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**Sudan University of Science and Technology**

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**Glutathione S Transferase Theta1 Enzyme Null Polymorphism in  
Patients with Chronic Lymphocytic Leukemia**

التغير الشكلي لغياب جين إنزيم الجلوتاثيون اس ترانسفيريز ثيتا 1 عند مرضى سرطان الدم  
الابيض الليمفاوي المزمن

A dissertation submitted for partial fulfillment for the requirement of M.Sc degree  
in medical laboratory science ( Hematology and Immunohematology)

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March 2016

# الآية

قال تعالى:

( إِنَّمَا أَمْرُهُ إِذَا أَرَادَ شَيْئًا أَنْ يَقُولَ لَهُ كُنْ فَيَكُونُ )

صدق الله العظيم

(يس الآية 82)

***Dedication***

*To my mother*

*To My father*

*To My husband*

*To My brothers*

*To My friends*

*To Mayar*

## **Acknowledgment**

Thanks for Allah would be before anything as without Allah will, this work would not be completed. Then, I have to extend a word of thanks for my supervisor who stands beside me and gave me help and support and his time to read my research, my grateful extend to Al Neelain University and my teacher Mohammed Alfatih who helped me and stood beside me and for all patients who gave me specimen to do this research, My grateful appreciation extended to my colleagues Marwa, Shima, Amel with whom I've had the honor to work.

Special thanks for my friend Khawla for her support and for flowcytometer center and it is top manager Dr. Osama Ali, Eiman and Yasmin for their help and support.

## **Abstract**

Glutathione S-transferases (GSTs), a superfamily of phase II metabolic enzymes, catalyze the conjugation of glutathione with reactive electrophiles and thus detoxify procarcinogens and carcinogens.

This is analytical case control study, conducted in Khartoum state during the period from March to August 2015 to evaluate the association of GSTT1 Null polymorphism in patients with chronic lymphocytic leukemia. A total of 37 patients diagnosed with CLL attended to the flowcytometer center , Khartoum-Sudan and 30 healthy volunteers as control group were enrolled in this study .Three milliliter (3ml) of venous blood was collected from each participant in ethylene diamine tetra acetic acid (EDTA) anticoagulant container . For molecular analysis DNA was extracted from blood samples by salting out method. The detection of GSTT1 genotype was done using allele specific polymerase chain reaction method.

The results were analyzed by statistical package for social sciences (SPSS) computer program.

Comparison of patients and controls showed that; among the 37 patients with chronic lymphocytic leukemia, 28 patients (75.7%) showed null genotype while 9 patients (24.3%) showed normal genotype for GSTT1 gene .while among 30 control 5(17%) had null genotypes and 25(83%) were normal for GSTT1 gene With significant statistical association(OR=15,95%CI and P.value=0,00.).

The result showed that the GSTT1 null genotype was significantly more common in elder age(P.value =0.00) and no significant association between sex and GSTT1 genotype in study group(P.value=0.37).

In summary results demonstrated that GSTT1 null polymorphism is a risk factor for CLL in Sudanese patients.

## المستخلص

جلوتاثيون اس ترانسفيريزيس هي عائله كبيره من انزيمات المرحلة الثانية للابيض تحفز اقتران الجلوتاثيون بالالكترونات التفاعليه وهكذا تزيل السموم المسرطنه.

هذه دراسة مقارنة حالة ضابطة تحليلية أجريت في ولاية الخرطوم في الفترة ما بين مارس الى اغسطس 2015 لتقييم علاقه غياب انزيم الجلوتاثيون اس ترانسفيريز ثيتا 1 في مرضى سرطان الدم الابيض الليمفاوى المزمن .تم إختيار سبعة وثلاثون مريض شخوصو بالمرض من الفلوسايتوميترسنتر ، الخرطوم ، السودان ' وثلاثون أصحاء وعوملوا كعينات ضابطه.

تم أخذ عينة وريدية 3 مل وضعت في وعاء بلاستيكي يحتوى على إيثايلين ثنائى الامين رباعى حامض الاستيك لمنع التخثر.

للتحليل الجزيئى تم استخلاص الحمض النووى منزوع الاوكسجين عن طريق الترسيب بواسطة الملح ، التحليل الجينى حدد بطريقة تفاعلات البلمرة المتسلسل.

تم تحليل النتائج بإستخدام برنامج الحزم الإحصائية للعلوم الإجتماعيه المحوسب.

مقارنة المرضى والعينات الضابطه اظهرت ان في 37 من مرضى سرطان الدم الابيض الليمفاوى المزمن ، 28 من المرضى 75.7% اظهروا غياب التركيب الجينى بينما 9 من المرضى 24.3% اظهروا تركيب جينى طبيعى للانزيم، في حين ان 30 من العينات الضابطه 5 (17%) لديهم غياب للتركيب الجينى و 25 (83%) لديهم تركيب جينى طبيعى للانزيم .

مع وجود علاقة ذات دلالة احصائية واضحه .حيث كانت القيمه الشاذه = 15 والقيمة المعنوية = 0.00.

وقد اظهرت النتائج ان غياب التركيب الجينى لانزيم الجلوتاثيون اس ترانسفيريز ثيتا 1 اكثر شيوعا في الاعداد الكبيرة (القيمة المعنوية = 0.00) و لا يوجد علاقة معنوية للجنس على الجين تحت الدراسة القيمة المعنوية = 0.37 بصورة مختصرة أظهرت الدراسة ان غياب الجين تحت الدراسه يشكل عامل خطوره لدى مرضى سرطان الدم الابيض الليمفاوى المزمن.

