Sudan University for Sciences and Technology

College of Graduate Studies

Association Between Angiotensin Converting Enzyme Gene Insertion/Deletion Polymorphism and Risk of Acute Lymphoblastic Leukemia

العلاقة بين تعدد الشكل الجيني إضافة / حذف للإنزيم المحول للأنجيوتينسين وخطرالإصابة بسرطان الدم اللمفاوي الحاد

A dissertation submitted for partial fulfillment for the requirement of MSc degree in Medical Laboratory Sciences

(Hematology and Immunohematology)

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الآية

قال تعالى

بسم الله الرحمن الرحيم

(رَبِّ قَدْ آتَيْتَنِي مِنَ الْمُلْكِ وَعَلَّمْتَنِي مِن تَأْوِيلِ الْأَحَادِيثِ ۚ فَاطِرَ السَّمَاوَاتِ وَالْأَرْضِ أَنتَ وَلِيِّي فِي الدُّنْيَا وَالْآخِرَةِ ۖ تَوَفَّنِي مُسْلِمًا وَأَلْحِقْنِي بِالصَّالِحِينَ)

صدق الله العظيم

سورة يوسف الآية (101)

Dedication

To the soul of my father asking Allah set him Eden.

To my precious mother.

To my lovely big brother (Abdalaziz).

To my lovely brother (Awad).

To wonder sisters and my all friends.

To Dr. Hind Elsiddig.

Acknowledgements

All thank and Praise to Allah to give me the health, strength, and patient to achieving this research . Great thanks to Dr. Elshazali Widaa Ali for his effort, support and supervision. Great thanks to teaching staff in Sudan university for Science and Technology. Grateful a lot to my teacher Sohair Ramadan for technical support and encouragement. Thank you so much to Mr Ahmed Osman to for help me in collection of the samples and Randa Hassan for help me for technical support. Grateful a lot to all the patients who participated in this study.

Abstract

Acute lymphoblastic leukemia is the most common malignancy in childhood and represents about 85% of childhood acute leukemia, also it occurs in adults but is uncommon 15% of adult acute leukemia.

Major Renin Angiotensin system components (RAS) and angiotensin converting enzyme (ACE) are present in human umbilical cord blood cells and BM, ACE serve as stimulates the proliferation of bone marrow hematopoietic progenitors, and involved in pathological neoplastic hematopoiesis and leukogenesis.

This study aimed to investigate the association between ACE gene I/D polymorphism and risk of acute lymphoblastic leukemia.

A total of 96 subjects were recruited for this case control study, 48 patients with acute lymphoblastic leukemia 48 healthy volunteers as a control group. Blood samples were collected from all participants in EDTA anticoagulant container, genomic DNA was extracted by salting out method, and the ACE I/D polymorphism was analyzed using polymerase chain reaction (PCR). Amplified fragments separated on 2% agarose gel stained with ethidium promide and demonstrated by gel documentation system.

Patients' data was collected from patients' medical files, and analyzed by statistical package for social sciences (SPSS), version11.5.

The DD genotype of ACE I/D polymorphism was the most frequent in both leukemic patients and control group (52.1% and 54.2% respectively) than ID genotype (47.9% and 45.8% respectively), also the II genotype was totally absent.

According to gender, there was no statically significant difference in genotypes distribution (*P.value* =0.404).

The results showed no statistically significant difference in mean of age in ALL patients when compared in the two ACE genotypes,(Mean± SD 12.7 ±11.5 and14.3 ±20. 4) for DD and ID respectively, *P.value*= (0. 726).

In conclusion, there was no statistically significant association between ACE I/D polymorphism and risk of acute lymphoblastic leukemia among Sudanese patients.

المستخلص

يعتبرسرطان الدم الحاد اللمفاوي لدى الاطفال من اكثرالسرطانات شيوعا في الاطفال بنسبة 85% ويسمى سرطان الدم الحاد اللمفاوي لدى الكبار.

يعتبرتعدد الشكل الجيني للإنزيم المحول للأنجيوتينسين من اهم مكونات نظام الرنين- انجيوتنسين.

يوجد جين الانجيوتنسين في الحبل السري لدى الانسان ويؤدي الى تحفيزانتاج وتكاثرخلاياالدم في النخاع الشوكي ,كمايلعب دورفي تمايزالخلاياالجذعيه الى خلايادم بيضاء وفي انتاج خلاياالدم البيضاءالسرطانية.

هدفت هذه الدراسة لفحص تعددالشكل الجيني إضافة/حذف وسرطان الدم اللمفاوي الحاد لدى الاطفال والكبار.

شملت الدراسة 96 شخص، 48 مريض مصاب بسرطان الدم اللمفاوي الحاد و 48أصحاء.أخذت عينة دم،أستخلص الحمض النووي (DNA).

تم تحليل تعدد الشكل الجيني للإنزيم المحو للأنجيوتينسين بتفاعل البلمرةالتسلسل, تم فصل الجزء المستهدف ال (DNA)بواسطة 2% من الجل وصبغ بالإثيديوم بروماید وأظهرت النتائج بنظام توثيق الجل.

معلومات المريض جمعت بواسطة ملفات المرضى الطبيه وحللت بواسطةالحزمةالإحصائية للعلوم الإجتماعية.

حذف/حذف نمط جيني هوالأكثرتكراربين المرضى والأصحاء(52,1%2 و45%) متتالية،يتبعه إضافة/حذف نمط جيني(47,9%و8 45%) متتالية،ولايوجدإضافة/إضافة نمط جيني.

