and family history of ALL, regression was used for examine GSTM1 null polymorphism as risk factorfor ALL.

Chapter Four

The Results

4.1 Demographic data

Study included 70 samples 40 of there were cases and 30 of them were control(Figure 4.1).The results showed that 26/40 (65%) of the samples were males and 14/40 (35%) were females(Figure 4.1).

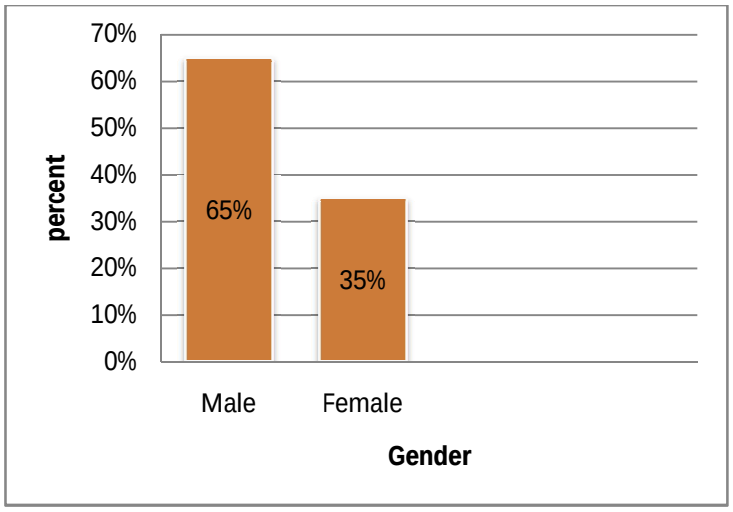


Figure (4.1) Gender distribution among the both group

The results showed that 6/40 (15%) of patients have family history formalignant disease and 34/40 (85%) have no family history(Figure 4.2).

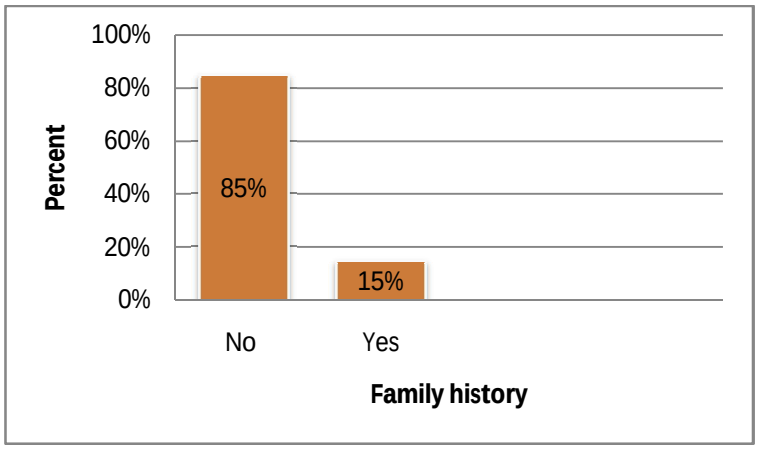


Figure (4.2) frequency of family history of disease among patient samples

The results showed that 30/40 (75%) of the cases were GSTM1 nullgenotype, and 10/40 (25%) of them had normal GSTM1genotypewhile16/30 (53%) of the controlhad GSTM1 null and 14/30 (47%) had normal GSTM1 genotype(Figure 4.3).

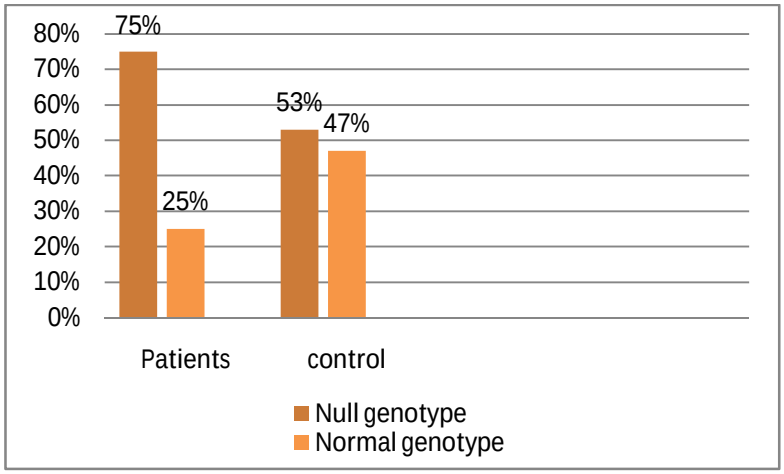


Figure (4.3) GSTM1 genotype among patients and control group

4.2 Comparisons of hematological findings in GSTM1 null group and normal GSTM1 group

Comparisons of Hb concentration, TWBCs, Platelets count, and Blast percentage in patients with GSTM1 genotype and those with null genotype showed no statistically significant differences (Table 4-1).

Table (4.1) The mean of hematological findings related to GSTM1 genotype among cases

Variables	Normal GSTM1		Null GSTM1		*P.value*
	Mean	SD	Mean	SD	
Blasts	**72.0**	**15.31**	**59.4**	**26.65**	**0.076**
Hb	**5.9**	**2.76**	**7.0**	**2.60**	**0.269**
TWBCs	**54.4**	**66.27**	**66.6**	**56.23**	**0.574**
Platelets	**42.1**	**41.34**	**60.6**	**94.81**	**0.556**

4.3Comparison of agein GSTM1 null group and normal GSTM1 group

The mean of age was not differ significantly higher (*p.value*= 0.373) in patients with normal genotype(mean±SD:7.8±4.05) compared to those with null genotype(mean±SD:5.6±3.10)(Table 4.2).

