

CHAPTER I INTRODUCTION

1.1 Statement of the problem

Cancer is a major public health problem in many parts of the world. In most economically developed countries, cancer is the second leading cause of death, following cardiovascular disease, and it is the third leading cause of death in developing countries following heart disease and diarrheal diseases [1]. The epidemiological data concerning cancer emphasizes low- and middle-income countries, which account for more than 70% of all cancer deaths [2]. WHO estimates that the number of deaths and new cases will continue to rise, to 12.4 and 21.4 million in 2030, increases of 69 and 72% from 2008 [3]. Currently, one in four deaths in the United States is due to cancer [4]. According to the data from the American Cancer Society, a total of 1,479,350 new cancer cases and 562,340 deaths from cancer are projected to occur in the United States in 2009 [4]. Cancer is the leading cause of death in Thailand as well [5]. The estimated number of new cancer cases in Thailand in the year 1999 was 31,582 in men and 33,678 in women. These correspond to age-standardized rates of 127.7 per 100,000 in men and 125.5 per 100,000 in women [5], and tend to increase in every year. To date, more than 100 types of cancer have been identified with approximately 10 leading cancer type found worldwide, such as lung & bronchus, prostate, breast, colon, and rectum cancer. In childhood, cancer is the second most common cause of death among children between the ages of 1 and 14 years

in the United States, surpassed only by accidents [4]. The incidence of childhood malignancy in Thailand is lower in comparison to the Western and regional countries [4, 6]. Moreover, Leukemia is the most common form of cancer in children for most countries except in Africa [4].

Leukemia is characterized by abnormal proliferation of blood cells. The four major types of leukemia are acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML), and chronic myelogenous leukemia (CML). Although affecting approximately 10 times more adults than children, leukemia is the most common cancer among children [7]. The incidence and mortality rates for leukemia are higher in whites than in people of other racial and ethnic groups, and men are more likely to develop leukemia than women [7]. In 2010, WHO estimated that 260,000 deaths would be caused by leukemia, and 360,000 new leukemic cases would be diagnosed, accounted for 2.8% and 3.4%, respectively [8]. In the United States, 44,790 new cases and 21,870 deaths from leukemia were reported in 2009. The incidence of leukemia in Thailand is relatively low by world standards. It is the eighth most common cancer in males and tenth in females [9]. The age-standardized incidence rates (ASR) of leukemia were 3.9 per 100,000 population in males and 3.2 per 100,000 population in females from 1998 to 2000 [9]. The age-specific rates of leukemia showed higher incidence rate in males than in females. In United States and Thailand, leukemia is the most common childhood cancer, approximately 26% [7] and 53% [5], respectively, compared to other childhood cancers. ALL accounts for approximately 70% of all childhood leukemias in the United States [10] and 74% in Thailand [5]. The most

common type of leukemia in adults is AML, followed by CLL, CML, and ALL in the United States [10], whereas AML has the highest incidence in Thailand, followed by ALL, CML, and CLL [9]. Although the incidence of leukemia has increased continuously, rapid diagnosis and proper treatment can increase survival rates in patients. Leukemia, like other cancers, results from mutations in the DNA [11]. These mutations may activate oncogenes or deactivate tumor suppressor genes, which cause the regulation of cell death, differentiation or division to be disrupted. The causes of mutations associated with leukemia are incompletely understood. However, people exposed to radiation or carcinogenic substances, or viral diseases, or have genetic factors associated chromosomal abnormalities, may be at risk to develop leukemia [12]. In many cases of leukemia, mutation in or overexpression of oncogenes or tumor suppressor genes are useful molecular markers for diagnosis, monitoring disease progression, and providing important information for the prognosis of the disease. The *Wilms' tumor 1 (WT1)* gene is an interesting model oncogene that has been studied extensively and used as a biological marker of leukemia.

The *Wilms' tumor 1 (WT1)* gene was first described as a tumor suppressor gene in Wilms' tumor [13]. It is located at chromosome loci 11p13, and produces a protein that functions as a transcription factor [14]. The *WT1* gene is expressed in many organs during mammalian embryonic development, and is also expressed in specific organs in adults [15]. In hematopoiesis, The *WT1* mRNA is found in bone marrow at one level, but the expression in normal mature blood cells is very low, suggesting that the *WT1* protein plays a role in early blood cell production [16]. In leukemic cells, it has been

demonstrated that the *WT1* gene is expressed in K562 and HL60 leukemic cell lines and downregulation of the WT1 protein can force these cells to initiate the differentiation process [17]. High expression of *WT1* is also found in acute leukemia; in both acute myeloid and lymphoid leukemia expression levels are approximately 1,000 to 10,000 times higher than in bone marrow and normal peripheral blood cells, and heterozygous *WT1* mutations can be detected in approximately 10-15% of acute leukemia cases [18, 19], suggesting that the *WT1* gene plays an important role in leukemogenesis as an oncogene [20].

The *WT1* mRNA encodes a 52-54 kDa protein with a zinc-finger (DNA-binding) domain at the C-terminal and a transcriptional regulatory domain at the N-terminal; *WT1* is thus a transcription factor [13, 14]. *WT1* is a complex gene that encodes at least 24 isoforms, resulting from alternative splicing. The four major isoforms of *WT1* protein identified to date result from two alternative splice sites in its transcript. It may influence the proliferation and differentiation of blood cells by differential regulation of genes for *TGF β* , *C-Myc*, *C-Myb*, *BCl-2*, and *retinoic acid receptor (RAR)* [16]. The *WT1* protein is found to be overexpressed in leukemic cells, and it is involved in cell proliferation, differentiation and apoptosis [21]. Due to the high level of expression of the *WT1* gene and *WT1* protein associated with leukemia, they have been used as biological markers for diagnosis and evaluation of minimal residual disease (MRD) of leukemia [18].