الإنزيم المحول للأنجيوتينسين لايؤثرعلي عمر حدوث سرطان الدم الحاد حيث كان متوسط العمرفي المرضى والاصحاء للأنماط الجينية المتوسط ± الإنحراف المعياري (12.7 ±11.5، 20.4 ±14.3) للحذف/حذف إضافة/حذف متتاليا،(القيمة =0,726).

الإنزيم المحول للأنجيوتينسين لايتاثر بنوع المصاب ذكر او انثى(القيمة =0,404).

لايوجد تفاعل بين الإنزيم المحول للأنجيوتينسين نمط جيني والعوامل المؤثرة في سرطان الدم الحاد كالعمرونوع المصاب ذكراوانثى.

لاتوجدعلاقة بين تعددالشكل الجيني للإنزيم المحول للأنجيوتينسين في المرضى السودانين المصابين بسرطان الدم اللمفاوي الحاد.

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List of Abbreviations

ACE	Angiotensin converting enzyme
ALL	Acute lymphoblastic leukemia
BM	Bone marrow
Bp	Base pair
CD	Cluster differentiates
CR	Complete Remission
FAB	French –American – British
I/D	Insertion/deletion
LFS	leukemia-free survival
MRD	Minimal residual disease detection
PAS	periodic acid – Schiff
PCR	Polymerase chain reaction
Ph	Philadelphia chromosome
RAS	Renin-angiotensin system
Rxn	Reaction
SCT	Stem cell transplantation
SPSS	Statistical package for social sciences
TE	Tris Ethylenediamine tetra acetate
WHO	world health organization

Introduction and Literature Review

1.1 Acute lymphoblastic leukemia (ALL)

ALL type of leukemia is type clonal malignant diseases of early hematopoietic progenitor cells, the lymphoblastic forms are characterized by homogeneous blast cell populations.(Porwit *et al* ., 2011),caused by an accumulation of lymphoblast in the bone marrow and blood, is the most common malignancy of childhood, characterized by 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. (Hoffbrand *et al.*, 2011).

ALL is malignant disease of the lymphoblastic system that is manifested by the slow but uncontrolled growth of abnormal cells, poorly differentiated lymphoid cells whose DNA synthesis time in significantly longer than in normal tissue. These abnormal lymphoid cells can be found in the bone marrow, spleen and lymph nodes. Normal bone marrow elements usually are replaced or displaced by the abnormal cells. (Hoffbrand *et al*., 2006).

Acute lymphoblastic leukemia is predominantly a malignant disease of children and occurs most common frequently between the ages of 2 and 10, although improved immunologic and cyto-chemical methods identification have increased the frequency of diagnosis in adult(Porwit *et al* ., 2011).

1.1.1 Epidemiology of Acute lymphoblastic leukemia

The median age at diagnosis for ALL is 13 years and approximately60% of cases are diagnosed under the age of 20 years. its the most common malignancy diagnosed in patients younger than 15 years, accounting for 23% of all cancers and 76% of all leukemia's in this age group are ALL, in general, the incidence is higher

in boys than in girls(T -cell ALL), except that girls have a slightly higher incidence of leukemia in the first year of life . The reported incidence of ALL is higher in northern and western Europe, North America, and lower in Asia, South America and Africa, Only a small proportion (< 5%) of patients with childhood ALL have underlying hereditary genetic abnormalities. Children with down syndrome have increased risk of developing ALL, mutations in the JAK2 gene, generally found in higher – risk ALL, fraternal twins and siblings of affected children are at two fold to four fold greater risk of leukemia during the first decade of life than are unrelated children (Hoffbrand *et al.*, 2011).

1.1.2 Classification

ALL classified by the French –American – British (FAB) schema and world health organization .FAB based on morphological features , WHO based on cytogenetic and immounologic marker classification (Porwit *et al* ., 2011).

1.1.2.1 Morphological classification (FAB)

1.1.2.1.1 Acute lymphoblastic leukemia (L1)

The blasts cells predominantly small, generally uniform , and lack variation in size creates homogeneous picture of similar cells. The chromatin is usually finely dispersed, nuclear round shape is regular, nucleoli not visible , if present are small and indistinct ,the cytoplasm usually scanty (high nuclear: cytoplasm ratio) and only slightly to moderately basophilic. The L1 type is the ALL that is common in childhood , with 74% of the cases occurring in children 15 years or younger (Firkin *et al*., 2006).

1.1.2.1.2 Acute lymphoblastic leukemia (L2)

Immature cells are more than twice the diameter of small lymphocyte ,there marked heterogeneity of cell size ,chromatin ranges from fine and dispersed to coarse and condensed ,thus presenting a mixed picture, nuclear cleaving and irregular shape , nucleoli are always visible and various sizes and numbers ,the degree of cytoplasmic basophilia is also variable, approximately 66% of the cases of ALL patients older than 15 years are of type L2 (Porwit *et al* ., 2011).

1.1.2.1. 3 Acute lymphoblastic leukemia (L3)(Burkitt type)

The burkitt form is relatively rare type of ALL 3%-5% of cases , the blast s are large , primitive appearing and present a characteristically homogeneous picture ,finely stippled chromatin, the nucleus is oval to round with regular ,one or more nucleoli are visible in most cells ,the cytoplasm is intensely basophilic and moderate abundant ,cytoplasmic is as well as nuclear vacuolation is prominent (Turgeon,2012).

1.1.2.2 Immunological classification

A panel of monoclonal antibodies differentiates of B-and T-lineage markers and lymphocyte maturation markers sub classify (Hoffbrand *et al*., 2011).

