Sudan University of Science and Technology

College of Graduate Studies

Association of Cytochrome P450 2 E1 (C1053T) and NADPH Quinone Oxide Reducatase 1(C609T) (C 465T) Genes Polymorphism with Acute Lymphoblastic Leukemia in Sudanese Patients

علاقة تعدد الأشكال الجينية للسيتوكروم ب 450 أي1(C1053) والناد المفسفر الهيدروجيني لأكسيد الكينون المختزل (C465T) (C609T) مع سرطان الدم الأبيض الليمفاوي الحاد عند المرضى السودانيين.

A thesis Submitted for Fulfillment of the Requirements for PhD Degree in Medical Laboratory Science (Hematology and Immunohematology)

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DECLARATION

I declare that this thesis is submitted to Sudan University of Science and Technology, College of Graduate studies for the degree of doctor of philosophy and has not been previously submitted by me for this degree or other degree at this university or any other universities or institutes.

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الاية

بسم الله الرحمن الرحيم

قال تعالي:

(وَفِي أَنفُسِكُم أَفَلَا تُبْصِرُونَ)

صدق الله العظيم

(سورة الذاريات الاية "21")

DEDICATION

To my family

To my friends

To my colleagues

To anyone really who helped me

I dedicate this work

With infinitive love

May

Acknowledgments

Abstract

This was retrospective analytical case control study conducted at SUST in Khartoum State during the period of May 2016-January 2019, aimed to investigate the association of cytochrome P450 2 E1 (C1053T) and NAD(P)H: quinone oxidoreducatase 1(C609T) (C 465T) genes polymorphism and development of acute lymphoblastic leukemia in Sudanese patients .

A total of 204 subjects were involved in this study, 102 ALL patients and 102 healthy volunteers as controls (1:1) matched age and sex. Five ml venous blood samples were collected from each subject in EDTA blood container, 2.5 ml of blood sample was analyzed by automated hematology analyzer for measurement of complete blood counts for case group and control group and flow cytometer used to identify ALL type and subtypes among patients group, the remaining 2.5 ml used for Polymerase chain − Restriction Fragment Length Polymorphism (PCR-RFLP) technique to detect genetic polymorphisms for CYP2E1(C1053T), NQO1 (C609T) and NQO1 (C465T). Data were analyzed by statistical package for social science (SPSS) computer program version 25. This study included 69(67.6%) males and 33(32.4%) females with male to female ratio 2.1:1. Age ranged from1-85 years with mean age (17.6±17.68) in both groups. Based on flowcytometery results B cell type account 82(80.4%), while T-ALL account 20 (19.6%). Complete blood count for B and T- ALL types in case group as follow: Mean TWBCs was (43.6 ± 67.29 $X10^9$/L) and (201.78 ± 261.26 $X10^9$/L), mean RBCs was ($2.90\pm0.86X10^{12}$/ L), and ($3.158\pm1017X10^9$/L), mean platelets was ($53.10\pm 53.26X10^9$), and ($74.70\pm 91.633X 10^9$/L), mean hemoglobin (Hb) level was (8.39 ± 2.43 g/dl), and (9.70 ± 2.76 g /dl) and mean blast% was (68.02 ± 19.91) and (71.25 ± 22.759) respectively, this study showed highly statistical significant association of mean total white blood count ($P= 0.000$) and mean of hemoglobin concentration ($P=0.038$) among T-ALL cases, while other parameters as RBCs , PLTs and Blast % were statistically insignificant with ALL subtypes ($P=0.287.0.168$ and 0.529) respectively. Complete blood count results among study group showed mean TWBCs in ALL patients was ($74.65\pm143.01X10^9$/L) while in control group was (8.89 ± 4.01 $X10^9$/L), mean RBCs ($2.95\pm0.93X10^{12}$/ L) in cases and ($4.58\pm 0.776X10^{12}$/L) in control group, platelets mean ($57.34\pm62.68X10^3$/L) and ($333.41\pm109.1X10^9$/L) respectively in cases and control. Mean hemoglobin (Hb) level was (8.65 ± 2.54 g/dl) for cases, (12.42 ± 2.05g /dl) for control, this difference was highly significant ($P= 0.000$).

CYP2E1 genotypes for wild type (CC) 95(93.1%), heterozygous type (CT) 7(6.9%), while homozygous mutant (TT) not detected 0(0%) among case group, while for control group were as follows: CC 98(96.1%), CT 4(3.9%) and for TT was not detected (0%). The hererozygosity was higher in patients when compared with controls (6.9% and 3.9%, respectively), Odd ratio was 1.805 (95% CI: 0.5117-6.369, P=0.3524); however, this difference was statistically insignificant and also for alleles was insignificantly associated with risk of ALL (OR=1.776, 95% CI: 0.5119-6.167, P= 0.3592).

NQO1 (C609 T) genotypes frequency for CC 76(74.5%), CT 18(17.7%) and 8(7.8%) for TT among cases, while for control group represented as CC 89(87.2%), CT 7(6.9%) and TT 6(5.9%). The hererozygosity was higher in patients compared with controls (17.7% and 6.9%) respectively, the odds ratio was 3.513 (95% CI: 1.327-9.301, P=0.0081), this difference was statistically significant, while for homozygous mutant frequency was also high in case (7.8%) than control (5.9%) with odd ratio 1.338 (95%CI 0.4638-3.862, P=0.5888), this difference was insignificant. Alleles frequency was significantly associated with risk of ALL (OR=1.840, 95% CI: 1.019-3.321), P= 0.0408).

Frequency of NQO1 (C465T) genotypes for CC, CT, and TT genotypes among patients with ALL were 72(70.6%), 23(22.5%) and 7(6.9%) respectively, while in control group were distributed as follows: 94(92.2%), 5(4.9%) and 3(2.9%) respectively. The hererozygosity was higher in patients compared with controls (22.5% and 4.9%, respectively); this difference was statistically highly significant, the odds ratio was 7.667 (95% CI: 2.539-23.15, P =0.0001) and for homozygous mutant was also high in case(6.9%) than control(2.9%) with odd ratio 4.667 (95%CI 0.9410-23.14, P = 0.0401), alleles frequency also significant associated with risk of ALL (OR=2.7, 95% CI: 1.427-5.107), P = 0.0017).

In conclusion: This study concluded that NQO1 (C609T) and (C465T) considered as risk factors for ALL development in Sudanese population, while CYP2E1 (C1053T) polymorphism was not associated with risk of ALL.

المستخلص

أجريت هذة الدراسة التراجعية التحليلية الحالة والحالة الضابطة في جامعة السودان للعلوم والتكنولوجيا بولاية الخرطوم في الفترة من مايو 2016 وحتي يناير2019 م ، وهدفت للكشف عن الإرتباط بين تعدد الإشكال الجينية للسيتوكروم ب2إي1(C1053T) والناد المفسفر الهيدروجيني لإنزيم أكسيد الكينون المختزل 1 (C609T) و(465T C) وتطور سرطان الدم الأبيض الليمفاوي الحاد عند المرضي السودانيين.

شملت هذة الدراسة 204 شخص، منهم 102مصاب بسرطان الدم الأبيض الليمفاوى الحاد و102 شخص اصحاء متطوعين كحالات ضابطة متطابقة بنسبة (1:1) في النوع والعمر .

جمعت 5 مل من عينات الدم من كل المنتسبين في الدراسة في حاوية تحتوي علي مادة مانعة للتجلط،2.5 مل من العينة حللت بواسطة جهاز تحليل الدم الالي لقياس تعداد الدم الكامل للمرضي والمجموعة الضابطة وأستخدم جهاز الفلوسايتوميتر للتعرف علي أنواع سرطان الدم الأبيض الحاد لدي المرضي. و المتبقي 2.5 مل من عينة الدم أستخدمت تقنية القطع الجزيئ الطولي المتنوع – تفاعل البلمرة التسلسلي لقياس تنوع الطفرات الجينية. وحللت المعلومات إحصائيا بإستخدام الحزم الإحصائية للعلوم الإجتماعية النسخة 25.

تضمنت الدراسة 69(76.6%) من الذكور و33(32.4%) من الاناث بنسبة الذكور للاناث2.1:1، وتراوحت أعمارهم بين 1– 85 سنة بمتوسط (17.68±17.61) في كلا المجموعتين.

إعتمادا على نتائج الفلوسايتوميتر وجد تعداد الخلايا البائية حوالي (80.4 %)، بينما تعداد الخلايا التائية (19.6%). تعداد الدم الكامل للخلايا البائية والتائية للمرضي كالتالي: متوسط خلايا الدم البيضاء(67.29 ± 43.64 X 10^9 /ل) و(261.261 ± X201.78 10^9 /ل) ومتوسط خلايا الدم الحمراء(0.86 ± 2.90 X 10^9 /ل)و(1.17 ± 3.158 X 10^9 /ل)،متوسط تعداد الصفائح الدموية(53.26 ± 53.10

10^9 X 8.39 ± 2.43)،متوسط خضاب الدم(74.70 X 10^9 /ل)و(91.633) ل/ 10^9 X

±22.75) و (68.02% ±19.91) ومتوسط نسبة الخلايا البادئة (9.70 X 10^9 /ل ± 2.76)و/ل

71.25%) لكل من خلاياالدم البائية والتائية علي التوالي.

وجدت علاقة ذات دلالة إحصائية عالية لمتوسط تعداد خلايا الدم البيضاء(P = 0.000) و متوسط خضاب

الدم (P=0.038) عند مرضى النوع التائ لسرطان الدم الأبيض الليمفاوي الحاد بينما علاقة خلايا الدم

الحمراء، الصفائح الدموية و نسبة الخلايا البادئة ليست ذات دلالة إحصائية (0.529, 0.287,0.168=P)

علي التوالي. وعند مقارنة تعداد الدم الكامل بين المرضى والمجموعة الضابطة كانت النتيجة علي النحو

التالي: متوسط تعداد خلايا الدم البيضاء لدي المرضى (143.01 ± 74.65 X 10^9 /ل) مقارنة ب

X2.95 ± 0. 93) في المجموعة الضابطة ، متوسط كريات الدم الحمراء (8.89X 10^9 /ل ± 4.01)

10^{12} /ل) في المرضي و(77. 0 ±X4.58 10^{12} /ل) ، متوسط تعداد الصفائح الدموية للمرضي

والمجموعة الضابطة (62.68 ± 57.34 X 10^9 /ل) و(109.10 ± 333.41X 10^9 /ل) على

التوالي ومتوسط مستوي خضاب الدم (2.54 ± 8.65 جرام /دسم) عند المرضى و(2.05 ± 12.42جرام

/دسم) فى المجموعة الضابطة، وهذا الإختلاف كان ذو دلالة إحصائية عالية(P = 0.000).

تردد المظهر الجيني لكروم الخلوي الطبيعي لدى المرضى 95 (93.1%) ،النوع المختلط 7 (6.9%) و

المتجانس الطفرة وكانت سالبة للنوع المتنحي المتماثل0(0%)، و في المجموعة الضابطة كما يلي 98

(96.1%) ، 4 (4.9%) و 0 (0%) علي التوالي. أظهرت الدراسة أن تجانس الطفرة عاليا في المرضى

مقارنة بالمجموعة الضابطة (6.9% و 3.9%) علي التوالي (النسبة الشاذة 1.805 و القيمة الاحتمالية

0.3524) لكن هذا الاختلاف ليس له دلالة إحصائية وكذلك بالنسبة للأليلات (النسبة الشاذة 1.776 والقيمة الاحتمالية 0.3592).

نسبة تكرار الطفرة الجينية (609) وسط المرضى 76(74.5%) للطبيعي، 18 (17.7%) للمختلط و 8 (7.8%) للمتجانس بينما في المجموعة الضابطة 89 (87.2%)، 7 (6.9%) و 6 (5.9%) علي التوالي. وجد التجانس المختلط عاليا في المرضى مقارنة بالمجموعة الضابطة (17.7% و 6.9%) علي التوالي (النسبة الشاذة 3.513 (95%CI:1.327−9.301) والقيمة الإحتمالية = 0.0081) لذلك يعتبر هذا الأختلاف ذو دلالة إحصائية مترافقة لتطور سرطان الدم الأبيض الليمفاوي الحاد. نسبة تردد الطفرة المتجانسة في المرضى أعلى منه في المجموعة الضابطة (7.8،%5.9%) (النسبة الشاذة 1.338 "95%Cl 0.4638_3.862" ،القيمة الاحتمالية 0.588) هذا الاختلاف ليس له دلالة إحصائية بينما تردد الأليلات أظهر علاقة ذات دلالة إحصائية مع سرطان الدم الأبيض الحاد الليمفاوي (القيمة الإحتمالية 0.0408= والنسبة الشاذة 1.840 ,95%CI:1.019−3.3219).

نسبة تردد الطفرة الجينية (465) للتجانس المتماثل ، المختلف و المتباين وسط مرضى سرطان الدم الأبيض الليمفاوي الحاد (70.6%) 72 ، (22.5%)، 23 و (6.9%) 7 تتابعيا بينما في المجموعة الضابطة (92.2%) 94 للأليل المشابه الطبيعي ، (4.9%) 5 للأليل المتباين المختلط و (2.9%) 3 للأليل المتشابه المتنحي. و وجد الاختلاف الجيني عاليا عند المرضى عند مقارنة بالمجموعة الضابطة (22.5%و4.9%) تتابعياً ذو دلالة احصائية عالية (النسبة الشاذة 7.667 (23.15−95%CI:2.539 والقيمة الاحتمالية = 0.0001) بينما الطفرة المتجانسة عاليةً أيضاً عند المرضى (6.9%) مقارنة بالمجموعة الضابطة (2.9%) (النسبة الشاذة 4.667 "23.14−95% Cl:0.9410" ،القيمة الإحتمالية =0.0401

)بينما الأليل (النسبة الشاذة 2.7 (1.427−5.107:CI%95" والقيمة الاحتمالية= 0.0017) له علاقة ذات دلالة إحصائية بينه وبين الإصابة.

خلصت هذة الدراسة الي أن الطفرة الجينيه CYP2E1 (C1053T) ليست عامل خطورة للإصابة بسرطان الدم الليمفاوي الحاد، بينما الطفرات الجينية NQO1 (C465 T) و NQO1(C609T) تعتبر عوامل ذات للإصابة بسرطان الدم الليمفاوي الحاد.

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List of Abbreviations

ALL	Acute Lymphoblastic Leukemia
B-ALL	B- Cell Acute Lymphoblastic Leukemia
c DNA	complementary Deoxy Ribonucleic Acid
cALL	common ALL
CBC	Complete Blood Counts
CD	Clusters of Differentiation
CDKi	Cycline Dependent Kinase inhibitors
CYP2E1	Cytochrome 2E1
DC	Direct Current
DNA	Deoxy Ribonucleic Acid
EBF1	Early B-cell Factor1
EGIL	European Group for the Immunological classification of Leukemias
FAB	French American British Classification
FISH	Fluorescent In Situ Hybridization
FITC	Fluorescein IsoThioCyanate
FSC	Forward Scatter
GSTs	Glutathione S-Transferase
GWA	Genome- Wide Association
HGB	Hemoglobin
Ig	Immunoglobulins
IKZF1	IKAROS family Zinc Finger 1
LED	Light Emitting Diode
MLL	Mixed Lineage Leukemia
NCBI	National Center of Biotechnology Information
NGS	Next Generation Sequencing
NQO1	NAD (P) H: quinone oxidoreducatase 1
OEHHA	Office of Environmental Health Hazard Assessment
PAHs	Polycyclic Aromatic Hydrocarbons
PAX5	Paired box 5

PBP	Peripheral Blood Picture
PCR	Polymerase Chain Reaction
Ph	Philadelphia Chromosome
PLTs	Platelets
RBCs	Red Blood Cells
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
RT-PCR	Real Time PCR
SCC	Side Scatter
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical Package for Social Science
SRV	Sample Rotator valve
T-ALL	T-Cell Acute Lymphoblastic Leukemia
TdT	Terminal Deoxynucleotidyl Transferase
TLC	Total Leukocytes Count
TWBCs	Total White Blood Cells
UNSCEAR	United Nations Scientific Committee on the Effects of Atomic Radiation
W H O	World Health Organization

Chapter I
Introduction

1.1 Introduction:

Leukemia is a common malignancy around the world comprising 30% of malignant tumors in children (Chiang, 2010). Many investigators reported that occurrence of leukemia is differ depending on ethnic groups, geographic areas and many causative agents such as infectious and/or environmental factors. (Demoury *et al*., 2012; Nyari *et al*., 2013).

Acute lymphocytic Leukemia (ALL) is white blood cells malignant disease resulting from accumulation of genetic alteration of B or T lymphoid precursor cells (Okamoto *et al.,* 2010), characterized by clonal proliferation of lymphoid cells which are blocked at an early stage of differentiation, the most common type of ALL is B cells linage (Pui *et al*., 2004; Pui *et al*., 2011).

Annual rate of ALL about 3 to 4 cases per 100.000 children less than 15 years of age (Ribera and Oriol, 2009; Scheurer *et al*., 2015), high peak of ALL among children of 2 to 5 years, males more affected often than females except in infant (Margolin *et al*., 2011).

In Sudan, according to Sudan Ministry of Health Leukemias are the second top ten cancers, there were about 7059 cases, 3594 in Khartoum State, about 600 cases are ALL (Sudan National Cancer Registry, 2010-2014).

In the last three decades, there has been a significantly improvement in treatment outcome of ALL in children, 70% to 80% of children can get cured of their disease, a situation that is different in adults with ALL, since only 30% -35% of them may cure (Stock *et al*., 2000; Stock, 2010; Downing *et al*., 2012).

Genetic polymorphisms are defined as natural genetic variation that occurs randomly in general population; the most common type is the single nucleotide polymorphisms (SNPs), which consist of single base pair substitution depending on where it is located. SNPs can interfere with gene's function affecting the metabolic pathways (Li *et al*., 2012).

The polymorphisms genes coding for xenobiotic metabolizing enzymes or proteins maintaining DNA stability are important determinants of the individual's susceptibility to cancer which leading to increased of phase I xenobiotic activation which results in higher levels of carcinogen activation and oxidative stress, while polymorphisms resulting in higher phase II xenobiotic detoxification leads to more efficient detoxification of activated carcinogens and protection against oxidative stress, also polymorphisms lowering the maintenance of DNA stability also lead to higher risk for cancer development (Melega *et al*., 2013).

The frequency of the polymorphism varies and genotypes have been associated with the loss of the enzyme activity and enhanced genotoxicity and believed to be main factors in determining susceptibility to diseases associated with exposure to xenobiotic (Gundacker et al., 2007). There are different studies suggested that association of polymorphisms in genes involved in xenobiotic metabolism phase I and II in patients with leukemia (Gallegos et al., 2008; Lordelo et al., 2012). The most important enzymes in phase I are CYP450 dependent monooxgenases, which have isozymes super family, these isozymes transform chemicals into carcinogenic, for that their polymorphisms may be risk factor for development of ALL (Pakakasama et al., 2005).

CYP2E1 is the most conserved forms in the CYP2 family which generates secondary metabolic pathways lead to carcinogenesis, toxification, genotoxicity or mutagenesis in the body (Marmiroli and Maestri, 2008). The most frequent genetic polymorphism in CYP2E1 is the CYP2E1*5B variant (rs3813867/rs2031920, located in the 5'-flanking region of the gene with substitutions at positions G-1293C and C-1053T (Lan, 2004).

Several polymorphisms have been reported about CYP2E1 gene and transcriptional activity and its polymorphism association and susceptibility to a number of adult cancers, some researchers found 2.8 to 3.4 fold increased risk for childhood ALL with the presence of CYP2E1*5B allele (Krajinovic et al., 2002; Aydin et al., 2006).

The other polymorphism is NAD (P) H: quinone oxidoreducatase 1 (NQO1) is a homodimers with a molecular weight of 60 KDa and express in many tissues such as bone marrow. NQO1 located in chromosome 16 in 16q22 region (Dunna et al., 2011), there are two polymorphisms described in the NQO1 gene are C609T (NQO1*2) and C465T (NQO1*3) substitutions which lead to P187S and R139W amino acid replacements respectively, in which C substitute to T at 609 and 465. NQO1 polymorphism lead to reduce enzyme activity or complete loss of enzymatic activity due to protein instability (Krajinovic, 2005), there are several studies focus on the association between the NQO1 polymorphisms and increased risk of acute lymphoblastic leukemia (Bolufer et al., 2007; Abdel Aziz and Alqatary, 2013).

1.2 Hypothesis:

Polymorphisms in CYP2E1 (C1053T), NQO1 (C609T and C465T) are associated with high risk of development of (ALL).

1.3 Rationale

Acute lymphoblastic leukemia has increasing prevalence in Sudan based on National Cancer Institute records; there is many studies revealed association of acute lymphoblastic leukemia with certain genetic abnormality. The frequencies of CYP2E1*5B (C1053T) and NQO1 (C609T), NQO1 (C465T) polymorphic alleles have been reported in various cancers and there are several studies have been published on the relationship between these polymorphisms and various types of cancers. To the best of our knowledge, there are no published reports about the association between these polymorphisms and acute lymphoblastic leukemia in Sudan and data about these enzymes and their role in acute lymphoblastic leukemia is scantly, only one study on NOQ1 (C609T) and acute lymphocytic leukemia. Hence, this study aimed to detect the association of the CYP 2E1*5B (C1053T) and NQO1 (C609T and C465T) polymorphisms and susceptibility to acute lymphocytic leukemia. So this study may fill the gap regarding these polymorphisms and its association with Sudanese ALL.

1.4 Objectives

1.4.1 General objective

To study the association of cytochrome P450 2E1 (C1053T) and NADPH quinone oxidoreducatase 1 genes (C609T) (C465T) polymorphism with acute lymphoblastic leukemia in Sudanese patients.

1.4.2 Specific objectives

1- To measure hematological parameters in both case and healthy control by automated blood cell counter analyzer Sysmex KX 21N.

2- To detect single nucleotide polymorphisms of CYP2E1 (C1053T) and NQO1 (C609T, C465T) by PCR-RFLP.

3- To compare CYP2E1 (C1053T) and NQO1 (C609Tand C465T) genotypes and alleles among Sudanese patients with acute lymphoblastic leukemia and control group.

4- To compare CYP2E1 (C1053T) and NQO1 (C609Tand C465T) genotypes and alleles among children and adults in both cases and control group.

5- To screen for other possible SNPs at the selected region by DNA sequencing.

6- To correlate between CYP2E1 (C1053T), NQO1 (C609Tand C465T) polymorphisms and hematological parameter among ALL patients.

7- To compare CYP2E (C1053T) and NQO1 (C609T, C465T) sequences with reference sequences in Gene bank.

Chapter II

Literature Review

2.1 Acute Leukemia:

Acute leukemia is aggressive hematopoietic neoplasms with maturation arrest of the myeloid or lymphoid lineage cause accumulation of immature blast cells in the bone marrow leading to bone marrow failure and this reflects in peripheral blood as cytopenia and present of blast cells (Hoffbrand *et al*., 2016).

There are several types of leukemia, which are divided based mainly on whether the leukemia is acute (fast growing) or chronic (slow growing), and whether it starts in myeloid cells or lymphoid cells. Different types of leukemia have different treatment options and outlooks (Provan, 2003; Arber *et al*., 2016).