Chemotherapy is generally effective and widely used for leukemia treatment. It can produce long-term remission or an outright cure for many people. However, chemotherapy acts by killing abnormally fast-dividing cells, and thus affects cells that

divide rapidly under normal circumstances, causing side effects. Therefore, naturally occurring plant compounds that have cancer inhibitory effects but cause fewer side effects are a valuable alternative treatment for leukemias.

Since 1984, the World Health Organization's (WHO) has promoted a traditional medicine program for the development, teaching, and application of analytical methods that can be used to evaluate the safety and efficacy of various elements of traditional medicine in developed and developing countries [22]. In recent years, simple synthetic modifications of naturally obtained substances such as plant extracts have been developed [23]. In addition to antioxidant activity, medicinal plant extracts may have anticancer activity. Various active compounds or their semi-synthetic derivatives derived from medicinal plants have been assessed for their efficacy and tolerability in the treatment of many types of cancer. Examples include vinblastine and vincristine from *Catharanthus roseus*, or paclitaxel and docetaxel from *Taxus baccata* [24]. Several medicinal plant extracts have been found to be cytotoxic to leukemic cells, including guava extract, and basil leaf extract [25].

Kaffir lime (*Citrus hystrix*, DC.) is a member of the citrus family. In Thailand, it is known as ma-krut. It originated in Southeast Asia and is commonly used in asian cuisine and folk medicine. Scientific studies found that kaffir lime leaf and fruit extracts have several biological activities, such as antioxidant [26], free radical scavenging [27], antimicrobial [26, 27] and anti-inflammatory activities [28]. In the field of cancers, glyceroglycolipid substances in kaffir lime leaf could inhibit 12-O-tetradecanoylphorbol 13-acetate (TPA), a skin carcinogen [29]. The essential oil from kaffir lime fruits and

leaves had anti-proliferative activity on KB (cervical cancer) and P388 (mice leukemia) cell lines [25], and the crude ethanolic extract of kaffir lime leaf was cytotoxic to human leukemic cell lines. However, there has been no study of the inhibitory effect of kaffir lime leaf fraction extracts on human leukemic cells and *WT1* gene and WT1 protein expression.

In this study, the cytotoxic effect and the effect on *WT1* gene and WT1 protein expression in K562, Molt4, U937, and HL60 leukemic cell lines of crude kaffir lime leaf fractional extracts were studied.

1.2 Literature review

1.2.1 Cancer

Cancer is responsible for one in eight deaths worldwide [30]. By 2020, the world population is expected to have increased to 7.5 billion; of this number, approximately 15 million new cancer cases will be diagnosed, and 12 million cancer patients will die [31]. Cancer encompasses more than 100 distinct diseases with diverse risk factors and epidemiology, and can originate from almost any cell type. Cancer is a genetic disease.

The many types of cancer are caused by cellular genome alterations in the DNA of a somatic cell. These genetic changes affect the expression or function of genes controlling cell growth and differentiation, resulting in uncontrollable cancer cell proliferation, and producing malignant tumors that can invade beyond normal tissue boundaries and metastasize to distant organs [32, 33].

1.2.1.1 Causes of cancer

Within the past several decades, researchers have divided the causes of cancer into two groups: those with an environmental cause and those with a hereditary genetic cause. Cancer is primarily an environmental disease, though genetics influence the risk of some cancers [34]. Common environmental factors leading to cancer include tobacco, diet and obesity, infections, radiation, lack of physical activity, and environmental pollutants [34]. These environmental factors cause or enhance abnormalities in the genetic material of cells [35]. Cell reproduction is an extremely complex process that is regulated by several classes of genes, including oncogenes and tumor suppressor genes. Hereditary or acquired abnormalities in these regulatory genes can lead to the development of cancer. The importance of lifestyle factors in the development of cancer is shown in studies of monozygotic twins [36]. Only 5–10% of all cancers are due to an inherited gene defect. Various cancers have been linked to genetic defects. Although all cancers are a result of multiple mutations [37, 38], these mutations are due to interaction with the environment [39]. The life style factors affecting the incidence and mortality of cancer include tobacco, alcohol, diet, obesity, infectious agents, environmental pollutants, and radiation, which have a profound influenced in cancer development [40].

1.2.1.2 Risk factors of cancer

1.2.1.2.1 Tobacco

Smoking was identified in 1964 as the primary cause of lung cancer in the US [41]. The use of tobacco increases the risk of developing at least 14 types of cancer such

as lung, laryngeal, oropharyngeal, pancreatic, and renal pelvic. In addition, smoking accounts for about 25–30% of all deaths from cancer, and 87% of deaths from lung cancer [39]. Tobacco contains at least 50 carcinogens; for example, benzo(a)pyrene, a tobacco metabolite, has a direct etiologic association with lung cancer [42]. How smoking contributes to cancer is not fully understood, but many studies suggest the relation between cigarette smoke and inflammation. Tobacco smoke can induce activation of NF- κ B, an inflammatory marker [43, 44]. Several natural phytochemicals also inhibit the NF- κ B induced by various carcinogens [45]. Curcumin, derived from turmeric, can block the NF- κ B induced by cigarette smoke [43].

1.2.1.2.2 Alcohol

The first report of an association between alcohol and an increased risk of esophageal cancer was published in 1910 [46]. Chronic alcohol consumption is a risk factor for oral cavity, pharyngeal, hypopharyngeal, laryngeal, and esophageal cancer, as well as liver, pancreatic, mouth, and breast cancer [34]. The risk of hepatocellular carcinoma (HCC) increases with a daily intake of more than 60 g. and is two times greater with the presence of HCV infection [47]. Alcohol is believed to play a role in carcinogenesis through two processes. First, ethanol produces the free radicals that are responsible for alcohol-associated carcinogenesis through their binding to DNA and proteins, which then destroys folate and results in secondary hyperproliferation. Other mechanisms are the induction of cytochrome P-450E1, which is associated with enhanced production of free radicals, and enhanced activation of various procarcinogens

present in alcoholic beverages [34]. In addition, alcohol can activate the NF- κ B proinflammatory pathway [48], which can also contribute to tumorigenesis [49].