1.1.2.2.1 B- lineage (85%)

Pro B-ALL (early precursor B-ALL): HLA-DR+, TdT+, CD19+ (5% children; 11% adults) (Turgeon,2012).

Common ALL: HLA-DR+, TdT+, CD19+, CD10+ (65% children, 51%adults) (Hoffbrand *et al*., 2006).

Pre B-ALL (precursor B-ALL): HLA-DR+, TdT+, CD19+, CD10±,CytoplasmicIgM+. (15% children; 10% adults) (Provan *et al* .,2015).

B-cell ALL: HLA-DR+, TdT-, CD19+, CD10±, surface IgM+ (3% children; 4% adults) (Hoffbrand *et al*., 2011).

1.1.2.2.2 T- lineage (15%)

Pre-TALL: TdT+, cytoplasmic CD3+, CD7+ (1% children; 7% adults) (Turgeon,2012).

T-cell ALL: TdT+, cytoplasmic CD3+, CD1a/2/3+, CD5+ (11% Children, 17% adults) (Provan *et al* .,2015).

1.1.3 Etiology

In general unknown, Predisposing factors including :

Inherited factors ,Environmental influences.

1.1.3.1Inherited factors

The incidence of leukemia is greatly increasedin some genetic diseases, particularly (Down's syndrome) and high risk in twins (Hoffbrand *et al*., 2006).

1.1.3.2 Environmental influences

Chemicals as chronic exposure to benzene may cause bone marrow chromosome abnormalities, Drugs as alkylating agents (e.g. chlorambucil)if used to treat patients with lymphocytic or plasmacytic disorders, butis associated with a risk of the development of secondary leukemia.

Radiation, especially to the marrow, is leukaemogenic in survivors of the atom bomb explosions in Japan, viruses Infection Epstein-Barr virus EBV

(Firkin *et al.*, 2006).

1.1.4 Pathophysiology of acute lymphoblastic leukemia

Acute leukemia 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 lsteps resulting in: (i) an increased rate of proliferation, (ii) reduced apoptosis and (iii) a block in cellular differentiation (Hoffbrand *et al*.,2011). 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. If untreated these diseases are usually rapidly fatal but, paradoxically, they are also easier to cure than chronic leukemia. ALL is genetic changes are central to the development of leukemia. The dysregulation of genes encoding transcription factors and the resulting subversion of transcriptional pathways

that regulate haemopoietic cell homeostasis provides a mechanistic explanation for leukemogenesis (Hoffbrand *et al*.,2006).

Haemopoiesis can also be altered by the dysregulated activity of tyrosine kinases, as in the case of the BCR – ABL1 fusion gene. Alternatively, activating mutations in tyrosine kinase receptors for growth factors may confer a growth advantage to leukemic cells. This mechanism is exemplifed by mutations of FLT3 , which encodes a receptor tyrosine kinase expressed by immature haemopoietic cells that acts synergistically with other growth factors to stimulate proliferation of haemopoietic progenitor cells. These mutations typically involve small tandem duplications of amino acids that result in constitutive tyrosine kinase activity (Hoffbrand *et al.*,2006).

1.1.5 Clinical features

Acute presentation usual often critically ill due to: BM failure, organ infiltration (Porwit *et al*.,2011).

1.1.5.1Bone Marrow failure

symptoms that are seen of anaemia; (weakness, , breathlessness, light headedness, and Palpitations), Infection (particularly chest, mouth, perianal, skin, sweats), Hemorrhage: purpura, menorrhagia, and epistaxis, bleeding gums due to thrombocytopenia ,bone and joint pain are other common presenting complaints (Porwit*et al*.,2011).

1.1.5. 2 Organ infiltration

Moderate splenomegaly and hepatomegaly , lymphadenopathy may be prominent symptoms ,where lymphadenopathy and hepatomegaly are present in 75% of patients. tender bones meningeal syndrome produced by infiltration of leukemic cells into the tissues ,testicular swelling less common, leukemic cells can infiltrate many areas of the body (Provan *et al.*,2015).

1.1.6 Diagnosis of acute lymphoblastic leukemia

The identification of patients with ALL by investigations Hematological investigations, differential diagnosis (Hoffbrand*et al* ., 2006).

1.1.6.1 Hematological investigations

1.1.6 .1.1 Complete blood count (CBC)

The total white cell count maybe decreased, normal or increased to 200 x 10^9/L or more. Blood film examination typically shows a variable numbers of blast cells, cells distinguishes three subtypes of ALL (L1, L2 and L3) as classified by the (FAB) schema, this classification cannot accurately distinguish between

ALL and non – lymphoid acute leukemia, and has no prognostic or therapeutic relevance with contemporary therapy (Hoffbrand *et al* ., 2006).

1.1.6 .1.2 Bone marrow

BM is hypercellular with >20% leukemic blasts, this blast usually show monomorphic population blasts with marked decrease in normal hematopoietic precursors of all types (Barbara, 2006)

1.1.6.2 Differential diagnosis

1.1.6.2.1 Cytochemistry analysis

Cytochemical stains such as periodic acid – Schiff reacts positively with cells in over 70% of ALL cases, while myeloperoxidase, Sudan black and non – specificesterases, including α –naphthyl butyrate and α –naphthyl acetate esterase, are typically negative (Hoffbrand *et al*., 2006) .

1.1.6.2.2 Cytogenetics and Molecular studies

The demonstration that specific chromosomal abnormalities are associated with distinct subtypes of acute leukaemia .In ALL, cytogenetics also provides important information with cases are stratified according to the number of chromosomes in the tumour cell (ploidy) or by specific molecular abnormalities, hyperdiploid cells

have>50 chromosomes and generally have a good prognosis whereas hypodiploid cases carry a poor prognosis (provan,2007).