2.2 Classification of Acute leukemia:

There are three common classification of acute leukemia; first one is French American British classification (FAB) which based on cellular morphology and cytochemical stain results and > 30% of bone marrow blasts, this classification is easier to use and still widely taught (Hoffbrand *et al*., 2010). Second one is World Health Organization Classification of Acute Leukemia is now the standard for diagnosis, this classification according to lineage of neoplastic cells including four groups: myeloid, lymphoid, mast cell and histiocytic cell, this classification based on morphology, cytochemistry and immunophenotype of the neoplastic cells; however, it also uses genetic, clinical features and blast cell count of ≥ 20% to define acute leukemias (Vardiman *et al*., 2009). Third one is European Group for the Immunological classification of Leukemias (EGIL); this classification formulated guidelines for acute leukemia with biphenotypic marker expression; these criteria had been incorporated in the WHO guidelines for classifying acute leukemia of ambiguous lineage, this classification based on immunophenotype alone, suggesting criteria for identify leukemia as myeloid, T lineage, B lineage, or biphenotypic, it is distinguishing biphenotypic leukemia from AML with aberrant expression of lymphoid antigens, and from ALL with aberrant expression of myeloid antigens (Hoelzer *et al* 2002; Van den Ancker, 2010).

2.3 Acute lymphoblastic leukemia (ALL):

Acute lymphoblastic leukemia (ALL) is malignant disorder due to transformation and proliferation of lymphoid progenitor in bone marrow, blood and extramedullary sites, about 80% of ALL affecting children while in adult it is a devastating disorder (Shaikh *et al*., 2014; Paul *et al*., 2016; National Cancer Institute 2016).

ALL is the most common childhood cancer with peak prevalence between the ages of 2 and 5 years (Pui *et al*., 2008), constituting 75% of acute leukemia under six years of age. Adults' acute lymphoblastic leukemia is a heterogeneous disease, due to the difference in disease biology and associated genetic abnormalities (Sabir *et al*., 2012). ALL remains one of the most demanding and challenging adult malignancies, especially with respect to therapy (Pui *et al*., 2008). It may be of B- or T-lymphoid lineage, 80% - 85% of ALL in children are of precursor B-lineage, with good prognosis in children and high cure rate of approximately 80% (Jaffe, 2010).

ALL characterized by recurring chromosomal aberrations and gene mutations often within members of key cellular pathways including lymphoid development, cell cycle regulation, tumor suppression, regulation of apoptosis, cell signaling and drug responsiveness (Armstrong *et al*., 2005; Mullighan, 2009), although considered as important initiating events in leukaemogenesis, these alterations alone usually do not cause leukemia (Brassesco, 2008; Emerenciano, 2009), implicating that there are other etiologic factors such familial inherited genetic factors influence the risk of developing ALL (Hemminki *et al*., 2002).

There are three genome- wide association (GWA) studies have revealed that genetic variation likely influence risk of ALL (Mullighan, 2007; Trevino *et al*., 2009 Han *et al*., 2010). Furthermore genetic associations with ALL risk and outcome have been identified for polymorphisms in candidate genes, including genes mediating drug and folate metabolism, cell cycle checkpoints and DNA repair genes, xenobiotic metabolism genes, as well as transcription factors or regulators of transcription factors (Bolufer *et al*., 2006; Mullighan, 2009; Vijayakrishnan *et al*., 2010).

2.3.1 Pathophysiology:

ALL represents a clonal proliferation of immature lymphocyte precursors, cells may be B-cell precursors or T-cell precursors, basic abnormality is due to somatically acquired genetic mutation of lymphoid precursor at development stages, which give clone of malignant lymphocytes, that proliferation and differentiation are uncontrolled which reflecting blocking differentiation in cells

maturation, leading these cells to fail in develop to mature cells with increased resistance to apoptosis (Pui, 2009).

Pathobiology ALL is originate from various important genetic lesions in blood-progenitor cells that are committed to differentiate in the T-cell or B-cell pathway, including mutations that impart the capacity for unlimited self-renewal which lead to precise developmental arrest (Wang and Dick, 2005; Weinstein and Joe, 2006).

 In some studies in pediatric population identified genetic syndromes such as Fanconi anemia, Nijimegen breakdown syndrome and Down's syndrome (trisomy 21) results in 10 to 18 fold elevated risk for leukemia (Liang and Pui 2005; Buitenkamp 2014), ataxia telangiectasia, bloom's syndrome (Hsu *et al*., 2015), congenital X-linked a gammaglobulinemia, immunoglobulin A deficiency and common variable immunodeficiency are also at increased risk (Liang and Pui 2005). Also other factors are included such as exposure to pesticides, ionizing radiation and certain chemical solvent or infected with viruses as human immunodeficiency viruses and Epstein Barr Virus (Spector, 2006; Sehgal *et al.,*2010), the first mutation might arise in a haemopoietic stem cell possessing multilineage developmental capacity (Wang *et al*., 2004). Cells that implicated in ALL have clonal rearrangements in immunoglobulin or T-cell receptor genes and express antigen-receptor molecules and other differentiation linked cell surface glycoproteins that recapitulate those of immature lymphoid progenitor cells within the early developmental stages of normal T and B lymphocytes (Pui, 2009). Most cases are occur as *de novo* in healthy individuals, hall mark of ALL is chrosomal aberration, there is translocation such as t(12;21), t(1;9) t(9;22) and rearrangement of MLL (Mullighan *et al*., 2009).

 An another variant in Philadelphia (Ph) chromosome positive ALL but without BCR-ABL1rearrangement identified in more than 80% of cases, it is called Ph –like ALL, this variant have deletion in transcription factors which affect B-cell development such as IKAROS family Zinc Finger 1(IKZF1), early B-cell factor 1(EBF1), transcription factor 3(E2A) and paired box 5(PAX5) (Mullighan *et al*., 2007; Shah *et al*., 2013). In 90% of Ph –like ALL, Kinase –activating mutation such as rearrangement ABL1, JAK2, also activating mutations of IL7R and FLT3 and deletion of SH2B3 which encodes for JAK2 negative regulator LNK and this have significant therapeutic implications which respond to kinase inhibitors (Roberts *et al*., 2012; Roberts *et al*., 2014).

In 2013 Holmfeld described genetic basis of other subset with poor prognosis Hypodiploid ALL which exhibited activation of RasI and PI3K singling transduction pathways and this suggesting that these pathways may be target for treatment and therapy in aggressive Hypodiploid ALL (Holmfeld *et al* ., 2013).

ALL survival rate has increased in the last 5-year from 60% to 90% for children younger than 15 years and from 28% to 75% and for adolescents aged 15 to 19 years (Howlader *et al*., 2015).

Number of factors such as elevated white blood cell count, hypodiploidy, presence of the Philadelphia (Ph) chromosome or the mixed lineage leukemia (MLL)/11q23 rearrangement, five or more chromosomal aberrations, prolonged time to remission, as well as persistence of minimal residual disease (MRD) are associated with worse outcome (Stock, 2010; Hochberg *et al.* 2013; Inaba *et al.*, 2013).

2.3.2 Lymphocytes development:

Lymphocytes are the second type of leukocytes in adults (~20–40% of WBC); it is increases in children and in viral infections. Functionally, there are two main types of lymphocytes, B and T cells. B cells are the cells of the humoral immune system, known as antibodies mediator immunity, normally represent about 80% of total lymphocytes count, development of these cells occurs in the bone marrow and in lymph nodes, after antigen stimulation the B-cells developed to plasma cells, antibody-producing cells (Greenspan and Cavacini, 2019).

T cells account approximately 20% of lymphocytes, they are main cell-mediated immunity and regulating cells of the immune system by stimulate or inhibit the function of other cells of the immune system, including B cells, monocytes and macrophages, and other T cells. T cell precursors originate in the bone marrow but they develop and mature in the thymus, the majority of circulating lymphocytes are T cells; T helper cells, major regulatory cells of the immune system usually express CD4 and the other cell is T suppressor/cytotoxic cells expressed CD8, which destroy viral infected cells, intracellular pathogen and transplant rejection (Ng *et al*., 2010; Scheurer, 2015).

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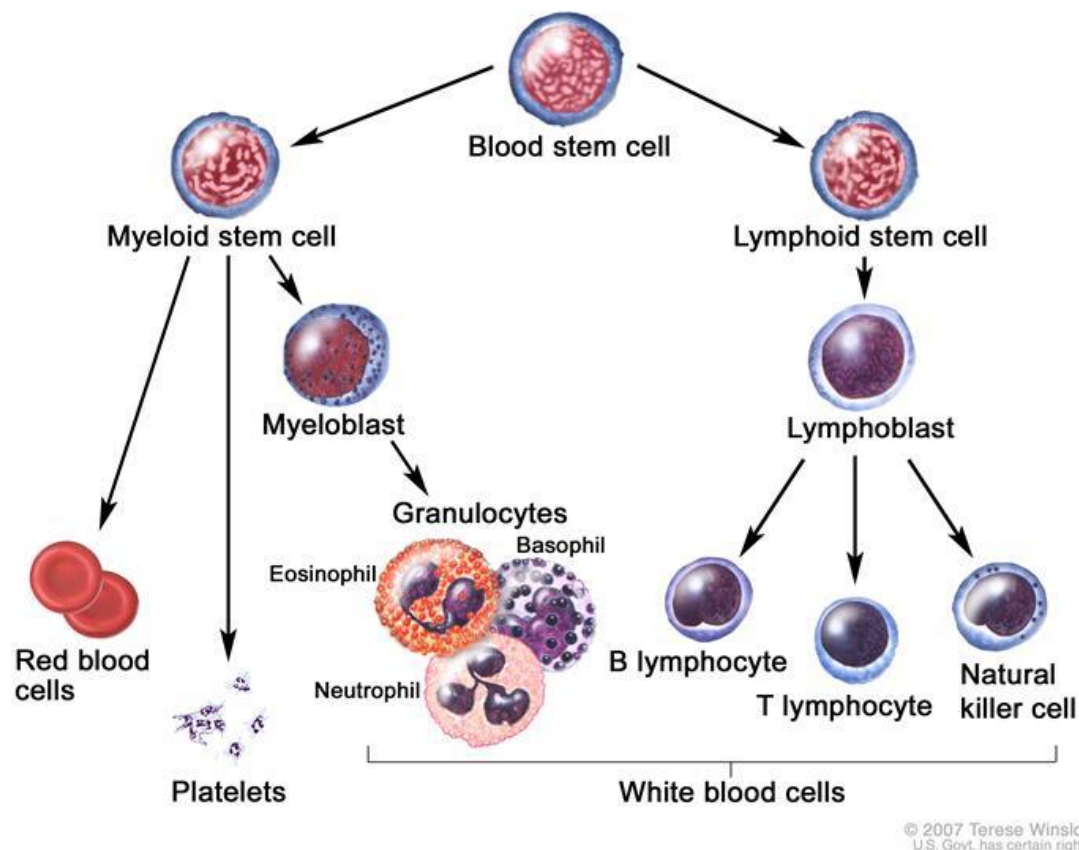
Figure 2-1: Blood cell development. Different blood and immune cell lineages, including T and B lymphocytes, differentiate from a common blood stem cell (National Cancer Institute, 2015).

2.3.3 Incidences of ALL:

ALL is a disease of childhood and adolescence, accounting for 25% of childhood cancers and up to 75% of childhood leukemia. The incidence peak of ALL is 2 and 5 years of age of childhood. Although ALL is rare in adults, risk increases with age over 40 years old; most adult patients are elder than 50 years of age (Hoffbrand, 2016), it is the most common malignancy in childhood and represents 85% of childhood acute leukemias (Cheok and Evans, 2006), the incidence is highest at 3–7 years (Pui *et al*., 2008), with 75% of cases occurring before the age of 6, represents the third part of pediatric cancer cases, accounting 75-80% of all cases of acute leukemia in children. Approximately, 75% of cases occur in children under six years of age and there is a frequency peak between 2 and 5 years (Campo *et al*. 2011), while in adults is uncommon represent about 15% of adult acute leukemias arise after the age of 40 years, ALL in adults is predominantly a disease of the elderly with 1.6 cases/100,000 people/year (Marks, 2015).

Race and ethnic differences may have a role; Hispanics are more likely to develop acute leukemia than Caucasians and African-Americans (Lim *et al*., 2014), 85 % of cases are B-cell lineage and 15 % T-cell ALL and males more than females (Paltiel *et al.*, 2004; Forero *et al*., 2013; Shahab and Raziq, 2014).

The incidence of ALL is not uniform around the world. It varies from 0.9 to 4.7 / 100,000 children per year (Zhang *et al*., 2011).

In USA ALL is estimated 1.6 /100000 of united state population (National Cancer Institute 2016), 6590 new cases were diagnosed, over 1400 deaths due to ALL, first peak in children while the second peak in age of 50, significant prognosis in pediatric patients, while elderly patient remains very poor, 30-40 % of adult cases with ALL achieving long term of remission (Jabbour *et al* ., 2015), the American Cancer Society's estimates for ALL in the United States for 2015 (including both children and adults) are about 6,250 new cases of ALL (3,100 in males and 3,150 in females), about 1,450 deaths from ALL (800 in males and 650 in females), the risk for developing ALL is highest in children younger than 5 years of age. The risk then declines slowly until the mid-20s, and begins to rise again slowly after age 50, about 4 of every 10 cases of ALL are in adults (American Cancer Society, 2015), age adjusted overall incidence of ALL in the United States is 1.7/ 100,000 individuals with peaks between ages 2 years and 5 years and again after age 50 years (Seiter, 2013; Seiter, 2014; Febbraro *et al*., 2015).

The incidence of ALL in England the prevalence of ALL is about 17% in England (Feltbower *et al*., 2009).

ALL represent 5.8% of total cancers in Saudi Arabia; male to female ratio among Saudis was 1.5:1 (Saudi Cancer Registry 2010).

In India leukemia represented 25 and 40%; 60 to 85% of all leukemias reported as Acute Lymphoblastic leukemia (Arora *et al*., 2009).

In Sudan leukemia are the second tops ten cancers there are about 7059 cases; 3594 in Khartoum state, gender-specific rate = 9.1 per 100,000 (Saeed *et al*., 2014), about 600 cases are acute lymphocytic leukemia (Sudan National Cancer Registry, 2010-2014). also regarding to previous study done in Sudan at Elobied Hospital in western Sudan during 2007 – 2008, leukemia represent 40% of study cases, 87% of these cases were ALL (Doumi *et al*., 2009).

2.3.4 Classification of ALL:

2.3.4.1 French-American-British classification (FAB):

French-American-British classification (FAB) for ALL has three subtypes: L1, L2, and L3. These subtypes of ALL are distinguished by size of leukemia cells: small cells in ALL-L1, large cells in ALL-L3, and mixture of small and large cells in ALL-L2, a among these three subtypes, ALL-L1 has the best prognosis (MoradiAmin *et al*., 2016).This classification based on cytoplasm morphology, including cell size, prominence of nucleoli, cytochemical stains and blast % ≥ 30 (Seiter, 2013).

2.3.4.1.1 ALL L1 Subtype:

In L1 ALL, the cells are small twice the diameter of a red cell, having high nuclear cytoplasmic ratio, nucleus is regular in shape with fine homogeneous chromatin, nucleoli maybe visible, scanty cytoplasm which may be slight to moderate basophilic and rarely intensely basophilic, and in some cases shows a variable degree of vaculation. In some cases small numbers of azurophilic granules may present, this type is more common in children (Chiaretti, 2014; Bain, 2016; Bain, 2017).

2.3.4.1.2 ALL L2 Subtype:

In L2 ALL, the blasts are larger and more heterogeneous, nucleocytoplasmic ratio is variable, cytoplasm shows a variable degree of basophilia and nuclei are irregular in shape with clefting, common folding and indentation with heterogence chromatin, nucleoli present and may be large. Variable degree of cytoplasmic vaculation may be present, in minor cases there are small numbers of azurophilic, peroxidase-negative, granules. L2 is more in adults (Hoffbrand, 2006; Chiaretti *et al*., 2014).

2.3.4.1.3 ALL L3 Subtype:

In L3 ALL, the blast cells are large and homogeneous, nucleocytoplasmic ratio is lower than in L1, nucleus is regular in shape, varying from round to oval, chromatin is uniform stippled or homogeneous with one nucleoli. In contrast to L1 and L2 ALL, cytoplasm is strongly basophilic with variable prominent vaculation (Hoffbrand, 2010; Chiaretti *et al*., 2014)

2.3.4.2 WHO Classification:

WHO in 1997 classified acute lymphoblastic leukemia based on morphology, cytogenetic to three types B lymphoblastic, T lymphoblastic and Burkitt cell leukemia (Harris *et al*., 1997), later in 2008 reviewed that Burkitt cell leukemia was eliminated as no longer seen separated from Burkitt

Lymphoma and B cell lymphoblastic leukemia divided to two subtypes: B-lymphoblastic leukemia/lymphoma and T-lymphoblastic leukemia/lymphoma (Vardiman *et al*., 2008).

In 2016 WHO added two new provisional to recurrent genetic abnormalities and refined the Hypodiploid either low Hypodiploid or Hypodiploid with TP53 mutations (Arber *et al*., 2016). Clinical history for leukemogenic therapy have been importance in the WHO 2008 classification of acute leukemia, also further details such as pre leukemic myeloid neoplasm, unrelated to Down's syndrome; history of myelodysplastic syndrome, recent therapy with growth factors may well be incorporated in the classification(Vardiman *et al*., 2008; Hasserjian, 2013).

Table (2-1) WHO classification of acute lymphoblastic leukemia based on

2016 revision of the World Health Organization

B-cell lymphoblastic leukemia /lymphoma, not otherwise specified
B-cell lymphoblastic leukemia /lymphoma, with recurrent genetic abnormalities
B-cell lymphoblastic leukemia /lymphoma with Hypodiploidy
B-cell lymphoblastic leukemia /lymphoma with Hyperdiploidy
B-cell lymphoblastic leukemia /lymphoma with t(9;22)(q34;q11.2)[BCR-ABLI]
B-cell lymphoblastic leukemia /lymphoma with t(v;11q23)[MLL rearranged]
B-cell lymphoblastic leukemia /lymphoma with t(12;21)(p13;q22)[ETV6-RUNX1]
B-cell lymphoblastic leukemia /lymphoma with t(1;19)(q23;p13.3)[TCF3-PBX1]
B-cell lymphoblastic leukemia /lymphoma with t(5;14) (q31;q32)[IL3-IGH]
B-cell lymphoblastic leukemia /lymphoma with intra chromosomal amplification of chromosome 21(Iamp21)
B-cell lymphoblastic leukemia /lymphoma with translocations involving tyrosine kinase or cytokine receptors (BCR-ABL1-like ALL)
T-cell lymphoblastic leukemia lymphomas
Early T-cell precursor lymphoblastic leukemia

2.3.4.3 European Group for the Immunological classification of Leukemias (EGIL) for ALL:

2.3.4.3.1 Precursor B-lymphoblastic leukemia:

Most common markers are HLA-DR+, TdT+, CD19+, and/or CD79a+, and/or CD22+, and/or CD34+, this type of ALL accounts 75% of adult cases and divided into subtypes:

a. Pro B-ALL expresses HLA-DR, TdT, and CD19. CD10-, cytoplasmic immunoglobulin- represents approximately 10% of adult ALL.

b. Common ALL account more than 50% of adult cases of ALL and characterized by the presence of CD10, negative cytoplasmic immunoglobulin.

c. Pre B-ALL is identified in 10% of adult cases, characterized by the expression of cytoplasmic immunoglobulin and CD10.

d. Mature B-ALL is found in approximately 4% of adult ALL patients, blast cells express surface membrane immunoglobulin (SmIg+) surface antigens of mature B cells, they typically TdT and CD34 negative and show L3 morphology. This overlaps with Burkitt lymphoma, which classified as mature B-cell neoplasms (Thalhammer, 2002; Ramirez *et al*., 2010; Chiaretti *et al*., 2014).

2.3.4.3.2 Precursor T-lymphoblastic leukemia:

Cells expressed TdT+ with cytoplasmic CD3+ and CD34+. This type of ALL accounts about 25% of adult cases and subdivided into:

a. Pro T-ALL CD2-, CD7+, CD4-, CD8- in 7% of adult ALL.

b. Pre T-ALL CD2+, CD7+, CD4-, CD8- in 7% of adult ALL.

c. Cortical T-ALL or Thymic ALL (Thy ALL) is CD1a+ and accounts for 17% of adult ALL CD7+, CD2+, CD5+, CD4+, CD8+

d. Mature T-ALL are surface CD3+, CD2+, CD7+, CD4 or 8, and TdT/CD34/CD1a- and make up approximately 1% of adult ALL (Hoelzer, 2002; Rothenberg, 2008; Hoffbrand, 2010).

2.3.4.4 Genetic Classification:

Genetic classification of ALL provides more biologic information than any other means. Approximately 75% of adult and childhood cases can be readily classified into prognostically or therapeutically relevant subgroups based on the modal chromosome number or DNA content estimated by flow cytometry, specific chromosomal rearrangements, and molecular genetics changes (Pullarkat *et al*., 2008).

2.3.5 Etiology of ALL:

The actual causes of ALL is remaining unknown, but there are several hypothesis about etiological associations of ALL which conflicting and unclear (Jiang *et al*., 2013).

Three factors that are known to influence and associated with risk of ALL development: exposure-independent characteristics, exposure to environmental agents, and genetic factors (Greaves, 2002; Kim *et al*., 2006).

2.3.5.1 Exposure-Independent Characteristics as Risk Factors:

There is few data and studies about exposure independent risk factors, some studies found increased risk with some factors such as Down syndrome (Maloney *et al*., 2010), which increased the risk of incidence ALL between 20 to 33-fold (Buitenkamp *et al*., 2014), also high birth weight is another factor associated with increased risk of childhood ALL in many studies (Okcu *et al*., 2002; Paltiel *et al*., 2004), statistic significant between birth weight and ALL risk is up to 26% (Hjalgrim *et a*l., 2004), also parents age, more than 40 years was also concerned as risk factor for their children to develop ALL and increased the chance of the disease (Dockerty *et al*., 2001).

2.3.5.2 Ionizing Radiation and Infections:

Ionizing radiation is one of the exposures factors, risk here is depending on the dose of radiation, the duration of exposure and the age of the individual at the exposure time (Mahoney *et al*., 2004). Exposure of pregnant women to diagnostic X-rays and postnatal exposure to therapeutic doses were shown to be risk factors for ALL (Infante-Rivard, 2002). So this explains only few percentage of leukemia in recent years because of the rarity of radiation treatments in children and diagnostic X-rays during pregnancy (Bunin, 2004). The potential effect of ionizing radiation exposure on children may occur during preconception, pregnancy, or the postnatal period (Unscear, 2008; Belson *et al*., 2007; Richardson *et al*., 2009).

Also infection was considered as a risk factor for ALL development, more over some studies suggested that viruses such as human T cell leukemia/ lymphoma virus type I, Epstein-Barr virus (EBV), and Cytomegalo virus (CMV), CMV and EBV are the popular viruses' causes infections in pediatric leukemic patients as a result of leukemia linked immuno suppression status. Also higher contact to herpes simplex virus types 1 and 2 among ALL children (Loutfy *et al*., 2017)**,** and bacteria as in Helicobacter pylori may have a part in the development of some types of leukemia, however this role in childhood ALL is uncertain (Greaves, 2002; Greaves, 2005). In

some postnatal studies there is suggestion that the exposure to infection in the first year of life decreases a child's ALL risk, while delayed exposure to infection may increases the risk (Kim *et al*., 2006).