1.2.1.2.3 Diets

In 1981, Doll and Peto (1981) estimated that approximately 30–35% of cancer deaths in the USA were linked to diet [50]. Heavy consumption of red meat is a risk factor for several cancers, especially for those of the gastrointestinal tract, but also for prostate [51], bladder [52], breast [53], and oral [54] cancers. The heterocyclic amines produced during the cooking of meat are carcinogens. Charcoal cooking and smoke curing of meat produces harmful carbon compounds having a strong cancerous effect as well [34]. Long-term exposure to food additives such as nitrite preservatives and azo dyes has been associated with the induction of carcinogenesis [55]. Saturated fatty acids, trans fatty acids, and refined sugars and flour have also been associated with various cancers. Several food carcinogens have been shown to activate inflammatory pathways.

1.2.1.2.4 Infectious agents

An estimated 17.8% of worldwide neoplasms are associated with infections [56]. Human papillomavirus, Epstein Barr virus, Kaposi's sarcoma associated herpes virus, human T-lymphotropic virus 1, HIV, HBV, and HCV are associated with risks for cervical cancer, anogenital cancer, skin cancer, nasopharyngeal cancer, Burkitt's lymphoma, Hodgkin's lymphoma, Kaposi's sarcoma, adult T-cell leukemia, B-cell lymphoma, and liver cancer [34].

Human papillomavirus is directly mutagenic by inducing the viral genes E6 and E7 (53), whereas HBV and HCV is indirectly mutagenic by generating reactive oxygen

species through chronic inflammation [57-60]. Human T-lymphotropic virus is directly mutagenic. However, other microorganisms, including selected parasites may also be involved, acting as cofactors and/or carcinogens such as *Helicobacter pylori* [61]. Infection-related inflammation is the major risk factor for cancer, and almost all viruses linked to cancer have been shown to activate the inflammatory marker, NF- κ B [62].

1.2.1.2.5 Environmental Pollution

Environmental pollution has been linked to various cancers. It includes outdoor air pollution by carbon particles associated with polycyclic aromatic hydrocarbons (PAHs); indoor air pollution by environmental tobacco, formaldehyde, and volatile organic compounds such as benzene and 1,3-butadiene; food pollution by food additives and by carcinogenic contaminants such as nitrates, pesticides, dioxins, and other organochlorines; carcinogenic metals and metalloids; pharmaceutical medicines; and cosmetics [63]. Numerous outdoor air pollutants such as PAHs increase the risk of cancers, especially lung cancer. Indoor air pollutants such as volatile organic compounds and pesticides increase the risk of childhood leukemia and lymphoma. Nitrates, in drinking water, can transform to mutagenic N-nitroso compounds, which increase the risk of lymphoma, leukemia, colorectal cancer, and bladder cancer [63].

1.2.1.2.6 Radiation

Up to 10% of total cancer cases may be induced by radiation [63], both ionizing and nonionizing, typically from radioactive substances and ultraviolet (UV), pulsed electromagnetic fields. Cancers induced by radiation include some types of leukemia, lymphoma, thyroid cancers, skin cancers, sarcomas, lung, and breast

carcinomas. Exposure to UV radiation is a major risk for various types of skin cancers including basal cell carcinoma, squamous cell carcinoma, and melanoma. An increased risk of cancers such as childhood leukemia, brain tumors and breast cancer has been attributed to electromagnetic field exposure [63].

1.2.1.3 Genetics of cancer

Tumorigenesis is a multistep process. It is characterized by progression of genetic alterations in a single line of cells that makes the cells increasingly less responsive to the body's normal tissues [68]. After they have become malignant, cancer cells continue to accumulate mutations that confer new properties on the cells. One of the first steps in the development of some malignant tumors is the formation of benign tumor, which is a tumor composed of cells that are no longer responsive to normal growth controls, but that lack the capability to invade normal tissue or metastasize to distant sites. Some benign tumors pose little chance to become malignant, but others, such as the benign polyps in the wall of the colon, are very likely to shift to the malignant state [68].

In tumor development, the genes implicated in carcinogenesis constitute a specific subset of the genome whose products are involved in the progression of a cell through the cell cycle, adhesion of a cell to its neighbors, apoptosis, and the repair of DNA damage.

Different genes tend to be mutated at different stages in the development of cancer [33].

The genes that have been implicated in carcinogenesis are divided into two broad categories: tumor-suppressor genes and oncogenes.

1.2.1.3.1 Tumor-suppressor genes

Tumor-suppressor genes encode proteins that restrain cell growth and prevent cell from becoming malignant [68]. The transformation of a normal cell to a cancer cell is accompanied by the loss of function of one or more tumor-suppressor genes. The genes that have been implicated as tumor suppressors in humans are those that encode transcription factors (*p53*, *WT1*), cell-cycle regulators (*RB*, *p16*), and signaling pathway regulators (*NF1*) [68] (Table 1). Most of the proteins encoded by tumor-suppressor genes act as negative regulators of cell proliferation, which is the elimination of uncontrolled cell growth. Some of these genes are related to the development of various cancers, whereas others play a role in the formation of one or a few cancer types [68].

1.2.1.3.2 Oncogenes

Oncogenes, on the other hand, encode proteins that promote the loss of growth control and the conversion of a cell to a malignant state [68]. As a result, oncogenes act as accelerators of cell proliferation and tumorigenesis. Proto-oncogenes which encode proteins that have various functions in normal cells can be converted into oncogenes by several mechanisms [68]:

- 1.) The gene can be mutated in a way that alters the properties of the gene product so that it can no longer carry out of the normal activity.
- 2.) A mutation in a nearby regulatory sequence can alter the expression of the gene, so that an excessive quantity of the gene product is produced.

3.) A chromosome rearrangement can occur that brings a DNA sequence from a distant site in the genome into close proximity of the gene, which can either alter the expression of the gene or the nature of the gene product.