The most common specific abnormality in childhood ALL (B-ALL) is the t(12; 21) (p13; q22) TEL –AMLl translocation ,the AMLl protein plays an important part in transcriptional control of haemopoiesis and this is repressed by the TEL-AMLl fusion protein, the frequency of the Philadelphia chromosome translocation t (9; 22) (q34;q11.2) BCR-ABL1,increases with age and carries a poor prognosis (Hoffbrand *et al* ., 2006). Translocations of chromosome t(v;11q23); involve the MLL gene and are seen particularly in cases of infant Leukemia, Using more sensitive molecular genetic tests, as well as fluorescence in situ hydridization (FISH) analysis, some cases normal by conventional cytogenetic testing are found to have fusion genesasBCR-ABL1 or other genetic abnormalities . In (T –ALL) , TCR and (in 20% the IGH gene) show rearrangement , cytogenetic changes often involve the TCR loci with different partner genes, the majority of cases have acquired genetic abnormalities that lead to activation of the NOTCH signaling pathway (Hoffbrand *et al* ., 2006).

1.1.6.2 .3 Immunophenotyping analysis

Leukaemic blasts are characterized by the aberrant expression of haemopoietic antigens on their cell surface, distinct patterns of antigen expression permit accurate discrimination between myeloblasts and lymphoblasts, allowing confident distinction between subtype of ALL. Immlmological markers for classification of ALL (Hoffbrand *et al.*, 2011),The B –lineage blast positive in CD17,Ccd 79,CD10,TdT, while all this markers negative in T- lineage blast.

The T–lineage blast positive in CD7,cCD3,CD2 , while all this markers negative in B – lineage blast also TdT Positive (provan , 2007).

1.1.7 Treatment of acute lymphoblastic leukemia

Treatment of ALL including :Supportive treatment, Chemotherapy treatment, and allogeneic stem cell transplantation (Hoffbrand *et al* ., 2006).

1.1.7. 1 Supportive treatment

Provide explanation and offer counseling—the word 'leukemia' and prospect of prolonged chemotherapy are often dis tressing. RBC and platelet transfusion support will continue through treatment Irradiated products for patients treated with purine analogues. Platelet transfusion to maintain count >10 × 10^9/L, unless septic, on antibiotics, hemorrhagic or other hemostatic abnormality (>20 × 10^9/L) (Firkin *et al*., 2006).

1.1.7. 2 Chemotherapy treatment

Adult ALL regimens have evolved from successful treatments for childhood ALL. Initial aim of chemotherapy is to eliminate leukaemic cells and achieve complete haematological remission (CR), defined as normal BM cellularity (blast cells <5% and normal representation of trilineage haematopoiesis), normalization of peripheral blood count with no blast cells,neutrophils ≥1.5 × 10^9/L, platelets ≥100×10^9/L and Hb>10g/dL. Leukaemia is undetectable by conventional morphology but may be demonstrated by more sensitive molecular techniques and CR is not synonymous with cure (Hoffbrand *et al* .,2011).

1.1.7. 3 Allogeneic stem cell transplantation

Ph+ ALL: allogeneic SCT transplant recommended in CR1 for all eligible patients (provan , 2007).

1.1.8 Detection of minimal residual disease

A slow reduction of the leukemic cell burden by remission induction therapy is associated with a poor treatment outcome.(Prowit *et al*., 2011).

Response to therapy, as assessed by morphological examination of bone marrow and peripheral blood smears, has limited sensitivity and accuracy. The advent of methods for detect in gminimal residual disease (MRD), which are at least 100 times more sensitive than conventional morphological techniques, has introduced a profoundly new way to monitor response to treatment. (Hoffbrand *et al* .,2011).

The most reliable methods for measuring MRD include flow cytometric profiling of aberrant immunophenotypes, PCR amplification of fusion transcripts and chromosomal breakpoints, and PCR amplification of antigen – receptor genes. The prognostic importance of MRD in childhood ALL has been unequivocally established by the results of numerous correlative studies (Provan *et al*., 2015).

MRD−ve status post-initial induction phase (28–35) days has very strong prognostic indications. MRD −ve patients carry a much better prognosis (Provan *et al*., 2015).

MRD can be repeated at additional time-points depending on the regimen used. MRD +ve patients have a very high risk of relapse and are candidates for allogeneic SCT (Provan *et al*., 2015).

1.1.9 Prognosis

Overall 75% of adults with ALL achieve a CR with a modern regimenand good supportive care; more intensive induction and consolidationreduces relapse risk but adds toxicity; results in patients >50 years areless good. Relapse rates remain high. median remission duration of CR is around 15 months. 35–40% ofadults with ALL treated with intensive chemotherapy survive 2 years, in contrast to high cure rate in childhood ALL, leukemia-free survival(LFS) in adult ALL in general is <30% at 5 years (patients >50 years10–20%) (Turgeon,2012).

LFS after chemotherapy in patients without adverse risk factors is >50% but <10% for very high-risk Ph/BCR-ABL+ ALL hence latter should have an allograft in 1st CR if possible (Hoffbrand *et al* .,2006).

1.1.9.1 Prognostic factors

Prognosis in adult ALL is much poorer than childhood ALL (cure rate80%) due to higher frequency of poor prognostic features and treatment-related toxicity. The most important prognostic factors are listed.These are useful for risk stratification to identify patients who require more intensive therapy and SCT in 1st CR (Provan *et al*., 2015).

1.1.9.1.1 Patient age

Patient age(<50 years CR >80%, LFS >30%, ≥50 years CR <60%,LFS <20%) (Provan,2007).