2.3.5.3 Chemical Exposure:

Exposure to chemicals has an important role in the etiology of all cancers, including childhood ALL due to modern world life style, there are lots of chances that individuals are exposed to chemical carcinogens such as air pollution, industrialization, automobile exhaust, occupation, tobacco smoke, food additives, drugs and cosmetics. This explains the higher level of cancer incidence in highly developed industrial countries (Bunin, 2004; Ramanakumar, 2007). Other sources of exposure can include local agricultural practices, contaminated frozen food, parental occupation, and pet products. Maternal occupational pesticide exposure during pregnancy and or paternal exposure to pesticide during occupational work around conception have been suggested to increase risk of leukemia in the offspring (Bailey *et al*., 2014; Bailey *et al*., 2015).

Pesticides is one of the chemicals that were shown to be associated with risk of childhood ALL. (Mason *et al.*, 2002), many reports conclude that exposure to indoor pesticides during pregnancy increased leukemia risk, also postnatal exposure of children to indoor pesticide was shown to be associated with ALL (Steffen *et al*., 2004). Hydrocarbons many household and industrial products including paints, paint removers, thinners, and solvents which dissolve other chemical substances are associated with risk of childhood ALL and the most important one is benzene, which used in the manufacture of paints and plastics and as a constituent in motor fuels and hobby glues (McHale *et al*., 2011). It is also formed during incomplete calcination of fossil fuels; these hydrocarbons can also found in air pollution, there is study in California suggested that children living in areas with high levels of point source of such pollutants were at an increased risk of developing leukemia (Reynolds *et al*., 2003).

British report found associations between birthplace of children with leukemia and proximity to industrial sites that release volatile organic compounds (Knox, 2005). Occupational study found excess risk for leukemia associated with cumulative benzene and benzene exposure intensities at lower levels (Glass, 2003).

Exposure to hazardous chemicals can also be occupational such as paints and pigments as in several studies producing risk factors for ALL and AML of more than 1.5 fold; another source of chemical exposure is alcohol, illicit drug use and cigarette. Most of chemicals exert their

carcinogenic effects after their bioactivation in the body by xenobiotic metabolizing enzymes, therefore the polymorphisms of the genes of these enzymes also play an important role in the risk of development of childhood ALL, for that attention should involve the exposure level and the xenobiotic-metabolizing which is different in children than adults (Greaves, 2002; Greaves, 2005; Kim *et al.,* 2006).

2.3.6 Signs and Symptoms of ALL:

Signs and symptoms are due to infiltration and accumulation of the bone marrow and organs by malignant blasts, include anemia associated with pale skin and mucous membranes, and easy fatigability, thrombocytopenia which indicated by petechial gum bleeding and occasionally retinal bleeding, bruising easily, hematoma are less common while neutropenia signs appear as infections, such as pneumonias and abscesses in some cases (Clarke *et al*., 2016), often some children have bone and joint pain most probably due to expansion of the medullary cavity by the malignant cells, hepatosplenomegaly and lymphadenopathy are also common in ALL, central nervous system can involved the leukemic meningitis is uncommon at original diagnosis, but the spinal fluid may be a site of relapse after treatment. Large meditational mass may be present in precursor T-cell ALL, respiratory distress due to compression of the trachea may be the present complaint. Hyperleukocytosis with leukostasis is less common in ALL (Munker *et al*., 2007; Clarke *et al*., 2016).

2.3.7 Laboratory Diagnoses of ALL:

 Diagnosis of ALL is based on morphology, immunophenotype, molecular analysis and cytogenetic analysis of the leukemic blast cells in the peripheral blood and bone marrow aspiration (Bhojwani *et al*., 2012; McGregor *et al*., 2012). The common genetic alterations in leukemic cells have contributed in understanding of the pathogenesis and prognosis of ALL (Iqbal *et al.,* 2007; Pui *et al*., 2008) also biochemical tests may reveal a raised serum uric acid, serum lactate dehydrogenase or, less commonly, hypocalcaemia. Liver and renal function tests are performed as a baseline before treatment begins (Hoffbrand *et al*., 2016),

2.3.7.1 Complete Blood   Count (CBC) and Peripheral Blood Picture (PBP):

Leukocyte count is variable maybe increased; decreased or normal, 50% of   ALL cases have leukocytosis due to leukemic lymphoblasts, so total leukocyte count is elevated, neutropenia may seen in some cases and markedly associated with risk of infection. Platelets count decreased, normocytic normochromic anemia and may be severe with anisocytosis and poikilocytosis,

nucleated red cells may be present (Hoffbrand *et al*., 2016). Morphological examination of peripheral blood smear usually reveals the presence of blasts, there is homogensity in lymphoblast morphology in pediatric patients while there is heterogeneity of lymphoblast in adult cases (Mondal *et al*., 2006; Chiaretti *et al*., 2014).

2.3.7.2 Bone Marrow Aspirate:

Lumbar puncture for cerebrospinal fluid examination should be performed and may show that the spinal fluid has an increased pressure and contains leukemic cells. hyper cellular bone marrow reveals replacement of normal hematopoietic cells by neoplastic lymphoid cells, > 20% lymphoblasts, more homogenous is frequently seen in pediatric patients while heterogeneous blast population seen in adult patients (Pitman and Huang, 2007; WHO, 2008).

2.3.7.3 Cytochemistry:

Differentiation between ALL and acute myelocytic leukemia (AML) is not possible by using Romanowsky stains, so cytochemistry stain help in identification of abnormal lymphoblast cells which negative for myeloperoxidase, sudan black B and nonspecific esterase stains, while PAS reaction is positive in over 70% of ALL cases (Pui, 2006; Chiaretti *et al*., 2014)

2.3.7.4 Immunophenotyping:

Immunophenotyping is essential parts of the diagnostic evaluation in which antibodies distinguish clusters of differentiation (CD) groups were used, but most leukocyte antigens lack specificity. Hence, a panel of antibodies is needed to establish the diagnosis and to distinguish between subclasses of leukemic cells. Typical panels include antibodies to at least one highly sensitive marker (CD19 for B-cell lineage, CD7 for T-cell lineage, and CD13 or CD33 for myeloid cells) and antibodies to a highly specific marker (Pui *et al*., 2006). Although ALL can be sub classified according to the recognized steps of normal maturation within the B-cell lineage or T-cell lineage (pre-T, mid-, and late thymocyte) pathways, the only distinctions of therapeutic importance at present are those between T-cell, mature B, and other B-cell lineage while subset of T-cell ALL, termed early T-cell precursor ALL, has been identified and associated with a dire prognosis with conventional chemotherapy (Coustan-Smith *et al*., 2009). Pattern of antigen expression at diagnosis is critically important for the detection of minimal residual disease by flow cytometry after treatment (Campana, 2009).

2.3.7.4 .1 Detection of ALL immunophenotyping by flowcytometery:

Flow cytometric immunophenotyping is essential tool in the distinction between acute and chronic leukemias and between ALL and AML, identification of lymphoblast (B-ALL or T-ALL), moreover the subtype of ALL (immunological classification) and assessing response to treatment, by the detection of minimal residual disease. The monoclonal antibodies used in FC are conjugated with fluorochromes which are stimulated by laser beam in the flowcytometer; these antibodies are against antigens found on the surface, cytoplasm or nuclei of the studies cells to identify their types (Gorczyca, 2007; Faderl *et al*., 2010).
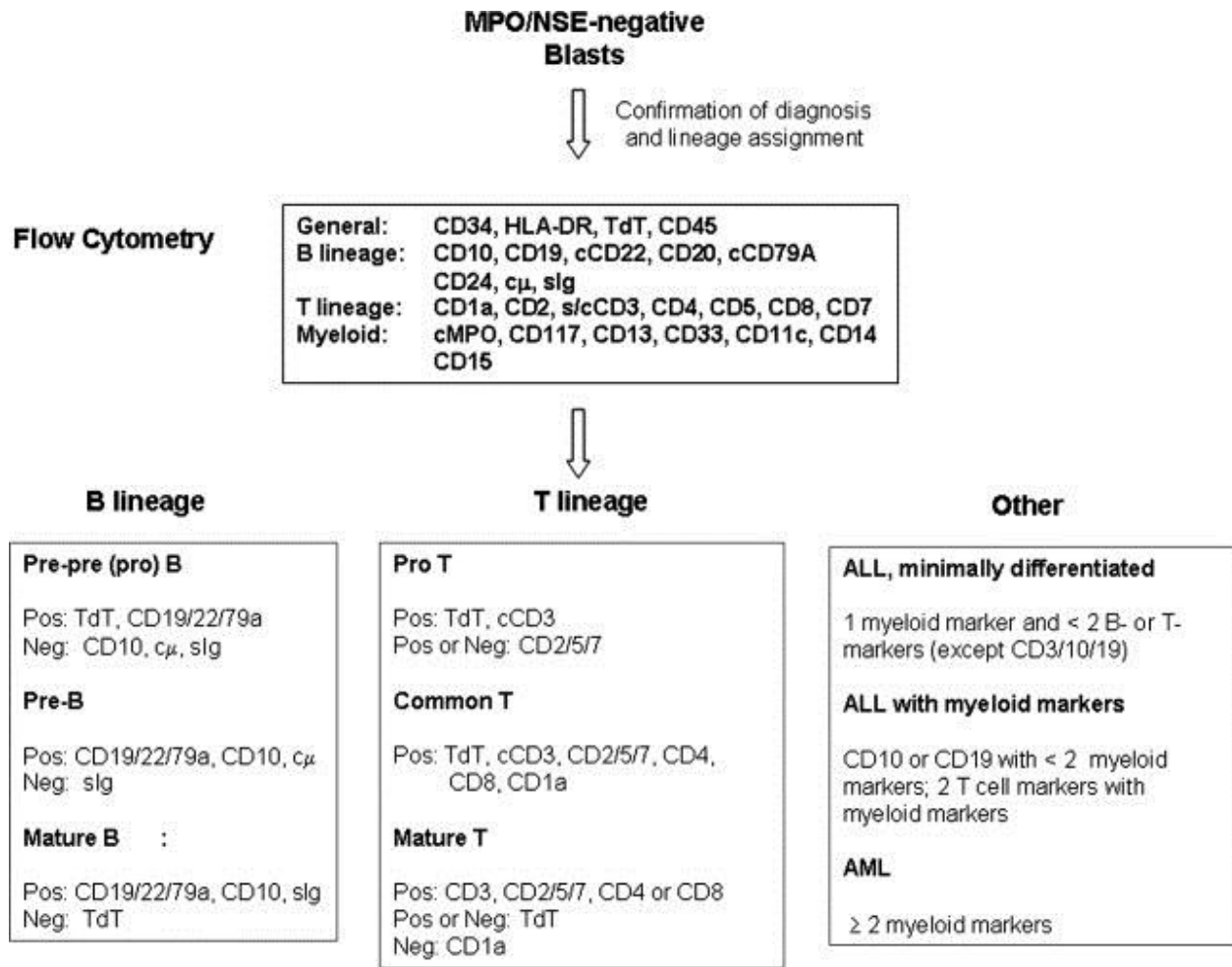
Figure 2-2: Flow Cytometric diagnosis of acute ALL (Faderl *et al*., 2010).

2.3.7.5 Cytogenetic and molecular analysis:

Cytogenetic and molecular technique reflects the genetic and epigenetic structure in both normal and abnormal status, major abnormalities are clonal translocations: t(9;22), t(4;11), t(8;14), t(1;19) or t(10;14) and presence of Philadelphia chromosome found in 5% of children and 25% of adults with ALL and is a very strong prognostic marker in both t(8;14) is associated with B-cell ALL (L3) and occurs in 5% of cases of dys-regulates the myc proto-oncogene, t(1;19) is associated with B-cell precursor ALL; t(4;11) occurs in 80% of infants with ALL and 6% of adults (Provan, 2003), also high frequency of BCR- ABL oncogen in pediatric acute lymphoblastic leukemia patients is sometimes common (Iqbal *et al*., 2012) and Loci on 7p12.2, 10q21.2 and 14q11.2 are associated with risk of childhood acute lymphoblastic leukemia (Papaemmanuil, 2009).

Karyotyping of ALL is very important to perform prognostic data, chromosomal translocations are found in about 75%of ALL patients (Ferrando, 2002; Ferrando, 2004). The impact of cytogenetic diagnosis in the management of hematological malignancies has improved over the past decade with the molecular techniques such as Southern blot, fluorescent in situ hybridization and polymerase chain reaction, also microarray studies are important in the research for the molecular classification of leukemias; however not yet tested in clinical practice (Mrózek *et al*., 2007). Genetic abnormalities have significance in prognostication such as FLT3-ITD, mutations in the NPM1, CEBPA, E26 transforming sequence related gene, ASXL1, IDH1 (iso-citrate dehydrogenase 1), IDH2 genes, partial tandem duplication of the mixed lineage leukemia (MLL) gene and in acute leukemia cytoplasmic gene (Iacobucci and Mullighan, 2017), these mutations have been assigned to be important in prognosis and in sub typing, for that these two have been incorporated in the 2008 WHO classification (Vardiman *et al*., 2008; Kohlmann *et al*., 2010). The genes encoding guanine nucleotide-binding protein gamma 11 and amphiregulin are found to be down regulated in AML, B- and T-ALL. However, the gene encoding ceruloplasmin is up regulated in AML, but not in B- and T-ALL (Haouas *et al*., 2010).

2.3.8 Prognosis:

The prognosis of childhood ALL has improved, over 95% of children show a complete remission and about 80% of children have disease-free survival for long time are cured. Patients who have good favorable prognostic factors can be treated less aggressively, while patients with poor

prognostic factors may be treated more aggressively. All adults with ALL are considered high risk (Iqbal and Tanveer, 2006; American Cancer Society, 2015).

Table 2-2: Prognostic factors of ALL (National Cancer Institute, 2015).

Factor	Favorable	Unfavorable
Age	2 to 10 years	Below 2 years or above 10 years
WBCs count	Low WBC count at diagnosis	WBC >50.000/µL
Phenotype	Precursor B cell	Precursor T cell Mature B cell
Chromosome number or DNA Index	Hyperdiploidy DNA Index>1.16	Pseudodiploidy Hypodiploidy Near tetraploidy
Chromosome abnormalities	t(12;21) Trisomy 4 and trisomy 10	c-MYC alterations{t(8;14): t(2;8):t(8;22)} MLL alteration(11q23),t(9;22) t(1;19)
Gender	Female	male
Ethnicity	Caucasian	African American, Hispanic
Time to complete remission	Short (7-14 days)	Prolonged time to remission or failure to achieve complete remission

2.3.9 Genetic polymorphism:

Genetic polymorphisms are defined as natural genetic variations that occur randomly in the general population and the most common type is the single nucleotide polymorphisms (SNPs), there are more than 10 million SNP polymorphisms in public databases. It is a single base pair change, such as substitution/ deletion/ insertion of one nucleotide, at a specific locus, generally consisting of two alleles, these SNPs can interfere gene's function, affecting metabolic pathways (Karathanasis, 2009; Faber *et al*., 2011; Li *et al*., 2012). There are three main pathways related to acute leukemia genetic susceptibility: xenobiotic system, DNA repair system, and cell regulation systems, these systems reported as risk factors in childhood leukaemia. Different studies suggested the association of polymorphisms in genes involved in xenobiotic metabolism phase I and II in patients with leukemia (Aydin *et al*., 2006; Gallegos *et al*., 2008; Lordelo *et al*., 2011).

2.3.9.1 Genetic polymorphisms in the xenobiotic metabolizing enzymes:

Xenobiotic metabolizing enzymes or drug metabolizing enzymes are responsible for protecting the organism by rapidly processing lipophilic chemicals to inert derivatives that can eliminated easily from the body through urine or bile (Grover *et al*., 2012). Besides detoxification, they also mediate the toxicity of chemicals by metabolic activation of procarcinogens and pro toxins, for that they thought to have a role in individual susceptibility to chemical which may lead to development of cancer and diseases, it consist of phase I enzymes represent by cytochrome P450 isoenzyme (CYP) and phase II enzymes, these enzymes possess genetic polymorphisms which may affect the individual's response to exposure (Deenen *et al*., 2011; Grover *et al*., 2012).

 Several studies suggest that hematological disorders may also be related to polymorphisms in genes encoding xenobiotic-metabolizing enzymes, such as cytochrome P4502E1 (CYP2E1), myeloperoxidase (MPO), NAD(P)H:quinone oxidoreducatase 1 (NQO1), and glutathione S-transferase (GST) (Zhang *et al*., 2007; Haro-García *et al*., 2012; Moreira and Gomes, 2012).

The major enzymes of phase I are cytochrome P450 dependent monooxgenases, which have roles in activate environmental chemicals into carcinogenic forms, hence their polymorphisms concerned as risk for ALL development. Another important member in relation to ALL development is NQO1, which detoxifies the carcinogenic metabolites of phase I, while among the members of phase II metabolism, polymorphisms of glutathione S-Transferase (GSTs) and N-acetyl Transferase (NATs) have been of great interest for the risk of development of ALL, as they have roles mainly in detoxification, and sometimes in activation of carcinogens and protection against oxidative stress (Brisson, 2015).

2.4 Cytochromes P450 (CYP 450):

Cytochromes P450 proteins are monooxgenases which include the most enzymes family which catalyze oxidative biotransformation of most drugs and other lipophilic xenobiotics, many studies investigated the association between CYPs to cancer due to the role of all CYPs enzymes in procarcinogenic bioactivation (Nelson, 2004; Guengerich, 2008; Zanger *et al.,*2008). Cytochrome P450 enzymes are the most important enzymes in phase I metabolism in mammals, responsible for most phase I reactions such as drugs degradation and elimination by the kidneys effectively, which usually happened by adding a hydroxyl group or hydrophilic group as amine or sulphydryl group, and usually involve hydrolysis, oxidation or reduction mechanisms (Hoskins *et al.,* 2009, Arinc, 2010).
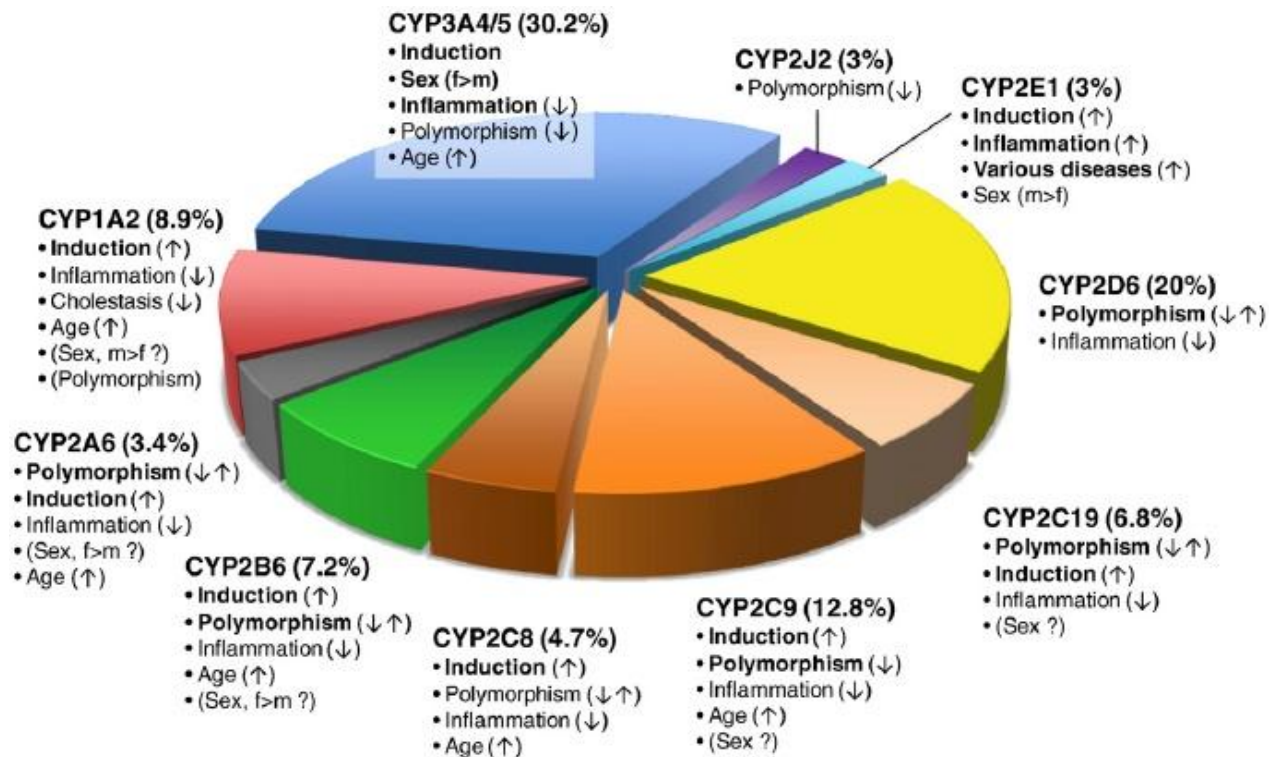
Figure 2-3: P450 isoforms and factors influencing variability drugs metabolized (Zanger, 2013).

2.4.1 CYP 2E1 Subfamily:

The human CYP2E1 gene is located in chromosome 10 (10q24.3), consist of 11,413 base pairs with nine axons and a typical TATA box, encodes 493 amino acid proteins. CYP2E1 belongs to the cytochrome P450 super family (Wang *et al*., 2010). It is a natural ethanol-inducible enzyme that is of great interest due to its role in the metabolism and bioactivation of many low molecular weight compounds, including ethanol, acetone, drugs like acetaminophen, isoniazid, chlorzoxazone, and fluorinated anesthetics and many procarcinogens like benzene, N-nitrosamines, vinyl chloride, and styrene (Ulusoy *et al*., 2007; Zhou *et al*., 2010), it is potent producer of reactive oxygen species (ROS); for that considered an important source of reactive oxygen species in alcohol-induced liver injury (Ogony *et al*., 2008)

CYP2E1 gene contains six restriction fragment length polymorphisms, of these are the two important RsaI polymorphism (CYP2E1∗5B; C-1053T substitution) and the 96-bp insertion in its 50-flanking region have drawn much interest (Morita *et al*., 2008;Wang *et al*., 2010; Zhou *et al*., 2010). RsaI polymorphism is known to have an effect in transcription level associating this polymorphism with others cancer such as gastric cancer (Feng *et al*., 2010), the variant type of this polymorphic site can enhance the transcription and increase the level of CYP2E1 enzymatic

activity in vitro (Nomura *et al*., 2003); responsible for the metabolism of xenobiotics, including toxic and therapeutic agents, and is involved in oxidative bioactivation of hydrophobic chemicals, such as benzene and acrylamide. It displays substrate for low molecular weight molecules and environmental toxicants as well as procarcinogenic, (Bolt *et al*., 2003; Lu and Cederbaum, 2008). Human CYP2E1 is expressed in liver and comprises approximately 10 % of human liver CYP 450 and to lesser extent in other organs and tissues, including human urothelial cells and exists in extra hepatic tissues, including the brain (Samochatova *et al*., 2009), it is metabolizes several precarcinogens including drugs, and solvents reactive metabolites, inactivates number of drugs and xenobiotics and also biactivates many xenobiotic substrates to their hepatyotoxic or carcinogenic forms (Ingelman 2001; Neafsey, 2009), Phenotypic polymorphism of CYP2E1 is cause interindividual drug metabolism differences, xenobiotics-induced liver injury discrepancy, or even severe adverse drug reaction (Cederbaum, 2006).