Most known proto-oncogenes play a role in the control of cell growth, including growth stimulation by external ligands, cell signal transduction, or progression through the cell cycle. Approximately 100 different oncogenes have been identified (Table 2). Any of these genetic alterations can cause a cell to become less responsive to normal growth controls, causing it to behave as a malignant cell [63].

The development of a human malignancy requires more than a single genetic alteration. Most tumors contain alterations in both tumor-suppressor genes and oncogenes, suggesting that the loss of tumor-suppressor function within a cell must be accompanied by the conversion of a proto-oncogene into an oncogene before the cell become malignant. Mutations in additional genes, such as those encoding cell adhesion molecules or extracellular protease, may be required before these cells acquire the full life-threatening phenotype. In general, the numbers of altered genes increase along with a progression in the malignant virulence of the tumor. Studies of colorectal carcinomas indicate, for example, that mutation in as many as seven different genes may be necessary for the development of the fully malignant tumor [63].

1.2.2 Leukemia

Leukemia, cancer of the blood, is a hematological malignancy. It was first described by the German pathologist Rudolf Virchow and John Bennett in 1845 [64]. The

Table1 Tumor-suppressor genes [63]

Gene	Primary tumor	Proposed function	Inherited syndrome
<i>APC</i>	Colorectal	Binds β -catenin acting as transcription factor	Familial adenomatous polyposis
<i>BRCA1</i>	Breast	Transcription factor, DNA repair	Familial breast cancer
<i>MSH2, MLH1</i>	Colorectal	Mismatch repair	HNPCC
<i>E-Cadherin</i>	Breast, colon, etc.	Cell adhesion molecule	Familial gastric cancer
<i>INM4a</i>	Melanoma, pancreatic	p16:Cdk inhibitor p19 ^{ARF} : stabilizer p53	Familial melanoma
<i>NF1</i>	Neurofiromas	Activates GTPase of RAS	Neurofibromatosis type 1
<i>NF2</i>	Meningiomas	Link membrane to cytoskeleton	Neurofibromatosis type 2
<i>P16(MTS1)</i>	Melanoma	Cdk inhibitor	Familial melanoma
<i>P53</i>	Sacromas, lymphomas, etc.	Transcription factor (cell cycle and apoptosis)	Li-Fraumeni syndrome
<i>PTEN</i>	Breast, thyroid	PIP ₃ phopphatase	Cowden disease
<i>RB</i>	Retinal	Binds E2F (cell cycle transcript regulation)	Retinoblastoma
<i>VHL</i>	Kidney	Regulates RNA pol II elongation	won Hippel-Lindau syndrome
<i>WT1</i>	Wilms' tumor of kidney	Transcription factor	Wilms' tumor

Table 2 Proto-oncogenes and human tumors: some consistent in crinations [63]

Proto-onogene	Neoplasm(s)	Lesion
<i>Abl</i>	Chronic myelogenous leukemia	Translocation
<i>bcl-2</i>	B-cell lymphoma	Translocation
<i>CYCD1</i>	Carcinoma of breast	Translocation
<i>Cdk4</i>	Sarcoma	Amplification
<i>erbB</i>	Squamous cell carcinoma; astrocytoma	Amplification
<i>neu/HER2</i>	Adenocarcinoma of breast, ovary, and stomach	Amplification
<i>Gip</i>	Carcinoma of ovary and adrenal gland	Point mutation
<i>Gsp</i>	Adenoma of pituitary gland; carcinoma of thyroid	Point mutation
<i>Myc</i>	Burkitt's lymphoma Carcinoma of lung, breast, and cervix	Translocation Amplification
<i>L-myc</i>	Carcinoma of lung	Amplification
<i>N-myc</i>	Neuroblastoma, small cell carcinoma of lung	Amplification
<i>H-ras</i>	Carcinoma of colon, lung, and pancreas; melanoma	Point mutation
<i>K-ras</i>	Acute myeloid and lymphoblastic leukemia; carcinoma of thyroid; melanoma	Point mutation
<i>N-ras</i>	Carcinoma of genitourinary tract and thyroid; melanoma	Point mutation
<i>Ret</i>	Carcinoma of thyroid	Rearrangement
<i>Trk</i>	Carcinoma of thyroid	Rearrangement

disease results from the neoplastic proliferation of hemopoietic or lymphoid cells. The cell in which the leukemic transformation occurs may be a precursor or pluripotential hemopoietic stem cell that can differentiate into both myeloid and lymphoid cells. Myeloid leukemia can arise in a lineage-restricted cell, in a multipotential stem cell capable of differentiating into cell of erythroid, granulocytic, monocytic, and megakaryocytic lineages or in a pluripotential lymphoid-myeloid stem cell [65]. This results in decreased production of normal blood cells. Leukemia can spread to other organs such as lymph nodes, spleen, liver, and central nervous system, and cause pathogenesis in these organs.

1.2.2.1 Causes of leukemia

Leukemia generally involves a series of genetic alterations rather than a single event. Genetic events contributing to malignant transformation include inappropriate expression of oncogenes and loss of function of tumor suppressor genes. Oncogenes may originate from mutation or dysregulation of normal cellular genes (proto-oncogenes), or may be novel hybrid genes resulting from fusion of parts of two genes. Genetic alterations leading to leukemic transformation often result from major alterations in the chromosomes and other changes, such as point mutations or partial duplications [65].

These mutations may occur spontaneously or as a result of exposure to radiation or carcinogenic substances, and are likely to be influenced by genetic factors.

1.2.2.2 Risk factors of leukemogenesis

1.2.2.2.1 Constitutional risk factors

A) Population-based factors

In most cases, the underlying mechanisms of population-based factors predisposing one to hematopoietic neoplasia remain obscure. Gender is important risk factor. Females are overrepresented among neonates and infants with acute leukemia, especially leukemia with *mixed-lineage leukemia (MLL)* rearrangement [65]. Males have more excess of cancer of the lymphatic and hematopoietic system than females [66]. Particular HLA variants predispose to or protect against hematologic malignancy [67, 68]. Moreover, a familial disease may appear at a progressively earlier age and run an unusually rapid course, especially when acquired risk factors are superimposed [69].

