بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Glutathione S TransferaseTheta1 Null Polymorphism among Patients with Acute Myeloid Leukemia

التغير الشكلي لغياب الجلوتاثيون اس ترانسفيريز ثيتا 1 لدى مرضى سرطان الدم الابيض الحاد الميلودي

A dissertation submitted for partial fulfillment for the requirement of M.Sc in Haematology

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الاية

بسم الله الرحمن الرحيم

قال تعالى:

{وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا}

صدق الله العظيم

سورة الإسراء الآية (85)

Dedication

To the candle which burns to light my life

MY mother

To the life who making our dreams become true

MY father

To those who have made it possible

MY teachers

To whom encouraged me

MY brothers, sisters, and friends.

Acknowledgment

My grateful thanks firstly for Allah who guided me to the strait way in my life. Then, I have to extend a word of thanks for my university, my college, 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 Mohamed Elfatih who help me and stands beside me a lot of thanks for my teatcher Mudathir Abdelrahim who help me many times and gave me recommendation and for all individuals who gave me specimen to do this research. My grateful appreciation should be extended to my colleagues Amel, Marwa and Eslam with whom I've had the honor to work.

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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 was conducted in Khartoum state during the period from March to August 2015 to evaluate the association of GSTT1 NULL polymorphism in patients with acute myeloid leukemia. A total 39 patients diagnosed with AML attended to the flowcytometer center, Khartoum-Sudan and 50 healthy volunteers as control group were enrolled in this study. Three milliliter (ml) 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 genetic polymorphism analysis for the GSTT1 was determined using polymerase chain reaction method.

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

The ages of case group ranged between2-85 years their mean 39; they were compared with 50 healthy volunteers as control group, their ages ranged between 25 - 55 years their mean 39. The highest frequency of sub class among case sample is M2 (23.1 %) while the lowest frequency of sub class among case sample is M6 (0 %).The mean of blasts in null cases (54 ± 20.4) which insignificantly decreased than gstt1 cases (57 ± 17.5) (P. value = 0.75).

The result showed the mean and STD for plts among patients with GSTT1 null genotype was (352.4 ± 132) while it was (90.4 ± 87) among those with normal genotype with insignificant difference (P. value = 0.74).

The result showed the mean and STD for TWBs among patients with GSTT1 null genotype was

(27.4 ± 4.4) while it was (35.6 ± 8.7) among those with normal genotype with insignificant difference (P. value = 0.42).

The result showed the mean and STD for Hb among patients with GSTT1 null genotype was

(8.8 ± 2.6) which insignificantly decreased than gstt1 cases (9.1 ± 2.7) (P. value = 0.83).

The mean of age in null cases (39 ± 22.7) which slightly decreased than gstt1 cases (42 ± 18.3) but without statistical significant (P. value = 0.68).

The rate of GSTT1 null polymorphism was 82.0 % in AML patients, while it was 22.0% in the control group and the difference was statistically significant (OR=3.5, P= 0.00).

The results of CBC and Blast percentage were taken from a patient file from flowcytometry center.

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

مستخلص البحث

هذه دراسة مقارنة حالة ضابطة تحليلية أجريت في ولاية الخرطوم في الفترة ما بين مارس إلى اغسطس 2015 لتقييم الإنزيم المحول للجلاتثيون في مرضى سرطان الدم الابيض الحاد الميلودي بالسودان تم إختيار تسعة وثلاثون مريض وفقاً لنظام إلاختيار المحدد وعوملوا كعينات اختيارية ،وخمسون اصحاء وفقاً لنظام الاختيار المحدد وعوملوا كعينات ضابطة .

تم أخذ عينة وريدية 3 مل وضعت في وعاء بلاستيكي يحتوي على إيثايلين ثنائ الامين رباعي حامض الأستيك لمنع التخثر. للتحليل الجزيئ تم استخلاص الحمض النووي منزوع الاوكسجين عن طريق الترسيب بواسطه الملح, التحليل الجيني حدد بطريقة تفاعلات البلمرة المتسلسل.

تم تحليل النتائج بإستخدام برنامج الحزم الإحصائية للعلوم الإجتماعية المحوسب، تراوحت أعمار المرضى مابين 2 – 85 سنة ومتوسطهم 39سنة تم مقارنتهم ب 50 من المتبرعين الأصحاء تراوحت أعمارهم ما بين25–55سنة ومتوسط أعمارهم 39 سنة .

أعلى تكرار لتفرع خلايا البلاست هو M2 23.1 %) وأقل تفرع لها هو M0 (0 %). متوسط البلاست عند المرضى الذين لا يملكون الانزيم هو (54± 20.4) وهو نقصان ليس له قيمه معنوية مقارنة بالذين يملكون الإنزيم الطبيعي (57 ± 17.5) (القيمة المعنوية = 0.75).

متوسط الصفائح الدموية لدى المرضى الذين لا يملكون الانزيم هو (352.4 ± 132) وهذه زيادة غير معنوية مقارنه بالمرضى الذين يملكون الإنزيم (90.4 ± 87) (القيمة المعنوية = 0.74).

متوسط كريات الدم البيضاء لدى المرضى الذين لا يملكون الانزيم هو (27.4± 4.4) وهذا نقصان غير معنوي مقارنه بالمرضى الذين يملكون الإنزيم (35.6 ± 8.7) (القيمة المعنوية = 0.42).

متوسط الخضاب لدى الحالات الذين لا يملكون الانزيم هو (8.8 ± 2.6) وهذا نقصان غير معنوي مقارنه بالحالات الذين يملكون الإنزيم (9.1 ± 2.7) (القيمة المعنوية = 0.83).