 CYP2E1 is of crucial importance for the risk of childhood ALL development because of its role in the metabolism of many environmental chemicals and effective generator of reactive oxygen species for that it associates with various cancers (Trafalis *et al*., 2010). CYP2E1 gene presses many polymorphisms in human and among them CYP2E1*5B (G1293 A/C1053T) polymorphisms are associated to increased risk of developing acute myeloid leukemia and acute lymphocytic leukemia (Ghanayem and Hoffler, 2007, Kanagal – Shamanna, 2012).
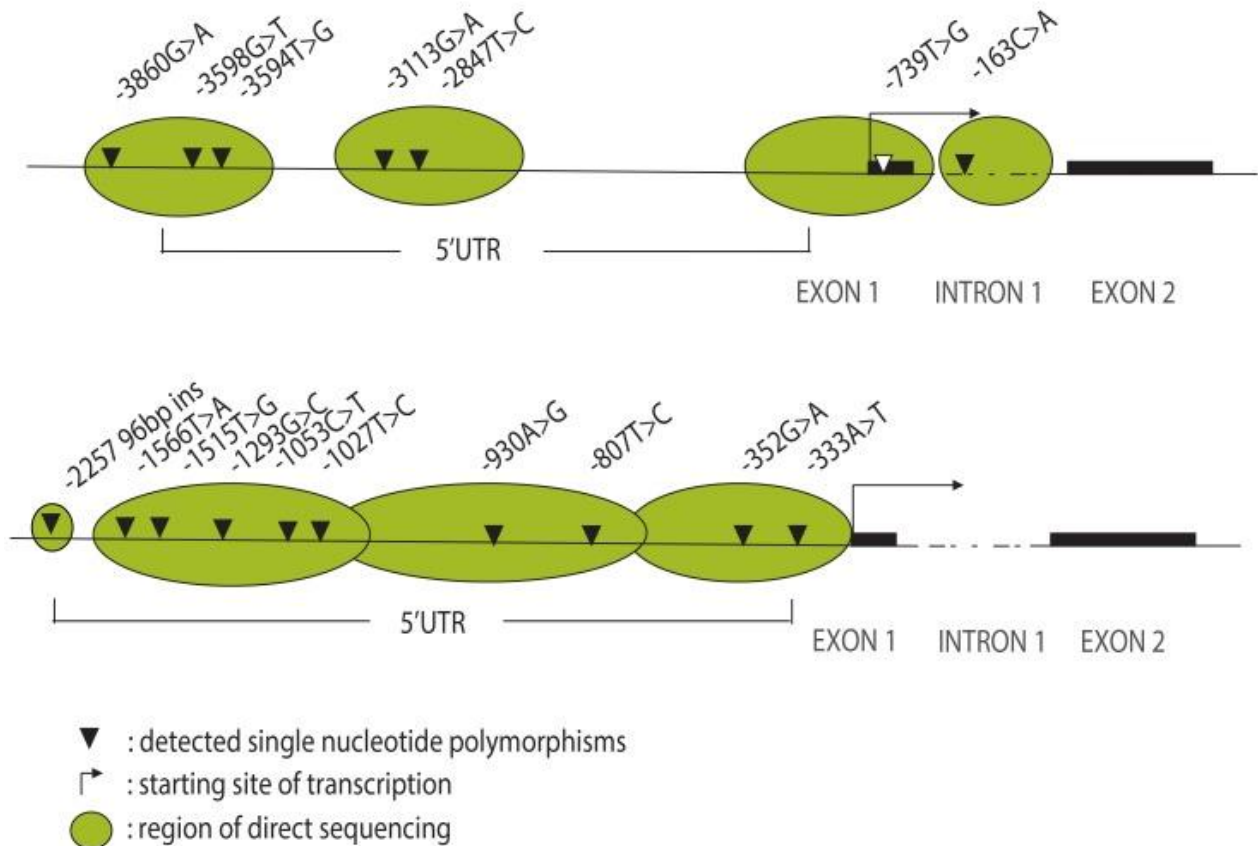
Figure 2-4: CYP2E1 SNPs (Yim *et al*., 2013)

CYP2E1 generates secondary metabolic pathways may have a carcinogenic action, toxicological, genotoxic or mutagenic in the body (Marmiroli and Maestri, 2008). Many studies on the association of CYP2E1 polymorphisms showed inconsistent results in increased risk for ALL with the presence of CYP2E1*5B allele (Krajinovic *et al*., 2002; Aydin *et al*., 2006).

Higher odds ratio observed in the case of postnatal maternal alcohol consumption during nursing period and presence of *5B allele for the risk of childhood ALL (Infante-Rivard *et al*., 2002). However, no relation was found in other reports (Bolufer *et al*., 2007; Canalle *et al*., 2004).

While others variants of CYP2E1 have not been investigated for ALL yet (Ulusoy *et al*., 2007).

2.4.2 NAD (P) H: quinone oxidoreducatase (NQO1):

NAD (P) H: quinone oxidoreducatase 1 (NQO1) is another important drug metabolizing enzyme that takes role in the metabolism of environmental chemicals; it is a member of the NAD (P) H dehydrogenase family and encodes a cytoplasmic 2-electron reducatase. NQO1 gene located at 16q22.1, 20 kb in length is composed of 6 axons and 5 intron having 17,881 base pairs, it is a flavoprotein which functions as a homodimer (North *et al*., 2011).

The physiological dimer has one catalytic site per monomer and each monomer consists of 273 amino acids. It is expressed in epithelial and endothelial tissues and at high levels throughout solid tumors. NQO1 is a mainly cytosolic enzyme and also found in smaller amounts in mitochondria, endoplasmic reticulum and nucleus (Ross, 2004; Chao *et al*., 2006).

NQO1 enzyme is generally considered as a detoxification enzyme because of its ability to reduce reactive quinones and quinone-imines to less reactive and less toxic hydroquinones by its unique ability to use NADH or NADPH as reducing cofactors, reduces quinones to hydroquinones (Winski *et al*.,2002; Principe *et al* ., 2011).

NQO1 expressed in bone marrow, where the expression was thought to be highly inducible by xenobiotics with quinine moieties and was up regulated during the times of oxidative or electrophilic stress (Winski *et al*., 2002; Ross *et al*., 2004).

 More over NQO1 catalyzes the reductive activation of quinoid chemotherapeutic agents and of environmental carcinogens; it is induced by synthetic antioxidants and cruciferous vegetables and offers protection against oxidative stress (Gong *et al*., 2008). Several studies have reported that NQO1 stabilized p53 transcription factor by a redox mechanism (Asher *et al*., 2002) or by protein to protein interaction (Anwar *et al*., 2003).

It is stabilizes p53 and protect it from degradation which may lead to resistance to drugs such as chemotherapy, stabilization of p53 by NQO1 in either ways could help explain the chemo protective effects of NQO1 in many different systems (Ross *et al*., 2004).

NQO1 protein protects cells against quinine-induced oxidative stress also it is involved in removal of quinone from biological systems as detoxification reaction: NAD (P) H+ quinone= NAD (p)$^+$ +hydroquinone this reaction ensures complete oxidation of substrate without formation of semiquinones and species with reactive oxygen radicals which deleterious to cells. Also involved in biosynthetic processes such as vitamin K-dependent gamma –carboxylation of glutamate residues in prothrombin synthesis it's able to detoxify vitamin K3 and protect cells against oxidative stress (Gong *et al*., 2008).

There are two polymorphisms in the NQO1 gene are C609T (NQO1*2) and C465T (NQO1*3) C465T causes reduction in enzyme activity, whereas the C609T results in complete loss of enzymatic activity due to protein instability (Krajinovic, 2005).

There have been more than 93 single nucleotide polymorphisms (SNPs) identified in the NQO1 gene. The most widely studied SNP of NQO1 is a C to T change at nucleotide position 609

(rs1800566), also known as NQO1*2 (Iida, *et al*., 2001), this polymorphism is a single nucleotide change from C to T at position 609 of the NQO1 cDNA coding for a proline to serine change at position 187 in the amino acid structure of the protein, results in a proline to serine amino acid change at codon 187(por187ser), which lead to loss of enzyme activity due to instability of the protein product (Ross, 2004), so lack of interaction of the NQO1*2 variant proteins with chaperons lead to aberrant folding and accelerated degradation through the ubiquitin proteosomal system, hence, individuals with homozygous NQO1*2/*2 genotype have no activity, and heterozygous individuals with NQO1*1/*2 genotype have reduced activity (Krajinovic *et al*., 2002).

NQO1 specifically derives attention for the risk of leukemias, because of its detoxification role in benzene metabolism. Several studies found an association between NQO1*2 polymorphism and infant leukemia and adult leukemia. (Naoe *et al*., 2000; Smith *et al*., 2001; Krajinovic *et al*., 2002), also there are correlation between NQO1*2 polymorphism and lymphomas (Soucek *et al*., 2002) and with therapy related acute myeloblastic leukemia acute lymphoblastic leukemias (Sirma *et al*., 2004) and also associated with an increased risk of tobacco related cancers and ALL (Lanciotti *et al*., 2005), several studies associate this SNP and increased susceptibility to develop leukemia and bladder cancer (Choi, 2007; Guo, 2010) also associated with cancer susceptibility (Lajin and Alackhar, 2013) and play a role in development of hepatocellular carcinoma (Akkiz *et al*., 2010).

Other polymorphisms were detected in NQO1 gene and were associated with the modification of the enzyme activity like the NQO1*3. The NQO1*3 polymorphism is located in exon 4 with a C to T substitution at location 465, which results in a nucleotide substitution of arginine to tryptophan at amino acid 139 (Krajinovic *et al*., 2002). However the frequency of NQO1*3 in different ethnic populations is generally low for that this polymorphism does not receive much attention in genetic epidemiological studies. The variant C609T polymorphism conferred an increased risk to develop leukemia, supporting the role for NQO1 substrates benzene, related compounds and oxidative stress as determinants of ALL (Eguchi-Ishimae *et al*., 2005).
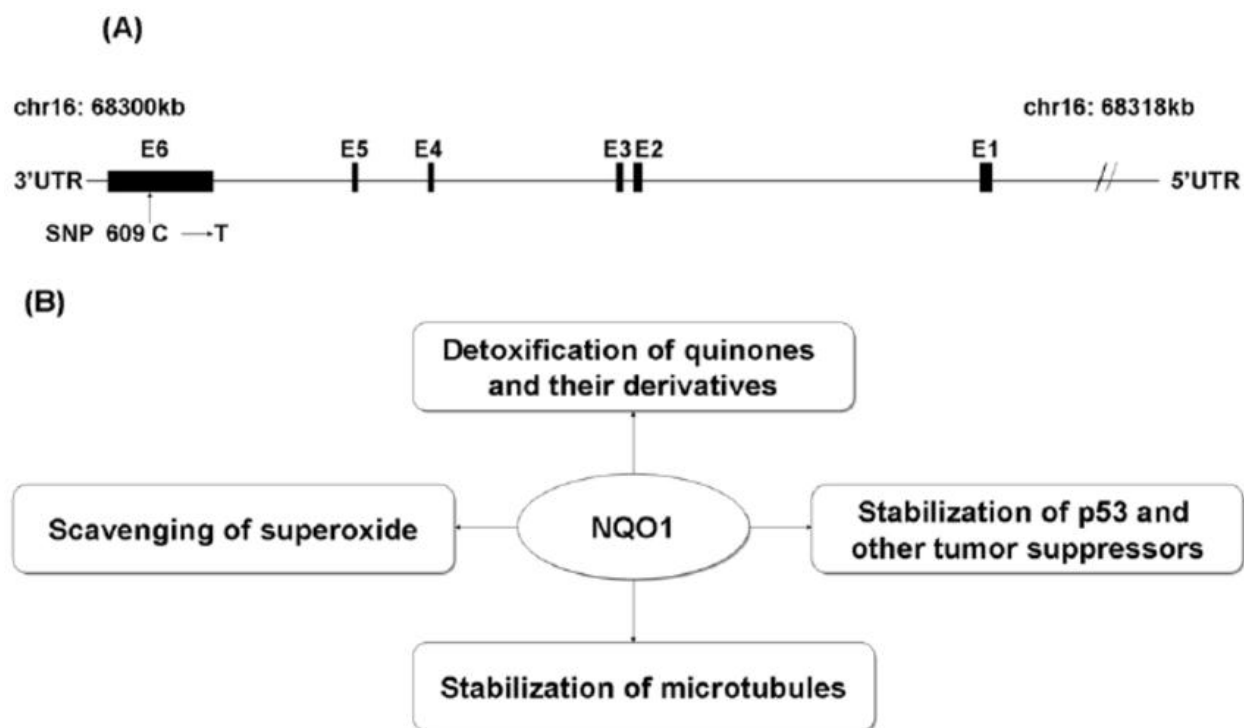
Figure 2-5: NQO1 gene function (Yu *et al*., (2012)

2.4.3 Previous studies:

The most gene polymorphisms that were investigated comprised the genes CYP1A1, CYP2D6, CYP2E1, CYP3A4, CYP3A5, EPHX1, GSTM1, GSTP1, GSTT1, MDR1, MPO, NAT1, NAT2, and NQO1. Majority of studies are from Asia (39.6%), followed by North America (25%), then Europe (20.8%), and South America (14.6%). The great difference between ethnic groups is well established, since the Asian population includes a wide range of people with distinct genetic backgrounds, such as Caucasians, Turkish, Indians, Japanese, Chinese, and Korean population, as in Americans have diverse ancestries, mainly Caucasian, Hispanic, and African. The majority of publications 75% investigated the genetic susceptibility in ALL only, and 25% in AML + ALL and regarding phase I metabolism, 14 publications (Aydin *et al* ., 2006; Yamaguti *et al*., 2010).

Another study found 2.8 to 3.4 fold increased risk for childhood ALL with the presence of CYP2E1*5B allele (Krajinovic *et al*., 2002), higher odds ratio 4.9 fold increased risk was observed in the case of postnatal maternal alcohol consumption during nursing period and presence of *5B allele for the risk of childhood ALL (Infante-Rivard *et al*., 2002).

Other study reported that there is no association found with development of acute lymphoblastic leukemia (Canalle *et al*., 2004; Bolufer *et al*., 2007).

Also other variants of CYP2E1 were not investigated for this ALL and the role of these polymorphisms in the risk of childhood ALL development still needs to be clarified and need more works up (Ulusoy *et al*., 2007).

Another study In Iraq reported that genetic polymorphisms in both NQO1 and CYP2E1 play a role in the development of leukemia. This is in accordance with the involvement of these variants in other types of leukemia, suggesting that the predicted higher level of CYP2E1 might be associated with an increased risk of childhood leukemia (Al-Marzoqi *et al.,* 2015).

It's revealed that NQO1 variants (NQO1*2) were associated with an increased risk of leukemia, particularly due to a higher prevalence of heterozygous individuals among cases relative to controls. Study conducted in France Canadians they found that *NQO1*, CYP2E1 variants influence an individual's susceptibility to childhood ALL. Because it is known that the frequency of these variants differs among population (Krajinovic *et al*., 2004).

CYP2E1 and NQO1 genetic polymorphisms play a role in the development of childhood ALL (Krajinovic *et al*., 2002).

Relevant studies were assess the association between the NQO1 C609T polymorphism and ALL risk (OR1.18, 95%=1.00-1.39, P=0.05).The allele frequency from a study for 95 Chinese was 0.51 for 187Ser, which was higher than those obtained from two Japanese studies (Naoe *et al*., 2000; Hamajima *et al*., 2002), also Caucasians have been reported to have a lower 187Ser allele frequency (Smith *et al*., 2001; Krajinovic *et al*., 2002). The significantly elevated risks of 187Ser allele have been reported for leukemia (Naoe *et al.*, 2000; Smith *et al*., 2001; Krajinovic *et al*., 2002).

In many studies there is association between the NQO1 polymorphisms and increased risk of ALL (Bolufer *et al.,* 2006), also study done in Tunis they found statistical evidence that NQO1*2 variant was associated with an increased risk of ALL in a Tunisian population. This ending suggests that leukaemogenesis of ALL is associated with carcinogen metabolism and consequently related to environmental exposures (Ouerhani *el al*., 2012).

Other study report that null or low NQO1 activity caused by inheritance of one or more mutant C609T alleles is associated with increased risk of acute leukemia in adults and this will provide further clues to its potential etiology in the general population (Stanulla *et al*., 2007). In multi-variant analysis, the heterozygous genotype (CT) of NQO1 C609T in combination with the heterozygous genotype (CT) of NQO1 C465T showed an increased risk for ALL (OR, 7.47; 95%

CI, 1.52-36.68), in which the main effect might have been created by the CT465 allele. Also, the CC 609/ CT 465 combined genotype was significantly associated with risk of ALL; again, this supports the results of our univariant analysis (Zaker *el al*., 2012). Family-based study suggested that the NQO1 C609T variant was associated with the risk of developing childhood ALL; another study performed in 2004 in Turkey did not support the role of the NQO1 C609T polymorphism in the increased risk of pediatric acute leukemia (Sirma*, et al.* 2004). A French-Canadian study showed that children carrying at least 1 mutant allele of the NQO1 C609T polymorphism had an increased risk of developing ALL, whereas individuals with wild-type Homozygotes seem to be protected against ALL (Krajinovic, *et al.* 2002; Silveira, *et al*. 2010). In meta- analysis, it was shown that the NQO1 C609T variant appeared to have no strong association with childhood ALL or AML but may be associated with mixed lineage leukemia–positive childhood leukemia. (Jeffrey *et al*., 2008).

Previous studies had also reported the NQO1 C609T polymorphism was associated with risk of childhood and adult ALL (Naoe *et al*., 2000; Krajinovic *et al*., 2002), T mutant allele of NQO1*2 was   more prevalent in children with ALL carrying mixed lineage leukemia (MLL) gene translocations as compared to controls (Smith *et al*., 2002). Moreover, the presence of mutant (T) allele conferred 2.7 fold increases in the risk of ALL / MLL positive cases. Contrary to earlier reports, two European studies reported lack of association between the NQO1 variant and the incidence of pediatric ALL, or AML. However, the modulating role of NQO1 in the pathogenesis of pediatric sporadic Burkitt lymphoma was suggested (Sirma *et al*., 2004; Kracht *et al*., 2004). Regarding NQO1 C609T, this results contrast with those of previous studies in other populations it indicate that the NQO1 C609T polymorphism is associated with the elevated risk of childhood ALL (Smith, 2002; Vijayakrishnan and Houlston, 2010).

NQO1 C/C genotypes among children living in Philippines to the risk of increased ALL leukaemogenesis increased 11.9-fold in the Philippines (Rimando *et al*., 2008).

Two Brazilian studies found significant associations of CYP1A1 variant alleles and ALL only with combined genotypes: CYP1A1*2 + CYP2E1*5B + GSTP1*B + GSTM1-null (Canalle *et al*., 2004), and CYP1A1*2A/*2B/*2C + NQO1 609-CT/ CT + TT (Yamaguti *et al*., 2010).

 Other variant alleles of CYP2E1, mainly *5B, *6 and *7B also have significant associations with development of ALL (Ulusoy *et al* ., 2007; Orsi *et al* ., 2012; Chokkalingam *et al* ., 2012), also

CYP2E1*5B was related to increased risk for ALL/AML in Canadians (Krajinovic *et al* ., 2002) and Turkish (Aydin *et al* ., 2006).

 Presence of at least two variant alleles (*5B and *6; *6 and *7B; or *5B, *6, and *7B) was reported to increased the risk for ALL in Turkish population (Ulusoy *et al.,* 2007), also the combined genotype CYP2E1*5B + CYP1A1*2 + GSTP1*B + GSTM1-null was associated with increased risk for ALL in Brazilians (Canalle *et al.,* 2004).

In Sudan there is study examined the association between NQO1 C609T polymorphism and risk of ALL, they indicated that mutant gene with low enzymatic activity is associated with increased risk of ALL and worse hematological feature (Abdulla and Kobara, 2015).

Chapter III

3. Materials and Methods

3.1 Study Design:

This study was analytical case control and hospital base study.

3.2 Study Area:

This study was conducted at Flowcytometery Center Khartoum and research lab at SUST- Sudan

3.3 Study Duration:

This study was conducted in period from May 2016 to January 2019

3.4 Study Population:

The study population was Sudanese acute lymphoblastic leukemia patients with different age groups whom attended flowcytometer lab and approved to participated in this study as cases and apparently healthy individuals were enrolled as control group matching (1:1).

3.5 Inclusion Criteria:

Acute lymphoblastic leukemia patients and healthy volunteers, both sexes and all age were included

3.6 Exclusion Criteria:

Other types of leukemic patients, immunecompramize patients and ALL patients who refused to percipient in this study were excluded.

3.7 Ethical Clearance:

The ethical approval was obtained from Sudan University of Science and Technology, College of Medical Laboratory Research Board, Ministry of Health Research Committee. After explains the purpose of the research with simple and clear words for the participant's, they were told that they have rights to voluntary, they signed inform consent and they can withdraw at any time without any deprivation. All participants have rights to no harm (privacy and confidentiality) by using coded questionnaire and the remaining samples were not be reused for other research and the data will be secured. All participants' has rights to benefit from the researcher knowledge and skills about acute lymphoblastic leukemia,

3.8 Sampling:

Non probability convenience sampling technique was used.

3.8.1 Sample size:

According to the following equation 102samples were collected for cases and 102 controls.

$$N= (Z^2 \times P (1-P) / e^2$$

Where Z = value from standard normal distribution corresponding to desired confidence level (Z=1.96 for 95% CI).

   P is expected true proportion

   e is desired precision

3.9 Data collection:

Data were collected through self and non self administrated questionnaire from all participants

3.10 Sample Processing:

A total of 204 blood samples were collected, 102samples from acute lymphocytic leukemia patients and 102 samples from healthy controls.

3.10.1 Sample Techniques:

Five ml blood was collected and distributed in two EDTA containers 2.5 ml for hematological tests and2.5ml for molecular technique.

3.10.2 Hematological Techniques:

Complete blood count (CBC) was measured using automated blood counter analyzer (Sysmex KX 21N).

3.10.2.1 Sysmex KX 21N

3.10.2.1.1 Principle:

Blood sample is aspirated, measured to a predetermined volume, diluted at the specified ratio, and then fed into each transducer. The transducer chamber has minute hole called the aperture .on both sides of aperture, there are the electrodes between which flows direct current (DC). Blood cells suspended in the sample pass through the aperture, causing DC resistance to change between the electrodes .As DC resistance change the blood cell size is detected as electric pulses. Blood cell count is calculated by counting the pulses and a histogram of blood cell sizes is plotted by determining the pulses sizes. Also, analyzing a histogram makes it possible to obtain various analysis data (Sysmex Corporation, 2012-2014).

3.10.2.1.2 Procedure of complete blood count:

Fully automated multichannel instruments require only that an appropriate blood sample is presented to the instrument and usually measure from 8 − 20 components for the basic CBC and

blood cell differential, impedance counting systems depends on the fact that red cells are poor conductors of electricity, whereas certain diluents are good conductors (Sysmex Corporation, 2012-2014).

Whole blood mode:

-Blood is aspirated from the sample probe into the sample rotor valve.

-6 micro litter of blood measured by the SRV is transferred to the WBCs TD chamber along with 1.994 ml of diluents .at the same time, 1.0 ml of WBCs /HGB lyse is added to prepare 1:500 dilution sample .

-Of the diluted / hemolyzed sample in the WBCs TD chamber, approximately 1 ml is transferred to the HGB flow cell.

- 500 micro litter of sample in the WBCs TD is aspirated through aperture; the pulses of the blood cells when passing through aperture are counted by the DC detection method.

- In the HGB flow cell , 555nm wave length beam irradiated from the light emitting diode (LED) is applied to the sample in the HBG flow cell. concentration of this sample is measured as absorbance , this absorbance is converted with that of the diluents along that was measured before addition of the sample, thereby calculating HGB (hemoglobin value) (Sysmex corporation, 2012-2014).

3.10.2.1.3 Sysmex Reagents:

Stromatolyser – WH: Lysing reagent lyse red blood cells to aid the counting and characterization on immature white blood cells.