## Abbreviation

ALL	Acute Lymphoid Leukemia
AML	Acute Myeloid Leukemia
CD	Cluster of Differentiation
CLL	Chronic Lymphocytic Leukemia
DNA	Deoxyribo Nucleic Acid
DW	Distle Water
EDTA	Ethylene Diamin Tetra Acidic Acid
FAB	French American British
FISH	Fluorescent In Situ Hybridization
GSTT	Glutathione S Transferase Thita
Ig	Immunoglobulin
L	Lymphoblast
M	Myeloblast
OR	Odd Ratio
PCR	Polymerase Chain Reaction
RCLB	Red Cell Lysis Buffer
SPSS	Statistical Package for Social Sciences
TE	Tris Edta
WCLB	White Cell Lysis Buffer
WHO	World Health Organization

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# **Chapter One**

## **Introduction and Literature Review**

### **1.1 Introduction**

The leukaemias are a group of disorders characterized by the accumulation of malignant white cells in the bone marrow and blood. These abnormal cells cause symptoms because of:

bone marrow failure(i.e. anaemia, neutropenia, thrombocytopenia) and infiltration of organs (e.g. liver, spleen, lymphnodes, meninges, brain, skin or testes).The main classification is into four types acute and chronic leukaemias, which are further subdivided into lymphoid or myeloid (Hoffbrand *et al.* 2006).

Chronic lymphocytic leukaemia accounts for about 25% of all leukaemias. In adults over the age of 50 years it is the most common form, particularly in the West. In the Far East, its incidence is low. CLL affects twice as many males as females, with a peak incidence between 60 and 80 years. CLL is rarely diagnosed below the age of 40 years and is even more rare below 30years. In the last 20 years ,they have seen several patients in their late 20s. In such cases, a diagnosis of follicular lymphoma needs to be excluded. Of all the leukaemias, CLL has the highest familial incidence, which can be documented in 5–10% of patients (Hoffbrand ,*et al.* 2005).

Glutathione S-transferase (GST) theta1 (GSTT1), is a member of a superfamily of proteins that catalyze the conjugation of reduced glutathione to a variety of electrophilic and hydrophobic compounds. Human GSTs can be divided into five main classes : alpha, mu, pi, theta, and zeta. The theta class includes GSTT1, GSTT2, and GSTT2B. GSTT1 and GSTT2/GSTT2B share 55% amino acid sequence identity and may play a role in human carcinogenesis. The

GSTT1 gene is haplotype-specific and is absent from 38% of the population. Alternative splicing of this gene results in multiple transcript variants. ([WWW.ncbi.nlm.nih.gov/gene/2952](http://WWW.ncbi.nlm.nih.gov/gene/2952)).

GSTT1 modulate the induction of other enzymes and proteins important for cellular functions, such as DNA repair. This class of enzymes is there fore important for maintaining cellular genomic integrity and, as a result, may play an important role in cancer susceptibility (Dadbrnpour *et al.* 2005).

## **1.2 Literature review**

### **1.2.1 Leukemia**

Leukemia is caused by the mutation of the bone marrow pluripotent or most primitive stem cells. This neoplastic expansion results in abnormal, leukemic cells and impaired production of normal red blood cells, neutrophils, and platelets. As the mutant cell line takes hold and normal hematopoiesis is inhibited, the leukemic cells spill into the peripheral blood and invade the reticuloendothelial tissue, specifically the spleen, liver, lymph nodes, and, at times, central nervous system. The leukemic stem cells have atypical growth and maturation capability. The mutant clone may demonstrate unique morphologic, cytogenetic, and immunophenotypic features that can be used to aid in the classification of the particular type of leukemia. Many of the leukemias have similar clinical features, but regardless of the subtype, the disease is fatal if left untreated (Ciesla, 2007).

The main classification is into four types: acute and chronic leukaemias, which are further subdivided into lymphoid or myeloid (Hoffbrand *et al.* 2006).

#### **1.2.1.1 Acute leukaemia**

Acute leukaemias are usually aggressive diseases in which malignant transformation occurs in the haemopoietic stem cell or early progenitors. Genetic damage is believed to involve several key biochemical steps resulting in an increased rate of proliferation, reduced apoptosis and a block in cellular differentiation. Together these events cause accumulation of the early bone marrow haemopoietic cells which are known as blast cells. The dominant clinical feature of these diseases is usually bone marrow failure caused by accumulation of blast cells although organ infiltration also occurs. If untreated these diseases are usually rapidly fatal but, paradoxically, they are also easier to cure than chronic leukaemias. Acute leukaemia is defined as the presence of over 20% of blast cells in the blood or bone marrow at clinical presentation. It

can be diagnosed with even less than 20% blasts if specific leukaemia-associated cytogenetic or molecular genetic abnormalities are present. It is further subdivided into acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia(ALL) on the basis of whether the blasts are shown to be myeloblasts or lymphoblast.

Classification of acute myeloid (AML) and acute lymphoblastic (ALL) leukaemia according to the French-American-British (FAB) groups.

