Chapter one

1. Introduction and literature review

1.1 Introduction of leukemia

The Leukemias are a group of blood disorders that characterized by the accumulation of malignant white cells in the bone marrow and blood. These abnormal cells cause symptoms because of: (i) bone marrow failure (ie: anemia, neutropenia, thrombocytopenia) and (ii) infiltration of organs (e.g.: liver, spleen, lymph nodes).

The main classification of Leukemia is acute and chronic leukemias, which are further subdivided in to lymphoid or myeloid. (Hoffbrand, *et al*. 2006).

1.2. Literature review

1.2.1. Acute Myeloid leukemia (AML):

Acute myeloid leukemia (AML), also known as acute myelocytic leukemia, acute myelogenous leukemia, acute granulocytic leukemia, and acute non lymphocytic leukemia."Acute" means that the leukemia can progress quickly, and if not treated, would probably be fatal in a few months. AML is a cancer of the myeloid line of blood cells, characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells AML starts in the bone marrow (the soft inner part of the bones, where new blood cells are made), in most cases it quickly moves into the blood. It can sometimes spread to other parts of the body including the lymph nodes, liver, spleen, central nervous system (brain and spinal cord), and testicles (in males) (Jemal, *et al*. 2002).

1.2.2. Risk factors for AML:

A risk factor is something that affects the chance of getting a disease, it is often very hard to know how much that risk factor may have contributed to the cancer. There are a few known risk factors for AML. (Le Beau, *et al.* 1986)

1.2.2.1. Smoking:

The only proven lifestyle-related risk factor for AML is smoking. Cancer causing substances in tobacco smoke are absorbed by the lungs and spread through the bloodstream to many parts of the body (Le Beau, *et al.* 1986)

1.2.2.2. Certain chemical exposures

The risk of AML can be increased by Long-term exposure to certain chemicals. Benzene is a risk factor for AML. Benzene is a solvent used in the rubber industry, oil refineries, chemical plants, shoe manufacturing, and gasoline related industries, and is also present in cigarette smoke, and some glues, cleaning products, detergents, art supplies, and paint strippers (Le Beau, *et al.* 1986)

Some studies have linked heavy workplace formaldehyde exposure to AML risk, but this link was not seen in other studies. Patients with other cancers who are treated with certain chemotherapy drugs are more likely to develop AML. Some of the drugs linked with these secondary (treatment related) leukemias include mechlorethamine, procarbazine, chlorambucil, melphalan, etoposide, teniposide and cyclophosphamide. Combining these drugs with radiation therapy further increases the risk. Most secondary cases of AML occur within 10 years after treatment of Hodgkin disease, non-Hodgkin lymphoma, or childhood acute lymphocytic leukemia (ALL). Secondary leukemias also sometimes

occur after treatment of breast, ovarian, or other cancers (Thirman, *et al.* 1993).

1.2.2.3. Radiation exposure

High-dose radiation exposure (such as being a survivor of an atomic bomb blast or nuclear reactor accident) increases the risk of developing AML. Japanese atomic bomb survivors had a greatly increased risk of developing acute leukemia, usually within 6 to 8 years after exposure. The possible risks of leukemia from exposure to lower levels of radiation, such as from radiation therapy, x-rays, or CT scans, are not well-defined. If a fetus is exposed to radiation within the first months of development, it may carry an increased risk of leukemia, but the extent of the risk is not clear. If there is an increased risk it is likely to be small, but to be safe, most doctors try to limit a person's exposure to radiation as much as possible (Yoshinaga, *et al.* 2004).

1.2.2.4. Certain blood disorders:

Patients with certain blood disorders seem to be at increased risk for getting AML. These include chronic myeloproliferative disorders such as polycythemia vera, essential thrombocytopenia, and idiopathic myelofibrosis. Chronic myelogenous leukemia (CML) is another type of myeloproliferative disorder, and some patients with CML later develop a form of AML. The risk of developing AML is increased further if treatment for these disorders includes some types of chemotherapy or radiation. Some patients who have a myelodysplastic syndrome (a pre-leukemic condition) may develop AML. These conditions cause defects in blood cell formation, and over a period of years may evolve into leukemia. Patients who have a myelodysplastic syndrome and develop AML typically have a poor prognosis (Bjergaard, *et al*. 2002).

1.2.2.5. Congenital syndromes (present at birth):

AML does not appear to be an inherited disease. It is rare for it to run in families, there are some congenital syndromes with genetic changes that seem to raise the risk of AML these include: Down syndrome, Fanconi anemia, Bloom syndrome, Ataxia-telangiectasia and Blackfan-Diamond syndrome (Jackowska, *et al*. 2006).

1.2.2.6. Having an identical twin with AML:

This risk is largely confined to the first year of life. As mentioned above, most cases of AML are not thought to have a strong genetic link. Increased risk among identical twins may be due to leukemia cells being passed from one fetus to the other while still in the womb (Greaves, *et al*. 2002).

1.2.2.7. Gender:

AML is more common in males than in females, but the reasons for this are not clear . (Greaves, *et al*. 2002).

1.2.2.8. Uncertain, unproven or controversial risk factors

Other factors that have been studied for a possible link to AML include: Exposure to electromagnetic fields (such as living near power lines), Workplace exposure to diesel, gasoline, and certain other chemicals and solvents and Exposure to herbicides or pesticides. none of these factors has been linked conclusively to AML. Research in these areas is ongoing.

1.2.3. Statistics of AML:

The American Cancer Society's estimates for leukemia in the United States for 2012 that About 47,150 new cases of leukemia (all kinds) and 23,540 deaths from leukemia (all kinds), about 13,780 new cases of acute myeloid leukemia (AML). Most will be in adults, about 10,200 deaths from AML. Almost all will be in adults. Acute myeloid leukemia is generally a disease of older people and is uncommon before the age of

40. The average age of a patient with AML is about 67 years. AML is slightly more common among men than among women. The lifetime risk of getting AML for the average man is about 1 in 232; for the average woman the risk is about 1 in 278 (Greaves, *et al*. 2002).

1.2.4. Classification of AML

The two most commonly used classification schemata for AML, are the older French-American-British (FAB) system and the newer World Health Organization (WHO) system.

1.2.4.1. French-American-British classification:

The French-American-British (FAB) classification system divides AML into 8 subtypes, M0 through to M7, based on the type of cell from which the leukemia developed and its degree of maturity. This is done by examining the appearance of the malignant cells under light microscopy and/or by using cytogenetics to characterize any underlying chromosoml abnormalities. The subtypes have varying prognoses and responses to therapy. Although the WHO classification may be more useful, the FAB system is still widely used (Bennett, *et al.* 1976).

Table (1-1): FAB classification of AML (Bennett, *et al.* 1976).

Type	Name	Cytogenetics	Percentage of adult AML patients
M0	**minimally differentiated acute myeloblastic leukemia**		**5%**
M1	**acute myeloblastic leukemia, without maturation**		**15%**
M2	**acute myeloblastic leukemia, with granulocytic maturation**	**t(8;21)(q22;q22), t(6;9)**	**25%**
M3	**promyelocytic, or acute promyelocytic leukemia (APL)**	**t(15;17)**	**10%**
M4	**acute myelomonocytic leukemia**	**inv(16)(p13q22), del(16q)**	**20%**
M4eo	**myelomonocytic together with bone marrow eosinophilia**	**inv(16), t(16;16)**	**5%**
M5	**acute monoblastic leukemia (M5a) or acute monocytic leukemia (M5b)**	**del (11q), t(9;11), t(11;19)**	**10%**
M6	**acute erythroid leukemias, including erythroleukemia (M6a) and very rare pure erythroid leukemia (M6b)**		**5%**
M7	**acute megakaryoblastic leukemia**	**t(1;22)**	**5%**

1.2.4.2. World Health Organization classification:

The FAB classification system is useful and is still commonly used to group AML into subtypes. But it doesn't take into account many of the factors that are known to impact prognosis (outlook). The World Health

Organization (WHO) has proposed a newer system that includes some of these factors to try to help better classify cases of AML based on a patient's outlook (Vardiman, *et al*. 2002). Not all doctors use this new system. The WHO classification system divides AML into several broad groups: (Harris, *et al*. 1999).

1.2.4.2.1 AML with certain genetic abnormalities

A. AML with a translocation between chromosomes 8 and 21
B. AML with a translocation or inversion in chromosome 16
C. AML with changes in chromosome 11
D. APL (M3), which usually has translocation between chromosomes 15 and 17 . (Harris, *et al*. 1999).

1.2.4.2.2 AML with multilineage dysplasia (more than one abnormal myeloid cell type is involved)

1) **AML related to previous chemotherapy or radiation**
2) **AML not otherwise specified** (includes cases of AML that don't fall into one of the above groups; similar to the FAB classification)

A. Undifferentiated AML…… ..(M0)
B. AML with minimal maturation.... (M1)
C. AML with maturation……....(M2)
D. Acute myelomonocytic leukemia...(M4)
E. Acute monocytic leukemia……... (M5)
F. Acute erythroid leukemia ……..(M6)
G. Acute megakaryoblastic leukemia (M7)
H. Acute basophilic leukemia
I. Acute panmyelosis with fibrosis
J. Myeloid sarcoma (also known as granulocytic sarcoma or chloroma)

1.2.4.2.3Undifferentiated or biphenotypic acute leukemias

(Leukemias that have both lymphocytic and myeloid features). Sometimes called ALL with myeloid markers, AML with lymphoid markers, or mixed lineage leukemias. (Harris, *et al*. 1999).