متوسط أعمار الحالات الذين لا يملكون الانزيم هو (39± 22.7) وهذا نقصان بسيط غير معنوي مقارنه بالحالات الذين يملكون الإنزيم (42± 18.3) (القيمة المعنوية = 0.68).

معدل غياب الجين تحت الدراسة 82% وسط المرضى بينما عدم وجوده بين العينات الضابطة 22% وهذا له فرق إحصائ ذا قيمة حيث كانت القيمة الشاذة =3.5 والقيمة المعنوية = 0.00 .

نتائج ال CBC ونسبة البلاست أخذت من ملفات المرضى وذلك من مركز الفلوسايتوميتر بالخرطوم . السودان.

بصورة مختصرة أظهرت الدراسة أن غياب الجين تحت الدراسه يشكل عامل خطورة لدى مرضى سرطان الدم الأبيض الميلودي.

Abbreviations

ALL	Acute Lymphoid leukemia
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
CBC	Complete blood count
CD	Cluster of differentiation
CR	Complete remission
Dl	Deciliter
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamin tetra acidic acid
FAB	French American British
G	Gram
GSTT	Glutathione s transferase thita
Hb	Hemoglobin
L	lymphoblast
M	Myeloblast
OR	Odd ratio
PAS	Periodic acid shciff
PCR	Polymerase chain reaction
Plts	Platelets
P.V	Polycythemia Vera
RNA	Ribo nucleic acid
SD	Stander deviation
SPSS	Statistical package for social sciences
T	Translocation
Twbcs	Total White Blood Cells
V	Vertion
WHO	World Health Organization

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Chapter one

Introduction and literature review

Introduction and literature review

1.1 Introduction

Leukaemia is a disease resulting from the neoplastic proliferation of haemopoietic or lymphoid cells. It results from mutation of a single stem cell, the progeny of which form a clone of leukaemic cells. Usually there is a series of genetic alterations rather than a single event. Genetic events contributing to malignant transformation include inappropriate expression of oncogenes and loss of function of tumour suppressor genes. Oncogenes may be normal cellular genes – proto-oncogenes, that have mutated or are dysregulated – or may be novel hybrid genes resulting from fusion of parts of two genes. The cell in which the leukaemic transformation occurs may be a lymphoid precursor, a myeloid precursor or a pluripotent haemopoietic stem cell capable of differentiating into both myeloid and lymphoid cells. Myeloid leukaemias can arise in a lineage-restricted cell, in a multipotent stem cell capable of differentiating into cells of erythroid, granulocytic, monocytic and megakaryocytic lineages or in a pluripotent lymphoid–myeloid stem cell. Genetic alterations leading to leukaemic transformation often result from major alterations in the chromosomes, which can be detected by microscopic examination of the chromosomes of cells in metaphase. Other changes, such as point mutations or partial duplications, are at a submicroscopic level but can be recognized by analysis of deoxynucleic acid (DNA) or ribonucleic acid (RNA). Neoplastic cells are genetically unstable so that further mutations can occur in cells of the clone. If a new mutation gives the progeny of that cell a growth or survival advantage it tends to replace the parent clone. Such clonal evolution can lead to transformation into a more aggressive or treatment refractory form of the disease with an associated worsening of prognosis. A series of mutations can occur with progressive worsening of prognosis at each stage (Bain, 2010).

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. Two widespread genetic polymorphisms deletions that involve in the GSTT1 and GSTM1 genes, namely Glutathione S-transferase M1 (GSTM1) and glutathione S-transferase T1 (GSTT1) null polymorphisms, have been reported to lead to abrogation of enzyme activity. The frequencies of GSTs polymorphic alleles, especially GSTT1 and GSTM1, have been reported in various cancers (Laila and, 2010).

1.2 Literature review

Leukaemias are a very heterogeneous group of diseases, which differ from each other in aetiology, pathogenesis, prognosis and responsiveness to treatment. Accurate diagnosis and classification are necessary for the identification of specific biological entities and underpin scientific advances in this field. The detailed characterization of haematological neoplasms is also essential for the optimal management of individual patients. Many systems for the classification of leukaemia have been proposed. Between 1976 and 1999, a collaborative group of French, American and British haematologists (the FAB group) proposed a number of classifications, which became widely accepted throughout the world. In 2001, a quarter of a century after the first FAB proposals, a World Health Organization (WHO) expert group proposed an updated system for the classification of leukaemia and lymphoma incorporating clinical features, haematological and histological features, immunophenotyping and the results of cytogenetic and, to a lesser extent, molecular genetic analysis. In 2008 a further updating of the WHO classification incorporated new knowledge and gave a greater importance to molecular genetics features (Bain, 2010).

1.2.1 Classification of leukaemia

The main classification is into four types: acute and chronic leukaemias, which are further subdivided into lymphoid or myeloid ,acute myeloid leukaemia: Mo-M7,acute lymphoblastic leukaemia: L1-L3,chronic myeloid leukaemias and chronic lymphoid leukaemias.(Hoffbrand and, *et al* 2006).