M0 undifferentiated

M1 without maturation

M2 with granulocytic maturation

M3 acute promyelocytic

M4 granulocytic and monocytic maturation

M5 monoblastic (M5.) or monocytic (MSb)

M6 erythroleukaemia

M7 megakaryoblastic

## **ALL**

L1 blast cells small, uniform high nuclear to cytoplasmic ratio

L2 blast cells larger, heterogeneous; lower nuclear to cytoplasmic ratio

L3 vacuolated blasts, basophilic cytoplasm (usually B-ALL) (Hoffbrand *et al.* 2006).

### **1.2.1.2 Chronic leukemia**

#### **1.2.1.2.1Chronic myeloid leukemia**

Chronic myeloid leukaemia is a clonal malignant myeloproliferative disorder believed to originate in a single abnormal haemopoietic stem cell. The progeny of this abnormal stem cell proliferate over months or years such that ,by the time the leukaemia is diagnosed, the bone marrow is grossly hyper cellular and the number of leucocytes is greatly increased in the peripheral blood. Normal

blood cell production is almost completely replaced by leukaemia cells, which, however, still function almost normally. Chronic myeloid leukaemia has an annual incidence of 1 to 1.5 per 100 000 of the population (in the United Kingdom about 700 new cases each year), with no clear geographical variation. Presentation may be at any age, but the peak incidence is at age 50-70 years, with a slight male predominance. This leukaemia is very rare in children. Most cases of chronic myeloid leukaemia occurs sporadically. The only known predisposing factor is irradiation, as shown by studies of Japanese survivors of the atomic bombs and in patients who received radiotherapy for ankylosing spondylitis. The clinical course of chronic myeloid leukaemia can be divided into a chronic or “stable” phase and an advanced phase, the latter term covering both accelerated and blastic phases. Most patients present with chronic phase disease, which lasts on average 4-5 years. In about two-thirds of patients the chronic phase transforms gradually into an accelerated phase, characterised by a moderate increase in blast cells, increasing anaemia or thrombocytosis, or other features not compatible with chronic phase disease. After a variable number of months this accelerated phase progresses to frank acute blastic transformation. The remaining one-third of patients move abruptly from chronic phase to an acute blastic phase (or blastic crisis) without an intervening phase of acceleration. (Provan.2003)

#### **1.2.1.2.2 Chronic lymphocytic leukaemia**

Chronic lymphocytic leukaemia (CLL) is by far the most common of the chronic lymphoid leukaemias and has a peak incidence between 60 and 80 years of age. The aetiology is unknown but there are geographical variations in incidence. It is the most common of the leukaemias in the Western world but rare in the Far East. In contrast to other forms of leukaemia there is no higher incidence after previous chemotherapy or radiotherapy. There is a seven fold increased risk of CLL in the close relatives of patients. The tumour cell appears to be a relatively mature B cell with weak surface expression of



immunoglobulin (Ig) M or IgD. The cells accumulate in the blood, bone marrow, liver, spleen and lymph nodes as a result of a prolonged lifespan with impaired apoptosis (Hoffbrand *et al.* 2006).

#### **1.2.1.2.2 .1 Clinical features**

CLL occurs principally in persons over 50 years of age (median age at presentation: 65-70 years). It is twice as common in males as compared to females. First-degree relatives of the patient have significantly increased risk of developing CLL and other lymphoid malignancies. Patient may present with weakness, fatigue, and weight loss, repeated infections (due to hypogammaglobulinaemia) and symptoms related to anaemia or thrombocytopaenia. Generalized lymphadenopathy is the most common presenting feature; mild to moderate splenomegaly is present in two-thirds of cases. About 25% of patients are asymptomatic and are discovered incidentally on clinical or laboratory examination (Kawthalkar. 2006).

#### **1.2.1.2.2 .2 Laboratory features**

##### **Peripheral blood examination**

Anaemia develops with progressive marrow replacement by tumour cells and is normocytic and normochromic. Other causes of anaemia in CLL include hypersplenism and autoimmune haemolysis. Autoimmune haemolytic anaemia occurs in about 10% of patients and is characterised by mild (antiglobulin) hyperbilirubinaemia, increased reticulocytes and spherocytes, and positive Coombs' test. Total leucocyte count is increased and is usually more than 50,000/cmm with >80% of cells being lymphocytes. Diagnosis of CLL should be considered if lymphocyte count is >5000/cmm in the absence of any other underlying cause in majority of cases, >90% of neoplastic cells are small, mature looking lymphocytes with high N/C ratio, scanty cytoplasm and dense, clumped chromatin. Nucleoli are not seen or are inconspicuous. In about 15% of cases, in addition to small, mature-looking lymphocytes, >10% (but <55%) cells

are prolymphocytes; this category is designated as CLL/PL. 'Smudge' or basket cells are a characteristic feature of CLL and are produced during spreading of blood film because of fragility of lymphocytes. Platelet count may be normal or decreased. Thrombocytopaenia becomes severe with progressive replacement of bone marrow by leukaemic cells. Other causes of thrombocytopaenia are immune destruction of platelets and hypersplenism (Kawthalkar. 2006).

### **Bone marrow examination**

National Cancer Institute criteria for diagnosis of CLL require lymphocyte count above 5000/cmm and bone marrow lymphocytes more than 30%. Assessment of pattern of infiltration of neoplastic cells in bone marrow has prognostic importance. Four patterns of infiltration can be recognised on bone marrow trephine biopsy—interstitial, nodular diffuse, and a combination of these. Diffuse pattern is associated with aggressive disease and worse prognosis. Nodular pattern is associated with a favourable prognosis (Kawthalkar. 2006).