1.2.4.3. Uncommon phenotypes of acute myeloid leukemia:

The morphologic subtypes of AML include many exceedingly rare types not included in the FAB system. All of them except acute myeloid dendritic cell leukemia acute eosinophilic leukemia and are included in the WHO classification. The following list shows these subtypes:

a. Acute basophilic leukemia
b. Acute eosinophilic leukemia
c. Mast cell leukemia
d. Acute myeloid dendritic cell leukemia
e. Acute panmyelosis with myelofibrosis
f. Myeloid sarcoma. (Harris, *et al*. 1999).

1.2.5. Sign and symptoms of Acute Myeloid Leukaemia:

AML can cause many different signs and symptoms. Some occur more commonly with certain subtypes.

1.2.5.1. Generalized symptoms

Patients with AML often have several non-specific (generalized) symptoms. These can include weight loss, fatigue, fever, night sweats, and loss of appetite. These are not specific to AML, and more often caused by something other than leukemia . (Vardiman, *et al.* 2002).

1.2.5.2. Symptoms caused by low blood cells:

Most signs and symptoms of AML result from a shortage of normal blood cells, which happens when the leukemia cells crowd out the normal blood-making cells in the bone marrow. As a result, people do not have enough normal red blood cells, white blood cells, and blood

platelets. These shortages show up on blood tests, but they can also cause symptoms (Thirman, *et al.* 1993).

a. **Anemia** is a shortage of red blood cells. It can cause a person to feel tired, weak, cold, dizzy, or lightheaded, and can cause headaches and shortness of breath. (Vardiman, *et al.* 2002).
b. **Leukopenia** is a shortage of normal white blood cells, increases the risk of infections. A common term you may hear is neutropenia, which refers specifically to low levels of neutrophils (a type of granulocyte). Patients with AML may have high white blood cell counts due to excess numbers of leukemia cells, but these cells do not protect against infection the way normal white blood cells do. Fevers and recurring infections are some of the most common symptoms of AML. (Thirman, *et al.* 1993).
c. **Thrombocytopenia** is a shortage of blood platelets can lead to excess bruising, bleeding, frequent or severe nosebleeds and bleeding gums. (Thirman, *et al.* 1993).

1.2.5.3. Symptoms caused by high numbers of leukemia cells

The cancer cells in AML (blasts) are bigger than normal white blood cells and have more trouble going through tiny blood vessels. If the blast count gets very high, these cells clog up blood vessels and make it hard for normal red blood cells (and oxygen) to get to tissues. This is called leukostasis. Some of the symptoms are like what is seen with a stroke, and include headache, weakness in one side of the body, slurred speech, confusion, and sleepiness. When blood vessels in the lung are affected, patients have problems with shortness of breath. Blood vessels in the eye can be affected as well, leading to blurry vision or even loss of vision. Leukostasis is rare, but it is a medical emergency that needs to be treated right away . (Harris, *et al*. 1999).

1.2.5.4. Other Signs and symptoms

a. Bone or joint pain

Some patients have bone pain or joint pain caused by the buildup of leukemia cells in these areas. (Harris, *et al*. 1999).

b. Swelling in the abdomen:

Leukemia cells may collect in the liver and spleen, causing them to enlarge. This may be noticed as a fullness or swelling of the belly. The lower ribs usually cover these organs, but when they are enlarged the doctor can feel them. (Harris, *et al*. 1999).

c. Spread to the skin:

If leukemia cells spread to the skin, they can cause lumps or spots that may look like common rashes. A tumor-like collection of AML cells under the skin or other parts of the body is called a chloroma or granulocytic sarcoma. (Harris, *et al*. 1999).

d. Spread to the gums:

Certain types of AML may spread to the gums, causing swelling, pain, and bleeding.

e. Spread to other organs:

Sometimes, leukemia cells may spread to other organs. Spread to central nervous system (brain and spinal cord) can cause headaches, weakness, seizures, vomiting, trouble with balance, facial numbness or blurred vision. On rare occasions AML may spread to the eyes, testicles, kidneys, or other organs (Martin, *et al*. 2004).

f. Enlarged lymph nodes:

In rare cases, AML may spread to lymph nodes. Affected nodes in the neck, groin, under arm areas, or above the collarbone may be felt as lumps under the skin. (Martin, *et al*. 2004).

1.2.6. Diagnosis of Acute Myeloid Leukaemia:

The first clue to a diagnosis of AML is typically an abnormal result on a complete blood count. While an excess of abnormal white blood cells (leukocytosis) is a common finding, and leukemic blasts are sometimes seen, AML can also present with isolated decreases in platelets, red blood cells, or even with a low white blood cell count (leukopenia). While a presumptive diagnosis of AML can be made via examination of the peripheral blood smear when there are circulating leukemic blasts, a definitive diagnosis usually requires an adequate bone marrow aspiration and biopsy (Abeloff, Martin, *et al*. 2004).

Marrow or blood is examined via light microscopy, as well as flow cytometry, to diagnose the presence of leukemia, to differentiate AML from other types of leukemia (e.g. acute lymphoblastic leukemia), and to classify the subtype of disease. A sample of marrow or blood is typically tested for chromosomal.abnormalities by routine cytogenetics or fluoresc ent in situ hybridization. Genetic studies may also be performed to look for specific mutations in genes such as FLT3, nucleophosmin, and KIT, which may influence the outcome of the disease (Baldus CD et al, 2007). Cytochemical stains on blood and bone marrow smears are helpful in the distinction of AML from ALL, and in subclassification of AML. The combination of a myeloperoxidase or Sudan black stain and a nonspecific esterase stain will provide the desired information in most cases. The myeloperoxidase or Sudan black reactions are most useful in establishing the identity of AML and distinguishing it from ALL. The nonspecific esterase stain is used to identify a monocytic component in AMLs and to distinguish poorly differentiated monoblastic leukemia from ALL (Vardiman, *et al.* 2002). The diagnosis and classification of AML can be challenging, and should be performed by a qualified hemato

pathologist or hematologist. In straightforward cases, the presence of certain morphologic features (such as Auer rods) or specific flow cytometry results can distinguish AML from other leukemias; however, in the absence of such features, diagnosis may be more difficult (Abeloff, Martin et al, 2004). According to the widely used WHO criteria, the diagnosis of AML is established by demonstrating involvement of more than 20% of the blood and/or bone marrow by leukemic myeloblasts (Harris N et al, 1999). The French–American British (FAB) classification is a bit more stringent, requiring a blast percentage of at least 30% in bone marrow (BM) or peripheral blood (PB) for the diagnosis of AML (Amin, *et al*. 2005).

AML must be carefully differentiated from "preleukemic" conditions suc h as myelodysplastic or myeloproliferative syndromes, which are treated differently. Because acute promyelocytic leukemia (APL) has the highest curability and requires a unique form of treatment, it is important to quickly establish or exclude the diagnosis of this subtype of leukemia. Fluorescent in situ hybridization performed on blood or bone marrow is often used for this purpose, as it readily identifies the chromosomal translocation (t[15; 17]) that characterizes APL (Grimwade, *et al*. 1996).

1.2.7. Treatment of Acute Myeloid Leukemia

First-line treatment of AML consists primarily of chemotherapy, and is divided.into.two.phases.**induction** and **post.remission** (or **consolidatio**) therapy. The goal of induction therapy is to achieve a complete remission by reducing the number of leukemic cells to an undetectable level; the goal of consolidation therapy is to eliminate any residual undetectable disease and achieve a cure. Hematopoietic stem cell transplantation is usually considered if induction chemotherapy fails or after a patient

relapses, although transplantation is also used as front-line therapy for patients with high-risk disease (Bishop, *et al*. 1997).

1.2.7.1. Induction

All FAB subtypes except M3 are usually given induction chemotherapy with cytarabine (ara-C) and an anthracycline (such as daunorubicin or idarubicin) This induction chemotherapy regimen is known as "7+3" (or "3+7"), because the cytarabine is given as a continuous IV infusion for seven consecutive days while the anthracycline is given for three consecutive days as an IV push. Up to 70% of patients will achieve a remission with these protocol (Bishop J et al 1997) .Other alternative induction regimens, including high-dose cytarabine alone or investigational agents may also be used (Weick JK et al, 1996, Bishop JF et al 1996). Because of the toxic effects of therapy, including myelosuppression and an increased risk of infection, induction chemotherapy may not be offered to the very elderly, and the options may include less intense chemotherapy or palliative care. The M3 subtype of AML, also known as acute promyelocytic leukemia (APL), is almost universally treated with the drug all-trans-retinoic acid (ATRA) in addition to induction chemotherapy, usually an anthracycline Care must be taken to prevent disseminated intravascular coagulation (DIC), complicating the treatment of APL when the promyelocytes release the contents of their granules into the peripheral circulation. APL is eminently curable, with well-documented treatment protocols. The goal of the induction phase is to reach a complete remission. Complete remission does not mean the disease has been cured; rather, it signifies no disease can be detected with available diagnostic methods. Complete remission is obtained in about 50%–75% of newly diagnosed adults, although this may vary based on the prognostic factors described

above. The length of remission depends on the prognostic features of the original leukemia. In general, all remissions will fail without additional consolidation therapy. (Bishop, *et al*. 1997).