1.2.2 Acute myelogenous leukemia (AML)

Acute myelogenous leukemia (AML) is the result of a somatic mutation in a pluripotential stem cell or a slightly more differentiated progenitor cell. Exposure to very high doses of radiation or chronic exposure to benzene increases the incidence of the disease. A small but increasing proportion of cases develop after the exposure of a patient with lymphoma or a nonhematologic cancer to intensive chemotherapy. The mutant cell gains a growth and/or survival advantage in relationship to the normal pool of stem cells. As the progeny of the mutant cell proliferates to form about ten billion cells or more, normal hematopoiesis is inhibited and normal red cell, neutrophil, and platelet blood levels fall. The resultant anemia leads to weakness, exertional limitations, and pallor; the thrombocytopenia to spontaneous hemorrhage, usually in the skin; and the neutropenia and monocytopenia to poor wound healing

and minor infections. Severe infection usually does not occur at diagnosis but will if the disease progresses for lack of treatment or if chemotherapy-induced impairment of neutrophil and monocyte blood cell levels is superimposed. The diagnosis is made by measurement of the blood cell counts and examination of blood and marrow cells and is based on the identification of blast cells in the marrow and blood. The diagnosis of AML is enhanced in some cases by identification of myeloperoxidase activity in blast cells by cytochemistry or a specific antibody test and by identifying characteristic CD antigens on the blast cells (e.g., CD13, CD33). The leukemic stem cell is capable of imperfect differentiation and maturation, and the clone may contain cells that have the morphologic or immunophenotypic features of erythroblasts, megakaryocytes, monocytes, eosinophils, or rarely basophils in addition to myeloblasts or promyelocytes. When one cell line is sufficiently dominant, the leukemia may be referred to as acute erythroblastic, acute megakaryocytic, acute monocytic, and so on. Certain cytogenetic alterations are very frequent. These include t (8; 21), t (15; 17), inversion 16, trisomy 8, and deletions of all or part of chromosome 5 or 7; t (15; 17) is uniquely associated with acute promyelocytic leukemia. AML usually is treated with cytarabine and an anthracycline antibiotic, although other drugs may be added or substituted in poor-prognosis, refractory, or relapsed patients. High-dose chemotherapy and either autologous stem cell infusion or allogeneic stem cell transplantation may be used in an effort to treat relapse or those at high risk to relapse after chemotherapy alone. The probability of remission ranges from about 75 percent in children to less than 25 percent in octagenarians. The probability for cure decreases from about 35 percent in children to virtually zero in octogenarians (Ernest and, *et al* 2000).

1.2.2.1 The WHO classification of acute myeloid leukemia (AML):

Acute myeloid leukaemia with recurrent genetic abnormalities, AML with t (8; 21) (q22; q22)/AML1-ETO fusion, AML with abnormal bone marrow eosinophils and in v (16) (p13q22) or t(16;15)(p13;q22)/CBFB-MYH11 fusion, acute promyelocytic leukaemia with t (15; 17) (q22; q12)/PML-RARA fusion, and variants, AML with 11q23 rearrangement and MLL abnormality, AML with multilineage dysplasia, following a myelodysplastic syndrome or a myelodysplastic/myeloproliferative syndrome without antecedent myelodysplastic syndrome, therapy-related AML and

myelodysplastic syndrome, alkylating agent-related, topoisomerase II-inhibitor-related, AML not otherwise categorized AML, minimally differentiated (resembles FAB M0), AML without maturation (resembles FAB M1), AML with maturation (resembles FAB M2), acute myelomonocytic leukaemia (resembles FAB M4), acute monoblastic and acute monocytic leukaemia (resembles FAB M5a, M5b), acute erythroid leukaemia, Erythroleukaemia (resembles FAB M6), pure erythroid leukaemia, acute megakaryoblastic leukaemia (resembles FAB M7), acute basophilic leukaemia, acute panmyelosis with myelofibrosis and Myeloid sarcoma (granulocytic or monocytic) (Bain and Gupta, 2003).

1.2.2.2Clinical features

Symptoms related to bone marrow infiltration and suppression of normal hematopoiesis is common. Fever is present in 15 to 20% of patients; this should be presumed due to infection and treated as such until proven otherwise but may be due to hypermetabolism. Physical examination may show mild splenomegaly; prominent lymphadenopathy is unusual. Tissue involvement is more common in AML than in ALL. Skin involvement occurs in approximately 10% of patients, particularly in patients with monocytic subtypes; it presents as violaceus nontender plaques or nodules. Involvement of the gums is common, and patients may present initially to the dentist, complaining of bleeding gums. Leukemic meningitis occurs in approximately 5 to 7% of patients at diagnosis and is more common with high WBC counts and monocytic subtypes. Hyperleukocytosis with leukostasis is also more common in AML than ALL. There is a risk of leukostasis with blast counts ≥50,000/_L, and the risk increases significantly with blast counts ≥100,000/_L. Hyperleukocytosis with leukostasis represents a medical emergency, and prompt lowering of the blast count with leukapheresis should be performed as soon as possible. Metabolic complications of AML may include hyperuricemia, hyper- or hypokalemia, hyperphosphatemia, and the tumor lysis syndrome with acute renal failure. Patients with high blast counts may have spurious hypoglycemia or hypoxemia if blood samples are not analyzed promptly, due to consumption of glucose or oxygen by the blasts. Spurious hyperkalemia may also occur. Disseminated intravascular coagulation may occur in AML. It is most frequent in acute promyelocytic leukemia (APL) but may also occur with other types (William, 2002).

1.2.2.3 Laboratory

Anemia and thrombocytopenia are almost always present. The platelet count given by automated hematology analyzers may be spuriously increased due to fragments of the leukemic blasts, which are counted as platelets by the instrument. Examination of a blood smear gives a more accurate assessment of the platelet count in this circumstance. The white cell count is variable; it is elevated in more than half of patients and may exceed 100,000/_L, but may also be decreased. Blasts are usually present on blood smear but occasionally may be absent or hard to find. Careful examination of a blood smear for circulating blasts is required. The blasts in AML tend to be larger and more variable in appearance than those in ALL, with more irregular nuclei, but may be impossible to distinguish from lymphoblasts. The only morphologic feature that absolutely confirms myeloid lineage is the presence of Auer rods (William, 2002).