B) Genetic factors

Constitutional autosomal anomalies are known to predispose to hematopoietic neoplasia. Many resemble the genetic anomalies commonly seen as acquired anomalies in hematopoietic malignancy. Karyotypic abnormalities have been found associated with hematopoietic malignancy, involving almost one-half of the somatic chromosomes and both sex chromosomes [70, 71]. Inherited mutations and polymorphisms of several genes are known to confer an increased risk of hematopoietic malignancy. The involved genes include those encoding *N*-acetyl transferase, *AML 1*, *NF*, *p53*, and others [72-74].

C) Chromosomal instability and bone marrow failure syndromes

The chromosomal instability syndromes are characterized by an inability to synthesize and/or repair DNA correctly. Fanconi's anemia carries an increased risk of

clonal myeloid and lymphoid disease, as well as therapy-related malignancy. Bloom's syndrome, Griscelli's Syndrome, and Werner's syndrome are other rare chromosomal instability disorders with a propensity to develop acute leukemia.

The bone marrow failure syndromes are characterized by the early onset of cytopenias and an increased risk of acute leukemia. In some cases, the underlying genetic defect has been discovered. These disorders include congenital hypoplastic anemia, familial sideroblastic anemia, severe congenital neutropenia, and others.

D) Dysmorphic syndromes

Dysmorphism or congenital anomalies, which may be attributable to either constitutional or acquire factors, are associated with an incidence of hematopoietic malignancy. Specific dysmorphic disorders reported with hematology neoplasia include Adam-Oliver syndrome, cardiac anomalies, cleft lip and palate, Dubowitz's syndrome, familial microcephaly, Marfan's syndromes, Noonan's syndrome, and others.

E) Other constitutional hematopoietic disorders

Preexisting constitutional hematopoietic disorders are present in some patients with hematopoietic neoplasia. It is tempting to speculate that chronic stimulation of hematopoiesis is the unifying characteristic in these disorders. Possibly the constant demand for stem cell renewal creates an increased opportunity for the genetic aberrations that initiate clonal disease.

Hemoglobinopathies reported in patients with hematopoietic neoplasia include β -thalassemia, hemoglobin S, and others [75, 76]. Other hematopoietic disorders reported with hematologic neoplasia include glucose-6-phosphate dehydrogenase deficiency,

hemophilia, hereditary spherocytosis, platelet storage pool deficiency, and pyruvate kinase deficiency. Moreover, cystic fibrosis may also predispose patients to acute leukemia [77].

1.2.2.2 Acquired risk factors

A) Environment factors

The environment contains numerous risk factors for hematologic neoplasia. Many of these substances are found in the workplace, contributing to an increased risk of leukemia in some occupations. Environment agents and substances implicated in hematopoietic oncogenesis include arsenic, cadmium, tri-halomethanes, chloroform, zinc, asbestos and related minerals, benzene, paints, petroleum products, organic solvents, pesticides and other agricultural chemicals, radiation from solar and earth sources, nuclear reactors and weapons, therapeutic devices, ethanol, and tobacco.

B) Acquired hematopoietic diseases

As in the case of constitutional hematopoietic disorders, it is tempting to speculate that acquired hematopoietic disorders require an increased, long-term demand for hematopoietic stem cells and thus increase the chance of a genetic event leading to neoplasia. In some cases, increases in hematopoietic stem cells and clonal emergence caused by increased cell cycling have been documented. Hematopoietic disorders, which appear to carry increased risk of leukemia, include aplastic anemia, pure red cell aplasia, immune-mediated thrombocytopenia, and pernicious anemia.

C) Infectious and immune-related factors

Infectious and immune disorders have long been implicated in leukemogenesis. Infectious agents associated with an increased risk of hematologic neoplasia include cytomegalovirus, Epstein-Barr virus, dengue virus, hepatitis virus, human herpes virus 6, human immunodeficiency virus (HIV), human T lymphotropic virus type I, influenza virus, Parvovirus B19, rubella virus, and varicella virus. Hypoimmune states are associated with an increased risk of leukemia. Such states include Kawasaki's disease, HIV infection, and tonsillectomy.

D) Medical interventions

Numerous therapies have been implicated in leukemogenesis. Antibiotic and anti-inflammatory agents have occasionally been implicated in leukemogenesis [78]. In most cases, these drugs are no longer commonly used. Antineoplastic agents are well-known carcinogens and are especially potent when used in patients with constitutional risk factors and those with leukemia and lymphoma [79].

Granulocyte and granulocyte-monocyte-colony-stimulating factors are associated with the onset and rapid progression of leukemia. Caution must be used in making a diagnosis of high-grade acute leukemia in patients on growth factors because clonal expansion may reverse upon drug withdrawal.

Other medical interventions involved in leukemogenesis are UV light therapy, radiotherapy, immunosuppressive agents, and stem cell transplantation. For stem cell transplantation, leukemia usually arises in host cells, but may also occur in donor cells [80].

E) Pregnancy and birth-related factors

Abundant evidence indicates that childhood leukemia arises in the uterus, likely from intrauterine exposure to environment risk factors. It is often difficult to separate intrauterine from other risk factors. Parental risk factors for childhood leukemia include exposure to environmental carcinogens, maternal age younger than 21 or older than 40 years, and maternal consumption of dietary bioflavonoids [81]. Nonparental risk factors include birth weight less than 2,500 units or more than 4,000 g, delivery by cesarean section, multiple birth, and low neonatal serum thyroid-stimulating hormone level.

F) Other acquired risk factors

Acute leukemia in the elderly resembles that seen in patients with therapy and toxin induced disease. In addition, increased body weight may be directly related to the risk of acute leukemia in adults, as it is in newborns.