1.2.7.2. Consolidation:

Even after complete remission is achieved, leukemic cells likely remain in numbers too small to be detected with current diagnostic techniques. If no further post remission or consolidation therapy is given, almost all patients will eventually relapse. Therefore, more therapy is necessary to eliminate non detectable disease and prevent relapse that is, to achieve a cure. The specific type of post remission therapy is individualized based on a patient's prognostic factors and general health. For good-prognosis leukemias (i.e. inv (16), t (8; 21), and t (15; 17)), patients will typically undergo an additional three to five courses of intensive chemotherapy, known as consolidation chemotherapy. For patients at high risk of relapse (e.g. those with high-risk cytogenetics, underlying MDS, or therapy-related AML), allogeneic stem cell transplantation is usually recommended if the patient is able to tolerate a transplant and has a suitable donor. The best post remission therapy for intermediate-risk AML (normal cytogenetics or cytogenetic changes not falling into good-risk or high-risk groups) is less clear and depends on the specific situation, including the age and overall health of the patient, the patient's personal values, and whether a suitable stem cell donor is available.

For patients who are not eligible for a stem cell transplant, immunotheray with a combination of histamine dihydrochloride (Ceplene) and interleuk in 2 (Proleukin) after the completion of consolidation has been shown to reduce the absolute relapse risk by 14%, translating to a 50% increase in the likelihood of maintained remission. (Bishop, *et al*. 1997).

1.2.7.3. Relapsed AML:

For patients with relapsed AML, the only proven potentially curative therapy is a hematopoietic stem cell transplant, if one has not already been performed. In 2000, the monoclonal antibody-linked cytotoxicagent gemtuzumab ozogamicin (Mylotarg) was approved in the United States for patients aged more than 60 years with relapsed AML who are not candidates for high-dose chemotherapy. Patients with relapsed AML who are not candidates for stem cell transplantation, or who have relapsed after a stem cell transplant, may be offered treatment in a clinical trial, as conventional treatment options are limited. Agents under investigation include cytotoxic drugs such as clofarabine, as well as targeted therapies, such as farnesyl transferase inhibitors, decitabine, and inhibitors of MDR1 (multidrug-resistance protein). Since treatment options for relapsed AML are so limited, palliative care may be offered. For relapsed acute promyelocytic leukemia (APL), arsenic trioxide has been tested in trials and approved by the Food and Drug Administration. Like ATRA, arsenic trioxide does not work with other subtypes of AML.

1.2.8. Genetics of Acute Myeloid Leukemia

The diagnosis, prognosis, and treatment of acute myeloid leukemia (AML) has been transformed over the past 15 years from a disease defined, classed, and staged based on histological characteristics alone to a disease classified largely based on genetic, genomic, and molecular characteristics. The risk pattern in AML is determined not only by cytogenetic abnormalities, such as chromosomal deletions, duplications, or substitutions, but also by the elucidation of certain molecular mutations leading to over- or under-expressions of one of many proteins. Cytogenetic studies performed on bone marrow in patients with AML play a crucial role in characterizing the leukemia, helping determine

disease aggressiveness, response to treatment, and prognosis. For example, the finding of a translocation between chromosomes 15 and 17, or t (15; 17), is associated with a diagnosis of acute promyelocytic leukemia (APL), a subtype of AML that is treated and monitored differently than other subtypes. (Fenaux, *et al*. 1999)

The commonly used French American British (FAB) classification as well as the more recent World Health Organization (WHO) classification use a variety of factors to classify AML as poor-risk, intermediate-risk, and better-risk disease. In general, better-risk disease is associated with long-term survival of up to 65%, medium-risk disease is associated with long-term survival of about 25%, and poor-risk disease is associated with long-term survival of less than 10%. (Harris, *et al*. 1997) (Grimwade, *et al*. 1998).

Table (1-2): Risk category and genetics of AML (Grimwade, *et al*. 1998).

Risk Category	**Abnormality**	**5-year survival**	**Relapse rate**
Good	t(8;21), t(15;17), inv(16)	70%	33%
Intermediate	Normal, +8, +21, +22, del(7q), del(9q), Abnormal 11q23, all other structural or numerical changes	48%	50%
Poor	-5, -7, del(5q), Abnormal 3q, Complex cytogenetics	15%	78%

In addition to cytogenetic abnormalities, several molecular abnormalities have been shown to have prognostic importance in patients with AML. *FLT3* is the most commonly mutated gene in AML and appears to be activated in one third of AML cases. Internal tandem duplications (ITDs) in the juxta membrane domain of *FLT3* are seen in 25% of AML cases, while others show mutations in the activation loop of *FLT3*. Patients with *FLT3* -ITD tend to have a poor prognosis Mutation in *NPM1* is generally favorable; patients with this mutation show increased response to chemotherapy and improved survival. However, if present together with the *FLT3* mutation, this survival benefit is negated. Mutations in *CEBPA* are detected in 15% of patients with normal cytogenetics and are associated with a longer remission duration and longer overall survival. Of note, the presence of *c-KIT* mutations in patients with otherwise favorable cytogenetic markers (e.g., t (8:21), inv (16)) confers a higher risk of relapse. Other molecular markers, such as *ERG* and *BAALC*, have been suggested to be predictive of risk and response to treatment. However, tests for these markers are not routinely available so they are not typically included in classification genetic and molecular systems.(Thiede, *et al*. 2002).

1.2.9. William's Tumor 1 Gene:

In 1899, Max Wilms made a significant discovery when he first described a young patient with malignant neoplasm of the kidney. Williams' tumor (nephroblastoma) affects 1:10,000 children and accounts for ~8% of all pediatric malignancies (Hastie, *et al*. 1994)

1.2.9.1. Structure of WT1 gene:

The WT1 gene, first cloned in 1990, is located at chromosome11p13 (Call KM et al, 1990). The gene encodes for 10 exons and generates a 3 kb mRNA (Figure 1). Although several transcriptional modifications can occur, there are two predominant alternative splicing events. These include splicing of exon 5 (17 amino acids), and of a stretch of nine nucleotides (three amino acids, lysine, threoine, and serine (KTS)) in the 30 end of exon 9. Alternative splicing of these two sites gives rise to four different protein isoforms designated A, B, C and D, or (-/-), (+/-), (-/+) and (+/+), representing the presence or absence of exon 5 and KTS insert, respectively.5 Under normal physiological conditions, the expression of KTS(+) / KTS(-) ratio is maintained at approximately 2:1(Haber DA, et al 1991). In addition to these major alternatively spliced forms, a N-terminally truncated WT1 referred to as AWT1 has recently been described. This arises as a result of gene transcription initiating within a promoter in intron 1.(Dallosso AR et al 2004). The N-terminal domain of WT1 is comprised of proline– glutamine-rich sequences and is involved in RNA and protein interactions (Figure 1)

This domain is critical for the transcriptional regulatory function of WT1, as deletion studies reveal both transcriptional repression and activation domains. The C-terminal domain of WT1 is composed of four Kru¨pple-like Cysteine2-Histidine2 zinc fingers, which permit binding to target DNA sequences but are also involved in RNA and protein interactions. The AWT1 isoform differs from WT1 in that it lacks the first 147 amino acids that contain the repression domain (Menke, *et al*. 1998).

1.2.9.2.WT1 isoform

A total of 24 different isoforms resulting from combinations of alternative splicing events, alternative startcodons and RNA editing were

described soon after cloning of the gene. The conservation of WT1 isoforms through evolution can provide insights in their function and their importance.

For instance, the RNA editing of WT1 has only been described in mouse and human samples, whereas the exon 5 variants and alternative start codons are conserved throughout mammals. As of yet, there are no data published on the conservation of alternative start isoforms. The only variation that is known to be conserved throughout all vertebrates is +KTS (Haber, *et al*. 1990).

1.2.9.3. Function of WT1 gene

The first function described for WT1 was a role in transcriptional regulation. In addition of that there is a wealth of circumstantial evidence pointing to a role for WT1 in RNA metabolism possibly splicing, mediated via Zn-finger 1 and with some specificity for the KTS isoform.

Recent data suggest the role of WT1 might not be limited to transcriptional regulation Anecdotal evidence from many labs showed that some endogenous Wt1 protein could be found in the cytoplasm, but for a long time, this was dismissed as an antibody-staining artefact. It has now been found that, in fact, 10–25% of endogenous Wt1 in murine kidney and differentiated ES cells is indeed. Many hypothesis suggest that WT1 may have either pro or anti- differentiation function during development, depending on the tissue and context.

WT1 is required for the development of the kidney and it necessary for normal kidney function and normal formation of the heart. (Haber *et al*,1990 ; Niksic , *et al*. 2004 ; Scharnhorst, *et al*. 2001).

1.2.9.4. Normal expression of WT1 gene:

WT1 is an important regulatory molecule involved in cell growth and development. It is expressed in a tissue specific manner. In the

developing embryo, WT1 expression is found primarily in the urogenital system (Menke AL et al 1998). In adult tissues, WT1 expression is found in the urogenital system, the central nervous system and in tissues involved in hematopoiesis, including the bone marrow and lymph nodes (Rivera , *et al*. 2005).