1.2.2.3.1 Bone marrow

The bone marrow is typically hypercellular, with a predominance of blasts or other immature cells. Normal hematopoietic precursors are decreased. Cytochemical stains may be performed on peripheral blood (if there is a high circulating blast count) or on bone marrow aspirate smears. By FAB criteria, staining for myeloperoxidase and/or Sudan black B must be present in ≥3% of blasts in AML, except for the M0 subtype. Staining for the naphthol ASD chloroacetate (“specific”) esterase may be seen in AML with granulocytic differentiation, and staining for the _-naphthyl butyrate (“nonspecific”) esterase may be seen in AML with monocytic differentiation. Finely granular cytoplasmic staining with the PAS reaction is common in AML and must not be interpreted as ALL. Coarse “block” positivity resembling that seen in ALL may be present in erythroleukemia and in megakaryocytic leukemia (William, 2002).

1.2.2.3.2 Immunophenotype

Immunophenotyping by flow cytometry is most useful in identifying myeloid lineage and distinguishing between AML and ALL. Phenotyping can suggest specific subtypes of AML, particularly megakaryocytic leukemia, but definitive subclassification usually requires correlation with morphology and cytochemical stains (William, 2002).

1.2.2.3.3 Cytogenetics

Cytogenetic analysis has become critical in the diagnosis and treatment of AML.As in ALL, recurring chromosomal abnormalities can be divided into reciprocal translocations and alterations in chromosome number.Many of the genes involved in translocations in AML encode DNA-binding transcription factors or regulatory components of transcriptional complexes. Several of the recurring translocations have been associated with specific subtypes of AML (although the correlations are imperfect) and with prognosis. Indeed, cytogenetic results are among the most powerful prognostic factors in AML (William, 2002).

1.2.2.4 Prognosis

The overall long-term disease-free survival of patients less than 65 years old with AML is approximately 40%. The prognosis is worse for older patients and those with secondary AML. Patients with AML can be divided into three broad prognostic groups, predominantly on the basis of cytogenetic results:

Favorable prognostic group: This group includes patients <60 years old with the t (8; 21), t (15; 17), inv (16), or t (16; 16) cytogenetic abnormalities, no previous hematologic disease, and AML that is not therapy related. This group makes up ~20% of patients <60 years. They have a high CR rate (>85%) and a relatively low risk of relapse (30 to 40%), (William, 2002).

Unfavorable prognostic group: This group includes patients with cytogenetic abnormalities involving more than two chromosomes, monosomies of chromosomes 5 or 7, deletion of the long arm of 5 (del5q), or abnormalities of the long arm of chromosome 3. Patients with abnormalities involving chromosome 11q23 (MLL gene) are sometimes also considered to be in this group. These abnormalities are more often present in older individuals and patients with secondary AML. This group makes up ~15% of patients who are 15 to 60 years old. They tend to have a lower CR rate and a higher relapse rate, and survival at 5 years is <20%. No current treatment approach is considered satisfactory for these patients. Patients over 60 years of age generally have an unfavorable prognosis, with 5-year-survival rates <10% (William, 2002).

Intermediate (standard) prognostic group: Patients in this group have either a normal karyotype or chromosomal abnormalities not included in the other groups (William, 2002).

1.2.3GSTT1 glutathione S-transferase theta 1

The Glutathione-s-transferases (GSTs) [8, 29] are a family of enzymes known to play important roles in the detoxification of several carcinogens found in tobacco smoke. GSTs are dimeric proteins that catalyze conjugation reactions between glutathione and tobacco smoke substrates, such as aromatic heterocyclic radicals and epoxides. Conjugation facilitates excretion and thus constitutes a detoxification step. In addition to their role in phase II detoxification step, GSTs also modulate the induction of other enzymes and proteins involved in 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 (Asha, 2013)

1.2.3.1 Classification

Protein sequence and structure are important additional classification criteria for the three superfamilies (cytosolic, mitochondrial, and MAPEG) of GSTs: while classes from the cytosolic superfamily of GSTs possess more than 40% sequence homology, those from other classes may have less than 25%. Cytosolic GSTs are divided into 13 classes based upon their structure: alpha, beta, delta, epsilon, zeta, theta, mu, nu, pi, sigma, tau, phi, and omega. Mitochondrial GSTs are in class kappa. The MAPEG superfamily of microsomal GSTs consists of subgroups designated I-IV, between which amino acid sequences share less than 20% identity. Human cytosolic GSTs belong to the alpha, zeta, theta, mu, pi, sigma, and omega classes, while six isozymes belonging to classes I, II, and IV of the MAPEG superfamily are known to exist. (Oakley, 2011) (Eaton, Bammler 1999) (Josephy, 2010).

1.2.3.2 Function

The activity of GSTs is dependent upon a steady supply of GSH from the synthetic of GSH from the cell. The primary role of GSTs is to detoxify xenobiotics by catalyzing the nucleophilic attack enzymes gamma-glutamylcysteine synthetase and glutathione synthetase, as well as the action of specific transporters to remove conjugates by GSH on electrophilic carbon, sulfur, or nitrogen atoms of said nonpolar xenobiotic substrates, thereby preventing their interaction with crucial cellular proteins and nucleic acids.(Josephy, 2010).(Hayes and *et al* 2005).

Although best known for their ability to conjugate xenobiotics to GSH and thereby detoxify cellular environments, GSTs are also capable of binding nonsubstrate

ligands, with important cell signaling implications. Several GST isozymes from various classes have been shown to inhibit the function of a kinase involved in the MAPK pathway that regulates cell proliferation and death, preventing the kinase from carrying out its role in facilitating the signaling cascade. (Laborde, 2010).

1.3 Previous studies:

A previous study done to examine the association between GSTT1 null polymorphism and polycythaemia vera in Sudanese patients (Mohamed and, *et al;* 2015), and they found that the GSTT1 null polymorphism was detected in 23% of cases (17%male and 5% female), and the association between GSTT null and polycythaemia vera is not statistically significance (P value=0.64) (Mohamed and, *et al* 2015).