Other acquired risk factors involved in leukemogenesis are connective tissue diseases, endocrine disorders, hormones and potent hematopoietic growth factors, inflammatory bowel disease, renal disorders, and other tumors.

1.2.2.3 Genes involved in leukemia

Approximately 50% of de novo acute leukemias have distinctive molecular abnormalities, most frequently chromosomal translocations. These translocations typically involve genes that are involved in transcription and differentiation. According to most translocations, they usually occur in chronic leukemia and malignant lymphomas and affect proto-oncogenes. Proto-oncogenes are constitutively expressed by influence of the

antigen-receptor gene enhancers or promoters, resulting in overexpression of normal protein. Other molecular mechanisms such as point mutations, gene deletions, and methylation have been implicated in leukemogenesis [65].

There are many genes that are involved in leukemogenesis. As a result of chromosome translocations, many types of fusion genes occur such as the *PML-RAR α* and *bcr-abl* fusion genes [82, 83]. The *PML-RAR α* fusion gene plays a role in neoplastic transformation and inhibits myeloid differentiation, found in AML. Other examples of fusion genes in AML are *AML1-ETO (RUNX1-MTG8)*, *HOX* fusion genes [84]. The *bcr-abl* fusion gene or Philadelphia chromosome play a role in CML. Moreover, there are many genetic alterations found in AML including internal tandem duplication of the *FLT3* gene, mutations in the *NPM1* gene, partial tandem duplication of the *MLL* gene, high expression of the *BAALC* gene, and mutations in the *CEBPA* gene [85]. Distinct expression profiles for ALL include *T-ALL*, *E2A-PBX1*, *BCR-ABL*, *TEL-AML1*, and *MLL* rearrangement [86].

In addition, mutations of proto-oncogenes or tumor suppressor genes commonly have been identified in leukemia. Genes that are usually affected in AML and ALL include *c-ras* gene family, *Rbl*, and *p53* [87]. Another gene involved in leukemia is *Wilms' tumor 1 (WT1)* [20].

1.2.2.4 Classifications of leukemia

Leukemias are broadly divided into: (i) acute leukemia, which if untreated, leads to death in weeks or months; and (ii) chronic leukemia, which if untreated, leads to death

in months or years. They are further divided into lymphoid and myeloid leukemia. Thus four types of leukemia are acute myeloblastic leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelocytic leukemia (CML), and chronic lymphoblastic leukemia (CLL). Acute leukemia is characterized by a defect in maturation, leading to an imbalance between proliferation and maturation; since cells of the leukemic clone continue to proliferate without maturing to end cells and dying there is continued expansion of the leukemic clone and immature cells predominate. Chronic leukemia is characterized by an expanded pool of proliferating cells that retain their capacity to differentiate to end cells [65].

The clinical manifestations of the leukemias are due directly or indirectly, to the proliferation of leukemic cells and their infiltration into normal tissues. Increased cell proliferation has metabolic consequences and infiltrating cells also disturb tissue function. Anemia, neutropenia and thrombocytopenia are important consequences of infiltration of the bone marrow, which in turn can lead to infection and hemorrhage [65].

1.2.2.4.1 Acute myeloblastic leukemia (AML)

AML is the result of a sequence of somatic mutations in multipotential primitive cells, or in some cases more differentiated progenitor cells. The neoplastic proliferation seen in AML consists of myeloblasts or partially differentiated myeloid cells. Approximately 11,000 new cases of AML occur annually, representing in approximately 35% of new leukemia cases in the USA. The mortality rate from AML is approximately 0.5 per 100,000 persons younger than 10 years and increases progressively to 20 per

100,000 persons in their 90s. AML accounts for 15 to 20% of acute leukemia in childhood and 80% of acute leukemia in adults [88]. It is slightly more common in males and has high incidence in Europeans and Africans.

Anemia is constant feature of AML. Thrombocytopenia is nearly always present. More than half of patients have a platelet count less than 50,000/ μL at the time of diagnosis [88]. The total leukocyte count is less than 5,000 cells/ μL , and the absolute neutrophils count is less than 1,000 cells/ μL , in more than half of cases. Myeloblast always presents in blood but may be infrequent in leukopenic patients. Signs and symptoms signaling the onset of AML include pallor, fatigue, weakness, easy bruising, hemorrhage, and fever. Palpable splenomegaly and hepatomegaly occurs in approximately one third of patients. Lymphadenopathy is uncommon, except in the monocytic variant of AML [88].

1.2.2.4.1.1 Prognosis

Acute myeloid leukemia is a curable disease. Age at presentation and diagnostic karyotype are among the most important independent prognostic factors in adult patients with AML [89]. AML occurring in adults older than 55 years of age is associated with an extremely poor prognosis with an overall survival (OS) of less than 20% at 5 year [90, 91]. A number of previous studies have identified diagnostic cytogenetics as a key determinant of outcome in AML [92, 93]. The largest study to date, which considered 1,612 children and younger adults (mostly younger than 55 years) from the UK Medical Research Council (MRC) AML10 trial, defined 3 cytogenetic prognostic groups that predicted response to induction therapy, relapse risk (RR), and OS. Patients with

t(8;21)(q22;q22), t(15;17)(q22;q21), or inv(16)(p13q22) were found to have a relatively favorable prognosis; in the absence of these changes, the presence of a complex karyotype, monosomies of chromosome 5 or 7 (-5, -7), deletions of the long arm of chromosome del(5q), or 3q abnormalities predict an adverse prognosis. The remaining patients, including those with normal karyotype or structural or numerical changes not encompassed by the favorable or adverse risk groups, have an intermediate prognosis [94]. About half of AML patients have normal cytogenetics; these fall into an intermediate risk group. A number of other cytogenetic abnormalities are known to associate with a poor prognosis and a high risk of relapse after treatment [95, 96]. The cytogenetics and prognosis of AML patients younger and older than 60 years of age are shown in Tables 3 and 4, respectively [94, 95].