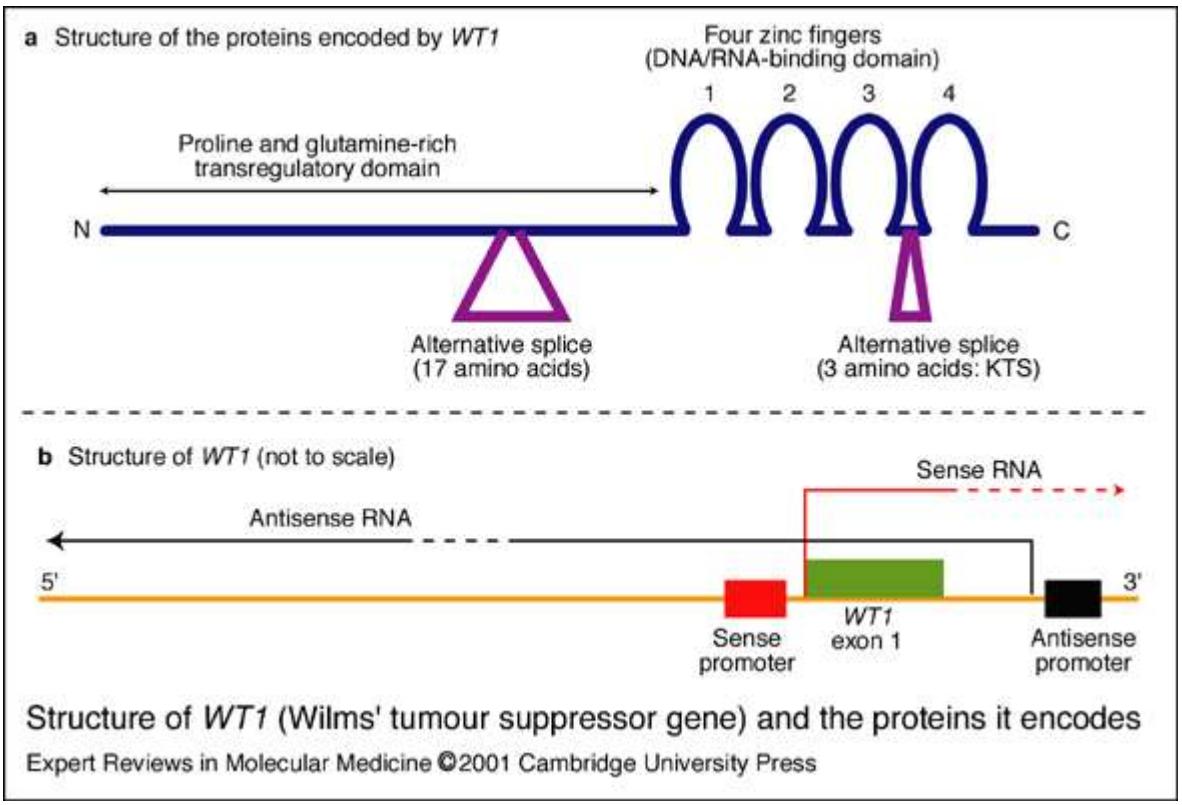

Fig (1-1): Schematic diagram of the WT1 structure

1.2.9.5. WT1 in normal hematopoiesis

In normal human bone marrow, WT1 is expressed at extremely low levels and is confined to the primitive CD34 population of cells, (Hosen N et al, 2002, Baird PN et al 1997). To ascertain the effect of WT1 on these early cell populations, studies have been carried out in mouse and human cells with different results. Gene knockout of WT1 in mice is embryonically lethal; however, this is not due to a hematopoietic defect as assessed by bone marrow and peripheral blood morphology. This is further confirmed by the finding that fetal liver cells from WT1 deficient

animals can reconstitute the hematopoietic system of irradiated adult mice thus indicating that these cells are capable of the full range of proliferation and differentiation (Alberta JA et al, 2003, King-Underwood L et al 2005). However, when chimeric mice are generated from ES cells lacking WT1, the WT1 null ES cells do not compete with wild-type WT1 expressing cells in contributing to bone marrow and blood cell products, (Alberta JA et al, 2003). This suggests that WT1 plays a role in the self-renewal of early murine hematopoietic cells. This is further supported by studies of Nishida et al,(Nishida S et al, 2006). who forced the expression of WT1 in hematopoietic progenitor cells by placing WT1 downstream of the tec promoter. This resulted in increased bone marrow cellularity and increased numbers of BFU-E, CFU-GM and CFU-GEMM; production of mature cells was normal in these mice. It is of note that these mice did not develop spontaneous leukemia. However, when the bone marrow was infected with a virus carrying AML1-ETO, the animals developed leukemia. The authors conclude that in this model, WT1 serves to maintain the self-renewal of cells, whereas the 'second hit' blocks differentiation, resulting in the development of leukemia. In human hematopoietic cells, WT1 appears to behave as a tumor suppressor gene as the overexpression of WT1 in early human bone marrow cells leads to growth arrest and reduced colony formation, (Ellisen, *et al*. 2001, Svedberg, *et al*. 2001). This effect is due to the zinc fingers, asdeletion of this domain abolishes the effect, These results indicate the need for caution and qualification, when drawing conclusios as to the role of WT1(Svensson, *et al*. 2005).

1.2.9.6. WT1, the tumor suppressor:

WT1 was initially discovered as a tumor suppressor in William's tumor (WT), a pediatric kidney malignancy that affect approximately 1:10 000 children. Mutations of WT1 associated with WT are found almost

exclusively in the sporadic form of the disease at a low frequency (15%),(Little Wells clinical, 1997). The abnormalities include large deletions and intragenic mutations (10 and 5% of all WT cases, respectively), (Little M, Wells clinical, 1997). However, the majority of WT express wild-type WT1, sometimes to high levels, (Rivera MN, Haber DA, 2005). These findings suggest that WT1 mutations are important only in a small fraction of cases. Furthermore, in WT that express wildtype WT1, it is not known whether the persistent expression of WT1 contributes to the development of the disease or is just a reflection of tumor ontogeny. In contrast to uncomplicated WT, three related syndromes are associated with heterozygous mutations of WT1. These include proapoptotic genes such as BAK, (Morrison, *et al*. 2005). Also, WT1 can downregulate growth factor receptors such as the epidermal growth factor receptor (EGFR) and the insulin receptor, altering the balance of survival signals towards death, (Menke, *et al*. 1997).

1.2.9.7. WT1 in leukemia:

1.2.9.7.1. WT1 as a tumor suppressor in leukemia.

Two main findings support WT1 as a tumor suppressor in acute myeloid leukemia (AML). First, in one study approximately 10–15% of cases (4/36 patient samples) had mutations of WT1 in leukemic cell DNA (King-Underwood L et al, 1996). In that study, of the four patients carrying WT1 mutations, five different point mutations were found. These included four nucleotide insertions in exons 1 or 7, and one nonsense mutation in exon 9. These mutations are predicted to yield truncated or modified WT1 protein. Unfortunately, in the published manuscript, there was no assessment of WT1 RNA or protein in cells containing WT1 mutations. Thus, it is not known if these mutations contributed to the development of the disease and the behavior of the

cells. Second, very low levels of WT1 RNA can be found in some forms of AML. We assessed the expression of WT1 in the Valk et al, (Valk , *et al*. 2004). AML microarray expression data set of 285 AML patients and found that the majority of patients have high levels of WT1. However, some subgroups, such as cluster 5, have low to absent levels of WT1. In these patients, high levels of WT1 expression may not be well tolerated. This possibility is supported by the growth inhibiting effect of WT1. For example, when the WT1 KTS (+) isoform is expressed in a WT1-negative human myeloblastic leukemic cell line, M1, decreased tumor formation is observed when the cells are injected into immunocompromised mice, This is consistent with a tumor suppressor function of WT1 in some forms of AML (Smith, *et al*. 2000).

1.2.9.7.2. WT1 as an oncogene in leukemia:

WT1 is highly expressed in the bone marrow or peripheral blood of a variety of leukemias in comparison to normal bone marrow and normal progenitor cells. The gene has been wild type in sequence when tested, (Miyagi, *et al*. 1993, Miwa , *et al*. 1992). In general, the expression of WT1 varies between and within different forms of human leukemia. In chronic myelogenous leukemia, WT1 levels are usually low in the chronic phase but frequently are seen to increase as patients move into the accelerated and blast crisis phase of the disease, (Miwa , *et al*. 1992, Menssen , *et al*. 1995). In the acute leukemias, increased levels of WT1 can be found in both acute lymphoblastic and myeloblastic leukemia. Combining the results of several studies, WT1 RNA levels, as assessed by RT-PCR and Northern blotting (earlier studies), were elevated in a total of 354 of 476 (74%) AML patient samples, (Miyagi , *et al*. 1993, Brieger J et al, 1994 and Schmid , *et al*., 1997) For acute lymphoblastic leukemia, WT1 RNA was increased in 86 of 131 (66%) patient samples, (Miyagi , *et al*. 1993, Brieger J et al, 1994, Schmid , *et al*. 1997 and

Chiusa, *et al*. 2006). High levels of WT1 were also seen in some forms of myelodysplastic syndromes (MDS). In MDS, increased WT1 expression is associated with higher blast counts and portends an early progression to AML (Tamaki, *et al*. 1999).

1.2.9.7.3. WT1 and standard Chemotherapy in leukemic patients:

Elevated levels of WT1 in leukemia are associated with poor prognosis following standard chemotherapy treatment. Consistent with the cell survival and oncogenic roles of the WT1 protein, increased WT1 levels have prognostic significance and are associated with a poor response to therapy, (Chiusa , *et al*. 2006, Barragan , *et al*. 2004). In one study, blast cells from 139 AML patients less than 60 years old treated with a typical chemotherapy regimen were assessed for WT1 transcript levels. The probability of 3-year overall survival was 59% in patients with low WT1 levels and 21% in patients with high WT1 levels ($P = 0.046$), (Bergmann, *et al*. 1997). However, these studies are in contrast with the observations made by Schmid et al, (Schmid, *et al*. 1997). where in 125 de novo AML patients, WT1 RNA levels did not correlate with disease-free survival or clinical remission. This discrepancy may be due to differences in methodology in measuring WT1 and differences in the patient In a more recent study, WT1 was evaluated along with the proto-oncogene Bcl-2, as prognostic markers of AML treatment outcome, (Karakas, *et al*. 2002). In patients less than 60 years old treated with standard chemotherapy, high expression of both WT1 and Bcl-2 identified a group of patients with significantly poor overall survival ($P = 0.0029$), and high incidence of relapse ($P < 0.03$). In patients over 60 years old, WT1 and Bcl-2 levels as prognostic markers did not provide the same prognostic information. This may be due to different disease biology in older AML patients or due to the reduced intensity of chemotherapy often used in treating older patients. In general there is an association

between the levels of WT1 and Bcl-2, which is in keeping with the observation that WT1 can increase the expression of Bcl-2, However, there are also cases where WT1 levels are high and Bcl-2 is low, and vice versa. These observations suggest that in some cases the high expression of WT1 and Bcl-2 are linked, whereas in other cases these are independent events (May , *et al*. 1999).