1.1.9.1.2 Leucocyte count

The high leucocyte count(>30 × 109/L in B precursor-ALL; >100 $\times 10^9$/L in (T-ALL) poor risk (Hoffbrand *et al*.,2011).

1.1.9.1.3 Immunophenotype

pro-B-ALL and pro-T-ALL have poorer outcomes, common pre-B-ALL still poor, mature B-cell ALL and T-cell ALL have better outcomes due to the use of more intensive regimens(Hoffbrand *et al*., 2006).

1.1.9.1.4 Cytogenetic

Philadelphia (Ph) chromosome BCR-ABL+ very poor prognosis: <10% LFS after chemotherapy. Long time to CR (>4 weeks): poor risk (Provan, 2007).

1.1.9.1.5 Minimal residual disease level

High level after induction, persistent/increasing MRD during consolidation: poor risk (provan *et al*., 2015).

1.2 Angiotensin converting enzyme

Major Renin Angiotensin system components (RAS) and angiotensin converting enzyme (ACE) are present in human umbilical cord blood cells (Goker *et al*., 2005).Angiotensin II could serve in the erythroid and myeloid differentiation of stem cells.(Rodgers *et al*.,2000). On the other hand, there are preliminary data that local bone marrow RAS may be involved in pathological neoplastic hematopoiesis and leukogenesis (Abali *et al*.,2002)

(Wulf *et al*., 1998).Polymorphism based on the presence (insertion I) or absence (Deletion D) of a 287 base pair Alu repeat sequence in intron 16, resulting in three genotypes: DD homozygote, II homozygote and ID heterozygote (Rigat *et al*.,1990).ACE , may play a role in the regulation of

lymphocyte functions. The T-lymphocyte ACE levels of a given subject are highly reproducible when measured on two different occasions, but may vary widely between individuals in association with I/D polymorphism of the ACE gene.(Akalin *et al*.,2011).

1.2.1 Angiotensin converting enzyme I/D Polymorphism

The ACE gene is 21kb long, consisting of 26 exons and 25 introns and located in chromosome 17p23 (ELmubarak *et al* .,2015). It is characterized by an insertion /deletion polymorphism based on the presence (Insertion I) or absence (Deletion D) of a 287-bp Alu repeat sequence in intron 16, resulting in three genotypes DD homozygote, II homozygote and ID heterozygote (Rigat *et al*.,1990). the DD genotype is associated with a two fold increase in plasma ACE activity over that of II genotype, with intermediate level of heterozygote ID (mekki& Ali, 2015). The low ACE level associated with allel I, could rise anti proliferative effect and protect normal hematopoiesis against chemotherapy rather than decrease malignant clone proliferation (Hajek *et al* ., 2003).

1.3 Previous studies

Many previous studies investigated the association between the ACE I/D polymorphism and risk of acute lymphoblastic leukemia.

Akalin *et al* investigated the ACE insertion/deletion (I/D) gene polymorphisms in patients with hematological malignancies including acute lymphoblastic leukemia, the results showed about 12% from patients carrier DD genotype and about 87% carrier II genotype, also found 80.4% of the patents presented ID/II genotypes whereas it was 55.9% in the control group, ID and II genotypes were found to be associated with 3.2 folds increase risk of ALL (Akalin *et al* ., 2011). A study by ELmubarak *et al* showed that, the genotype DD was the most frequent among both patients and control groups, followed by the genotype ID, whereas the

genotype II was present in patients and completely absent in control group. There was a significant association between I allele of ACE and risk of AML among Sudanese population. (*P.value*: 0.017) (ELmubarak *et al* .,2015).

Gonzalez *et al* conducted case control study including 4150 women showed, the DD carriers showed a significantly increased risk of developing breast cancer when compared with the II carriers,(P.*value* 0.03) (Gonzalez*et al*.,2005). Gard investigated 101 Brazilian patients with breast cancer and showed that , I/D genotype was less frequently associated with breast cancer than were the DD or II genotypes (Gard,2010).

Toma *et al* screened colorectal cancer patients in a Romanian population, they showed no difference alleles frequency between cancer patients and controls (Toma *et al*.,2009).

Vairaktaris *et al* found the II genotype was more frequent in a sample of Greek and German oral squamous cell carcinoma patients than in matched controls (Vairaktaris *et al.*, 2009).

Rationale

Leukemia is most common childhood cancer and acute lymphoblastic leukemia (ALL) is the most common type, accounting for 75-80% of all cases . Childhood ALL comprises different biological subtypes , defined by cell morphology , immunophenotype, gene expression features and genetic abnormalities this is methods of identification have increased the frequency of diagnosis in adult ALL most frequently between the ages of 2 and 10 years (Hoffbrand *et al*., 2006).

High profiling of genetic alterations has transformed the genetic basis of ALL. it has been known for several decades that the majority of childhood ALL cases gross chromosomal alterations. These alterations are of key importance in both the pathogenesis and clinical management of ALL (Harrison. ,2009)

ACE may play a major role in physiological activities including cellular proliferation ,also ACE participate in the regulation of lymphocyte functions, but may vary widely between individuals in association with I/D polymorphism of the ACE gene (Akalin *et al* ., 2011).

Some studies reported an association between ACEI/D polymorphism and risk of some hematological and non hematological malignancies.

To our knowledge, there is no study in Sudan addressing ACE I/D polymorphism as risk factor for ALL patients.

Objectives

General objective

To examine the association of a angiotensin converting enzyme insertion/deletion (I/D) gene polymorphism with risk of acute lymphoblastic leukemia.