Cell Pack (diluents) volume is about 20 liter (Sysmex Corporation, 2012-2014).

3.10.2.1.4 Quality Control of Sysmex:

The reliability of this instrument and reagents was monitored by two quality control methods l using control materials of three types EIGHTCHECK-3WP-N (Normal), EIGHTCHECK-3WP-L (Low level) and EIGHTCHECK-3WP-H (High level) to monitor an instrument performance before analyzing sample and also by Levey -Jennings (L-J) chart which use the data from a single analysis of control blood as quality control data (Sysmex Corporation, 2012-2014).

3.10.2.2 Flowcytometry:

Immunophenotyping Flowcytometer was used to diagnose of ALL; Lymphocytes panel was used including CDs markers (Coulter EPICS XL –Mcl [TM] Flowcytometer –Miami, Florida –USA)

3.10.2.2.1 Principle:

Basic principle is the passage of cells in single file in front of laser beam so they can be detected, counted and sorted. Cell components are fluorescently labeled and then excited by the laser to emit light at varying wavelengths.

3.10.2.2.2 Reagent and fluid:

Sheath fluid (30X) is pre filtered, pH balanced phosphate buffered saline solution used for transporting particles through any Flowcytometer, it is easy to prepared, each bottle provides to prepare 20 liters of 1X sheath fluid.

3.10.2.2.3 Components of Flowcytometer:

Fluidics: is a transport particle in fluid stream to the laser beam for interrogation.

Light Scatter: is occurs when particles pass through laser beam, its FSC and SSC to differentiate cell type

Fluorescence: absorbs light energy over range of wavelength which is characteristic for that compound, one example of the fluorochromes is Fluorescein IsoThioCyanate (FITC).

Optical System: include (1) optical bench which provide stable surface that holds the light source and excitation and collection optics in fixed position. (2) Optical filters (3) signal detection (4) threshold.

Laser and Laser Alignment: gas laser consist of cylinder or plasma tubes filed with inert gas. The laser beam is focused on the sample core, the laser head is held in fixed position

Data Collection and display: by computer attach with the Flowcytometer instrument

3.10.2.2.4 Procedure:

Passage of cells in single file in front of the laser, so the cell can be detected, counted and sorting out, cell components are fluorescent labeled then excited by laser to emit light at different wavelengths, then the fluorescence measured to determine the amount and type of cell in the sample, up to thousands particles per second analysed when they pass through the liquid stream. The laser light beam is directed at hydrodynamic focused stream of fluid carrying the cells; several detectors are carefully placed around the stream at the point where the fluid passes through the laser light beam .one of these detectors is in the same line with the light beam and is measuring Forward Scatter (FSC), the other on is placed perpendicular to stream and measure the Side Scatter (SSC) .since fluorescent labels used to detect the different cells or cells components, suspended particles or cells. Ranged in size from 0.2 to 150micrometer, they pass through the

light beam and scatter it, and then the fluorescent labeled cell components are excited by the laser and emit light at longer wavelength than the light source, which then detected by the detectors, therefore pick up the scattered and fluorescent light. The brightness of each detector is adjusted for this detection using the light measurements, different information can gathered about the physical and chemical structure of the cells. Generally FSC detect the cell volume while the SSC reflect inner component of the cell then this data then analyzed by computer attach to the Flowcytometer using special software.

3.10.3 Molecular Technique:

3.10.3.1 DNA Extraction:

Genomic DNA was isolated using innuPREP blood DNA mini kit (Germany); the extraction procedure is based on combines of lysis of blood sample with subsequent binding of nucleic acid on to the surface of a spin filter membrane, after several washing steps, the nucleic acids are eluted from membrane by using elution buffer.

3.10.3.1.1Kit components:

Lysis Solution (SLS) 25 ml

Binding Solution (BL) 40 ml

Proteinase K for 2x1.5 ml working solution

Washing Solution (BS) 25 ml ready to use

Elution Buffer 6x2 ml

Spin Filter (red)

Receiver Tubes

Elution Tubes 1.5 ml

3.10.3.1.1 Procedure:

Lysis step :200 µl of blood sample were placed in 1.5 Ependorf tubes, 200 µl SLS , 20 µl was added to the tube, vortex for 10 seconds and incubated at 60 ^{0}C for 10 minutes , the second step is binding of DNA 350 µl BL ,added spin filter to receiver tube ,then sample added to spin filter ,centrifuged at 12000 rpm for one minutes. Third steps washing step 400 µl from washing reagent then centrifuged at 12000 rpm for one minutes and washing BS reagent 600 µl from it added to the sample tube, centrifuged at 12000 rpm for one minutes, another 600 µl from BS was added then centrifuged at 12000 rpm for one minutes. fourth step is remove ethanol by discard filtrate , centrifuged max speed for 3 minutes, the last step elution step by adding 200 µl elution buffer

(60^0C),incubated for 2 minutes at room temperature then centrifuged at 12000 rpm for one minute.

3.10.3.2 Determination of DNA quality and purity:

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

3.10.3.3 DNA Storage:

DNA was preserved at - 20ºC until PCR was performed.

3.10.3.4 Molecular Analysis:

3.10.3.4 .1 Detection of CYP2E1, NQO1 609 and NQO1 465 polymorphisms:

DNA samples were analysed for CYP2E1 and NQO1 609 and NQO1 465 polymorphisms using PCR - RFLP, the primers sequence used were as follow:-

 Table 3-1 Primers sequences

Locus	Mutation	Primers sequences	Product size
CYP2E1	C1053 T	F 5 CCAGTCGAGTCTACATTGTCA 3 R5 TTCATTCTGTCTTCTAACTGG 3	413
NQO1	C 609 T	F 5 ATTCTCTAGTGTGCCTGAG 3 R 5-AATCCTGCCTGGAAGTTTAG 3	319
NQO1	C465 T	F 5 CTAGCTTTACTCGGACCCACTC 3 R 5 GCAACAAGAGGGAAGCTCCTCCATC3	464

3.10.3.4 .1.1 Primer Reconstitution (Rehydration):

Each vial of the primers was reconstituted with PCR-grade water and vortex according to the instruction sheet which provided by Invitrogen life technology in-order to prepare 100 uM primer stock solution.

3.10.3.4 .1.2 Preparation of the Primer Working Solution:

Twenty micro liters of each primer was prepared from the 100 uM stock by dilution, then 100 uLof primer mixes were prepared and aliquot in different 0.2 mL PCR tubes, in -20 Cº till PCR analysis.

Master Mix: ready master mix (iNtRON BIOTECHNOLOGY).

3.10.3.4 .1.3 Preparation of the PCR Reaction Mix:

Before starting all reagents were allowed for thawing at room temperature, gently mixed by tube inverting and spin down, then a 25 uL reaction mixes were prepared.

3.10.3.4 .2 Polymerase Chain Reaction (PCR):

The CYP2E1 and NQO1 genes were determined using PCR and DNA sequencing analysis. PCR amplifying using previously specific designed primers were conducted. PCR reactions were prepared in a volume of 25mL .Amplification was performed using suitable conditions for each SNPS was described below.

3.10.3.4 .3 A garose Gel Electrophoresis:

1. Agarose (Sigma USA)   2. DNA ladder/marker (100-1500bp and 50-1000bp)

3. An electrophoresis chamber and power supply   4. Gel casting trays   5. Sample combs

6. Electrophoresis buffer (Tris-borate-EDTA "TBE")

7. Loading buffer (contains glycerol to allow the sample to "fall" into the sample wells and tracking dyes which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded).

8. Ethidium bromide (10mg/ml) (a fluorescent dye used for staining nucleic acids)

9. Trans illuminator an ultraviolet light box, which is used to visualize ethidium bromide-stained DNA in gels.

Procedure:

The agarose powder was mixed with the TBE buffer in concentration of 1%, and then heated in a microwave oven until completely melted. After cooling the ethidium bromide dye was added to the mixture in a concentration of 0.5 ug/ml and poured into the casting tray after fitting of the sample comb and allowed to solidify at room temperature.

1. After the gel solidified, the comb was removed, leaving wells in the solidified gel.

2. Then the tray laid on a horizontal position into the electrophoresis chamber and covered with the TBE running buffer.

3. The products pipette to the sample wells of the gel as well as the DNA ladder.

4. Then the power supply was turned on at 90 volts (current 40 amperes) for 30 minutes

5. After finishing of the running time the DNA fragments were visualized against the UV transilluminator where the ethidium bromide will intercalate within the DNA double strand and gave red shining clear bands, then a photo was taken to the gel for documentation.

6. Finally the agarose gels were discarded safely into a special basket previously prepared for the gels, because the harmful effect of the ethidium bromide as a carcinogenic substance according to Hengen PN methods for disposing of ethidium bromide.

3.10.3.4 .4 Genotyping of Single Nucleotide Polymorphisms by PCR-RLFP:

In this study two drug metabolizing genes, CYP2E1 and NQO1, were genotyped for their single nucleotide polymorphisms. Three SNPs one of CYP2E1, namely *5B (C1053T) and two SNPs for NQO1 polymorphisms C609T, C465T were identified by PCR amplification of SNP containing regions followed by appropriate restriction enzyme digestions. The details of these methods were described below. NEW ENGLAND BIOLABS Inc(NEB) (England) and Eppendorf Master Cycler (Hamburg, Germany) thermo cyclers were used for PCR.

Table 3-2: Restriction Enzymes.

Locus	Restriction enzyme	Description
CYP2E1(C1053T)	RsaI	Rsa I R0167s , size 1,000 units ,concentration 10,0000 units /ml
NQO1 (C609 T)	Hinf I	Hinf I R0155T , size 5,000 units ,concentration 50,000 units /ml
NQO1 (C 465 T)	Hap II	Hap II R0171s , size 2000 units , concentration 10,000units /ml

PCR mixture of 20μ was prepared using premix master mix tube (intron PCR premix kit {i-TagTM}) for each sample.

Table 3-3: PCR mixture:

Reagents	Volume
Double D.W	16 μl
Forward primer	1 μl
Reverse primer	1 μl
Template DNA	2 μl
Total reaction volume	20 μl

3.10.3.4.4.1 PCR protocol for amplification and Restriction Endonucleases Digestion of PCR Products for CYP2E1 (C1053T):

For amplification of 5 franking region for CYP2E1 the PCR protocol as follow:

Initial denaturation at 95$^{\text{o}}$C for 2 minutes, followed by 35 cycles; denaturation at 95 $^{\text{o}}$C for 30seconds, annealing at 56.2_63.2 $^{\text{o}}$C for 30 seconds, extension 72 $^{\text{o}}$C for 1 minute, final extension 72 $^{\text{o}}$C for 5 minutes, then PCR products were analyzed on 2% agarose gel, 5µL of PCR product was applied to the wells of the gel. 5 µL of DNA ladder (50-1000bp) was applied to one well. The gel was run until the bromophenol blue reached to the bottom of the gel, visualized under UV and photographed. After that PCR products were digested by restriction endonucleases, RsaI, the components of restriction enzyme digestion mixture are ; buffer1 µL, Restriction enzyme RsaI 1 µL , pcr product5 µL and sterile a apyrogen H2O 3 µL, RsaI mixtures were incubated at 37°C for 18 hours for complete digestion and then analyzed on 2.0% agarose gel. 5 µL of digestion product and applied to the wells of the gel. 5 µL of DNA ladder (50- 1000 bp) was applied to the first well of the gel. The gel was run until the bromophenol blue reached to the bottom of the gel for 30 minutes, visualized under UV and photographed.

3.10.3.4.4.2 PCR protocol for amplification and Restriction Endonucleases Digestion of PCR Products NQO1 (C609T):

For amplification of NQO1 (609) PCR protocol as follow: Initial denaturation at 95$^{\text{o}}$C for 5 minutes, followed by 35 cycles; denaturation at 95 $^{\text{o}}$C for 30seconds, annealing at 55$^{\text{o}}$C for 30 seconds, extension 72 $^{\text{o}}$C for 1 minute, final extension 72 $^{\text{o}}$C for 5 minutes, then PCR products were analyzed on 2% agarose gel, 5µL of PCR product was applied to the wells of the gel. 5 µL of DNA ladder (50-1000bp) was applied to one well. The gel was run until the bromophenol blue reached to the bottom of the gel, visualized under UV and photographed. After that PCR products were digested by restriction endonucleases HinfI, The components of restriction enzyme digestion mixture are; buffer1 µL, Restriction enzyme HinfI 1 µL, pcr product5 µL and sterile a apyrogen H2O 3 µL, HinfI mixtures were incubated at 37°C for 18 hours for complete digestion and then analyzed on 2.0% agarose gel. 5 µL of digestion product and applied to the wells of the gel. 5 µL of DNA ladder (50- 1000 bp) was applied to the first well of the gel. The gel was run until the bromophenol blue reached to the bottom of the gel for 30 minutes, visualized under UV and photographed.

3.10.3.4.4.3 PCR protocol for amplification and Restriction Endonucleases Digestion of PCR Products (C465T):

For amplification of NQO1 (609) PCR protocol as follow: Initial denaturation at 95$^{\text{o}}$C for 5 minutes, followed by 35 cycles; denaturation at 95 $^{\text{o}}$C for 30seconds, annealing at 61.2 $^{\text{o}}$C for 30

seconds, extension 72 ^{o}C for 1 minute, final extension 72 ^{o}C for 5 minutes, then PCR products were analyzed on 2% agarose gel, 5µL of PCR product was applied to the wells of the gel. 5 µL of DNA ladder (50-1000bp) was applied to one well. The gel was run until the bromophenol blue reached to the bottom of the gel, visualized under UV and photographed. After that PCR NQO1 C465T polymorphism was defined and PCR products were digested by restriction endonucleases, HpaII, and the components of restriction enzyme digestion mixture are, The components of restriction enzyme digestion mixture are; buffer1 µL, Restriction enzyme HinfI 1 µL, pcr product5 µL and sterile a apyrogen H2O 3 µL, HpaII mixtures were incubated at 37°C for 18 hours for complete digestion and then analyzed on 2.0% agarose gel. 5 µL of digestion product and applied to the wells of the gel. 5 µL of DNA ladder (50- 1000 bp) was applied to the first well of the gel. The gel was run until the bromophenol blue reached to the bottom of the gel for 30 minutes, visualized under UV and photographed.

3.10.4.5 DNA sequencing:

DNA purification and standard sequencing was performed for PCR products of CYP2E1, NQO1 609 and NQO1 465 genes by Bioneer Company (South- Korea).

3.10.4.6   Bioinformatics analysis

Firstly before uploading the sequences to NCBI we proofread the nucleotides chromatogram by using Finch TV software version 1.4.0 (http://www.geospiza.com/Products/finchtv.shtml) to ensure that all ambiguous sites are correctly called and determined the overall quality of it. Then nucleotides sequences were searched for sequence similarity using nucleotide BLAST (At schulet al., 1997) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using the BioEdit software (Hall, 1999).

3.10.4.7 Data analysis:

3.10.4.7.1 Statistical analysis:

Data were analyzed using statistical package for social science software (SPSS version .25), and presented in form of tables and figures. Frequencies mean and standard deviation were calculated. Chi square test were performed between qualitative variables. Independent T test performed   two quantitative and qualitative variables. A *P.value* of < 0.05 was considered as significant for all statistical tests in the present study. Odd ratio calculated with GraphPad Prism6 programme version 6.07.

4. The Results

4.1 Demographic data:

This study conducted in Khartoum state in the period from May 2016 to January 2019 to detect gene polymorphism associated with ALL. Two hundred and four individuals were included in this study. The study group (ALL patients) consists of 69(67.6%) male and 33(32.4%) female, M:F 2.1:1,age ranging from 1 to 85 years old with mean age (17.61±17.68) years; in case and control. The control group (healthy volunteers) was matching in sex and age. Immunophenotyping processed for ALL patients and complete blood count was also measured for all of the participants in patients and control. Both patients and control were investigated for three gene polymorphisms that considered being risk factors for development of acute lymphoblastic leukemia. (Figures 4-1 and 4-2).
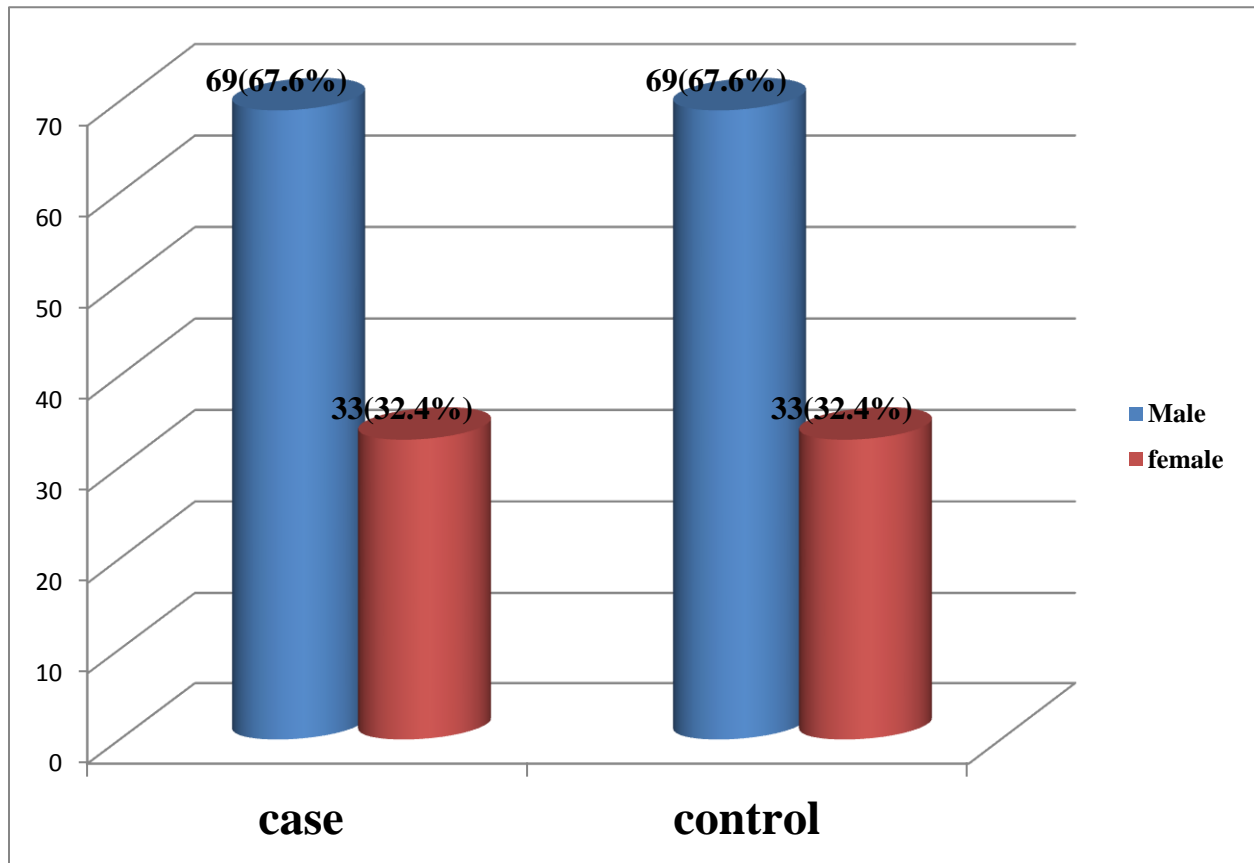
Figure: 4-1 Fequency of gender among case and control groups

The age group of study population is divided as shown in table 4-1,in both groups 64(62.7%) were 0-15 years old, 26(25.5%) 16-40 years old and >40 yrs old account12 (11.8%), (Figure4-2).
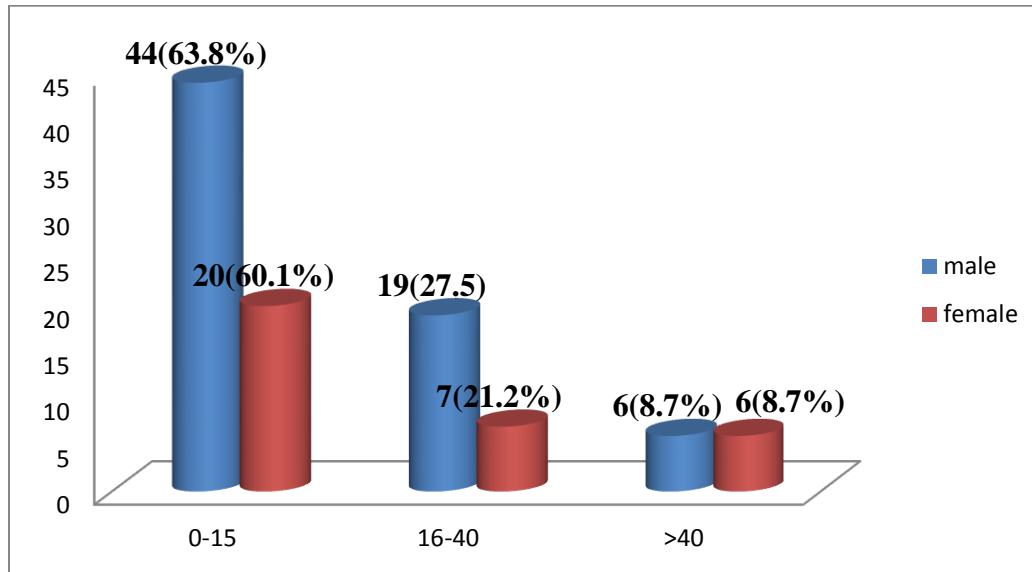
Figure 4-2: Frequency of gender and age group among case group

All volunteers were tested for complete blood cell counts. The results of blood count for ALL cases were as follows: mean total white cells (TWBCs) count $74.65\pm143.01X10^9$/L; mean red blood cell (RBCs) count $2.95\pm0.93X10^{12}$/L; mean platelets count $57.34\pm65.68X10^3$/L; mean hemoglobin (Hb) level 8.65 ± 2.54 g/dL. While for the control group: mean total white cells (TWBCs) count 8.89 ± 4.01 $X10^9$/L; mean RBCs count $4.58\pm0.776X10^{12}$/L; Mean platelet count $333.41\pm109.1X10^9$/L; mean Hb level 12.42 ± 2.05g /dL (table 4.1)

Table 4-1 Comparison of hematological parameters among study subjects

Variables	Cases((Mean ± SD)	control((Mean ± SD)	*P.value*
TWBCs(X10^9/L)	(74.6529 ± 143.01350)	(8.8929± 4.01338)	0.000
RBCs (X10^{12}/L)	(2.9580± 0.93183)	(4.5899± 0.77601)	0.000
PLTs (X10^9/L)	(57.3431± 62.68458)	(333.4118± 109.10449)	0.000
Hb (g/dl)	(8.6520± 2.54079)	(12.4227± 2.05574)	0.000

4.2 Immunophenotypic data:

Immunophenotypic for ALL patients processed by flowcytometer; B-cell ALL revealed 82(80.4%) while T – cell ALL represented 20 (19.6%), as shown in figure 4.3
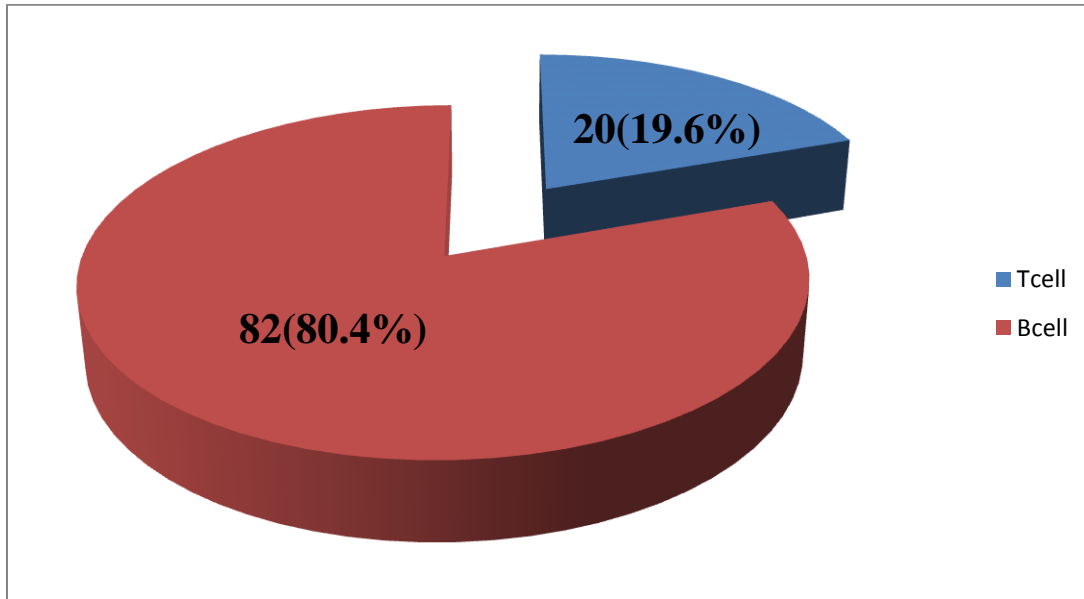
Figure 4-3 Frequency of ALL type among case group

B- cell ALL type was predominant than T-cell ALL type in children and adult, males also predominant in both type than female; 57(55.9%) of patients with B-ALL were children and 25(24.5%) were adults, while of those with T-ALL, 13(12.7%) were children 7 (6.9%) were adults; the association between types of ALL and age group was statistically insignificant (*P*=0.444). Males represent 54(52.9%) of patients with B-ALL and 15(14.7%) of those with T-ALL, whereas 28(27.5%) of patients with B-ALL and 5(4.9%) of those with T-ALL were females, the association between gender and type of ALL was statistically insignificant (*P*=0.308) (Table 4-2).