1.2.9.6.4. Clinical relevance of WT1 expression in adult leukemia

Large clinical studies in AML patients have evaluated WT1 as a prognostic marker at diagnosis and to predict relapse in clinical remission. In AML, WT1 expression did not correlate with age, antecedent MDS, or cytogenetic risk group. The presence or absence of WT1 RNA does not have prognostic significance in adult *de novo* AML. However, high levels of WT1 gene expression in acute leukemia was associated with lower CR rates and reduced overall survival and disease-free survival. Recently, Karakas et al have proposed that WT1 expression can be used as part of a prognostic index in newly diagnosed AML. (King et al , 1998; Miyagawa, *et al*. 1999).

Regarding the use of WT1 expression to predict disease recurrence for patients in CR, the data are less clear. In one study of leukemic patients, reappearance of WT1 mRNA preceded clinical relapse by a median of 7 months (range, 1–18 months) and blood was found to be more sensitive than marrow for detecting minimal residual disease. In another study involving 50 AML patients in CR with at least 3 years of follow-up, marrow WT1 expression antedated clinical relapse by up to 3 months. A third study evaluated the predictive value of WT1 determinations during CR for 44 *de novo* AML WT1-positive patients. Achieving a WT1-negative status did not affect overall survival or disease-free survival. Furthermore, 10/11 patients in continuous remission for more than 3 years had WT1 detected at least once and seven patients were repeatedly

positive. Some patients with WT1-positive leukemia at diagnosis relapsed with WT1-negative disease and some patients with WT1-negative leukemia had a WT1-positive relapse. (King et al , 1998; Miyagawa, *et al*. 1999; Falkenburg, *et al*. 1990)

1.3. Rationale:

The *WT1* gene is expressed in a limited set of tissues during fetal development, consistent with its critical role in early nephrogenesis and in the development of gonads. The gene is also expressed in acute leukemias and in CD34+ hematopoietic progenitor cells. The expression of this gene is down-regulated during differentiation of hematopoietic cells. These findings suggest that *WT1* plays an important role in hematopoiesis and in hematological malignancies. Subsequent studies revealed that WT1 is mutant in approximately 10 - 15% of primary leukemia, mainly in AML. (King, *et al.* 1996). All leukemias with *WT1* mutations were eventually refractory to treatment, suggesting that the *WT1* gene is associated with aggressive disease and had poor prognosis the association between WT1 mutation and prognosis remain un understood. (King, *et al.* 1998). According to the all of the above mentioned, we carried this study to detect WT1 mutation in Sudanese patients with AML because this study has not been done before in the Sudan, and also to answer the following questions: Does the WT1 mutation exist in Sudanese patients with acute myeloid leukemia as it's found in other population?, What is the type of WT1 mutation that exist in Sudanese patients with AML. Is there is any co relation between WT1 mutation and clinical outcome in Sudanese patients with AML? Is there is any link between the mutant WT1 gene and the development of leukemia in Sudan? And can we use the WT1 gene as the molecular marker for the detection of disease or selection of population at risk?

1.4. Objectives:

1.4.1. General objective

To detect William's Tumor 1 gene Mutation in patients with Acute Myeloid Leukemia in the Sudan.

1.4.2. Specific objectives

1. To detect the frequency of WT1 mutation in the Sudanese AML patients.

2. To assess the relationships of WT1 mutation to clinical outcome.

3. To evaluate the possible use of WT1 as a molecular marker for early diagnosis of patients with AML.

Chapter Two

2. Materials & Methods

2. 1.Study design

This is a cross - sectional hospital based study. The study was conducted in Radiation and Isotopes Center in Khartoum during the period of March 2012 to November 2013.

2.2. Study population:

- fifty one Sudanese patients with AML, 26 males and 25 females in the age range between 10 and 70 years.

2.3. Sampling design:

Simple random sample,

2.4. Sample collection

Five ml of blood were collected from patients in EDTA as anticoagulant.

2.5. Data collection:

-Data were collected using a questionnaire containing the demographic information and the clinical data of patients and Hospital were recorded.

2.6. Methods

2. 6.1. DNA extraction from blood:

Genomic DNA was extracted from peripheral blood lymphocytes of patients and controls. (Orita, *et al.* 1990)

Salting out method for DNA extraction from human blood:

Solutions and buffers:

1. PBS (1 mM KH2P04, 154mM NaCl, 5.6 mM Na2HP04; pH7.4)

in 1 liter:

KH2P04----------0.136 g

NaCl----------9.0 g

Na2HPO4----------0.795 g

2. Sucrose Triton X-lysing Buffer:

1 M Tris-Hcl, PH 8.0----------1.0 ml

1 M MgCl2 ----------0.5 ml

Triton X-100----------1.0 ml

Made up to 100 ml with distilled water, Autoclaved and kept at 4 degrees

11 g sucrose (D (+) saccharose) was added just before use (11 g/100mL). The solution cannot be stored with sucrose

T20E5, pH8

20 mM Tris-HC----------5.0 ml

5 mM EDTA----------4.0 ml

3. Proteinase K (stock of 10 mg/mL).

4. Saturated NaCl

100 mL of sterile water was taken and 40g NaCl was added to it slowly until absolutely saturated. It was agitated before use and NaCl was left to precipitate out. (Orita, *et al.* 1990)

Procedure:

Purification of DNA from frozen blood (for 2.5 ml sample). The blood was thawed at room temperature and then transferred to a sterile polypropylene tubes then it was diluted with 2 volume of PBS (1 mM KH2PO4, 154 mM NaCl, 5.6 mM Na2HPO4, pH 7.4) mixed by inverting the tubes and centrifuged at 2200 g for 10 min. The supernatant was poured off and the pellet (reddish) was resuspended in 9volume 12,5ml of Sucrose Triton X-100 Lysing Buffer (1 M Tris-HCl

10 ml, 1 M MgCl2 5 ml Triton X-100 10 ml Made up to 1 liter with distilled water). Sucrose was added just before use i.e. (11 g/100 mL). The mixture was vortexed and then placed on ice for 5 minutes. The mixture was spun for 5 minutes at 2200 g (2300 rpm in TH.4 rotor) and the supernatant was poured off. The pellet (pinkish or white) was resuspended in 1.5 mL of T20E5 (0.6X volume of original blood). 10% SDS was added to a final concentration of 1% (100 μL) then Protienase K was added (10 mg/mL) to a final concentration of 250 μg/mL (35 μL). (Orita, *et al.* 1990)

The mixture was mixed by inversion after adding each solution then the samples were incupated at 45°C overnight. 1 mL of saturated NaCl was added to each sample and mixed vigorously for 15 seconds then it was spun for 30 minutes at 2400 g. A white pellet was formed which consisted of protein precipitated by salt, the supernatant that contained the DNA was transferred to a new tube and precipitation of DNA was achieved by adding two volumes of absolute alcohol kept at room temperature. The solution was agitated gently and the DNA was spooled off and transfered to eppendorf tube. The DNA was washed in 70% ice-cold alcohol (1 mL), air dried and was dissolved in the appropriate volume of TE (100-250 μL) and stored at 4 degrees overnight to dissolve. (Orita, *et al.* 1990)

Management of DNA extraction:

Extracted DNA measured by using DNA reader Bio-photometer for (eppendorf AG company 2231-hamburg-made in Germany) model it was uses just 10 microliter of DNA sample and diluted by 990 micro litter of distilled water to convert the volume 1milletter measure from. (Orita, *et al.* 1990)

2.6.2. Polymerase chain reaction (PCR):

Primer design:

- a pair of two primers was designed using the published data for human William's Tumor gene chain sequence to amplify the DNA fragment that containing the mutation.
- Primer sequence

  F primer **5-GTGCTTAAAGCCTCCCTTCCTCTTAC-3‘**

  R primer **5‘-CTCTTGAACCATGTTTGCCCAAGAC-3‘**

PCR procedure:

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94°C for 30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules. (King, *et al.* 1998)

Annealing step: The reaction temperature is lowered to 55 °C for 1minute allowing annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis. (King, *et al.* 1998)

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 72 °C for 45 seconds. .At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The

extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. 35 cycles required for amplification (annealing, denaturatin and extension) (King, *et al.* 1998)

Standard PCR reaction mixture:

The experiment consists of DNA negative control in 0.5 ml PCR tube, all components necessary to make new DNA in the PCR process will be placed in 20ul total volume using maxime PCR premix kit (i- Taq), from iNtRoN Biotechnology – Korea. Table (2-1).