### **Immunophenotyping**

Immunophenotyping provides definitive diagnosis and should be done in all cases before beginning therapy. It is particularly helpful in situations where lymphocytosis is less than 5000/cmm or when lymphocyte morphology is atypical. CLL cells usually express membrane phenotype of early B cells. Characteristically CLL cells express CD19, CD20 (weak), CD5, CD23, weak surface membrane immunoglobulin, and absent reactivity with FMC7 and with CD2. A single light chain is expressed on the surface of cells supporting the clonal origin of lymphocytes (Kawthalkar. 2006).

### **Cytogenetic analysis**

In CLL, conventional cytogenetic analysis is difficult due to the presence of only a few dividing neoplastic lymphocytes. Advent of fluorescent in situ hybridization (FISH) (which can detect chromosomal abnormalities in non-

dividing cells) has led to the identification of cytogenetic abnormalities in majority of patients with CLL. There is no single cytogenetic abnormality specific for CLL. The common abnormalities include 13q-, 11q-, trisomy 12, 17p-, and complex abnormalities. Some chromosomal abnormalities are associated with a poor outcome such as 11q- or 17p-(Kawthalkar.2006).

### **Immunological studies:**

Hypogammaglobulinaemia is observed in two thirds of patients and becomes severe with progression of disease; it is associated with increased risk of bacterial infections. M band (monoclonal protein) is observed in about 5% of patients(Hoffbrand *et al.* 2006).

### **Diagnosis**

Diagnosis of chronic lymphocytic leukemia should be considered when an elderly patient presents with absolute lymphocytosis in peripheral blood >5000/cmm, lymphocytes are small, mature looking with high N/C ratio, round to oval nuclei and clumped chromatin; smudge cells is a characteristic feature, immunophenotyping of such lymphocytes reveals B cell markers, and bone marrow shows increased numbers (>30%) of mature, small lymphocytes(Hoffbrand *et al.* 2006).

### **Differential diagnosis**

#### **Reactive lymphocytosis**

Reactive lymphocytosis occurs in infections by viruses (such as Epstein-Barr virus, cytomegalovirus, hepatitis, influenza), tuberculosis, toxoplasmosis, and rickettsia. Reactive lymphocytosis is transient and lymphocyte count is usually less than 5000/cmm. Morphology of reactive lymphocytes (large size, abundant cytoplasm, scalloping, dark blue edges) is also helpful. In doubtful cases surfacemarker analysis for monoclonality can be done (Kawthalkar. 2006).

### 1.2.1.2.2.3 Complications of CLL

**Infections:** Patients with CLL have increased risk of bacterial, viral, and fungal infections, due to disease itself (from hypogammaglobulinaemia) or following therapy (from neutropaenia and depletion of T lymphocytes).

**Autoimmune haemolytic anaemia and thrombocytopenic purpura.**

**Second malignancies:** There is increased risk of second malignancies in CLL such as skin cancer and solid tumours.

**Aggressive transformation:** Progression to a more aggressive disorder can occur such as prolymphocytic leukaemia or Richter's syndrome. Richter's syndrome is development of a diffuse large cell lymphoma in a patient with pre-existing CLL. It occurs in approx. 3 to 5% of cases. It should be suspected when patient develops unexplained fever, weight loss, and localized lymphadenopathy, particularly abdominal, and elevation of lactate dehydrogenase. It is refractory to chemotherapy and median survival is about 4 months (Kawthalkar. 2006).

### 1.2.1.2.2.4 Prognosis

**Staging:** prognosis depends primarily on the stage of the disease at diagnosis. There are two main staging systems for CLL: Rai (1975) and Binet (1981).

**Other prognostic factors:** There is a correlation between disease stage and median survival. However, the staging systems cannot accurately predict those patients in early stage who will have disease progression and those who will remain indolent. About 50% of patients in early stage will develop more advanced disease. Also, there is marked variation in disease progression amongst patients with similar stages. Assessment of risk of progression of disease can be done from various factors (Kawthalkar. 2006).

**Table(1.1) Staging system of CLL**

<b>Binet stages</b>	<b>Rai stages</b>
A: <3 lymphoid areas enlarged B: ≥3 lymphoid areas enlarged C: Anaemia (Haemoglobin<10 gm/dl) and/or splenomegaly thrombocytopaenia (Platelet count <1 lac/cmm)	0: Lymphocytosis only I: Lymphadenopathy II: Hepatomegaly and/or ± lymphadenopathy III: Haemoglobin<11 gm/dl IV: Platelet count <1,00,000/cmm.

### **1.2.1.2.2.5 Treatment**

Treatment is principally symptomatic and is not curative .Early stage disease is relatively benign and patients are often asymptomatic. Median survival of these patients is >10 years and therefore no treatment is usually indicated. Early institution of therapy does not improve survival and increases the risk of second cancers and development of resistance to treatment. Treatment is indicated when patient develops systemic symptoms related to disease (e.g. fever,weight loss, fatigue), evidence of disease progression (progressive worsening of anaemia or thrombocytopaenia, progressive enlargement of lymph nodes or spleen, lymphocyte doubling time<12 months), or presence of massively enlarged lymph nodes or spleen.Various forms of therapy for suppression of disease include chemotherapy, corticosteroids, andradio therapy. Chlorambucil is the commonly used drug in CLL. It is used either as high dose intermittent therapy every 4 to 6 weeks or as a continuous low dose treatment. Although both forms of therapy areequally effective, intermittent therapy is less toxic to bone marrow. Treatment, however, does not affect overall survival. Other chemotherapeutic drugs that are employed are—fludarabine,

CHOP(cyclophosphamide, hydroxydaunomycin [daunorubicin], oncovin [vincristine], and prednisolone),pentostatin (2,3 deoxycoformycin), and cladribine (2 chlorodeoxyadenosine). Fludarabineisincreasingly being used since it has been shown to produce a better and more durable response.Corticosteroids are helpful for treatment of autoimmune haemolytic anaemia and immune thrombocytopenia. Local radiotherapy is given for treatment of splenic/lymph node enlargement causing compression problems. Splenectomy is indicated for hypersplenism, painful splenomegaly,and for immune haemolytic anaemia or thrombocytopaenia resistant to corticosteroids (Kawthalkar. 2006).