Table (4.2) The mean of age among the case

Variables	Normal GSTM1		Null GSTM1		*P.value*
	Mean	SD	Mean	SD	
Age	**6.5**	**3.10**	**7.8**	**4.05**	**0.373**

4.4 Correlation between gender and GSTM1 polymorphism

The results showed no statistically significant correlation between genderand GSTM1 polymorphism (Table 4.3).

Table (4.3) Correlation between gender and GSTM1 polymorphism

Gender	GSTM1		Total	*p.value*
	Null	Present		
Male	18 45%	8 20%	26 65%	o.262
Female	12 30%	2 5%	14 35%	
Total	30 75%	10 25%	40 100%	

4.5 Correlation between family history of Malignant disease and GSTM1 polymorphism

The results showed that no statistically significant correlation between family history and the GSTM1 polymorphism (*p.value*=0.620) (Table 4.4).

Table(4.4) Correlation between family history andGSTM1polymorphism

Previous history	GSTM1		Total	*p.value*
	Null	Present		
Yes	5 12.5%	1 2.5%	6 15%	0.620
No	25 62.5%	9 22.5%	34 85%	
Total	30 75%	10 25%	40 100%	

4.6Association of ALL and GSTM1 null polymorphism

The result showed that GSTM1 null genotype was not a risk factor for ALL and there is a no statistically significant Associationbetween GSTM1 null polymorphism and childhood ALL (OR: 1.34, 95%C I :1.11 - 1.39, P=0.059).

Chapter Five

Discussion, Conclusion and Recommendations

5.1 Discussion

Heterogeneity in patients response to chemotherapy is consistently observed across differences in drug disposition and effects is emerging as a tool to predict efficacy and toxicity of drugs. Glutathione S transferase are involved in the metabolism and toxicity from cytotoxic chemotherapy (Mossallam*etal.*, 2006) .

Genetically based differences in carcinogen metabolism have been related to polymorphisms of the cytochrome P450IA1 gene (CYPIA1) and the null genotypes of glutathione S-transferase classes mu and theta (GSTM1 and GSTT1). Thus, it is unlikely that polymorphisms of *GSTM1*, *GSTT1*, or *CYPIA1* represent susceptibility factors for breast cancer in Caucasians or African-Americans (Bailey*et al.*,1998).

This case control study was investigated the association between GSTM1 Null polymorphism and childhood acute lymphoblastic leukemia.

The characteristic features of the study population showed the frequency of gender of male 26(65 %),to female 14 (35%).

Our study didn't find a significant association between the GSTM1 null polymorphism and ALL (OR: 1.34, 95%CI:(1.11 - 1.39, P=0.059) and this findings is in agreement with previous study done byMoulik*et al*,who reported that there was no significant association between GSTM1 null and ALL(*O.R.* = 1.29), also disagree with reports ofstudy by Chen*etal.*

The mean of blasts percentage in GSTM1 null cases was insignificantly lower than in those with normal GSTM1 genotype (*P.value*= 0.076).

The mean of Hb, TWBCs and platelets was higher among patients with GSTM1 null genotype than those with normal GSTM1 genotype and the difference was not statistically significant(*P. value*: 0.269 , 0.574 & 0.556respectively),and this findings is in agreement with previous study done byBakhiet*et al*who reported that there were no significant association betweenpatient genotype and TWBCS count *p.value*(0.418) Hemoglobin(Hb),*p.value*(0.463), Red Blood Cells (RBCs) *p.value*(0.458), Hematocrit(HCT%) *p.value*(0.610) and platelets (Plts) *p.value*(0.339). The mean of age in GSTM1 null cases not statistically significant higher than in normal GSTM1 cases (*P.value*= 0.373). There was not statistically significant association between GSTM1null polymorphism and gender *(P.value* = 0.262).

There is insignificant association between GSTM1 null polymorphism and family history of ALL (*P.value* = 0.620).

5.2 Conclusions

- There is statistically insignificant association between GSTM1 null polymorphism and ALL.
- Nostatistically significant correlation was foundbetween GSTM1 null polymorphism and each of age, gender, and family history of malignant disease.
- No statistically insignificant difference was found in mean of blast,Hb concentration, white blood cell counts, and platelets countsin patients with nullpolymorphism and those with GSTM1 gene.

5.3 Recommendations

- Another study should be conducted with larger sample size to Supportthisstudy of GSTM1 null polymorphism with ALL.
- Another study should be conducted including information on the subtype of childhood ALL is demanded to clarify the relationship between the GSTM1 polymorphisms and subtypes of childhood ALL.

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Appendixes

Appendix 1:

Questionnaire

Sudan University of Science and Technology

College of Graduate Studies

Glutathione S Transferase Mu1 Null Polymorphism among Childhood Patients with Acute Lymphoid Leukemia

I D: ……

Age:……..

Gender: …….

Do you have history of ALL in family: Yes( ) No( )

Hematological parameters :

HB:……..

TWBCs:…….

Platelets:…….

Blast:……..

GSTM1 genotype: Null( ) Present( )

Appendix 2:

Reagent

Red Cell Lysis Buffer (RCLB):

Add 8.3 gm of NH4CL, 1gm KHCO3, 1.8 ml 5% EDTA and 1liter of distilled water.

White Cell Lysis Buffer (WCLB):

1.576 gmTris-HCL, 1.088 gm EDTA, 0.0292 gmNaCl, 0.2%SDS, and 100 ml distilled water.

TE buffer:

2.42 Tris base, 0.57 ml acetic acid, 50µl EDTA (0.01 M), and 100 ml distilled water.

6 M NaCl:

35 gm of NaCl added to 1 Liter of distilled water

Appendix 3: Images

TECHNE

(TC 312)

Gel electrophoresis