Table (2-1): Standard PCR mixture

Component concn.	**Final conc.**	**Purpose**
DNA template	100ng/ µl	The DNA which will be amplified by the PCR Reaction.
10x taq buffer	1X	keeps the master mix at the proper pH so the PCR reaction will take place.
2.5mM Deoxynucleotides	200uM	provide both the energy and nucleosides for the Synthesis of DNA. It is important to add equal amounts of each nucleotide (dATP, dTTP, dCTP, dGTP) to the master mix to prevent mismatches of bases
10Pmol/µl Primers	10 Pmol	Short pieces of DNA (20-30 bases) that bind to the DNA template allowing Taq DNA polymerase enzyme to initiate incorporation of the deoxynucleotides.
5U/1ul taq polymerase	1u/0.25 µl	A heat stable enzyme that adds the deoxynucleotides to the DNA template.

Agarose gel electrophoresis requirements for PCR product:

To confirm the presence of amplifiable DNA in the samples, the specificity of the PCR is typically analyzed by evaluating the production of target fragment by gel electrophoresis of 5.0 µl PCR product on 2% agarose gel stained with ethidium bromide.

Procedure:

- 1.5% Agarose gel (1.5 gram of agarose – 100ml 1X TBE buffer) was prepared.
- The mixture was heated until the Agarose completely dissolved , was cooled to 60c and 4.0 µl of ethidium bromide was added, then the gel was poured on the gel tank and left it to solidify.
- 3.0 µl of PCR product was loaded on the gel.
- Gel ran at 100 volts for 15 min.
- The gel was viewed in the UV system (UV Transilluminator JY – 02S) and photographed.

2.6.3. Restriction Fragment Length Polymorphism Analysis (RFLPs):

- Restriction enzyme used AflIII, New England Biolabs Inc.
- We used the following digestion mixture for RFLP, Table (2-2)

Table (2-2): Digestion mixture for RFLP

NO	Items used	Amount used for one sample
1.	10X Buffer	5.0 µl
2.	PCR Product	10 µl
3.	Restriction enzyme AflIII	0.5 µl (5.0 unit)
4.	Distilled water	20 µl
	Total volume	**35.5 µl**

- The samples were incubated at 37c for 60 min.
- 2.0 % Agarose gel (2.0 gram of Agarose – 100ml 1X TBE buffer) was prepared.
- The mixture was heated until the Agarose completely dissolved , cooled to 60c and 4.0 µl of ethidium bromide was added, then the gel was poured on the gel tank and left it to solidify.
- 10 µl of digested PCR product was loaded on the gel.
- Gel ran at 100 volts for 30 min.
- The gel was viewed in the UV system (UV Transilluminator JY – 02S) and photographed.

RFLP interpretation

- Homozygous wild type (Normal) was cut into two bands 165 bp and 49 bp.
- Homozygous mutant type was uncut gave band 214 bp.
- Heterozygous mutant type was cut into three bands 214 bp, 165 bp and 49 bp.

2.7. Ethical consideration

A written informed consent presented to all patients who volunteer to participate and the nature of the study was explained to them. The volunteers were asked to sign the consent form when they were agreed to take part in the study.

2.8. Data Analysis:

The results were entered into the computer and analyzed using the SPSS program version 17 for windows.

Chapter Three

3. Results

This study was included 51 Sudanese patients with AML. The age of AML patients range between 10 to 70 years, mean age (2.51), Table (3-3), the male / female percentage of AML patients was 26 (51 %) males/ with mean age (2.60) and 25 (49.0%) females with mean age (2.42), Table (3-1), Table (3-2).

Table (3-4) shown the tribal group distribution among the study population were 38 (74.5%) from AS, 3 (5.9%) from NS and 10 (19.6%) from NK tribal group.

Genomic DNA was extracted from blood lymphocytes using saluting out method, the quality of the extracted DNA were checked on 1% Agarose gel by electrophoresis Fig (3-9).

PCR amplification for the required area of the WT1 gene was done using the specific primers designed for exon 7 of the gene, the PCR product was 214bp, the results were shown in fig (3-10).

RFLP analysis was done for all PCR products of Patients samples using (AflIII) restriction enzyme, the results were shown in Fig (3-11).

Table (3-5) shown genotyping distribution among the study population were 27 (52.9%) were normal (A/A) of WT1 mutation, 22 (43.1%) were heterozygous (A/G) of WT1 gene mutation and only 2 (3.9%) of patients were homozygous (G/G) of WT1 gene mutation.

Table (3-1): summarizes the frequencies of gender among the study population.

Gender	Frequency	Percent
Male	26	51.0 %
Female	25	49.0 %
Total	**51**	**100 %**

Table (3-2): mean of age among study population.

Patients sex	Mean	Frequency	Std. Deviation
Male	2.60	25	1.000
Female	2.42	26	1.102
Total	**2.51**	**51**	**1.046**

Table (3-3): Distribution of age among study population.

Group	Sex	Age groups					Total
		1 – 15	15 – 30	30 – 45	45 – 60	60 – 75	
Patients	**Male**	06	08	09	03	00	**26**
	Female	05	07	09	01	03	**25**

Table (3-4): shown tribal group distribution among sex of the study population.

Tribal group	Patients Sex		Total	(%)
	Male	Female		
AS (Afro Asiatic)	19	19	**38**	**74.5**
NS (Nilo Saharan)	2	1	**3**	**5.9**
NK (Niger Kordofanian)	4	6	**10**	**19.6**
Total	**25**	**26**	**51**	**100%**

Table (3-5): Genotyping distribution among study population in exon 7 of WT1 gene using RFLP technique restriction enzyme (AflIII).

WT1 A/A: Homozygote Normal gene.

WT1 A/G: Heterozygote mutant gene.

WT1 G/G: Homozygote mutant gene

genotyping	Frequency	Percentage (%)
A/A	27	52.9
A/G	22	43.1
G/G	2	3.9
Total	**51**	**100**

Table (3-6): Genotyping frequency among sex of the study population.

Genotyping	Patients Gender		Total
	Male	Female	
A/A	13	14	**27**
A/G	12	10	**22**
G/G	0	2	**2**
Total	**25**	**26**	**51**

Table (3-7): Shown mutation Frequency among tribal group of the study population

Tribal group	patients genotyping Frequencies			Total
	A/A	A/G	G/G	
AS (Afro Asiatic)	18	19	1	38
NS (Nilo Saharan)	3	0	0	3
NK (Niger Kordofanian)	6	3	1	10
Total	27	22	2	51

Table (3-8): Shown Allele frequencies among the study population

Allele A: Normal allele **Allele G: Abnormal allele**

Alleles	Frequency	Percentage (%)
Normal allele	76	74.5
Abnormal allele	26	25.5
Total	**102**	**100**

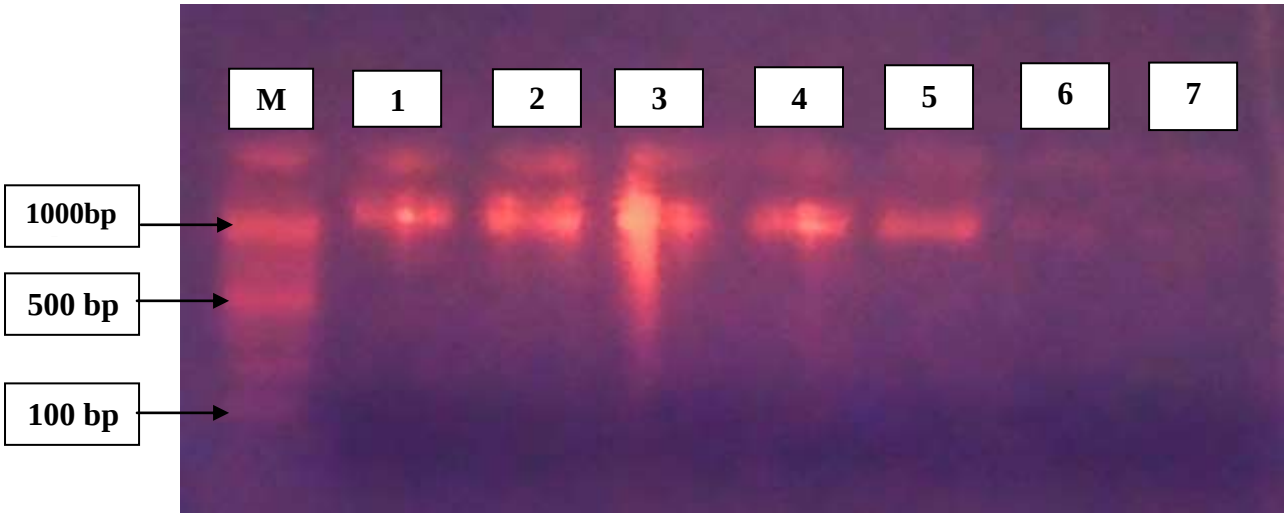

Figure (3-9): shown genomic DNA, lane 1-5 positive DNA, lane 6-7: Negative DNA, M: DNA marker.