### **1.2.2Glutathion S-transferase theta1**

The human glutathione S-transferases (GSTs) are a family of enzymes known to act in the body as the defense systems for neutralize free radicals. They play an important role in the detoxification of electrophiles by glutathione conjugation. For example, the function of the GST enzymes has traditionally been considered to be the detoxification of several carcinogens found in tobacco smoke. There is a wide range of electrophilic substrates both endogenous (e.g. by-products of reactive oxygen species activity) and exogenous (e.g. polycyclic aromatic hydrocarbons). GSTs are dimeric proteins that catalyze conjugation reactions between glutathione and tobacco smoke substrates, such as aromatic heterocyclic radicals and epoxides .In addition to their role in phase II detoxification, GSTs also modulate the induction of other enzymes and proteins important for cellular functions, such as DNA repair. This class of enzymes is therefore important for maintaining cellular genomic integrity and, as a result, may play an important role in cancer susceptibility .The loci encoding the GST enzymes located on at least seven chromosomes. This multi gene family divided in seven families (Alpha, Mu, Pi, Theta, Sigma, Zeta, and Omega) with functions ranging from detoxification to biosynthesis and cell signaling. Many of the GST genes are polymorphic, therefore, there has been substantial interest

in studying the associations between particular allelic variants with altered risk of a variety of diseases. Several GST polymorphisms have been associated with an increased or decreased susceptibility to several diseases (Dadbrnpour *et al.*2013).

The theta class gene, GSTT1, is located on chromosome 22. It has a common null polymorphism (GSTT1\_0 allele) in which the entire gene is deleted with an allele frequency of ~40% in Caucasians (Rajagopal *et al.* 2005).

### 1.3 Previous study

Study conducted by Martin *et al* in( 2002) was aimed to study the relationship between glutathione s-transferase M1, T1, and P1 polymorphisms and chronic lymphocytic leukemia they found that the frequency of the GSTT1 null alleles in control subjects was 23% (66/270) while in CLL patients it was 30%(41/138).

Study done by Rajagopal *et al* (2005) in UK aimed to study the glutathion S-transferaseT1 polymorphisms and it's association with outcome in colorectal cancer they found that the GSTT1 null genotype was significantly more common in cases (96/361,26.6%) than in controls(158/881,17.9% ;p=0,006,odds ratio (OR) =1.65 , 95%.

Study done by Kassogue *et a l*(2015) in Morocco aimed to study the association of glutathione S-transferase (GSTM1 and GSTT1) genes with chronic myeloid leukemia, they found that the GSTT1null genotype found in 17.4% in patients against 9.7% in the control group (OR 95% CI, 1.97, Pvalue=0.13.

Study done by Guven *et al* (2015) in Turkia , aimed to study the role of glutathioneS-transferase M1, T1, and P1 gene polymorphisms in childhood acute lymphoblastic leukemia susceptibility in Turkish population. They found that no difference in the prevelance of GSTT1 null genotype between the childhood ALL patients and the controles (Pvalue 0.71 OR 0.90).

Study done by Alves *et al* (2002) in north Portugal, aimed to study the GSTM1 and GSTT1 genetic polymorphisms and susceptibility to acute lymphoblastic leukemia in children from north portugal, they found that the GSTT1 defferences were not significant (Pvalue=0.528).



## **1.4 Objectives:**

### **1.4.1 General objective**

- To study the association between GSTT1null genotype and CLL.

### **1.4.2 Specific objectives**

- To determine the frequency of GSTT1null genotype among Sudanese patients with CLL.
- To evaluate the role of GSTT1 polymorphisms as a risk factor for CLL.
- To correlate the presence of GSTT1 null genotype with patients age, and gender.

## **1.5 Rationale**

There is many studies revealed association of CLL with certain genetic abnormality. Inherited absence of alleles (null genotype) in GSTT1 genes result in lack of enzymatic activity. The frequencies of GSTs polymorphic alleles, especially GSTT1 and GSTM1 have been reported in various cancers and there are several studies have been published on the relationship between GSTT1 null polymorphism and various types of cancers. To our knowledge there are no published reports about the association between GSTT1null polymorphism and CLL in Sudan so this study will fill the gab regarding this polymorphism and it is association with CLL in Sudanese patients.

## **Chapter Two**

### **Materials and methods**

#### **2.1 Study design**

Case control study

#### **2.2 Study area**

This study was conducted at Flowcytometer center, Khartoum-Sudan.

#### **2.3 Study period**

This study was conducted in the period from March to August 2015.

#### **2.4 Study population**

Chronic lymphocytic leukemia patients who referred to the floctometry center in Khartoum between March to August 2015.