Specific objectives

1- To determine the frequency of ACE I/D genotypic variants in patients with ALL using polymerase chain reaction (PCR).
2- To compare the genotype distribution between subjects with ALL and healthy controls
3- To investigate the association between ACE(I/D) polymorphism and risk of ALL.
4- To associate ACE (I/D) polymorphism with patients gender .
5- To compare age of incidence according to ACE genotypes.

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Study design

This was a hospital -based case control study.

2.1.2 Study area and duration

Study was conducted at Radiation and Isotopes Centre, Wad madni and Flow cytometer center, Khartoum, Sudan, from January 2017- May2017.

2.1.3 Study population

Acute lymphoblastic leukemia (ALL) patients and healthy volunteers.

2.1.4 Inclusion criteria

ALL patients diagnosed with ALL using cytochemical stains and immune - phenotyping were included.

2.1.5 Exclusion criteria

No Exclusion criteria.

2.1.6 Ethical consideration

This study was approved by faculty of medical laboratory science, Sudan University of Science and Technology. Verbal informed consent was obtained from each participant before samples collection..

2.1.7 Data collection

Patients data (age, sex) were collected from patients medical files.

2.1.8 Sample collection

Blood samples (3ml) were collected from each patients upon their consent in EDTA container.

2.2 Methods

2.2.1 Laboratory methods

2.2.1.1 DNA extraction

Nine hundred microliter of red cell lysis buffer was added to 300 µl of whole blood in 1.5 ml eppendorf tube.

The mixture was centrifuged at 8000 rpm for 3 minutes and the supernatant was discarded.

This step was repeated 2-3 times till RBCs lysis was complete and a white pellet of WBCs was obtained.

To the cell pellet, 300µlof white cell lysis buffer and 40µlof 10%Sodium dodecyl sulphate were added, Mixed thoroughly ,and incubated at 37˚c for 5 minutes. At the end of incubation, 100µl of 6MNaCl was added, vortexed and centrifuged at 8000 rpm for 5 minutes to precipitate the proteins.

To Precipitate of DNA, The supernatant was transferred into a new eppendorf tube containing 300µlof isopropanol. DNA was precipitated by inverting the eppendorf tube slowly. Further, the eppendorf tube were centrifuged at 8000rpm for 10 minutes to pellet down the DNA. Supernatant was discarded, 70% ethanol was added and mixed slowly to remove any excess salts, the tubes were centrifuged at 8000 rpm for 5 minutes to pellet down the DNA. Supernatant was discarded and DNA air-dried. After thorough drying, 50µl of TE buffer was added to dissolve the DNA,1% agarose gel electrophoresis use to detect genomic DNA isolated from human blood samples (Sugana *et al*., 2014).

2.2.1.2 Polymerase chain reaction

Allele specific PCR was used for molecular detection of ACE insertion /deletion polymorphism. The sequences of the forward, reverse and internal primers are shown in (Table 2.1).

Table 2.1 primer sequences and length.

Name of the gene	Primer sequences	Possible bands		
		DD	**ID**	**II**
ACE	Forward-5'-CTG GAG ACC ACT CCC ATC CTT TCT - 3'	190bp	190bp 490bp	490bp
	Reverse-5'-GAT GTG GCC ATC ACA TTC GTC GTC AGA T- 3'			
	Internal-5'-TGG GAT TAC AGG CGT GAT ACA G - 3'			

Table 2.2PCR reaction mixture

PCR reaction mixture	Volume
Template DNA	2µ/L
Primer (F: 100Pmol/µL)	1µ/L
Primer (R: 100Pmol/µL)	1µ/L
Primer (I: 100Pmol/µL)	1µ/L
Distilled water	15µ/L
Total reaction volume	20µ/L

The amplification process consisted of initial denaturation of 94˚Cfor 3 min, 35 cycles each consist of 94˚C for 30sec,57˚C for 30 sec,72˚C for 30 secthen final extension at 72˚C for 5 min.

2.2.1.3 Agarose gel electrophoresis

PCR products were electrophoresed on 2% agarose gel containing ethidium bromide and analyzed under UV light. 5µL of the PCR product were loaded on the gel along with 4µL of 100bpdeoxyribonucleic acid (DNA) ladder applied with each batch of patients samples.

2.2.1.4 Interpretation of results

The sizes of the different fragments obtained were 490 bp (II), 190 bp (DD), and 490 and 190bp (ID) figure(2.1).

2.2.2 Statistical analysis

Data was analyzed by statistical package for social sciences (SPSS), version 11.5. Qualitative data was represented as frequency and Percentage. Quantitative data was presented as mean ± SD. Association between qualitative variables was tested using Pearson's Chi square ($\chi 2$) and Fisher's exact tests. Binary logistic regression analysis was used to investigate the association between genotypes and risk of ALL. The alleles frequencies were tested using the conventional formula (Allele frequency= homozygous+1/2X heterozygous).