Table 4-2: Association of ALL types with patients' demographic data

Demographic data		B-ALL	T-ALL	*P.value*
Age type	Children	57(55.9%)	13(12.7%)	0.444
	Adults	25(24.5%)	7(6.9%)	
Gender	Male	54(52.9%)	15(14.7%)	0.308
	Female	28(27.5%)	5(4.9%)	

In this study there are eight subtypes of ALL among case group , B-cell line involve 5 subtypes; early pre B cell 43(42.2%) , pre B-cell 15 (14.7%) ,pro B-cell 2(2%) ,common B cell 21(20.6%), Burkitt B cell 1(1%) , while T-cell linage include 3subtypes; mature T cell 1(1%),cortical T cell 13(12.7%), pre T cell6(5.9%) .Type of B cell ALL was early pre B cell 28(27.5%) male, while in female15 (14.7%), second one is common B cell with frequency of 15(14.7%) in male, in female 6 (5.9%) ,then pre B cell9(8.8%)in male and 6(5.9%) in female while pro B cell, account 1(1%) in male and female and Burkitt B cell 1(1%)in male and 0(0%) female. While in T-cell subtypes the most common Cortical T cell 10(9.8%) in male and 3(2.9%) female, while pre T cell male 4(3.9%) and female2 (2%).And the least frequency was mature T cell male 1(1%), female 0(0%) Table4-3

Table 4-3: Gender distribution in B- and T-ALL subtypes

Gender	Early pre B cell	Pre B cell	Pro B cell	Common B cell	Burkitt B cell	Mature T cell	Cortical T cell	Pre T cell	Total
Male	28(27.5%)	9(8.8)	1(1%)	15(14.7%)	1(1%)	1(1%)	10(9.8%)	4(3.9%)	69(67.6%)
Female	15(14.7)	6(5.9)	1(1%)	6(5.9%)	0(0%)	0(0%)	3(2.9%)	2(2%)	33(32.4%)
Total	43(42.2%)	15(14.7%)	2(2%)	21(20.6%)	1(1%)	1(1%)	13(12.7%)	6(5.9%)	102(100%)

Comparison of hematological parameters among ALL types showed highly statistical significant among ALL types and mean total white blood count (*P*.value 0.000) and mean of hemoglobin concentration (*p*=0.038), while other parameters RBCs, PLTs and Blast % were (*P*=0.287, *P*=0.168, *P*=0.529) respectively as in table 4.4.

Table 4-4: Comparison of hematological parameters and ALL types among case group

Parameter	B-cell (Mean ± SD) N=82	T-cell (Mean ± SD) N=20	*P*.value
TWBCsX10^9/L	43.646 ±67.290	201.780±261.263	0.000
RBCsX10^{12}/L	2.909 ±0.8638	3.158 ±1.175	0.287
PLTsX10^9/L	53.109±53.267	74.700±91.633	0.168
Hb g/dl	8.3951±2.4328	9.7050±2.7628	0.038
Blast%	68.02±19.91	71.25±22.759	0.529

4.3Genetic Analysis:

4.3.1 Genotyping for CYP2E1*5B (C1053T) Polymorphism:

In C-1053T single nucleotide polymorphism of CYP2E1 gene is occupied with C in wild type allele (CC), while the same location is T in mutated allele (TT). In wild type allele, the PCR product bears a recognition site for restriction endonucleases RsaI (recognition site: (5'-GT↓AC-3'),digestion with RsaI cuts the PCR product with (CC) producing two fragments (351 bp and 62 bp),while for (TT) there is no suitable sequence that RsaI can recognized so digestion of PCR products give one fragment (413bp) and for heterozygotes allele (CT) three bands (413bp, 351 bp and 62 bp) would be observed. Throughout this study, a homozygous mutated individual was not detected; size of pcr product in figure (4-4) and digestion pattern of RsaI showed in agarose gel photo in Figure (4-5).
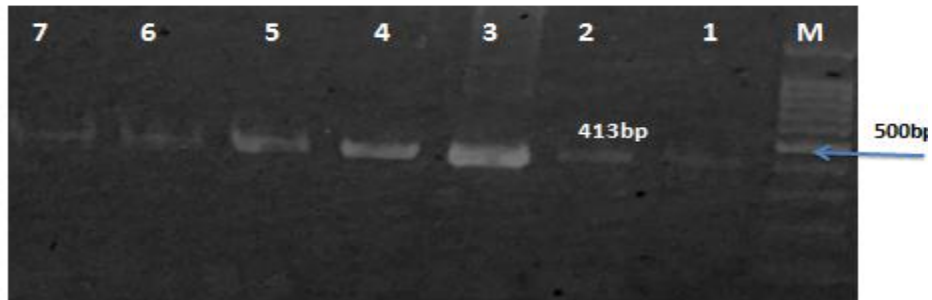
Figure 4-4 CYP2E1 (C1053T) PCR product 413 bp
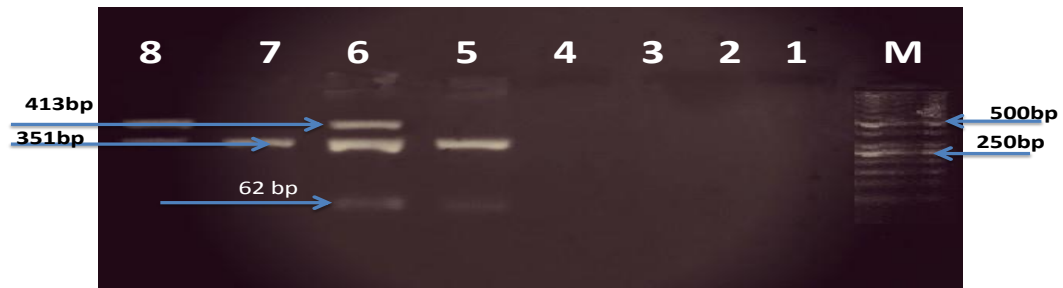
Figure 4-5 RsaI digestion of CYP2E1 in agarose gel 2%: Lane M DNA ladder (50-1000 bp), Lan (5and 7) homozygous wild type with bands of (351, 62 bp) Lane (6 and8) is a heterozygote with (413, 351, 62 bp) bands.

Frequency of The CYP2E1 genotypes for CC, CT, and TT genotypes among cases revealed 95(93.1%), 7(6.7%) and 0(0%) respectively, while for control group 98(96.1%) for   CC, 4(3.9%) (CT) and 0(0%) for (TT), the hererozygosity was higher in patients when compared with controls (6.7% and 3.9%, respectively). Odds Ratio (OR) was 1805; however, this difference was statistically insignificant (95% CI: 0.5117-6.369, p=0.3524). Allele's frequency was insignificant associated with risk of childhood ALL (*P*= 0.3592), with OR=1.776, (95% CI: 0.7289-17.32). (Table 4-5).

Table 4-5: Genotypes frequencies of CYP2E1 in patient and control samples.

CYP2E1*5B RsaI RFLP		Patients N(102) %	Control N(102) %	P.value	OR(95%CL)
Genotypes	CC	95 (93.1%)	98 (96.1%)		
	CT	7(6.9%)	4 (3.9%)		1.805
	TT	0(0%)	0(0%)	0.3524	(0.5117-6.369)
Allele					
	C	197(96.6%)	200(98%)	0.3592	1.776
	T	7(3.4%)	4(2%)		(0.5119-6.167)

Frequency of genotypes and alleles were analysed for children and adults in case and control; children represent 70(68.6%) and adults 32(31.4%), with insignificant association for genotypes among children and adults respectively in case and control (P=0.446, OR 1.718, 95%CI 0.3943-7.485) and (P=0.554, OR 2.067:95%CI 0.1778-24.02), alleles frequency for children and adults in both group also showed insignificant association (P=0.473, OR 1.691, 95%CI 0.3962-7.220) and (P=0.559, OR 2.032:95%CI 0.1795-23.00) respectively as shown in table (4-6).

Table 4-6: Association of CYP2E1 (C1053T) genotype among child and adult among study Population

	Genotypes		*P.value*	OR(95%CI)
	Case	**Control**		
Child(N70)	CC 65(92.9%)	CC 67 (95.7%)		1.718(0.3943-7.485)
			0.466	
	CT 5(7.1%)	CT 3 (4.3%)		
	TT 0(0%00	TT 0 (0%)		
Adult(N32)	CC 30(93.75%)	CC 31(96.9%)		2.067(0.1778-24.02)
			0.5543	
	CT 2(6.25%)	CT 1(3.1%)		
	TT 0(0%)	TT 0(0%)		
	Alleles			
Child	C 135(96.4%)	C 137(97.9%)	0.4731	1.691(0.3962-7.220)
	T 5 (3.6%)	T 3(2.1%)		
Adult	C 62(96.9%)	C 63(98.4%)	0.5591	2.032(0.1795-23.00)
	T 2(3.1%)	T 1(1.6%)		

Hematological parameters compared with wild type (CC) and mutant types (CT+TT) in cases showed significant association in mean PLTs and RBCs (P=0.050 and P=0.032) respectively, while for mean Hb, mean TWBCs and Blast% were insignificant (P=0.320, P= 0.530 and P=0.798) respectively, while in control group mean TWBCs(P= 0.008) and RBCs(P= 0.000) were highly significant, while other parameters mean Hb and PLTs showed insignificant association (P=0.236 and P=0.821) respectively. Table (4-7).

Table 4-7: Comparison of haematological characteristic between ALL patients with wild type and those with mutant types (CYP2E1 C1053T).

Parameter	Wild type(CC)	Mutant type (CT+TT)	P. value
Hb (Mean±SD g/dl)	(8.506±2.482)	(10.628±2.692)	0.320
TWBC(Mean±SDX10^9/L)	(77.083±147.448)	(41.671±48.159)	0.530
Platelets(Mean±SDX10^9/L)	(58.863±64.487)	(36.714±21.406)	0.050
RBCs(Mean±SD X10^9/L)	(2.904±0.902)	(3.685±1.089)	0.032
Blasts (Mean ±SD %)	(68.80±20.249)	(66.71±24.315)	0.798

4.3.2 Genotyping for NQO1 (C609T) Polymorphism

Single nucleotide polymorphism of NQO1 609 gene is occupied with C in wild type allele, while the same location is T in mutated allele. In wild type (CC) allele, with C in position not recognition site for restriction endonucleases HinfI (5'-GT↓A N TC-3'), so digestion with wild type allele, producing one fragment (319 bp), while in the mutated allele (TT) give two fragment (155bp and164 bp) and heterozygotes (CT) three bands detected (319 bp, 155 bp and 154 bp) Representated in agarose gel photo given in Figure (4-6).
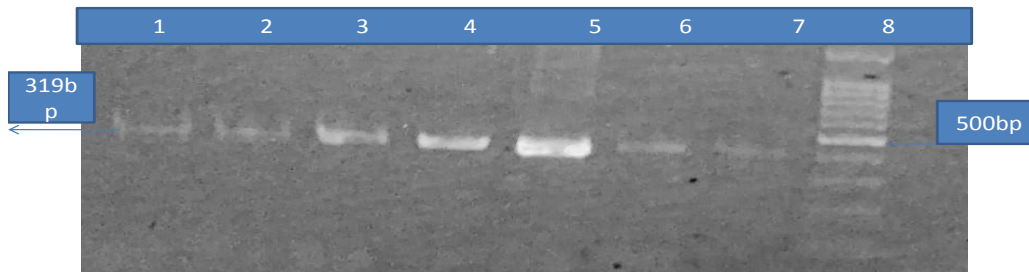
Figure 4-6 NQO(C 609 T) PCR product 319 bp
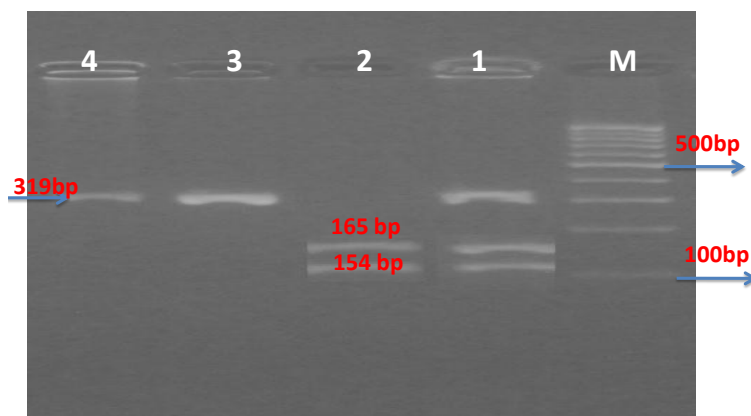
Figure 4-7: PCR product for HinfI digestion of NQO1 (C609T). Lane 3and 4 homozygous wild type CC (319bp), lane 2 homozygous mutant TT (155bp, 164bp), lane 1 heterozygous mutant CT (319bp, 155bp,164 bp), lane M DNA ladder (100-1500 bp).

Frequency of The NQO1 (C609T) genotypes for CC, CT, and TT genotypes among the 102 patients with ALL were 76(74.5%), 18(17.6%) and 8(7.8%) respectively, while for control group were distributed as follows: 89(87.2%) wild type (CC), 6(5.9%) heterozygous (CT) and 7(6.9%) homozygous mutants (TT). (Table 4.10), the hererozygosity was higher in patients when compared with controls (17.6% and 5.9%, respectively), the odds ratio was 3.513; however, this difference was statistically significant (95% CI: 1.327-9.301, p=0.0081) and alleles frequency was also significantly associated with risk of ALL (p= 0.0408, OR=1.840, 95% CI: 1.019-3.321).Table (4-8).

Table 4-8: Genotypes frequencies of NQO1 609 in patients and control samples.

NQO1 609 HinfI RFLP		Patients N(102) %	Control N(102) %	OR (95% CI)	*P. value*
Wild type	CC	76 (74.5%)	89(87.2%)		
Heterozygotes CT		18 (17.7%)	7 (6.9%)	3.513(1.327-9.301)	0.0081
Mutant	TT	8 (7.8%)	6(5.9%)	1.338(0.4638-3.862)	0.5888
Allele	C	170(83.3%)	185(90.7%)	1.840(1.019-3.321)	0.0408
	T	34(16.7%)	19(9.3%)		

Frequency of genotypes among children and adults showed; heterozygosity among children insignificant (p=0.1276, OR 2.0226; 95% CI 0.7806-6.350), while in adult significant association detected (P= 0.0352, OR 7.826;95% CI 0.8797-69.66).Homozygosity for children and adults have insignificant association(*P*= 0.8709, OR 1.113 ;95% CI 0.3051- 4.161 , *P*= 0.2211, OR 3.913; 95% CI 0.3815- 40.14) respectively and for alleles in children insignificant(P=0.295, OR 1.445; 95%CI 0.735-2.886), while in adult represented significant association(P=0.0134, OR 4.692; 95%CI 1.255-17.54), table 4-9.

Table 4-9: Association of NQO1 (C 609 T) genotypes and alleles among children and adults in study population

|	Genotypes		*P.value*	OR(95%CI)
	Case	Control		
Child(N70)	CC 53(75.71%)	CC 59(84.3%)	0.1276	2.226(0.7806-6.350)
	CT 12(17.14%)	CT 6(8.6%)		
	TT 5(7.14%)	TT 5(7.1%)	0.8709	1.113(0.3051-4.061)
Adult(N32)	CC 23(71.9%)	CC 30(93.8%)	0.0352	7.826(0.8797-69.66)
	CT 6(18.7%)	CT 1(3.1%)		
	TT 3(9.4%)	TT 1(3.1%)	0.2211	3.913(0.3815-40.14)
	Alleles			
Child	C 118(84.3%)	C 124(88.6%)	0.295	1.445(0.735-2.886)
	T 22(15.7%)	T 16(11.4%)		
Adult	C 52(81.2%)	C 61(95.3%)	0.0134	4.692(1.255-17.54)
	T 12(18.8%)	T 3(4.7%)		

Hematological parameters compared with wild type (CC) and mutant types (CT+TT) in cases and control showed insignificant association in mean Hb, TWBCs, PLTs, RBCs and blasts in cases (*P*=0.997, *P*=0.773, *P*=0.721, *P*=0.762 and *P*=0.515) respectively and for control group (*P*=0.764, *P*=0.443, *P*=0.304 and P=0.568) respectively. Table (4-10).

Table 4-10: Comparison of haematological characteristic between ALL patients group with wild type and those with mutant types (NQO1 C609T).

Parameter	Wild type(CC)	Mutant type (CT+TT)	*P*. value
Hb (Mean±SD g/dl)	(8.651±2.558)	(8.653±2.538)	0.997
TWBC(Mean±SDX10^9/L)	(72.248±140.098)	(81.680±153.876)	0.773
Platelets(Mean±SDX10^9/L)	(56.039±65.639)	(61.153±54.118)	0.721
RBCs(Mean±SD X10^9/L)	(3.941±0.9531)	(3.006±0.938)	0.762
Blasts (Mean ±SD %)	(67.88±19.955)	(70.92±21.976)	0.515

4.3.3 Genotyping for NQO1 (C465T) Polymorphism

Single nucleotide polymorphism of NQO1 465 gene is occupied with C in wild type allele (CC), while the same location is T in mutated allele (TT) recognition site for restriction endonucleases HpaII (5'-C↓CGG-3'), the PCR product digested by HpaII give 2 bands for wild type (353bp, 112bp), three bands for heterozygote allele (353bp,112bp, 464bp) and for mutant type there is no suitable sequence that HpaII can recognize so the products give one fragment of (464bp). Figure (4-8 and 4-9).

Figure 4-9 NQO1 (C465T) PCR product 465 bp
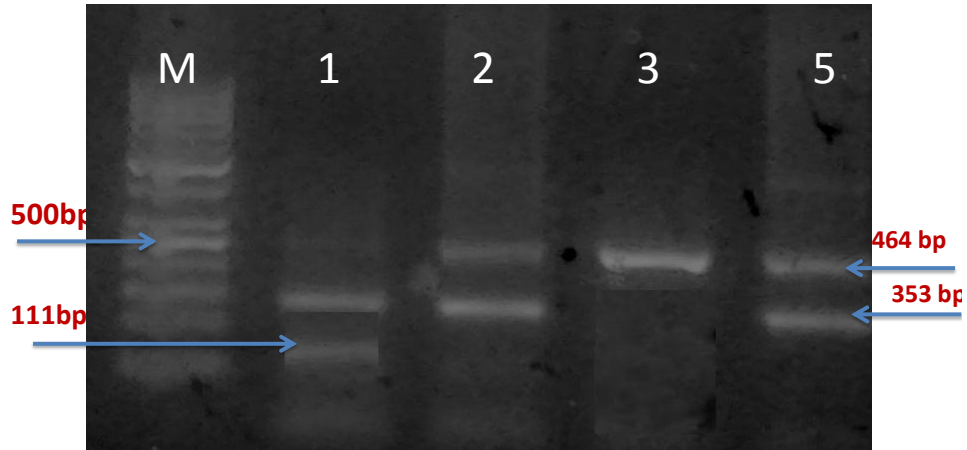
Figure 4-10: Agarose gel (2%) of HpaII digestion NQO1 (C465T). Lane M: DNA ladder (100-1500 bp). Lane1 wild type CC (353bp, 111pb), lane 2, 5 heterozygous CT (353bp, 111bp and 464bp), lane 3 homozygous mutant type TT (464bp)

Frequency of The NQO1 (C465T) genotypes among cases were CC 72(70.6%), CT 23(22.5%) and TT 7(6.9%), while in control: CC 96(94.1%), CT 4(3.9%) and TT 2(2%). (Table 4-10), the hererozygosity of genotypes was higher in patients than controls (22.5% and 3.9%, respectively), this difference was statistically highly significant (P=0.0001) with odds ratio was 7.667, (95% CI: 2.539-23.15), also homozygote mutant genotypes was higher in case (6.9%) than control (2%), this was also significant association (P=0.0401), with odd ratio 4.667 (95%CI 0.9410-23.14). The alleles frequency also showed statistical significant associated with risk of ALL development (P= 0.0017, OR=2.7, 95% CI: 1.427-5.107) (table 4-11).

Table 4-11: Genotypes frequencies of NQO1(C 465 T) in patients and control groups.

NQO1 C 465 T HpaII RFLP	Patients N(102) %	Control N(102) %	OR (95% CI)	P.value
Wild type CC	72(70.6%)	94(92.2%)		
Heterozygotes CT	23(22.5%)	5(4.9%)	7.667(2.539-23.15)	0.0001
Mutant TT	7(6.9%)	3(2.9%)	4.667(0.9410-23.14)	0.0401
Allele C	167(81.9%)	193(94.6%)	2.7(1.427-5.107)	0.0017
T	37(18.1%)	11(5.4%)		

Frequency of genotypes among children and adults showed highly significant for heterozygosity among children (*P*=0.0006, OR 6.26; 95% CI 1.98-19.73), while in adult insignificant association detected (*P*= 0.0858, OR 5.76; 95% CI 0.63-52.64).Homozygosity for children and adults have insignificant association(*P*= 0.0668, OR 4.17 ;95% CI 0.80- 21.62 , *P*= 0.9207, OR 1.15; 95% CI 0.0.69- 19.4) respectively and for alleles frequency in children highly significant(*P*=0.0001, OR 4.5; 95%CI 0.1.98-10.22), while adult represented insignificant association(*P*=0.2882, OR 2.1; 95%CI 0.51-8.96), table 4-12.