Study done by Ghadai and, *et al* in 2006 they study the Glutathione S-Transferase GSTM1 and GSTT1 polymorphisms in adult acute myeloid leukemia; its impact on toxicity and response to chemotherapy they found no significant, between GSTT1 null genotype and toxic death during induction and between GSTM1 present genotype and lower rate of CR (Ghadai and, *et al* 2006).

On other hand significance differences was gained by Teresa and *et al*; GST deletions predicted poor response to chemotherapy (P =0.04), and also and shorter survival (P =0.04) (Teresa and *et al*; 2015).

Also a study done by Beray Kiran and *et al* in 2010. They study the *GST* (*GSTM1*, *GSTT1*, and *GSTP1*) polymorphisms in the genetic susceptibility of Turkish patients to cervical cancer. They found that In the patient group, statistical significance was determined for gravidity (p=0.03), parity (p=0.01), and the number of living children (p=0.01) compared to the control group, and the gene frequency of GSTM1, GSTT1, and GSTP1 polymorphisms was evaluated they observed that GSTM1 and GSTT1 null genotype frequencies were 54.3% and 32.6%, no statistical variation was determined between the control and patient groups in terms of GSTM1, GSTT1, and GSTP1 polymorphisms (p>0.05).Finally they concluded thatGSTT1, GSTM1, and GSTP1 polymorphisms are not associated with cervical cancer in their study group (Beray Kiran and *et al* in 2010).

Chapter Two

Rationale and Objective

2.1 Rationale

AML has increasing prevalence in Sudan according to observation; there is many studies revealed association of AMLwith 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 AML in Sudan; so this study may fill the gab regarding this polymorphism and its association with AML in patients .

Objectives:

General objective

- To study the association between GSTT1 null genotype and AML.

Specific objectives

- To determine the frequency of GSTT1 null genotype among Sudanese patients with AML
- To evaluate the role of GSTT1 polymorphisms as a risk factor for AML.
- To correlate the GSTT1 null genotype to the patient`s haematological parameters.
- To correlate the presence of GSTT1 null genotype with patient`s age, and gender.

Chapter three

Materials and Methods

Materials and Methods

3.1 Study design

A case control study conducted in Khartoum state in 2015 to evaluate the effects of GSTT1 polymorphisms in patients with AML.

3.2 Study population

Thirty nine AML patients referring to flowcytometery center-khartoum, Sudan during the period from March-August 2015 were recruited to participate in this study as well as 50 apparently healthy volunteers were enrolled as control group.

3.3 Inclusion criteria

Patients with AML and resident in Sudan.

3.4 Exclusion criteria

Patient gets transformation to AML.

3.5 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.

3.6 Data collection

Data was collected using non self-administered pre-coded check list which was specifically designed to obtain information.

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 in sample.

3.9 Data analysis

Data of this research was analyzed using the statistical package for the social sciences (SPSS) version 16 (independent t test, chi-square and regression).

3.10 Sample

Venous blood (3ml) was collected in plastic vacocontainer containing EDTA anticoagulant for DNA extraction.

3.11 Methodology

3.11.1 Complete Blood Count (CBC) and blast percentage

The results of CBC and Blast percentage were taken from a patient file from flowcytometry center.

3.11.2 DNA extraction by salting out method

3.11.2.1 Principle steps

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 50 µL D.W.

3.11.2.2 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 1000µl of RCLB (repeated until clear pellet was obtained). WCLB, 10µlprotinase K and 10 µl SDS were added to the clear white pellets. The mixture was incubated for 2 hours at 56^0C. 100 µl 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 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 5minutes. 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.

3.11.3 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 gel electrophoresis.

3.11.4DNAstorage

DNA was transferred into 1 ml eppindroff tube and preserved at -20°C until PCR is performed.

3.11.5 Molecular analysis

3.11.5.1 Detection of GSTT1 / null genotype

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

Table (3.1) Oligonucleotides sequences for GSTT1

Primer direction	**Sequence**	**Product size (bp)**
Forward primer	5'TTC CTT ACT GGT CCT CAC ATC TC3'	480
Reverse primer	5'TCA CCG GAT CAG GCC AGCA3'	

PCR mixture of 20 µl was prepared using premix master mix tubes (Maxime^{TM}PCR premix Kit{i-Taq}) for each sample, with positive and negative controls in sterile eppindroff tube as follow:-

Table (3.2) PCR mixture

Reagents	**Volume**
Double D.W	8µl
forward primer	1µl
Reverse primer	1µl
Template DNA	10 µl
Total reaction volume	20µl

Optimized cycling protocol for PCR analysis of GSTT1 on the TECHN (TC412) as follow:

Table (3.3) PCR protocol:

Profile	Temperature	Time duration	Number of Cycles
Initial Denaturation	94°C	10 minutes	1
Denaturation	95°C	1 minutes	45
Annealing	62°C	1 minutes	
Extension	72°C	1 minutes	
Extension Final	72°C	10 minutes	

3.11.5.2 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 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 Four

The Results

4. Results

Table (4.1) frequency of case and control samples. Show 39 patients and 50 controls.

Samples	Frequency
Cases	39
Controls	50

Table (4.2) GSTT1 genotype among all subjects

Showed that 48% of the samples were null and 52% of the samples were GSTT1 normal genotype.

Samples	Frequency	Percent
Null	43	48
GSTT1	46	52
Total	89	100

Table (4.3) Gender distribution among the samples

Show 56% of the samples are males and 44%of the samples are female.