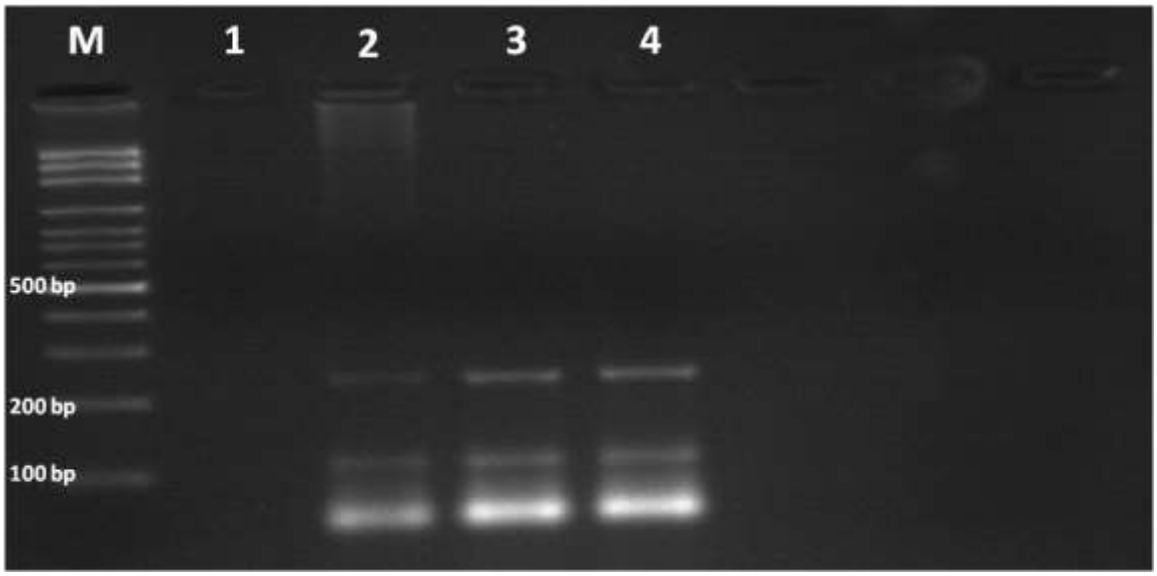

Figure (3-10): PCR amplification of Exon (7) of WT1 gene, M: DNA marker Lane1: Negative PCR, Lane 2 – 4 shown positive PCR amplification of exon 7 of WT1 gene give band 214pb.

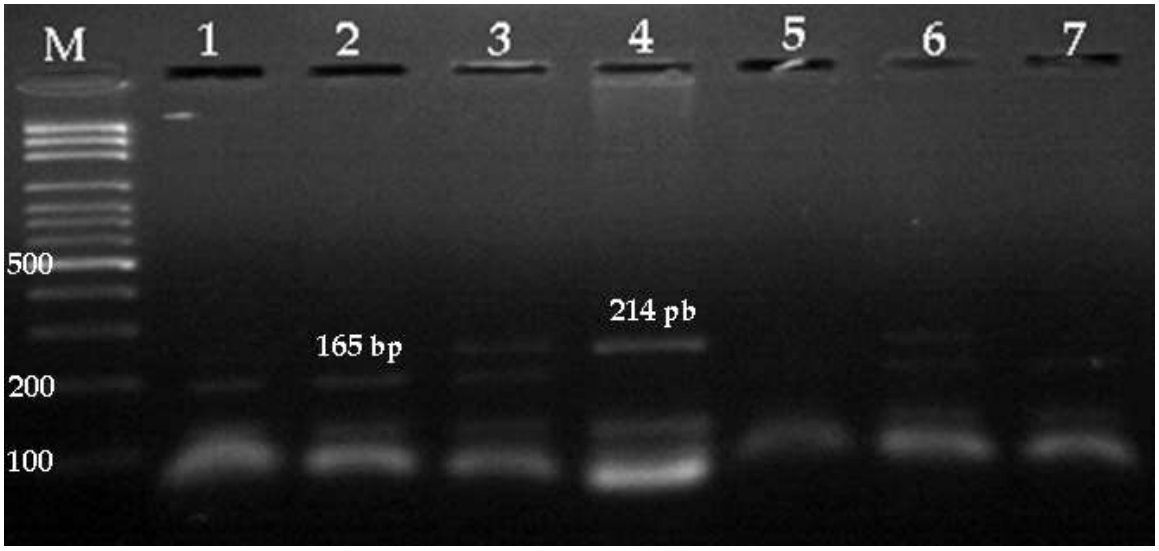

Figure (3-11): Shown RFLP for samples 1 – 7, M: DNA marker Lane 1, 2 & 7 shown normal allele (cut band 165pb), lane 3& 6 shown heterozygote mutant allele (cut into 3 band 214,165 & 49 pb), lane 4 shown Homozygote mutant allele (un cut band 214 pb).

Chapter Four

4. Discussion , Conclusion and Recommendations

4.1 Discussion

We used PCR based genotyping technique to detect the mutation of WT1 gene in Sudanese patients with AML. This is specific and sensitive method despite of the expensive of primers kits and restriction enzyme used.

The use of RFLP analysis revealed that 27 (52.9%) were normal (A/A) of WT1 mutation, 22 (43.1%) were heterozygous (A/G) of WT1 gene mutation and only 2 (3.9%) of patients were homozygous (G/G) of WT1 gene mutation. Table (3-5), Fig (3-5).

In this study we have found the mutation of WT1gene in 24 of 51 (47.1%), AML cases, This mutation frequency is not equivalent to the previous studies of WT1 gene mutation in AML, Subsequent studies in United States revealed that WT1 is mutant in approximately 10 - 15% of primary leukemia, mainly in AML (King, *et al* . 1998).

L. King-Underwood et al, found mutation of WT1 gene in 3 of 20 (15%) cases of AML (King, *et al.* 1996).

And also found that this mutation frequency is not equivalent to that found in sporadic William's Tumors, suggests that WT1 may be not equally important in both tumor types in Sudanese. (King, *et al.* 1996).
This study had assessed for the first time the frequency of the mutant WT1gene (47.1%) in Sudanese Patients with AML, Table (3-4), (King, *et al* . 1998).

It was observed that the frequency of the mutant WT1 gene was significantly higher in Sudanese AML patients, comparing to the other population from previous studies that has been done. (King, *et al* . 1998).

Yang, *et al*. 2007. Stated that in one study approximately 10 – 15 % of cases (4/36 patient samples) had mutations of WT1 in leukemic cell DNA (King-Underwood , *et al.* 1996). In that study, of the four patients carrying WT1 mutations, five different point mutations were found. These included four nucleotide insertions in exons 1 or 7, and one nonsense mutation in exon 9. These mutations are predicted to yield truncated or modified WT1 protein. Unfortunately, in the published manuscript, there was no assessment of WT1 RNA or protein in cells containing WT1 mutations. Thus, it is not known if these mutations contributed to the development of the disease and the behavior of the cells. (King, *et al*. 1998).

WT1 is highly expressed in the bone marrow or peripheral blood of a variety of leukemias in comparison to normal bone marrow and normal progenitor cells. The gene has been wild type in sequence when tested, (Miyagi , *et al.* 1993, Miwa , *et al.* 1992). In general, the expression of WT1 varies between and within different forms of human leukemia. In chronic myelogenous leukemia, WT1 levels are usually low in the chronic phase but frequently are seen to increase as patients move into the accelerated and blast crisis phase of the disease, (Miwa , *et al.* 1992, Menssen , *et al.* 1995). In the acute leukemias, increased levels of WT1 can be found in both acute lymphoblastic and myeloblastic leukemia. Combining the results of several studies, WT1 RNA levels, as assessed by RT-PCR and Northern blotting (earlier studies), were elevated in a total of 354 of 476 (74%) AML patient samples, (Miyagi T et al, 1993, Brieger, *et al.* 1994 and , *et al.*, 1997) For acute lymphoblastic leukemia, WT1 RNA was increased in 86 of 131 (66%) patient samples, (Miyagi T et al, 1993, Brieger , *et al.* 1994, Schmid , *et al.* al, 1997 and Chiusa, *et al.* 2006). High levels of WT1 were also seen in some forms of

myelodysplastic syndromes (MDS), (Tamaki H et al 1999). In MDS, increased WT1 expression is associated with higher blast counts and portends an early progression to AML. (King, *et al*. 1998).

The result suggest a possible role of WT1 gene mutation in the development of AML in Sudanese and that is because of the heterozygous nature of the mutation , this gene act as an oncogene that inherited as dominant allele (only one mutant allele could causes the disease). These finding is supported by L. King-Underwood and K. Pritchard-Jones, they stated that it is now clear that a substantial minority (< 30%) of Wilms' tumors retain one normal *WT1* allele, suggesting that in some cases, heterozygous mutation is sufficient for tumorigenesis (King, *et al*. 1998).

4.2 Conclusion:

From the results obtained from this study, we may conclude the following:

1. AML is a significantly health problem in Sudan.

2. The frequency of WT1 gene mutation was found to be 47.1% (twenty two out of fifty one of cases were heterozygous and only two out of fifty one were homozygous for W T1 gene mutation in Sudanese patient with AML, and most of the infected population was from Afro-asiatic tribal group.

3. There was an apparent relationship between WT1gene mutation and the development of AML in Sudanese population.

4.3 Recommendations :

We recommends the following:

1- A large studies including large number of patients samples with detailed clinical data including all pathological and treatment data of AML patients for assess the relationship of patient's response to chemotherapy and WT1 gene mutation.

2- Other molecular tests should be done to detect exactly the type of WT1 gene mutation found in Sudanese AML patient e.g. DNA sequencing.

References

Jemal A, Thomas A, Murray T, Thun M (2002). "Cancer statistics, 2002". CA.Cancer.J.Clin 52 (1):2347.doi:10.3322/canjclin.52.1.23. PMID 1181 4064

Bennett J, Catovsky D, Daniel M, Flandrin G, Galton D, Gralnick H, Sultan C (1976). "Proposals for the classification of the acute leukaemias. French-American-British (FAB).co-operative.group". Br.J.Haematol 33 (4):451–8.
Vardiman JW, Harris NL, Brunning RD (2002). "The World Health Organization (WHO) classification of the myeloid neoplasms". Blood 100 (7): 2292–302.doi:10.1182/blood-2002-04-1199.