Table 4-12: Distribution of NQO1 (C465T) genotypes among children and adults among study population

	Genotypes				*P.value*	OR(95%CI)
	Case		**Control**			
Child(N70)	CC	46 (65.7)	CC	64(91.4%)		
	CT	18(25.7%)	CT	4(5.7%)	0.0006	6.26 (1.98-19.73)
	TT	6 (8.6%)	TT	2(2.9%)		
					0.0668	4.17 (0.80-21.62)
Adult(N32)	CC	26(81.3%)	CC	30(93.8%)		
	CT	5 (15.6%)	CT	1 (3.1%)	0.0858	5.76(0.63-52.64)
	TT	1 (3.1%)	TT	1(3.1%)		
					0.9207	1.15(0.69-19.4)
	Alleles					
Child	C	110(78.6%)	C	132(94.3%)		
	T	30(21.4%)	T	8(5.7%)	0.0001	4.5(1.98-10.22)
Adult	C	57(89.1%)	C	61(95.3%)		
	T	6(9.4%)	T	3(4.7%)	0.2882	2.1(0.51-8.96)

Hematological parameters compared with wild type (CC) and mutant types (CT+TT) for NQO (C465T) in cases and control showed insignificant association mean Hb, TWBCs, PLTs, RBCs and blasts in cases (*P*=0.649, *P*=0.638, *P*=0.968, *P*=0.851 and *P*=0.298) respectively and for control group (*P*=0.475, *P*=0.521, *P*=0.616 and P=0.574) respectively. Table (4-13).

Table 4-13 Comparison of haematological characteristic between ALL patients with wild type and those with mutant types (NQO1 C465T).

Parameter	Wild type(CC)	Mutant type (CT+TT)	*P*. value
Hb (Mean±SD g/dl)	(8.7264±2.44552)	(8.4733±2.79185)	0.649
TWBC(Mean±SDX10^9/L)	(78.9847±156.43764)	(64.2567±105.63133)	0.638
Platelets(Mean±SDX10^9/L)	(57.1806±61.56419)	(57.7333±66.37352)	0.968
RBCs(Mean±SD X10^9/L)	(2.9468± 0.87453)	(2.9850±1.07265)	0.851
Blasts (Mean ±SD %)	(67.29±20.948)	(71.93±19.033)	0.298

4.3 Bioinformatics and Sequencing Analysis:

DNA sequencing, 54 samples were successively sequenced by BGI, China.

Results of sequencing for all sequencing samples (54 samples) confirm the PCR-RLFLP results.

For CYP2E1, 14 samples showed 99% identity with CYP2E1 from NCBI database with accession number (NG_008383.1).figure (4.11-4.12)
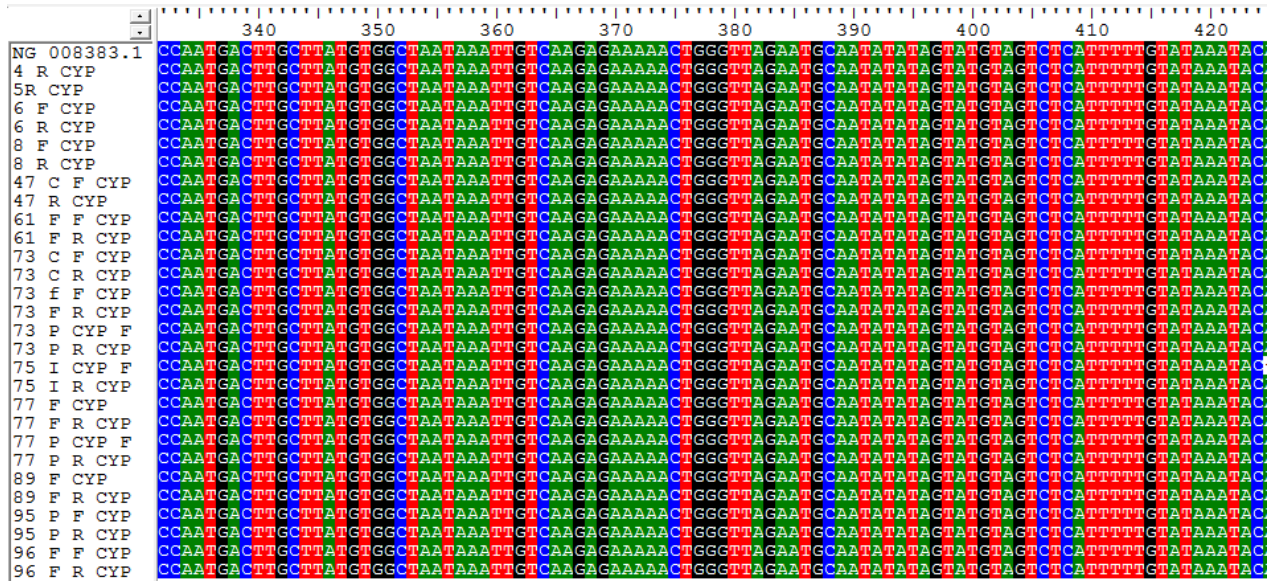
Figure 4-11: Bio-Edit multiple sequence alignment of CYP2E1 gene compared to reference (NG_008383.1) CYP2E1 gene from Gene bank.
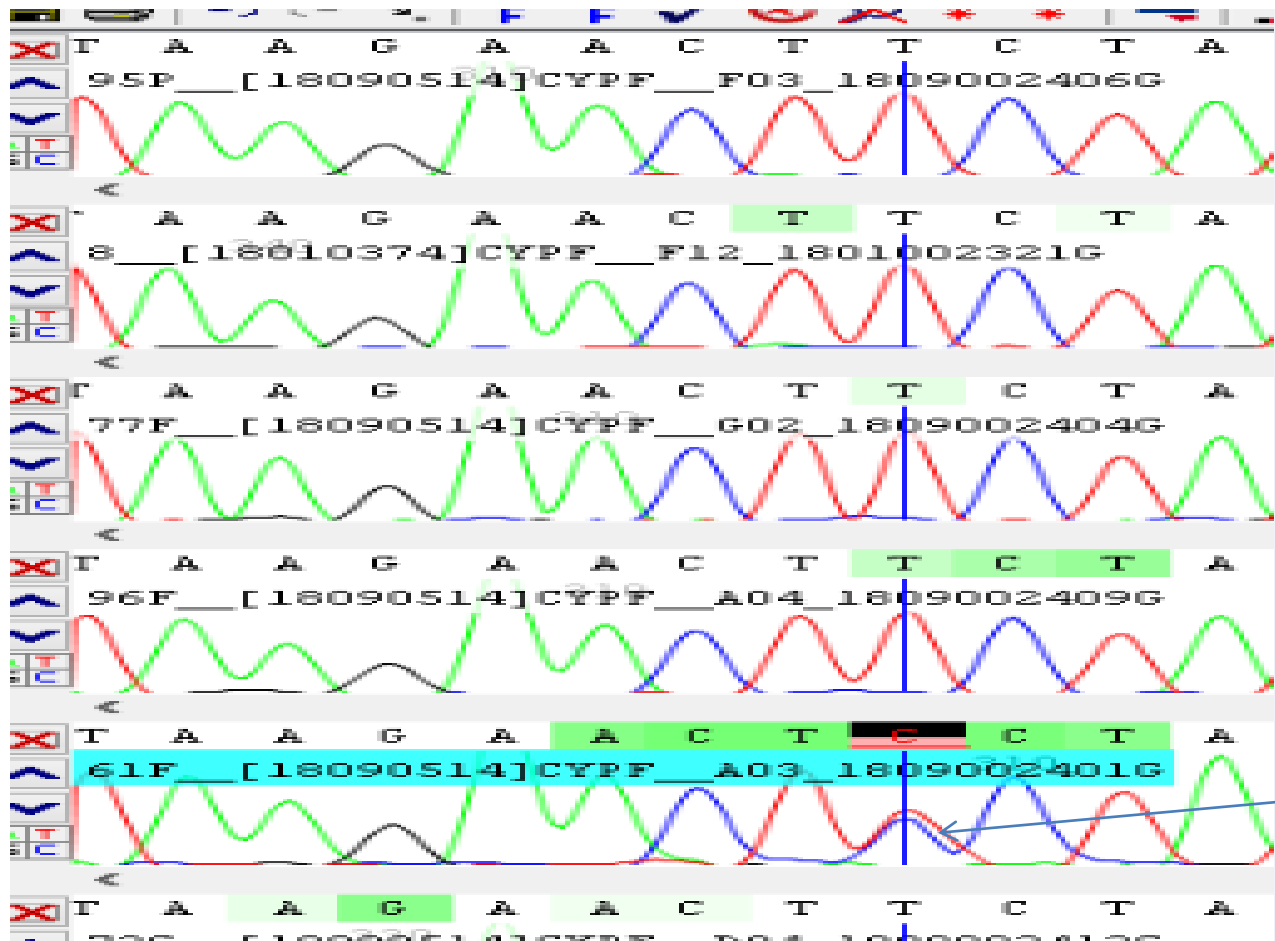
Figure 4-12: Sequences chromatogram viewed by Codon code aligner program, which showed CYP2E1 (C1053T) heterozygous CT compared to samples with wild type CC.

Twenty samples for NQO1 (C609T) and 20 samples for NQO1 (C465T) showed 100% similarity with NQO1 reference (NG_011504.1) from NCBI database,
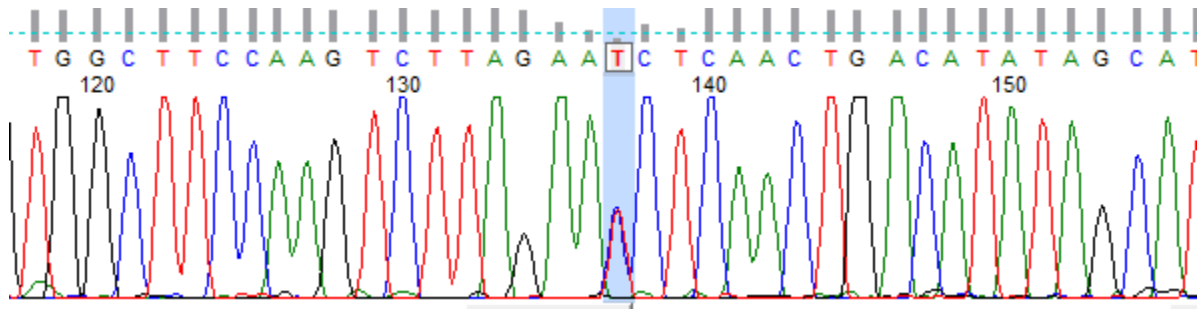
Figure 4-13: Sequences chromatogram viewed by Finch TV program, which showed heterozygous CT in NQO1 609.
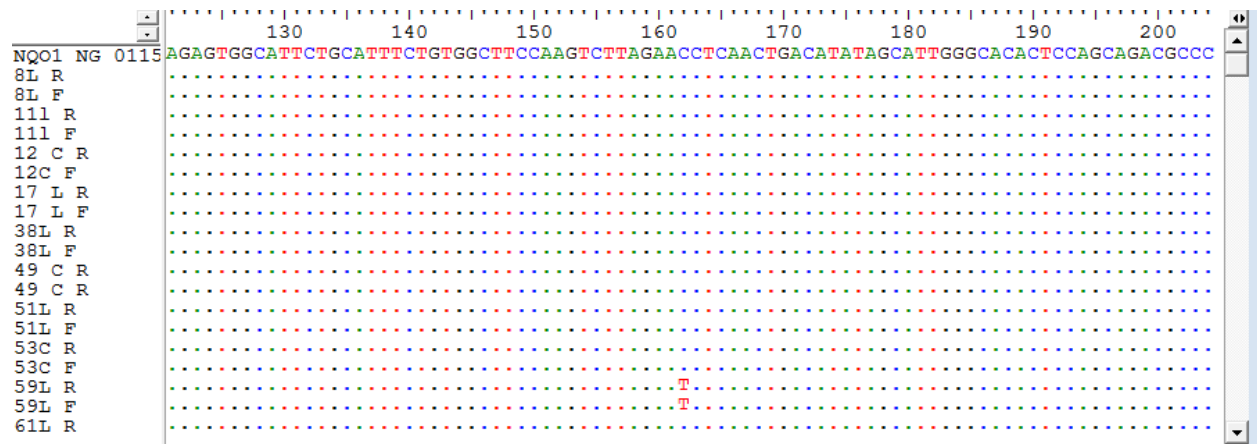
Figure 4-14: BioEdit multiple sequence alignment showed SNPs in two samples (C609T) when compared with ref. sequence (NG_011504.1).
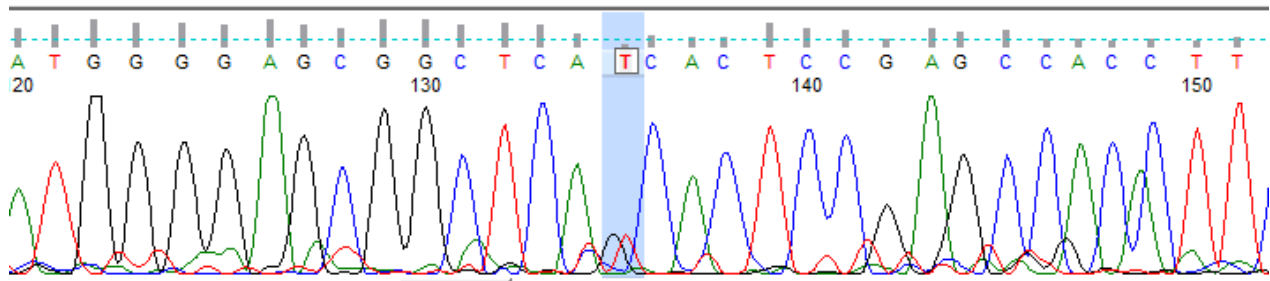
Figure 4-15: Sequences chromatogram viewed by Finch TV program, which showed heterozygosity (CT) in NQO1 (C465T).
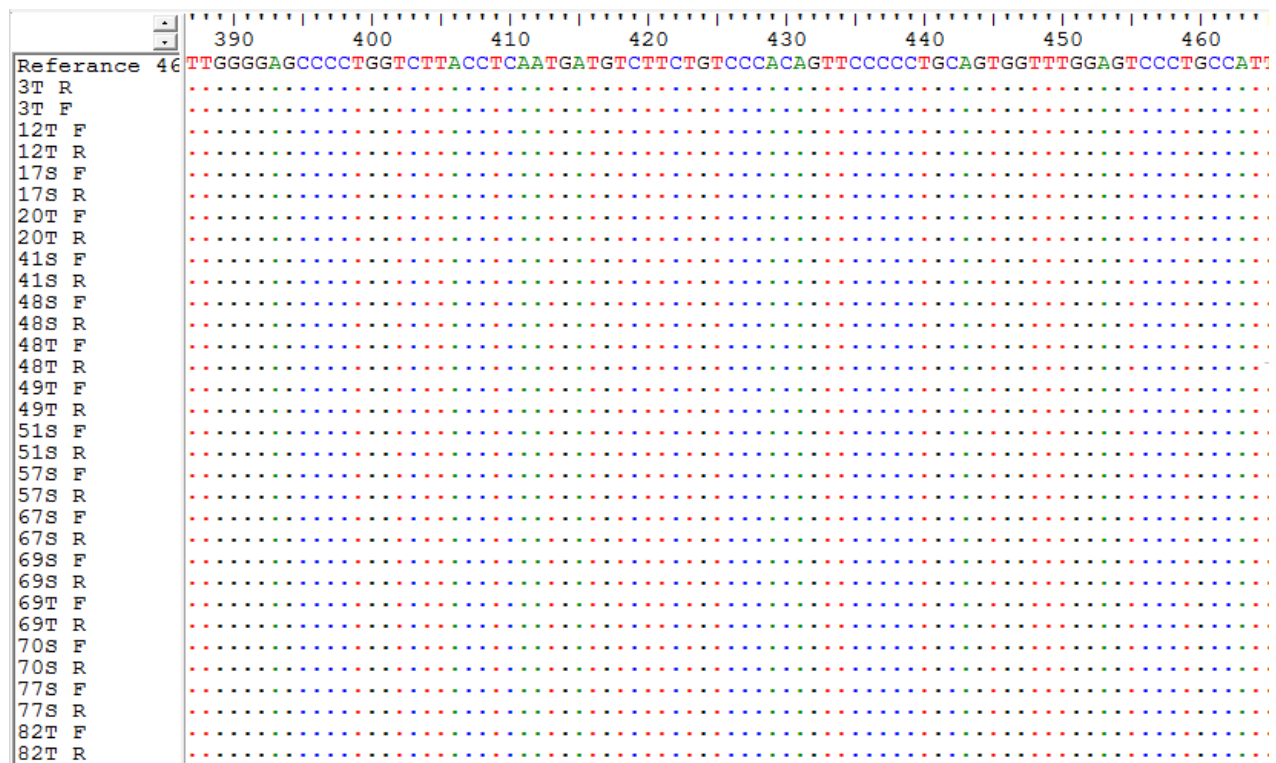
Figure 4-16: BioEdit multiple sequence alignment showed similarity of NQO1 (C465T) when compared with ref. sequence (NG_011504.1).
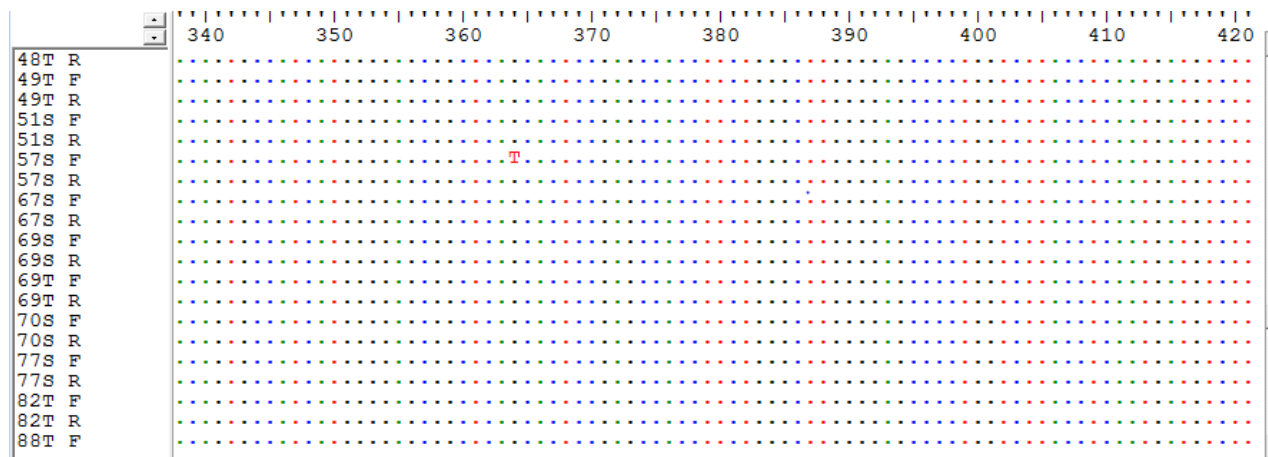
Figure 4-17: BioEdit multiple sequence alignment showed mutant sample NQO1 (C465T) when compared with ref. sequence (NG_011504.1).

Chapter Five
5 Discussion, Conclusion and Recommendations

5.1Discussion:

Acute lymphoblastic leukemia (ALL) being the most common malignancy in children, ALL affects different age groups, with a peak at 2-5 years; therefore age is an important predictor of disease development (Johnston *et al*., 2010), its etiology remains poorly understood, there are few hypothesis that both environmental and genetic factors contribute to the initiation of Leukemogenesis (Healy *et al*., 2010).

Single nucleotide polymorphisms (SNPs) been under focusing, there are several gene polymorphisms have been of great interest for the risk of development of ALL, for their important roles mainly in detoxification, and sometimes in activation of carcinogens and protection against oxidative stress; however they thought to be associated with development of ALL (Brisson, 2015).

This study focus in CYP2E1 (C1053T) and NQO1 (C609T and C465T) as risk factors for development of ALL due to their roles in the metabolism of many environmental chemicals. Two hundred and four participants enrolled in this study, 102 were ALL patients as case group and 102 healthy volunteers as control matched in sex and age, most of them were males, with male/female ratio about 2.1:1. This finding in agreement with results observed by Jawaid *et al*. (2017) who reported that males were predominant than females , also by Sultan *et al*. (2016) and Shahab and Raziq, 2014 in Pakistan, their finding showed that most ALL populations under study were males with 2:1 ratio, also it was consistent with study done in Brazilian patients by De Sousa *et al*. (2015) and De França *et al*. (2014) and similar to Hayakawa *et al*. (2014) in Japan.

The present study showed that most predominant age was 0-15 years among study groups, this finding similar to report published by American Cancer Society (2008), also similar to study done in Sudan by Ebrahim *et al*. (2017), they found that two third of their cases were at age between 6 - 16 yrs.

In current study complete blood count results among study group showed leukocytosis, anemia, thrombocytopenia and high blast percentage, this finding were agree with study done in Egypt by Hanna (2015), showed a significant elevation in TLC and bone marrow blast count while hemoglobin and platelets count were significant decreased and also similar to Ebrahim *et al*. (2017) they revealed leukocytosis, anemia and thrombocytopenia, also similar result reported by

Barakat *et al*. (2010) in Egypt and with another study done by Yasmeen and Ashraf in Pakistan (2009).

In this study B- ALL is more frequent than T- ALL, this outcome agree with study done in Jordan by Abbasi *et al*. (2015), also similar finding found in study done in Brazil done by De Andrade Alves *et al*. (2012) also agree with a previous study done by Shrestha *et al.* (2013) in Nepal and with Italian study done by Chiaretti *et al* (2014), on other hand this study disagree with study done in Iran by Pahloosye *et al*. (2011) and with study in Pakistan done by by Mushtaq *et al*. (2013), they both found that frequency of T-cell documented to be greater than B-Cell ALL.

This study showed highly association of mean total white blood count (*P*= 0.000) and mean of hemoglobin concentration (*p*=0.038) among T-ALL cases, while other parameters as RBCs , PLTs and Blast % were statistically insignificant with ALL subtypes (p=0.287.0.168 and 0.529) respectively, this finding agree with study done in Brazil by De sousa *et al*. (2015), they found that patients with T- ALL presented significantly higher Hb levels and total white blood cells count at diagnosis than patients with B-ALL, also agree with another study done in Iraq by Jaafar and Kadhom in (2018) they reported that WBCs count was significantly higher in T-cell ALL when compared to B-cell ALL (P < 0.01).

In this study, the most common B cells ALL subtypes is early pre B cell ,followed by common B cell, then pre B cell, pro B cell, , while Burkitt leukemia was less , this result slightly similar to study done in Sudan by Abdalla *et al*. (2016) and agree with another study in Saudi Arabia by Abdelaziz and Alqatary (2013) they reported that the most common were pre-B ALL, then pre-B ALL, followed   by pro-B ALL.

In this study for T cell types, cortical T cells leukemia is the commonest one followed by pre T cell while mature T cells was less, this result is similar to study done for T cell ALL frequency in Sudan by Ebrahim *et al*. (2017) they found that cortical T-ALL was the most predominant subtype among Sudanese patients followed by pre T-ALL and other subtypes were minority among cases. This result similar to previous study done by Proleskovskaia *et al*. (2002) they demonstrated a higher incidence of the cortical subtype T-ALL followed mature T-ALL subtype.