Gender	Frequency	Percent
Males	50	56
Females	39	44
Total	89	100

Table (4.4) frequency of sub class among case sample

Cases	Frequency	Percent
M0	5	12.8
M1	4	10.3
M2	9	23.1
M3	5	12.8
M4	7	17.9
M5	5	12.8
M6	0	0
M7	4	10.3
Total	39	100

Table (4.5) GSTT1 genotype among AML patients

Show 82%of the case samples are null and 18% of the samples are gstt1

GSTT1 genotype	Frequency	Percent
Null	32	82%
Normal	7	18%
Total	39	100

Table (4.6) GSTT1 genotype among control subjects

Show 82%of the case samples are null and 18% of the samples are gstt1

GSTT1 genotype	Frequency	Percent
Null	11	22%
Normal	39	78 %
Total	50	100

4.1 Comparisons of mean (blast) results between null group and gstt1 group

Table (4.6) showed that the mean of blast among patients with normal GSTT1 genotype was 57 % while it was 54% among those with null genotype with insignificant difference (P. value = 0.75).

Table (4.7) the blast percentage among cases

GSTT1	Blast %		
	Mean	STD	P. value
Null	54	20.4	0.75
GSTT1	57	17.5	

4.2 Comparisons of mean (plts) results between null group and gstt1 group

Table (4.7) show insignificant mean of plts in null cases (352.4 ± 132) when compared with gstt1 cases (90.4 ± 87) (P. value = 0.74).

Table (4.8) the mean of PLT count x10^9/l among the cases

GSTT1	PLTX 10^9 / L		
	Mean	STD	P. value
Null	352.4	132	0.74
GSTT1	90.4	87	

4.3 Comparisons of mean TWBCs count X10^9/l results between null group and gstt1 group

Table (4.7) show insignificant mean of TWBCs count in null cases (27.4 ± 4.4) when compared with gstt1 cases (35.6 ± 8.7) (P. value = 0.42).

Table (4.9) the mean of TWBCs among the cases

GSTT1	TWBs X 10 ^{9} / L		
	Mean	STD	P. value
Null	27.4	4.4	0.42
GSTT1	35.6	8.7	

4.4 Comparisons of mean Hb g/dl results between null group and GSTT1 group

Table (4.9) show insignificant mean of hb in null cases (8.8 ± 2.6) when compared with GSTT1 cases (9.1 ± 2.7) (P. value = 0.83).

Table (4.10) the mean of Hb g/dl among the cases

GSTT1	Hb g/dl		
	Mean	STD	P. value
Null	8.8	2.6	0.83
GSTT1	9.1	2.7	

4.5 comparisons of mean (age) results between null group and gstt1 group:

Table (4.10) show insignificant mean of age in null cases (38.5 ± 22.7) when compared with gstt1 cases (42.3 ±18.3) (P. value = 0.68).

Table (4.11) the mean of age among the cases

GSTT1	Age years		
	Mean	STD	P. value
Null	38.5	22.7	0.68
GSTT1	42.3	18.3	

4.6 Regression analysis

The results showed that GSTT1 null genotype is a risk factor for AML and there is significant association between GSTT1 null polymorphism and AML (P.Value:0.00, OR: 3.5).

Chapter Five

Discussion, conclusion and recommendations

Discussion, conclusion and recommendations

5.1 Discussion

Homozygotes for the null alleles (deletion) of GSTM1 and GSTT1 lack activity of the respective enzymes (Strange and Fryer, 1999).

This decrease the reactivity of electrophilic substrates, which may affect the functions within cellular macromolecules, such as nucleonic acid, lipid and protein. So, the genetically determined differences in metabolism, related to GST enzymes, have been reported to be associated with various cancer susceptibilities. (Kim and,et al 2000).

Positive associations were found in certain types of cancers while not found in others.

Case control study was conducted to determine the frequency of GSTT1 null genotype among Sudanese patients with AML and to examine the association between GSTT1 polymorphisms and frequency of null polymorphism.

In my study we found that the GSTT1 null genotype was a risk factor for AML (OR=3.5, 95% CI= 1.8-4.3, P= 0.00).and this disagree with reports of Mohammed .S and *et al* who reported no significant association between GSTT1 and P.V.

The highest frequency of sub class of AML patients was M2 (23.1 %) while the lowest frequency of sub class among cases was M6 (0 %).The mean of blasts in null cases insignificantly lower than in gstt1 cases. The mean of plts, TWBCs and Hb was lower among patients with null genotype than those with normal genotype and the difference was statistically insignificant, the p. value respectively was (0.74), (0.42) and (0.83).

5.2 Conclusion

- ✓ GSTT1 null genotype is a risk factor for AML and there is significant association between GSTT1 null polymorphism and AML.
- ✓ GSTT1 null genotype is associated with insignificant lower HB and white cell count.
- ✓ GSTT1 null genotype is associated with insignificant higher platelet counts.

5.3 Recommendations

The percent study recommended that:

- Another study should be conducted with larger sample size for obvious information.
- GSTT1 null genotype detection should be established to be done among risk groups.
- Another study should be done to evaluate the prognostic value of GSTT1 null genotype.

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APPENDICES

Appendices

Appendix 1: Questionnaire.