A recent published report suggests that adult AML patients diagnosed with a distinct group of cytogenetic abnormalities, which are multiple autosomal monosomies or a single autosomal monosomy in the presence of one of more structural abnormalities, defined a new subgroup of cytogenetic abnormalities or monosomy karyotype (MK), associated with an unfavorable prognosis [96, 97]. Moreover, FLT3 internal tandem duplications (ITDs) [98] and elevated lactate dehydrogenase levels have been shown to confer a poor prognosis in AML [99].

Table 3 Survival and relapse risk by cytogenetic abnormalities in AML patients under 60 years of age [94]

Risk	Category abnormality	5-year survival (%)	Relapse rate (%)
Favorable	t(8;21), t(15;17), inv(16)	70	33
Intermediate	Normal,+8, +21, +22, del(7q), del(9q), Abnormal 11q23, all other chromosomal changes	48	50
Adverse	-5, -7, del(5q), Abnormal 3q, Complex cytogenetics	15	78

Table 4 Survival and relapse risk by cytogenetic abnormalities in AML patients over 60 years of age [95]

Risk	Category abnormality	5-year survival (%)	Relapse rate (%)
Favorable	t(15;17)	38	26
	t(8;21)	35	84
	inv(16)	17	89
Intermediate	No abnormality	5	95
	Sole +8	15	78
	11q23*	0	100
	Other intermediate	11	84
Adverse	Noncomplex adverse	7	81
	Complex (with no favorable)	2	91

1.2.2.4.1.2 The FAB categories and other morphological categories of acute myeloid leukemia

One of the criteria for AML diagnosis is FAB classification. The FAB group suggested that this be based on a peripheral blood differential count and a 500-cell bone marrow differential count, supplemented when necessary by cytochemistry and immunophenotyping; with the greater availability of immunophenotyping. AML is categorized as acute myeloblastic leukemia without (M1) and with (M2) maturation, acute hypergranular promyelocytic leukemia and its variant (M3 and M3V), acute myelomonocytic leukemia (M4), acute monoblastic (M5a) and monocytic (M5b) leukemia, acute erythroleukemia (M6), and acute megakaryoblastic leukemia (M7). M0 is AML without maturation and with minimal evidence of myeloid differentiation [100]. Among the various cytochemical reactions which could help to distinguish different leukemia cell types, five cytoenzymatic reactions have been selected, namely myeloperoxidase (MPO), alkaline phosphatase (APL), acid phosphatase (ACP), non-specific esterase (NSE), and naphthol AS-D chloroacetate esterase (NASDCA). In addition the results from the Sudan black B (SB) and period acid-schiff (PAS) reactions are mentioned [101]. Immunophenotyping is indicated in all cases of acute leukemia that are not obviously myeloid, in order to recognize all cases of FAB M0 and M7 AML. A possible further indication is the recognition of an immunophenotype that is likely to indicate a specific subtype of acute leukemia or that will be useful for subsequent monitoring of minimal residual disease.

A) Acute myeloid leukemia with minimal evidence of myeloid differentiation (M0 AML)

The blasts in M0 AML usually resemble M1 myeloblasts or L2 lymphoblasts. In a minority of cases they resemble the monoblasts of M5 AML. Associated dysplastic features in erythroid and megakaryocyte lineages may provide indirect evidence that a leukemia is myeloid not lymphoid. Dysplastic features are present in up to a quarter of cases. For the diagnosis of acute myeloid leukemia of M0 AML, blasts should be found in more than 30% of bone marrow nucleated and non-erythroid cells. Blasts are demonstrated to be myeloblasts by immunological markers.

Immunophenotyping is now widely used for identifying cases of M0 AML. Immunophenotyping shows that the most specific lymphoid markers, CD3 and CD22, are not expressed in M0 AML. However, there may be expression of less specific lymphoid-associated antigens such as CD2, CD4, CD7, CD10, and CD19, in addition to CD34, human leukocyte antigen DR (HLA-DR) and terminal deoxynucleotidyl transferase (TdT). CD7 is more often expressed than in other FAB categories of AML [102].

Cytochemical reactions defining M0 acute myeloid leukemia are fewer than 3% of blasts being positive for MPO, SBB and chloroacetate esterase (CAE). Similarly, blast cells do not show NSE activity.

M0 AML has been associated with older age, higher WBC, and adverse cytogenetic abnormalities. The molecular genetic abnormalities recognized include a high incidence of mutations of the *RUNX1 (AML1)* gene [65]. In children M0 AML has been

associated with a lower WBC, more frequent occurrence of -5/del(5q), and +21, and more frequent hypodiploidy [103].

B) Acute myeloid leukemia without maturation (M1 AML)

M1 blasts are usually medium to large in size, a round or oval nucleus, one or more nucleoli, and cytoplasm that sometimes contains Auer rods. For the diagnosis of acute myeloid leukemia of M1 AML, blasts must be $\geq 30\%$ of bone marrow cells and $\geq 90\%$ of bone marrow non-erythroid cells. Promonocytes to monocytes should be $\leq 10\%$ of non-erythroid cells. Bone marrow maturing granulocytic component (promyelocytes to polymorphonuclear leukocytes) should be $\leq 10\%$ of non-erythroid cells. Lymphocytes, plasma cells, macrophages, and mast cells are also excluded from the count. Auer rods may be found in M1 AML. In children, the presence of Auer rods is associated with a better prognosis [104]. The M1 category accounts for 15-20% of AML cases.

M1 AML blasts are usually positive for CD13, CD33, and HLA-DR and may express CD34. The translocations associated with M1 AML are t(9;22) and t(11;19).