In the current study genotypes of CYP2E (C1053T) showed high heterozygous in both case and control group which was higher in patients when compared with controls, the odds ratio was 1.805; however, this difference was not statistically significant (95% CI: 0.5117-6.369, p=0.3524)

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and for *5B allele also was not statistically significantly associated with risk of OR=1.776, (95% CI: 0.7289-17.32, *P*= 0.3592) , also for children insignificant association with increased risk 1.7 fold and in dult increased up to 2 fold, this results is similar to Turkish study, conducted by Ulusoy *et al*. (2007) they found increased risk up to 1.7 even the difference was not statistically insignificant, same result found in study done in Ribeirao Preto Brazil by Canalle *et al*. (2004) they found no significant association between the CYP2E1 *5 B alleles and ALL in children. Also this current study disagree with study done by Aydin *et al*. (2006) in Turkia they reported that the difference for *5 genotype frequency was significant for both pediatric and adult ALL patients (OR 3.4, 95% CI 1.3–9.1; *P*= 0.01 and OR 4.4, 95% CI1.3–14.8; *P*= 0.01, respectively as well as another study performed in a French Canadian population by Krajinovic *et al*. (2002) showed that carriers of the CYP2E1*5 variant were more frequent among cases than in controls suggesting CYP2E1*5B might be associated with an increased risk of ALL (OR 2.8, 95% CI 1.2–6.7). Also Infante-Rivard *et al*. (2002) observed higher odds ratio 4.9 fold increased risk of ALL. Tumer *et al*.(2010),investigated the possible association others polymorphisms in combination wit h CYP2E1*5B, they found increased risk of childhood acute lymphoblastic leukemia up to 3.7 fold and Ulusoy *et al*. (2007) also investigated possible association of CYP2E1*5B and other variants *6B and *7B in Turkish population reported increased risk of childhood ALL 2.9 fold.

 Regarding NQO1 C609T, results indicate that the NQO1 C609T polymorphism is associated with the elevated risk of childhood ALL , the heterozygous was higher in patients when compared with controls (17.6% and 5.9%, respectively), the odds ratio was 3.513; however, this difference was statistically significant (95% CI: 1.327-9.301, *P*=0.0081) and alleles frequency was also significantly associated with risk of ALL (*P*= 0.0408, OR=1.840, 95% CI: 1.019-3.321), this findings is in agreement with study done in Sudan by Abdalla and Kobar (2015), they found 2.9 fold increased risk of ALL for those carrying NQO1 609CT heterozygous genotype (OR 2.878, *P*= 0.040), also similar with Infante-Rivard *et al*. (2007) in Canada suggested that the NQO1 C609T variant was associated with the risk of developing childhood ALL (OR 1.39, 95% CI: 1.07, 1.79). Another French-Canadian study by Krajinovic *et al*. (2002) showed that children carrying at least 1 mutant allele of the NQO1 C609T polymorphism had an increased risk of developing ALL. The association between the NQO1 C609T polymorphism and ALL risk was analyzed also by Wang and his follows in (2014) they found that this polymorphism increase ALL risk, also they compare the alleles frequency and concluded that there was increased ALL

risk too, also Ouerhani *et al* (2012) reported that genetic polymorphisms of NQO1, CYP1A1 and TPMT and susceptibility to ALL in a Tunisian population increased the risk 1.26 fold.

A study done in American population by Smith *et al.*(2002) was also indicate that the NQO1 C609T polymorphism is associated with the elevated risk of childhood ALL (OR = 2.47, 95% CI: 1.08, 5.68), as well as in Italian study done by Lanciotti *etal* (2005) (OR = 5.55, 95% CI: 1.81, 16.98). Meta-analysis included 28 relevant studies in Asian population done by He *et al*. (2017), stated that NQO1 C609T polymorphism was associated with the risk of ALL (OR =1.18, 95% CI 1.00–1.39, *P*=0.05).

Sirma, 2004 in Turkey did not support the role of the NQO1 C609T polymorphism in the increased risk of pediatric acute leukemia. Moreover, in Brazilian children, the NQO1 and myeloperoxidase polymorphisms were shown to have a protective function against leukaemogenesis as reported by Silveira *et al*. (2010). In addition to meta- analysis study done by Jeffrey *et al*. (2008) showed that the NQO1 C609T variant appeared to have no strong association with childhood ALL or AML but may be associated with mixed lineage leukemia– positive childhood leukemia ALL.

NQO1 C609T genotype heterozygosity (p=0.1276, OR 2.0226; 95% CI 0.7806-6.350) and homozygosity (*P*= 0.8709, OR 1.113; 95% CI 0.3051- 4.161) among children in this study showed insignificant association with increased risk 2.02, 1.13 respectively and for alleles in children (*P*=0.295, OR 1.445; 95%CI 0.735-2.886), risk increased 1.4 fold. This outcome similar to study done by Zaker *et al*. (2012) in Iran suggests that the mutant allele of the NQO1 C609T polymorphism is not associated with increased risk of pediatric ALL, (CT 1.01,TT 0.95 and alleles 0.93). The same results observed by Abdelaziz and Alqatary in (2013) in Saudi Arabia, they deduced NQO1 C609T polymorphism is not associated with increased risk of pediatric ALL, this in contrast with Krajinovic *et al*. (2002) they found that children carrying at least one NQO1 mutant allele were at increased risk of developing ALL (OR 1.7, 95% CI, 1.2–2.4), whereas individuals homozygous for the wild-type allele seem to be protected against ALL, another study in France Clavel *et al*. (2005) revealed that NQO1609 increased the risk 2.01fold, also similar results in Indian study done by Dunna *et al*. (2011) suggested that the TT genotype might be considered as a risk genotype for development of acute leukemia, also in Filipin, Rimando and his colleagues, (2008) they clarified that the genetic contribution of GSTM1 null and NQO1C/C genotypes among children increased ALL leukaemogenesis 11.9-fold.

Lack of agreement between these studies might be due to differences in the duration of the exposure to the NQO1 substrates variation and small sample sizes in some studies, as well as the demographic stratification that exists in these kinds of studies.

For NQO1 C465T in this study the hererozygosity of genotypes was higher in patients than controls (22.5% and 3.9%, respectively), this difference was statistically highly significant (P=0.0001) with odds ratio was 7.667, (95% CI: 2.539-23.15), also homozygote mutant genotypes was higher in case (6.9%) than control (2%), this was also significantly associated (P=0.0401), with odd ratio 4.667 (95%CI 0.9410-23.14). The allele's frequency also showed statistical significant associated with risk of ALL development (P= 0.0017, OR=2.7, 95% CI: 1.427-5.107). In the current study the risk increased in children with genotype heterozygosity 6.26 fold while for alleles 4.5 fold this is similar to study done by Zaker *et al*. (2012), reported that NQO1 C465T variant showed a significant association with increased risk of ALL in children for heterozygosity high odd ratio 6.40 while for alleles 6.09 fold and also to study done by Abdelaziz and Alqatary in 2013 in Saudi Arabia showed a significant association with increased risk of ALL in children. there were relevant studies assessed the association between the NQO1 C465T polymorphism and the ALL risk, two of them did not find a significant association Eguch *et al*. 2005 and Krajinovic *et al*. (2002), another one found an increased risk of ALL for the NQO1 C465T polymorphism(aker *et al*. (2012).

In the present study comparison of haematological parameters between ALL patients with wild type and those with mutant types (NQO1 C609T) showed statistically insignificant association, which disagree with previous study done in Sudan by Abdulla *et al*. (2015) they observed a statistically significant reduction in the mean Hb level and RBCs count in patients with mutant genotypes than in wild type patients (P= 0.000 and 0.003), WBCs count was significantly higher in patients with mutant type when compared to those with the wild type (P= 0.000).

5.2. Conclusions:

On the bases of outcomes, we conclude:

ALL is more common in children than adults also males more affected than female (2.1:1).

B –cell ALL type is predominant than T-cell type, and most common B- ALL subtype was early pre B cell ALL ,while for T-cell subtype was cortical T cell.

CYP2E1*5B (C1053T), is not a risk factor for developing ALL.

NQO1 (C609T) as significant risk of ALL.

NQO1 (C465T) polymorphism is a risk factor for development of ALL.

As a result, genetic susceptibility clearly contributes to ALL risk.

5.3. Recommendations

On the bases of this study, recommend:

Effect of environmental agents such as benzene, pollution, paints risk factors for ALL need more attention and investigations.

An additional research should be done, including information on the geographical area and ethnic groups demanded to clarify the relationship between CYP2E1*5B and NQO1 polymorphisms and these factor in Sudan.

Further studies with large sample size are still needed to investigate the impact of these mutations on Sudanese population, which in turn would help to explain these finding more accurately.

Other variants of CYP2E1 have not been investigated for this disease yet and there is no study in Sudan about CYP2E1*5B. So the role of CYP2E1*5B and for NQO1 (C609T, C465T) polymorphisms as risk ALL development still needs to be clarify.

Performing further studies to investigate the relation between these polymorphisms and other genes mutations related to ALL.

Next Generation Sequencing (NGS) need to perform to detect any SNPs in whole genome in Sudanese population.

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APPENDICES

Appendix I

Custom Digest

Linear Sequence: *unnamed sequence*

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Sequence digested with: RsaI

Cleavage code
X | blunt end cut
| 5′ extension
| 3′ extension
| cuts 1 strand

Enzyme name code
Available from NEB
Has other supplier
Not commercially available
*: cleavage affected by CpG meth.
#: cleavage affected by other meth.
(enz.name): ambiguous site

```
                                          RsaI
5′... AAGATTCATTGTTAATATAAAAGTACAAAATTGCAACCTATGAATTAAGAA ... 3′
      |1    |    |10   |    |20   |    |30   |    |40   |    |50
3′... TTCTAAGTAACAATTATATTTTCATGTTTTAACGTTGGATACTTAATTCTT ... 5′
                                          RsaI
```

Sequencing digesting and recognition site with RsaI restriction enzyme

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Custom Digest

Linear Sequence: *unnamed sequence*

[Back to main display]

Help   Comments

Sequence digested with: HinfI

Cleavage code
blunt end cut
5' extension
3' extension
cuts 1 strand

Enzyme name code
Available from NEB
Has other supplier
Not commercially available
*: cleavage affected by CpG meth.
#: cleavage affected by other meth.
(enz.name): ambiguous site

HinfI

5'... TTTCTGTGGCTTCCAAGTCTTAGAATCTCAACTGACATATAGCATTGGGCA ... 3'
⊢1 | ⊢10 | ⊢20 | ⊢30 | ⊢40 | ⊢50
3'... AAAGACACCGAAGGTTCAGAATCTTAGAGTTGACTGTATATCGTAACCCGT ... 5'

HinfI

Main options
New custom digest
View gel
Print

Display
Highlight bases
All enzymes

List
Enzymes & sites
Fragments

Sequencing digesting and recognition site with HinfI restriction enzyme

Linear Sequence: *unnamed sequence*

Sequence digested with: HpaII

Cleavage code		Enzyme name code
⚡	blunt end cut	Available from NEB
◥	5′ extension	Has other supplier
◢	3′ extension	Not commercially available
▼	cuts 1 strand	*: cleavage affected by CpG meth.
		#: cleavage affected by other meth.
		(enz.name): ambiguous site

```
                                                *HpaII
                                               ┌
5′... TGCCATGTATGACAAAGGACCCTTCCGGGTAGGTGGATGGTTCTGAATGCT ... 3′
      ├1       |      ├10      |     ├20      |     ├30      |     ├40      |     ├50
3′... ACGGTACATACTGTTTCCTGGGAAGGCCCATCCACCTACCAAGACTTACGA ... 5′
                                               └
                                                *HpaII
```

Main options	Display	List
New custom digest	Highlight bases	Enzymes & sites
View gel	All enzymes	Fragments
Print		

Sequencing digesting and recognition site with HpaII restriction enzyme

Homo sapiens cytochrome P450 family 2 subfamily E member 1 (CYP2E1), RefSeqGene on chromosome 10
Sequence ID: NG_008383.1 Length: 18754 Number of Matches: 6

Range 1: 2791 to 3373 GenBank Graphics   ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1038 bits(1150)	0.0	580/583(99%)	0/583(0%)	Plus/Plus

```
Query  1     CACCCTGTCGTCCTGATTATTTCACCTTGTCACGGGCAGAGGCACAGGCACCCTGTCGTC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2791  CACCCTGTCGTCCTGATTATTTCACCTTGTCACGGGCAGAGGCACAGGCACCCTGTCGTC  2850

Query  61    CTGATTATTTCACCTTGTCACGGACAGAGGCACAGGCACCCTGTCGTCCTGATTATTTCA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2851  CTGATTATTTCACCTTGTCACGGACAGAGGCACAGGCACCCTGTCGTCCTGATTATTTCA  2910

Query  121   CCTTGTCACAGGCACAGGCACCCTGTCGTCGTGATTATTTCACCTTGTCACAGGCACAGG  180
             |||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
Sbjct  2911  CCTTGTCACAGGCACAGGCACCCTGTCGTCCTGATTATTTCACCTTGTCACAGGCACAGG  2970

Query  181   CACTCTGTCGTCCTGATTATTTCACCTTGTCACGGGCATAGGCACAGGCACTCTGTCATC  240
             |||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||
Sbjct  2971  CACTCTGTCGTCCTGATTATTTCACCTTGTCACGGGCAGAGGCACAGGCACTCTGTCATC  3030

Query  241   CTGATTATTTCACCTTGTCCTAGAGTGTCCTGCCAATGGGACAGATGCAAAACAAATAAA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3031  CTGATTATTTCACCTTGTCCTAGAGTGTCCTGCCAATGGGACAGATGCAAAACAAATAAA  3090

Query  301   AGCCCCGGCTTCTGAAAAGAAGCACACAGAAATGTCATTATTTTCAAACGAGGTGTTCCC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3091  AGCCCCGGCTTCTGAAAAGAAGCACACAGAAATGTCATTATTTTCAAACGAGGTGTTCCC  3150

Query  361   GTATATAAAATTTGATGTTGGTTGGGCATCTAACAGTATTATGGCCAGAGGACTCAGACC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3151  GTATATAAAATTTGATGTTGGTTGGGCATCTAACAGTATTATGGCCAGAGGACTCAGACC  3210

Query  421   ACAGCTGCATCCCTGTGAGGCACACACTCTCCAGGGCACGCGGGTCCCGCCTGGGATGTG  480
             |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
Sbjct  3211  ACAGCTGCATCCCTGTGAGGCACAGACTCTCCAGGGCACGCGGGTCCCGCCTGGGATGTG  3270

Query  481   CACACTCAGGTGAGCTGCACAGACAAGGTGTCCTCAGCCCAGGGGAGCCAGAGGCCTGCT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3271  CACACTCAGGTGAGCTGCACAGACAAGGTGTCCTCAGCCCAGGGGAGCCAGAGGCCTGCT  3330

Query  541   CTGCCTCTCCACCCTGATGCTTCCTGTTCTCACCCCACCAAAG  583
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct  3331  CTGCCTCTCCACCCTGATGCTTCCTGTTCTCACCCCACCAAAG  3373
```

BLAST pair wise sequence alignment. Showed similarity99%

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Homo sapiens NAD(P)H quinone dehydrogenase 1 (NQO1), RefSeqGene on chromosome 16
Sequence ID: NG_011504.1 Length: 24230 Number of Matches: 1

Range 1: 16388 to 16765 GenBank Graphics   ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
699 bits(378)	0.0	378/378(100%)	0/378(0%)	Plus/Plus

```
Query  1      ATAATGATCTCTTTCCTTAAAGTGCTAACTCCCCAGGAGGAATGGGAAAGGTGTGAAGAG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16388  ATAATGATCTCTTTCCTTAAAGTGCTAACTCCCCAGGAGGAATGGGAAAGGTGTGAAGAG  16447

Query  61     GGGCTTCCCACACAGTGCCATCATGGGGAGCGGCTCAGCACTCCGAGCCACCTTCTGGGC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16448  GGGCTTCCCACACAGTGCCATCATGGGGAGCGGCTCAGCACTCCGAGCCACCTTCTGGGC  16507

Query  121    TTGGGGAGCCCCTGGTCTTACCTCAATGATGTCTTCTGTCCCACAGTTCCCCCTGCAGTG  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16508  TTGGGGAGCCCCTGGTCTTACCTCAATGATGTCTTCTGTCCCACAGTTCCCCCTGCAGTG  16567

Query  181    GTTTGGAGTCCCTGCCATTCTGAAAGGCTGGTTTGAGCGAGTGTTCATAGGAGAGTTTGC  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16568  GTTTGGAGTCCCTGCCATTCTGAAAGGCTGGTTTGAGCGAGTGTTCATAGGAGAGTTTGC  16627

Query  241    TTACACTTACGCTGCCATGTATGACAAAGGACCCTTCCGGGTAGGTGGATGGTTCTGAAT  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16628  TTACACTTACGCTGCCATGTATGACAAAGGACCCTTCCGGGTAGGTGGATGGTTCTGAAT  16687

Query  301    GCTCTGACAGCCAGCTTCTGGGTGGTCTGTCCTGATGCAGGGGtgtttgtttgtttgttt  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16688  GCTCTGACAGCCAGCTTCTGGGTGGTCTGTCCTGATGCAGGGGTGTTTGTTTGTTTGTTT  16747

Query  361    gtttgAGATGGAGCTTCC     378
              ||||||||||||||||||
Sbjct  16748  GTTTGAGATGGAGCTTCC     16765
```

BLAST pair wise sequence alignment. Showed similarity100%

Appendix II

Molecular reagents:
1- 10 X TBE buffer
Formula in grams per liter
Tris base………………………………… 108 gm
Boric acid……………………………….…..55gm
EDTA………………………….…................40 ml of 0.5M
Deionized water……………..………….…1 liter
Preparation
Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M
EDTA then dissolved into 1 liter deionized water pH 8.0.
2- 1X TBE buffer
Formula in ml per liter
10 X TBE……………………………………….10 ml
Deionized water………………………………….90 ml
Ten ml of 10 X TBE buffer was added to 90 ml deionizer water and heated until dissolved
Completely.
3- Ethidium bromide solution
Formula in grams per 1ml
Ethidium bromide……………………………10 mg
Deionized water………………………………I ml
Preparation
Twenty milligrams of ethidium bromide powder were dissolved into 1000 μl deionized water,
and kept into brown bottle.

4- Agarose gel
Preparation
- Amount of 2 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer
- Cooled to 55°C in water bath. 1.5 μl of Ethidium bromides stock (10 mg/ml) per 100 ml gel,
  solution for a final concentration of 0.5 ug/ml were added.
- Mixed well and poured on to the casting tray that has been taped up appropriately and was
  equipped with suitable comb to form well in place. Any bubbles were removed and the gel was
  allowed to set at room temperature.
- After solidification, the comb was gently removed and the spacer from the opened sides was
  removed.

Appendix III

Questionnaire

I D: …………………………………………………….

Age: ………………………………………………..

 Gender: ………………………………………….

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

Analysis:

1-Hematological Parameters:

HB: ……………………..   g/dl.

TWBCs: ………………….. $X10^9$/L.

RBCs: ………………………$X10^{12}$/L

Platelets: ………………….. $X10^9$/L.

 Blast: …………………….. %.

2-Flowcytometery:

B-ALL and Subtypes …………………………………………….

T-ALL and Subtypes……………………………………………..

3- Molecular Result:

CYP2E1(C 1053T) genotype: ………………………………………..

NQO1 (C609T) genotype…………………………………………

NQO1 (C 465T) genotype……………………………………………

بحث بعنوان علاقة تعدد اشكال جينات سيتوكروم ب 450 2أي 1 وقينون أوكسيدو ردكتييز1 مع سرطان الدم الليمفاوي الحاد عند المرضي السودانيين.استمارة الموافقة على المشاركة فى بحث عن التعرف على الجينات التى قد يكون لها دور في الإصابة بسرطان الدم الليمفاوي .

أنا دارس من كلية المختبرات الطبية بجامعة السودان بحثى يهدفالى التعرف على الجينات التى قد يكون لها دور في الإصابة بسرطان الدم الليمفاوي ولتعرف على هذه الجينات ونسبة انتشارها في وضع الاستراتيجيات المستقبليه لمواجهة لهذه المشكله. وحتى نستطيع ان نقوم بهذا الدور نود أن نطلب منك ان تشارك/ يشارك طفلك فى هذا البحث. واذا وافقت سيقوم الكادر الطبى المدرب بأخذ عينة دم لاجراء الفحوصات الللازمه لك/ لطفلك . واخذ هذة العينات لن يعرضك او يعرض طفلك للخطر وسنستعمل ادواة معقمه لاخذ تلك العينات. ونعلمك بان عدم موافقتك على المشاركة فى البحث اوعدم موافقتك لن يحرمك من حقك فى العلاج والرعاية الطبية المطلوبة.ونفيدك بانك لن تتلقى اى عائد مادى لمشاركتك فى البحث وستكون مشاركتك طوعية. نود اخطارك بان المعلومات الخاصة بك /بطفلك ستكون سرية ولن يطلع عليها الاخرون ماعدا القائمون علي هذا البحث .نود موافقتك / طفلك على المشاركة فى هذا البحث ويمكنك أن تسال وتستفسر عن البحث ومشاركتك فيه قبل موافقتك النهائية. ونرجو اثبات موافقتك بامضاء الاستمارة المرفقة بعد قرائتها بعنايه.

أنا...اؤكد فهمى لمحتوى الاستمارة وافيد بموافقتى على المشاركة/ مشاركة طفلى.......... على المشاركة فى البحث واعلم أنه من حقى رفض المشاركة و الانسحاب من المشاركة فى اى وقت ولن يؤثر ذلك فى حقوقى .
واوافق على اخذ العينه المطلوبة للبحث.
الامضاء...
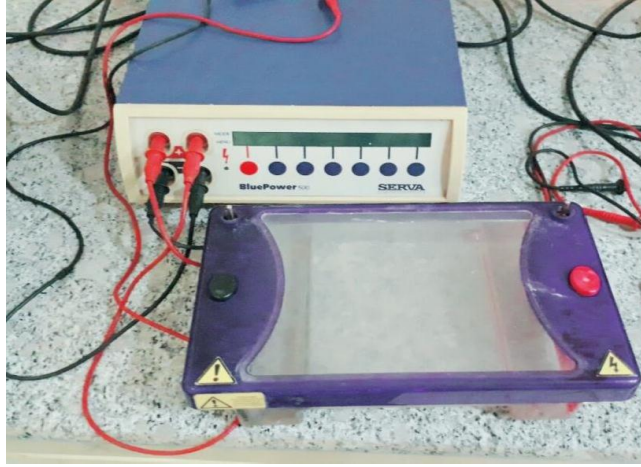الشاهد:...
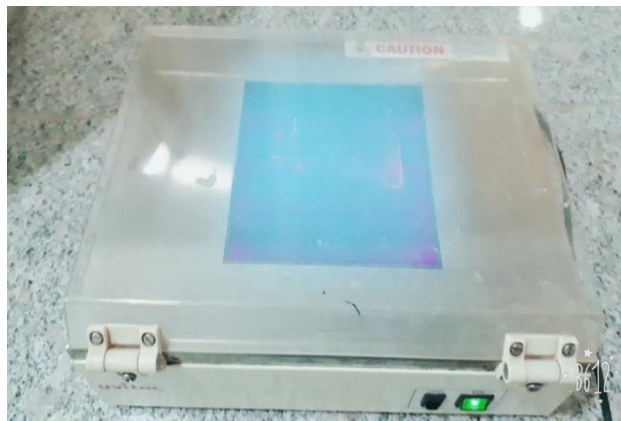
APPENDIX V

Sigma 1-14 Germany Microcentrifuge Device

CLASSIC K960 China Thermocycle Device

Gel Electrophoresis and Power Supply Device

UV Light Transilluminator Device

Sysmex KX 21N Hematology Analyzer

Beckman Coulter EPICS XL MCL Flowcytometer