1- Name: ----------

2- Number ----------

3- Age ----------

4- Gender ----------

5- Sub class----------

6- blast----------

7- Platelets ----------

8- Hemoglobin ----------

9- TWBCs ----------

10-GSTT1 ◯ null ◯

*signature ----------

*Date ----------

Appendix 2: Master Sheet

Cases

no	Subclass	Blast	Plts	twbcs	hb	age	Sex	Genotype
52.0	4.0	52.0	121.0	68.0	9.1	45	Female	GSTT1
113.0	3.0	74.0	41.0	22.5	10.4	25	Female	Null
28.0	4.0	85.0	30.0	63.9	12.7	72	Female	Null
78.0	1.0	87.0	69.0	3.4	13.7	30	Male	Null
127.0	7.0	23.0	400.0	8.9	8.0	55	Female	Null
108.0	0.0	26.0	22.0	9.3	8.0	4	Female	Null
21.0	2.0	52.0	278.0	4.3	9.7	43	Female	GSTT1
128.0	3.0	33.0	19.0	20.0	8.1	20	Male	Null
110.0	2.0	63.8	63.0	75.0	13.9	35	Female	Null
116.0	4.0	69.0	119.0	100.0	4.5	27	Female	Null
46.0	4.0	32.0	31.0	20.9	7.1	45	Male	Null
40.0	3.0	73.0	39.0	18.0	4.8	2	Female	Null
68.0	4.0	30.0	34.0	6.8	7.7	57	Male	Null
119.0	4.0	64.0	63.0	31.0	8.3	44	Female	Null
109.0	3.0	79.0	75.0	24.4	10.9	9	Female	Null
76.0	5.0	81.0	22.0	67.0	10.7	31	Male	Null
131.0	5.0	57.0	31.0	5.5	11.4	85	Male	Null
126.0	5.0	76.0	34.0	69.6	6.6	4	Male	Null
54.0	3.0	79.0	46.0	22.5	10.9	25	Male	GSTT1
122.0	5.0	85.0	30.0	63.9	12.7	72	Female	GSTT1

72.0	0.0	60.0	50.0	34.0	4.9	59	Female	Null
112.0	2.0	46.0	25.0	32.2	4.0	46	Male	GSTT1
120.0	1.0	90.0	87.0	21.4	12.4	28	Male	Null
111.0	2.0	40.0	2010.0	4.2	12.1	40	Female	Null
23.0	0.0	47.0	32.0	2.9	12.0	68	Female	Null
129.0	0.0	36.0	335.0	58.2	6.5	80	Male	Null
118.0	1.0	37.0	85.0	2.1	6.8	56	Female	Null
1.0	7.0	46.0	23.0	14.0	6.9	38	Male	Null
2.0	2.0	53.0	90.0	40.0	9.0	52	Female	Null
3.0	1.0	35.0	48.0	10.0	8.4	23	Female	Null
4.0	4.0	45.0	39.0	24.0	7.8	50	Male	GSTT1
5.0	5.0	28.0	88.0	11.0	10.2	62	Male	Null
6.0	0.0	41.0	70.0	34.6	9.2	15	Female	GSTT1
7.0	7.0	34.0	32.0	42.0	8.0	34	Male	Null
8.0	2.0	47.0	49.0	28.0	6.7	44	Female	Null
9.0	2.0	82.0	80.0	19.9	8.8	29	Female	Null
0.0	7.0	55.0	56.0	9.5	6.3	2	Female	Null
1.0	2.0	55.0	33.0	21.1	7.0	18	Male	Null
62.0	2.0	46.0	31.0	13.4	9.4	55	Female	Null

Control

control samples	Sex	Age/year	Genotype
1	Female	42	GSTT1
2	Female	25	Null
3	Male	27	GSTT1
4	Female	36	GSTT1
5	Male	25	GSTT1
6	Male	36	GSTT1
7	Male	28	GSTT1
8	Female	27	GSTT1
9	Female	29	Null
10	Male	48	GSTT1
11	Male	50	Null
12	Male	40	GSTT1
13	Male	55	GSTT1
14	Male	42	GSTT1
15	Male	54	GSTT1
16	Male	39	GSTT1
17	Male	51	GSTT1
18	Male	45	GSTT1
19	Male	51	GSTT1
20	Male	26	GSTT1

21	Male	29	GSTT1
22	Male	26	Null
23	Male	47	GSTT1
24	Male	38	GSTT1
25	Male	51	GSTT1
26	Male	54	GSTT1
27	Male	35	Null
28	Female	35	GSTT1
29	Female	26	GSTT1
30	Female	29	GSTT1
31	Male	52	GSTT1
32	Female	38	GSTT1
33	Female	25	GSTT1
34	Female	28	Null
35	Male	30	GSTT1
36	Female	41	GSTT1
37	Female	26	GSTT1
38	Male	50	GSTT1
39	Female	45	GSTT1
40	Male	42	GSTT1
41	Male	55	Null
42	Male	49	Null

43	Female	34	GSTT1
44	Male	40	Null
45	Male	45	GSTT1
46	Male	42	GSTT1
47	Female	30	Null
48	Male	48	GSTT1
49	Male	41	Null
50	Male	46	GSTT1

Appendix 3

Reagent:

Red cells lysis buffer (RCLB)

Add 8.3 gm of NH4CL, 1 gm KHCO3, 1.8 ml 5% EDTA and1 liter of distilled water.