#### **2.5 Inclusion criteria**

-Patients with chronic lymphocytic leukemia

-Both gender were included

-And all ages

Attended to (flowcytometer center).

#### **2.6 Exclusions criteria**

Any patient with chronic lymphocytic leukemia with another type of cancer were excluded from the study.

#### **2.7 Ethical consideration**

The consent of the selected individuals to the study was taken after being informed with all detailed objectives of the study and its health benefit in future.

#### **2.8 Data Collection**

The data were collected through a well designated questionnaire targeting information gender, age.

## **2.9 Sample collection**

Under sterile condition EDTA blood were taken from each individual, after oral permission this transport to Al Neelain university , the samples were stored at 4°C till DNA extraction.

## **2.10 Data analysis**

Data of this research was analyzed using the statistical package for the social science (SPSS) version 16.

## **2.11 Materials**

Reagents and equipment of DNA extraction by salting out method:

- Red cell lyses buffer(RCLB).
- White cell lyses buffer(WCLB).
- TE buffer.
- 6M NaCl.
- Proteinase K.
- Cold absolute ethanol.
- 70% ethanol.
- 1.5 appendorf tube.
- Yellow tips .
- Blue tips.
- White tips.
- Automatic pipette.
- Vortex.
- Centrifuge.
- Incubator

Reagents and equipments of electrophoresis:

- TBE buffer.
- Agarose.
- Ethidium bromide.

- Electrical current.
- Loading buffer.
- DNA ladder.

Reagents and equipments of polymerase chain reaction:

- Water.
- PCR buffer.
- MgCl.
- dNTPs.
- Forward primer.
- Reverse primer.
- Target DNA.
- Polymerase.
- Automatic pipette.
- tips.
- PCR machine.

## **2.12 Methodologies**

### **2.12.1DNA extraction by salting out method**

#### **2.12.1.1 Principle**

RBCs was haemolysed 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 50µl D.W.

#### **2.12.1.2 Procedure**

300µl of blood sample was placed in 1.5 ependorf tube , 1000µl 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 1000µl of RCLB (repeated until clear pellet was obtained). WCLB, 10µl proteinase k and

10µl SDS were added to the clear white pellets. The mixture was incubated for 2 hours at 56 C. 100µl of 6 M NaCl was added to precipitate the protein and mixed well by vortex. 200µl of ice cold chloroform were added to tube and centrifuged at 12000 rpm for 6 minutes. The aqueous phase was transferred carefully to clean eppendorf 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 600µl 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 50µl distilled water and leaved to dissolve overnight.

### **2.12.2 Determination of DNA quality and purity**

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

### **2.12.3 DNA storage**

DNA was transferred into 1 ml eppendorf tube and preserved at -30 C° until PCR is performed.

## 2.12.4 Molecular analysis

### 2.12.4.1 Detection of GSTT1 / null genotype

All patients with CLL were screened for the presence of GSTT1 null genotype using allele specific PCR. The primers sequence used were as follow:

**Table (2.1) Oligonucleotides sequences for GSTT1**

<b>Primer direction</b>	<b>Sequence</b>	<b>Product size(bp)</b>
Forward primer	5'TTCCTTACTGGTCCTCACATCTC3'	480
Reverse primer	5'TCACCGGATCAGGCCAGCA3'	

PCR mixture of 20  $\mu$ l was prepared using premix master mix tubes(Maxime™PCR premix kit{i-Tag}) for each sample, with positive and negative controls in sterile eppindroff tube as follow:

**Table (2.2) PCR mixture**

<b>Reagents</b>	<b>Volume</b>
Double D.W	8 $\mu$ l
Forward primer	1 $\mu$ l
Reverse primer	1 $\mu$ l
Template DNA	10 $\mu$ l
Total reaction volum	20 $\mu$ l

Optimized cycling protocol for PCR analysis of GSTT1on the TECHN (TC412) as follow:

**Table (2.3) PCR protocol**

<b>Profile</b>	<b>Temperature</b>	<b>Time duration</b>	<b>Number of Cycles</b>
<b>Initial Denaturation</b>	94°C	10 minutes	1
<b>Denaturation</b>	95°C	1minutes	45
<b>Annealing</b>	62°C	1minutes	
<b>Extension</b>	72°C	1minutes	
<b>Extension final</b>	72°C	10minutes	

#### **2.12.4.2 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 vsoltage applied to the gel was 100 volt with time duration of 45 minutes. 50 bp DNA ladder was used as molecular weight marker with each patch of samples. Finally, PCR product was demonstrated by gel documentation system (SYNGENE).



## **Chapter three**

### **Results**

#### **3.1. Demographic data**

A total of 67 Sudanese participants were enrolled in this study 37 of them were suffering from CLL and 30 of them were healthy volunteers as control group.

The age of patients range between 34-80 years with high frequency 78% seen in the age group of patients ranged between 50-70 years .

According to the sex , the results showed increased frequency of male patients 76% compared with female patients 24% ( table 3.1).while in control group the frequency of male 67% and female 33% (table 3.2).

#### **3.2. GSTT1 genotype results**

Statistical analysis of patients sample and control sample showed that genotyping of GSTT1 among the 37 patients with chronic lymphocytic leukemia, 28 patients (75.7%) showed null genotype while 9 patients (24.3%) showed normal genotype for GSTT1 gene. while among 30 control 5 (17%) had null genotypes and 25 (83%) were positive for GSTT1 gene. Statistical analysis for GSTT1 showed significant difference between patient and control, GSTT1 present in 24.3% and 83% respectively with P.value = 0.00 and 0.R=15 (Table 3.3).