Hoffman, Ronald (2005). Hematology: Basic Principles and Practice (4th. ed.). St. Louis, Mo.: Elsevier Churchill Livingstone. pp. 1074–75. ISBN 0-443-06629-9.

Abeloff, Martin (2004). Clinical Oncology (3rd. ed.). St. Louis, Mo.: Elsevier Churchill Livingstone. p. 2834. ISBN 0-443-06629-9.

Klepin HD, Balducci L. Acute my.elogenous leukemia in older adults Oncologist 222–23214 32-14:222, 2009

Baldus CD, Mrózek K, Marcucci G, Bloomfield CD (June 2007). "Clinical outcome of de novo acute myeloid leukaemia patients with normal cytogenetics is affected by molecular genetic alterations: a concise review". Br. J. Haematol. 137 (5):387400. doi:10.1111/j.13652141.2007.06566.x.

Abeloff, Martin et al. (2004), p. 2834. (clinical oncology book).3rd edition

Harris N, Jaffe E, Diebold J, Flandrin G, Muller-Hermelink H, Vardiman J, Lister T, Bloomfield C (1999). "The World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. Report of the Clinical.Advisory.Committee.meeting,.Airlie.House,.Virginia,November,.1997 ". Ann.Oncol 10 (12):1419–32.doi:10.1023/A:1008375931236.

Amin, H. M.; Yang, Y.; Shen, Y.; Estey, E. H.; Giles, F. J.; Pierce, S. A.; Kantarjian, H. M.; O'Brien, S. M. et al. (2005). "Having a higher blast percentage in circulation than bone marrow: Clinical implications in myelodysplastic syndrome and acute lymphoid and myeloid.leukemias". Leuke mia 19(9):1567.1572.

Grimwade D, Howe K, Langabeer S, Davies L, Oliver F, Walker H, Swirsky D, Wheatley K, Goldstone A, Burnett A, Solomon E (1996). "Establishing the presence of the t(15;17) in suspected acute promyelocytic leukaemia: cytogenetic, molecular and PML immunofluorescence assessment of patients entered into the M.R.C. ATRA trial. M.R.C. Adult Leukaemia Working Party". Br J Haematol 94(3): 557–73.

Bishop J (1997). "The treatment of adult acute myeloid leukemia". Semin Oncol 24 (1): 57–69.

Weick JK, Kopecky KJ, Appelbaum FR et al. (15 October 1996). "A randomized investigation of high-dose versus standard-dose cytosine arabinoside with daunorubicin in patients with previously untreated acute myeloid leukemia: a Southwest Oncology Group study" (PDF). Blood 88 (8): 2841–51.

Bishop JF, Matthews JP, Young GA et al. (1 March 1996). "A randomized study of high-dose cytarabine in induction in acute myeloid leukemia" (PDF). Blood 87 (5): 1710–7.

Estey E (2002)."Treatment of acute myelogenous leukemia". Oncology (Williston Park) 16 (3): 343–52, 355–6; discussion 357, 362, 365–6.

Cassileth PA, Harrington DP, Hines JD et al. (1988). "Maintenance chemotherapy prolongs remission duration in adult acute nonlymphocytic leukemia". J. Clin. Oncol. 6 (4): 583–7.

Mayer RJ, Davis RB, Schiffer CA et al. (1994). "Intensive postremission chemotherapy in adults with acute myeloid leukemia. Cancer and Leukemia GroupB". N.Engl.J.Med.331 (14):896.903.

Appelbaum FR, Baer MR, Carabasi MH et al. (2000). "NCCN Practice Guidelines for Acute Myelogenous Leukemia". Oncology (Williston Park, N.Y.) 14 (11A): 53–61.

Brune M, Castaigne S, Catalano J et al. (July 2006)."Improved leukemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukemia: results of a randomized phase 3 trial". Blood 108 (1): 88–96.

Appelbaum FR (2001)."Editorial:Who should be transplanted for AML?". Leu kemia 15 (4): 680–2.

Appelbaum FR (2002). "Keynote address: hematopoietic cell transplantation beyond.first.remission". Leukemia 16(2):157.9.

Sievers EL, Larson RA, Stadtmauer EA et al. (1 July 2001)."Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse". J. Clin. Oncol. 19 (13): 3244–54.

Soignet SL, Frankel SR, Douer D et al. (15 September 2001). "United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia". J. Clin. Oncol. 19(18): 3852–60.

Byrd JC, Mrózek K, Dodge RK, Carroll AJ, Edwards CG, Arthur DC, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood*. Dec 15 2002;100(13):4325-36.

Fenaux P, Chastang C, Chevret S, Sanz M, Dombret H, Archimbaud E, et al. A randomized comparison of all transretinoic acid (ATRA) followed by chemotherapy and ATRA plus chemotherapy and the role of maintenance therapy in newly diagnosed acute promyelocytic leukemia. The European APL Group. *Blood*. Aug 15 1999;94(4):1192-200.

Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med*. Oct 1985;103(4):620-5.

Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. *J Clin Oncol*. Dec 1999;17(12):3835-49.

Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research

Council Adult and Children's Leukaemia Working Parties. *Blood*. Oct 1 1998;92(7):2322-33.

National Comprehensive Cancer Network. NCCN Clinical Practice Guidelines in Oncology. Acute Myeloid Leukemia, Version 2.2012.

Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U, Platzbecker U, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood*. Jun 15 2002;99(12):4326-35

Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK, et al. The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. *Blood*. Mar 1 2008;111(5):2776-84

Bizzozero O, Johnson K, Ciocco A (1966). "Radiation-related leukemia in Hiroshima and Nagasaki, 1946–1964. I. Distribution, incidence and appearance time". N.Engl.J.Med274 (20):1095.101.

Le Beau M, Albain K, Larson R, Vardiman J, Davis E, Blough R, Golomb H, Rowley J (1986). "Clinical and cytogenetic correlations in 63 patients with therapy-related myelodysplastic syndromes and acute nonlymphocytic leukemia: further evidence for characteristic abnormalities of chromosomes no. 5 and 7". J Clin Oncol 4 (3): 325–45.

Thirman M, Gill H, Burnett R, Mbangkollo D, McCabe N, Kobayashi H, Ziemin-van der Poel S, Kaneko Y, Morgan R, Sandberg A (1993). "Rearrangement of the MLL gene in acute lymphoblastic and acute myeloid leukemias.with.11q23.chromosomal.translocations". N.Engl.J.Med 329 (13):90 9.14.

Rowley J, Golomb H, Vardiman J (1 October 1981)."Nonrandom chromosome abnormalities in acute leukemia and dysmyelopoietic syndromes in patients with.previously treated malignant disease". Blood 58 (4): 759–67.

Pedersen-Bjergaard J, Andersen M, Christiansen D, Nerlov C (2002). "Genetic pathways.in.therapy.related.myelodysplasia.and.acute.myeloidleukemia". Bloo d 99(.6): 1909–12. doi:10.1182/blood.V99.6.1909.

Congenital leukaemia and transient myeloproliferative disorder: diagnostic difficulties.case.reports].JackowskaT,Steczowicz.M.Pawelec.K,Pacholska.J.Med.Wieku.Rozwoj.2006.Jul-Sep;10(3.P.t1):595-601.

Hastie ND. The genetics of Wilms' tumor—a case of disrupted development. Annu Rev Genet 28523–558, 1994

Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA et al, Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 1990; 60509–520

Dallosso AR, Hancock AL, Brown KW, Williams AC, Jackson S, Malik K. Genomic imprinting at the WT1 gene involves a novel coding transcript (AWT1) that shows deregulation in Wilms' tumours. Hum Mol Genet 2004; 13: 405–415.

Menke AL, van der Eb AJ, Jochemsen AG. The Wilms' tumor 1 gene: oncogene or tumor suppressor gene? Int Rev Cytol 1998; 181: 151–212.

Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, Housman DE. Alternative splicing and genomic structure of the Wilms tumor gene WT1. Proc Natl Acad Sci USA 1991; 88: 9618–9622.

Rivera MN, Haber DA. Wilms' tumour: connecting tumorigenesis and organ development in the kidney. Nat Rev Cancer 2005;5: 699–712.

Appendices

Appendix (1)

PCR Machine Used in PCR Technique

Appendix (2)

UV system (UV Transilluminator JY – 02S)

Appendix (3)

بسم الله الرحمن الرحيم

Sudan University of Science and Technology
College of Graduate Studies and Scientific Research
Detection of William's Tumor 1gene Mutation in Sudanese Patients with Acute Myeloid Leukemia

Questionnaire

1.0 Patient Data

1.1 Date of birth _/___/____

1.2 Gender M ☐ F ☐
1.3 Patient hospital number_____
1.4 Patient Ethnicity _____
1.5Patient Education level_____
1.6Residence in last 10 years _____
1.7 Current residence _____
1.8 Occupation_____
1.9 Are you Smoking Y ☐ N ☐

2.0 Details of Hospitalization

2.1 Name of Hospital _____
2.2 Date of Admission _____/____/_____
2.3 Patient Primary diagnosis _____
2.4 patient sign and symptoms at admission_____
_____
2.5 Any other disease _____

3.0 Treatment details

3.1 When the patient start the treatment (chemotherapy)? ____/____/_____
3.2 what is the type of chemotherapy drugs given to the patient?
_____
_____
3.3 Did the patient have any drug resistance? Y ☐ N ☐
3.4 If yes, please specify _____ ☐
3.5 Did the patient rich the CR after the treatments? Y ☐ N ☐
3.6 For long period patient remain in CR_____
_____

3.7 Any patient history of relapse Y ☐ N ☐

Date:……. Signature:……