White cells lysis buffer

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

TE buffer:

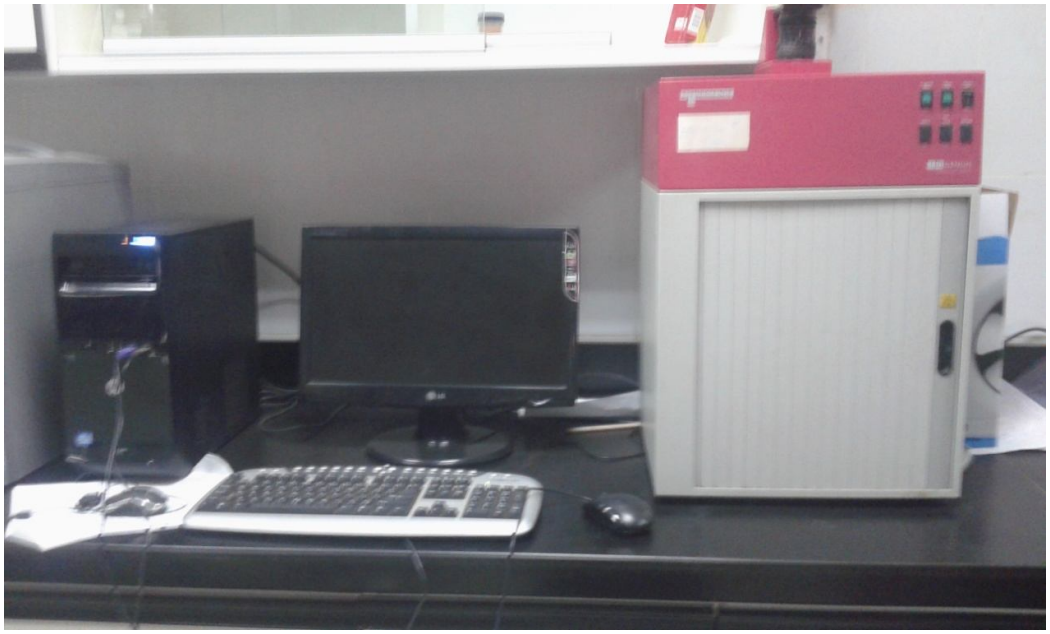
2.42 Tris base, 0.57 ml acetic acid, 50µl EDTA, (0.01M), and 100ml distilled water.

6 M NaCL:

35 gm of NaCL added to 1 liter of distilled water

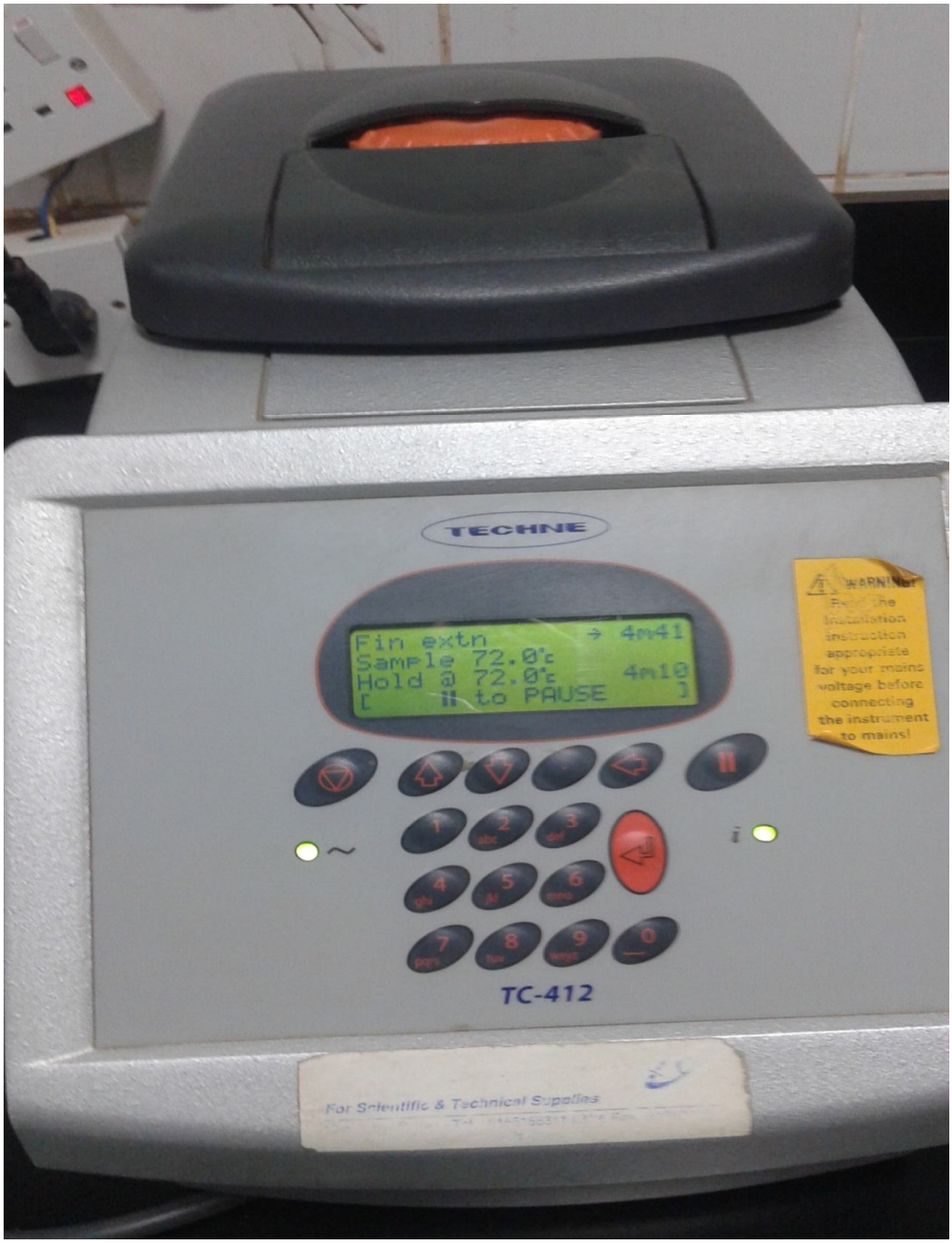
Appendix 4: images

SYNGENE



TECHN

(TC412)


TECHNE
Fin extn → 4m41
Sample 72.0°c
Hold @ 72.0°c 4m10
[⏸ to PAUSE]
TC-412
For Scientific & Technical Supplies

Gel electrophoresis