The results showed significant correlation between age and GSTT1 genotype P.value=0.00 ( table 3.4).

In table (3.5) the result showed no significant correlation between GSTT1 and gender P.value (0.37) .

**Table (3.1) Gender distribution among cases**

<b>Gender</b>	<b>Frequency</b>	<b>Percent</b>
Male	28	76
Female	9	24
Total	37	100

**Table (3.2) Gender distribution among control**

<b>Gender</b>	<b>Frequency</b>	<b>Percent</b>
Male	20	67
Female	10	33
Total	30	100

**Table (3.3) Genotype among Study groups**

<b>GSTT1</b>	<b>Sample</b>		<b>p.value</b>
	<b>Case</b>	<b>control</b>	
Null	28	5	0.00
Present	9	25	
Total	37	30	

**Table (3.4) The mean of age among case and controls**

<b>Age</b>	<b>GSTT1</b>	<b>N</b>	<b>Mean</b>	<b>Std, deviation</b>	<b>p.value</b>
	present	34	42	12.74	0.000
	Null	33	56	14.05	

**Table (3.5) Genotype among the gender**

<b>GSTT1</b>	<b>Sample</b>		<b>p.value</b>
	<b>Male</b>	<b>female</b>	
Null	26	8	0.37
Present	22	11	
Total	48	19	

## Chapter four

### Discussion, Conclusion, Recommendations

#### 4.1. Discussion

Glutathione s-transferase (GSTs) are a supergene family of phase2 enzymes whose role has been traditionally viewed as providing protection against chemical carcinogenesis. Differences in the activities of some GSTs are determined by genetic polymorphism .So the GSTT1 polymorphism that leads to expression of an enzyme with reduced activity.GSTT1 null genotype act as risk factor for chronic lymphocytic leukemia.

A total of 37 patients with chronic lymphocytic leukemia attended to flowcytometry center during the period of study were enrolled in this study. Most of them showed GSTT1 null while the remaining were showed GSTT1 present, most study patients are male.

This study showed that GSTT1 null genotype significantly increase the risk of developing chronic lymphocytic leukemia. The GSTT1 null in CLL patient more than four time than control, p.value (0.00) . My result is agree with Martin *et al*,(2002) they stated that carrying GSTT1null genotype significantly increase the risk of developing chronic lymphocytic leukemia. My findings agree with another study done by (Rajagopal *et al* .2005) found that the GSTT1 null genotype was significantly more common in cases (96/361,26.6%) than the controls (158/881, 17.9% p=0.0006,OR=1.7). My findings was disagree with study done in north Portugal by Alves *et al*( 2002) found the GSTT1 differences were not significant between patients and controles (Pvalue=0.528).

In this study the results showed the GSTT1 null polymorphism more common in elder age with significant Pvalue (0.00), and no significant association between GSTT1genotype and sex.

## **4.2.Conclusion**

- These results suggest that the GSTT1null polymorphism significantly increase the risk of developing chronic lymphocytic leukemia.
- There was significant corelation between age and GSTT1genotype in study group.
- There was insignificant corelation between sex and GSTT1genotype in study group.

### **4.3. Recommendations**

The percent study recommended that:

- \*Another study should be conducted with larger sample size .
- \*Another study should be done to evaluate the prognostic value of GSTT1 null genotype among patients with CLL.

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## **Appendix I**

### **Questionneire**

1-Name:.....

2-Number.....

3-Age.....

4-Gender.....

GSTT1(     )                      Null (     )

\*Signature.....

\*Date.....

## Appendix II

بسم الله الرحمن الرحيم

جامعة السودان للعلوم والتكنولوجيا

كلية الدراسات العليا

ماجستير مختبرات طبية

تخصص علم امراض الدم ومبحث المناعة الدموية

إقرار موافقة بالمشاركة

الإسم:.....

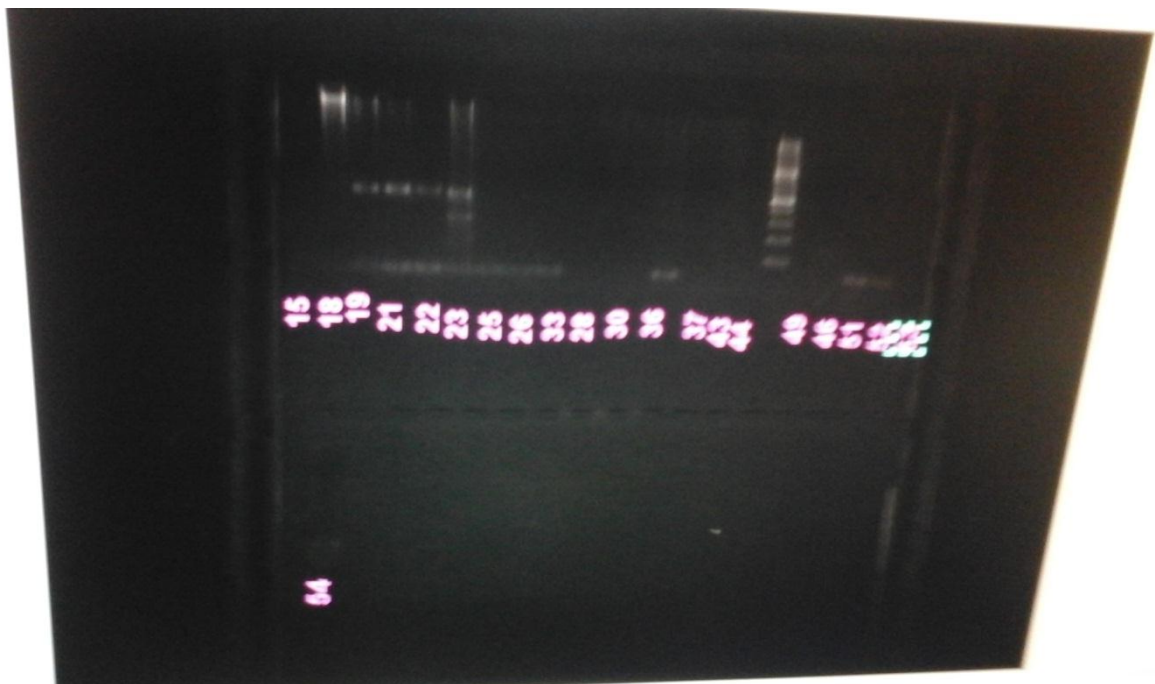
سوف يتم أخذ عينة من الدم (3مل) من الوريد بواسطة حقنة طعن وذلك بعد مسح منطقة العينة بواسطة مطهر . كل الأدوات المستخدمة لأخذ العينة معقمة ومتبع فيها وسائل السلامة المعملية