Chapter Three

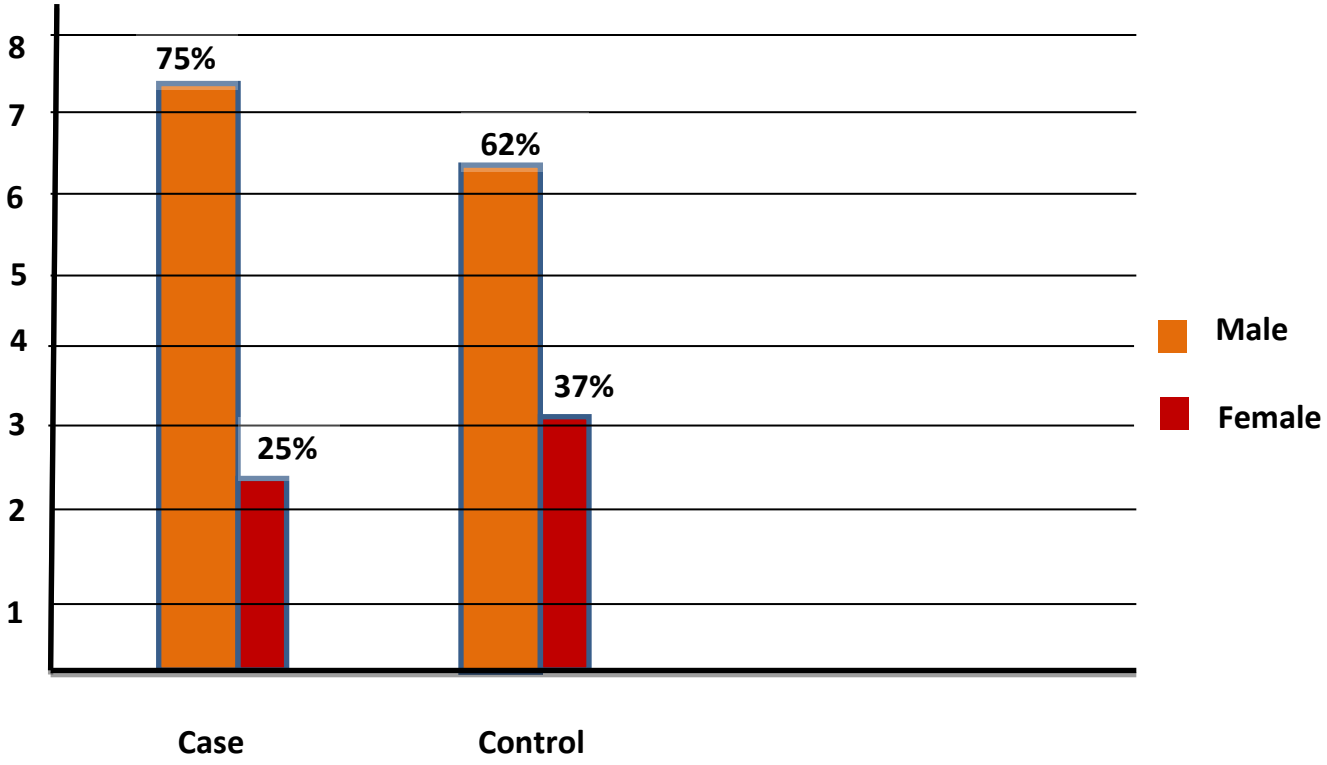
Results

A total of 96 Sudanese subjects were enrolled in this study, 48 patients with ALL and 48 healthy volunteers as a control group. The mean age of patients was 14 years and of controls was 17 years (Table 3.1).

Table 3.1 Comparison of mean age in patients and controls.

Variables	**Age (years)**	
	Mean	**SD**
Patients	14	16.2
Controls	17	17.1
Total	31	23.3

Thirty six (75%) of patients were males and 12 (25%) were females, while 30 (62%)of controls were males and 18 (37%) were females (Figure 1) .



Figer 3:1 Gender distribution in study groups.

DD genotype was the most frequent in both patients and control groups, the DD genotype was the most frequent in both patients and control groups, followed by ID genotype, while II genotype was totally absent.

There was no statistically significant difference in genotype distribution when compared in patients and control groups (Table 3.2).

Table3. 2 Genotype distribution in study groups.

Groups	**Genotype distribution**		***P. value***
	DD	ID	
Patients	25 (52.1 %)	23 (47.9 %)	0.398
Control	26 (54.2 %)	22(45.8 %)	

The results showed, no statistically significant association between each of ACE I/D genotypes and gender (Table3.3).

Table3.3 Association between ACE I/D polymorphic genotypes and gender.

Gender	**Genotype distribution**		***P. value***
	DD	ID	
Male	20(41.7%)	16(33.3%)	0.404
Female	5(10.4%)	7 (14.6%)	

comparison of mean age in patients with DD and ID genotypes showed no statistically significant difference (Table3.4).

Table3. 4 comparison of mean age according to genotype.

Age	**ACE polymorphism**		***P. value***
	DD	ID	
Mean	12..7	14.3	0.726
SD	11.5	20.4	

The logistic regression showed no interaction between ACE genotypes with age and gender in ALL patients (Table 3.5).

Table 3.5 Interaction between ACE I/D polymorphism with age and gender in ALL patients.

Variables	OR	95%C.I.		*p. value*
		Lower	Upper	
Age	2.286	0.193	27.0	0.512
Gender	1.570	0.466	6.568	0.407

The frequency of D allele was 0.76 in patients and 0.77 in control group, while the frequency of I allele was 0.24 in patients and 0.23in control group.

Chapter Four

Discussion, Conclusion, and Recommendations

4.1 Discussion

This study investigated the association of ACEI/D gene polymorphism with risk of ALL.

The results showed that, the genotype DD was the most common among both patients and control groups, followed by the genotype ID, whereas the genotype II was completely absent in both groups. There was no statistically significant association between ACE I/D polymorphism genotypes and age and gender and ALL (*P.value*=0.512 and 0. 407respectively).This result reported mean of age in patents and control (14 years and 17 years respectively).This in agreement with a study carried out by ELmubarak *et al*, who conducted study in Sudanese with AML, and reported that DD genotype was the most frequent among patients and controls, and found no statistically significant association between ACE I/D polymorphism and gender of AML patents. In contrast in the same study found statistically significant association between ACE I/D polymorphism and AML(*P.value*=0.017), also, found II genotype associated with increased risk of AML (ELmubarak *et al.*, 2015).

In contrast , our study disagrees with a study carried out by Akalin *et al* who conducted study in ALL patents and reported that, the genotype DD was the most frequent among control group compared with leukemic ALL .also, reported mean of age in patients and control (49 years and 30 years respectively), and found ID/II genotype frequency was significantly increased in patients with hematological disorder (*P.value* =0.008), compared to control group (Akalin*et al*., 2011). Studies investigated the association between ACE I/D gene polymorphism and non- hematological malignancies reported conflict results. A study by Gonzalez *et al* revealed that the DD carriers had a significantly increased

risk of developing breast cancer when compared with the II carriers (Gonzalez *et al* 2005). Sun *et al* showed that there was no significant difference in genotype distribution (DD, ID or II) between breast cancer patients and controls (Sun *et al*.,2011). A study conducted by Hibi *et al* found that there is no significant association between the ACE I/D polymorphism and the risk of gastric cancer (Hibi *et al*,2011). Recent study by Cheng did not find any association between the ACE gene I/D polymorphism and lung cancer in either genotype or allele distribution (Cheng *et al*.,2015).

These different findings may reflect variations in carcinogenesis process in different types of cancer particularly between solid tumors and hematological malignancies (ELmubarak *et al* .,2015).

4.2 Conclusion

- The DD genotype of ACE I/D polymorphism was more frequent inALL patients and control group than ID genotype, while the II genotype was absent in both groups.
- There was no statistically significant association between ACE I/D polymorphic genotypes and ALL risk.
- There was no interaction observed between ACE genotypes with age and gender with ALL patients.

4.3 Recommendations

- Further studies should be conducted to identify other sucsubility genetic factors, and conducted with large sample size.
- Future studies should be conducted to identify association between ACE I/D polymorphism with morphological and immunological classification.
- Future studies should be conducted to measure ACE enzyme plasma level and coupled with ACE genotypes.

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Appendix

Appendix (1): Preparation of reagents

Ethylenediamine –tetra-aceticacetate (EDTA)

Diapotassiumsalt 100g
Distilled water 1L

Red cells lysis buffer (RCLB)

Tris HCL0.605g
$MgCL_2$1.016 g
KCL0.372g
EDTA0.372g
Distilled water 500 ml

White cells lysis buffer (WCLB)

Tris HCL0.121g
KCL0.0 74g
$MgCL_2$1.203g
EDTA0.074g
Nacl0467g
Distilled water 100ml

SD Sodium dodecylsulphate 1g
Distilled water 10ml

6M Nacl Nacl8.765g

Distilled water 25ml

TE Buffer

Tris HCL 0.030g
EDTA0.009g
Distilled water 100 ml

1XTBE buffer:

TE Buffer 10ml

Distilled water 90 ml

Preparation of Agarose Gel (2%)

Two gram from agarose powder was dissolved in 50 ml from 1XTBE buffer and heated in microwave for 2 min (until agarose completely dissolved). then added 1.5 µL from ethidium bromide after cooling, mixed well and poured in to a casting tray that was taped up appropriately and was equipped with a sutable comb to from well in place. Any bubbles were removed and the gel allowed to set at room temperature. After solidification the comb was gently removed and spacer from the opened slides was removed.

Ethidium Bromide solution

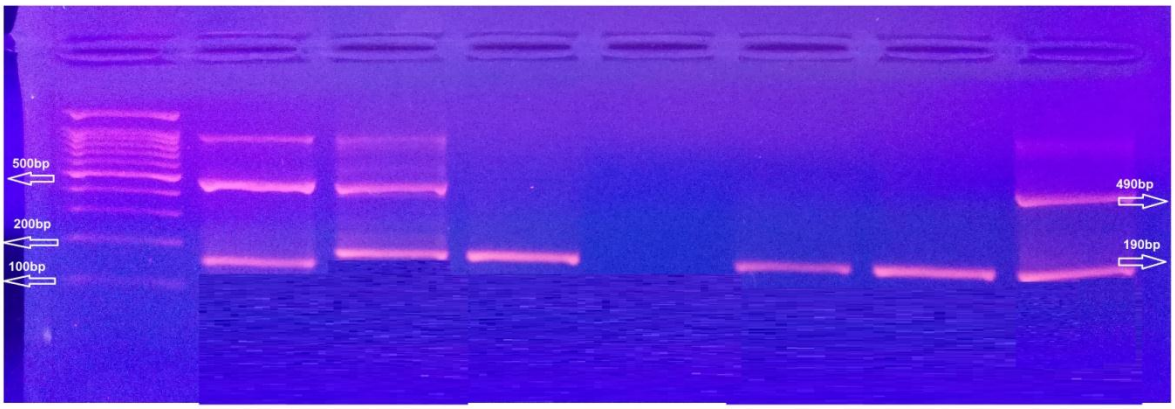
Ten milligrams of ethidium bromide powder were dissolved into 500 µL deionized water ,and kept into brown bottle.

Appendix (2)

Primers (forward, reverse, internal)

The stock solution were prepared by adding 250 µL from dH_2O then were pelleted at 14.000 for 5min and placed at 4˚C over night . The working solutions were prepared by adding 10 µL from stock solutions and 90 µL from dH2O, mixed and placed at -20 ˚C .

Appendix (3) Amplified fragments



Appendix (4): Instruments

1- PCR machine

2- Electrophoresis machine

3- UV light machine