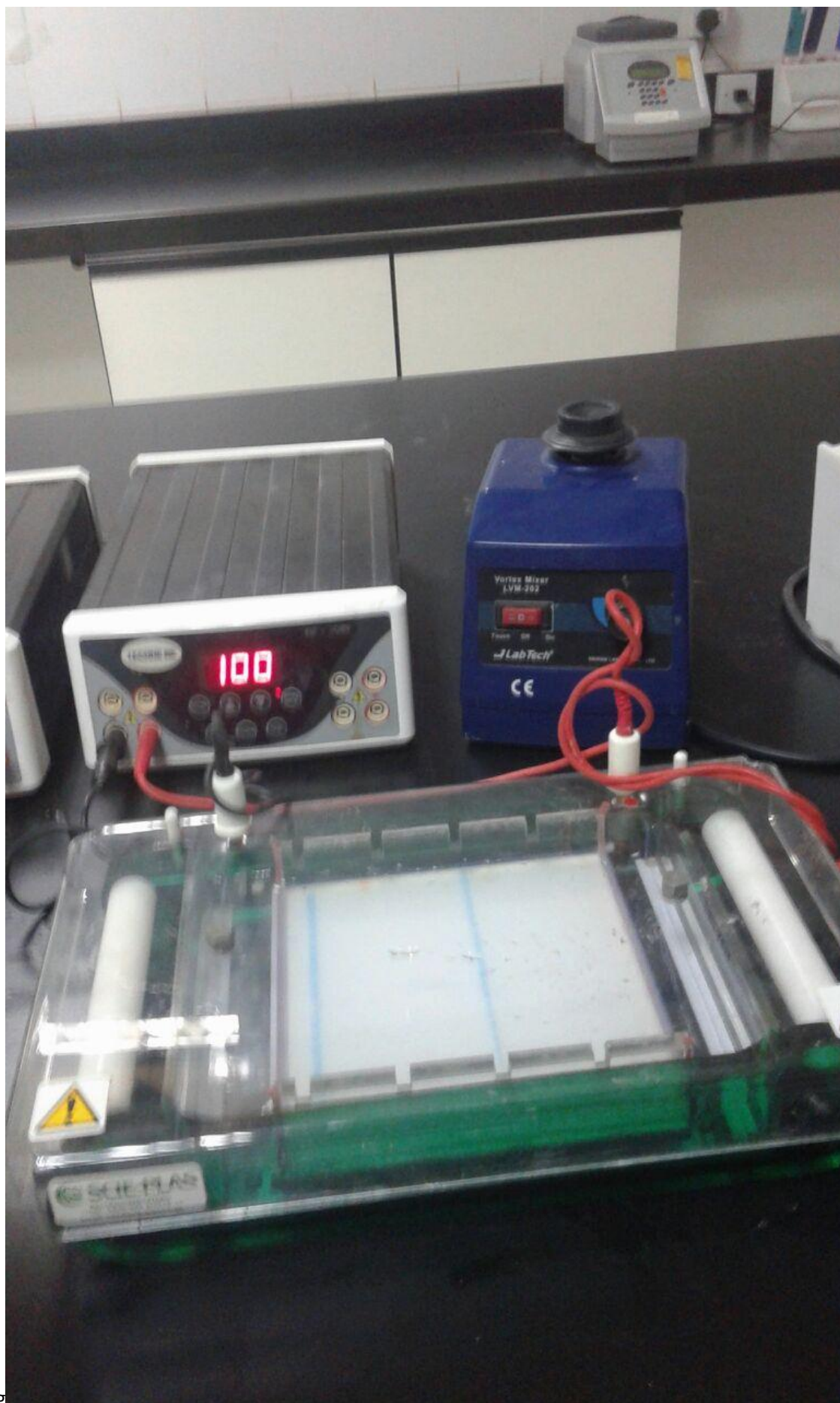
أوافق أنا المذكور اعلاه على أخذ عينة لإجراء الدراسة

الإمضاء.....

...التاريخ.....

## Gel electrophoresis









GSTT1