Cytochemical reactions in M1 AML are defined by a minimum of 3% of blasts being positive for MPO or SBB. M1 blasts are sometimes positive for CAE. In the case of α -naphthyl butyrate esterase (ANBE) the reaction is usually negative, whereas in the cases of naphthol AS-D acetate esterase (NASDA) there is usually a weak fluoride-resistant reaction. The PAS reaction is usually negative. Auer rods give positive MPO and SBB reaction, and occasional weak positive for PAS. The reaction for CAE is usually weak or negative

C) Acute myeloid leukemia with maturation (M2 AML)

In the criteria for M2 AML, blasts should be 30-89% of bone marrow cells. Bone marrow maturing granulocytic components (promyelocytes to polymorphonuclear leucocytes) are more than 10% of non-erythroid cells. Bone marrow monocytic components (monoblasts to monocytes) are less than 20% of non-erythroid cells.

Auer rods may be present. This form has been associated with a better prognosis in children [104], probably because of the association between Auer rods and t(8;21). Dysplastic features, such as hypo- or hypergranularity abnormalities of nuclear shape, are common in the differentiating granulocytic component of M2 AML.

Maturation of myeloblasts to promyelocytes occurs in both M2 and M3 AML, and promyelocytes are prominent in some cases of M2 AML. M2 AML is distinguished from M4 AML by the monocytic component in the bone marrow being less than 20% of non-erythroid cells and the lack of evidence of monocytic differentiation. In a minority of cases of M2 AML, eosinophilic or basophilic maturation occurs. Such cases may be designated M2Eo or M2Baso. The M2 subtype accounts for about 30% of cases of AML.

Myeloblasts of M2 AML always express HLA-DR, CD13, CD15, and CD33.

Expression of CD34 and CD117 are infrequently found.

Cytochemical reactions in M2 AML are the same as those in M1 AML, but generally reactions are stronger and involve a higher percentage of MPO and SBB stain positive cells. CAE is more often positive in M2 than M1, and Auer rods show the same staining characteristics as in M1 AML but are more numerous.

Table 5 The FAB classification of acute myeloblastic leukemia

M0	Myeloblastic without maturation or Undifferentiated leukemia Blasts > 90% (of nonerythroid cells) and conventional cytochemistry negative
M1	Myeloblastic with minimal maturation Blasts > 90% (of nonerythroid cells) and promyelocytes < 10%
M2	Myeloblastic with maturation Blasts 30-89% of nonerythroid cells; Granulocytic component > 10% and Monocytic component < 20%
M3	Promyelocytic Abnormal promyelocytes with heavy granulation and some with multiple Auer rods (faggots). Typical reniform or bilobed nucleus
M3 variant	Promyelocytic with usual hypogranular form
M4	Myelomonocytic Blasts > 30% , Monocytic component > 20% Monocytic + granulocytic component > 30% but < 80%
M4eo	M4 with eosinophilia > 5% Abnormal eosinophils with basophilic granules
M4baso	M4 with basophil maturation
M5a	Monoblastic Monoblast > 80%, granulocytic component < 20%
M5b	Promonocytic Monoblast < 80%, granulocytic component < 20%
M6	Erythroleukemia Erythropoetic component > 50%; Blasts of nonerythroid cells > 30%
M7	Megakaryoblastic Megakaryoblasts > 30%

D) Acute promyelocytic leukemia (M3 AML or APL)

Promyelocytes are the predominant immature cells in M3 AML. Myeloblasts are also increased, but not to the extent of promyelocytes. M3 AML is divided into two major morphologic subtypes: hypergranular and hypogranular. The hypergranular variant is characterized by the presence of atypical promyelocytes heavily loaded with azurophilic granules. Auer rods are often present and may appear in bundles in some of the promyelocytes (faggot cells). In the hypogranular variant, promyelocytes contain fewer and finer azurophilic granules. The nuclei are often lobulated, folded or convoluted, displaying a monocyte-like morphology. Auer rods are often present.

The leukemia cells in M3 AML are strongly MPO and SBB positive. A small proportion of cases may also demonstrate NSE reactivity. Moreover, CD13, CD33, CD11, and CD15 antigens are often expressed but HLA-DR and CD14 are negative.

The cytogenetic abnormality associated with M3 AML is t(15;17), which has been observed in over 90% of the cases. This translocation puts two genes, *PML* and *retinoic acid receptor- α* (*RAR- α*), together with a hybrid protein product that appears to block the myeloid differentiation process. Rare cases of M3 AML are associated with t(11;17).

E) Acute myelomonocytic leukemia (M4 AML or AMML)

In AMML, myeloblasts, monoblasts, and promonocytes are the predominant immature cells. Therefore, there is considerable polymorphism in cell size, nuclear shape, and the amount of cytoplasm. The proportion of the monocytic component in the leukemic population ranges from more than 20% to less than 80%. Auer rods may be

present but are infrequent. Patients with AMML often demonstrate an absolute peripheral blood monocytosis with the presence of immature forms .

Immunophenotypic studies reveal expression of a combination of granulocytic and monocytic markers such as CD11c, CD13, CD14, CD15, CD33, CD64, CD68, and HLA-DR. The leukemic cells in some cases may also express CD2, CD4 or TdT.

The most frequent cytogenetic findings in AMML are t(6;9) and 11q23 abnormalities. Approximately 15% to 30% of the cases are associated with atypical eosinophilia and abnormality of chromosome 16 (16q22). The eosinophils in this subtype (M4Eo AML) contain a mixture of eosinophilic or basophilic granules. The M4Eo subtype has a more favorable clinical outcome, but has a higher frequency of CNS involvement.

F) Acute monoblastic leukemia (M5 AML or AMoL)

AMoL accounts for about 10% of all AML cases. It is divided into two subtypes: M5a and M5b.

M5a demonstrates minimal morphological evidence of monocytic differentiation. Monoblasts account for 80% or more of the leukemic cells. They have a variable amount of gray-blue or deep blue cytoplasm, often round or oval nuclei and a single or a few very prominent nuclei.

M5b displays partial monocytic differentiation with a mixture of monoblasts, promyelocytes and more mature monocytic cells. Promonocytes are characterized but abundant pale blue cytoplasm, scattered azurophilic granules, folded or lobulated nuclei,

and finely dispersed chromatin. Nucleoli are usually prominent. Auer rods are detected in a small proportion of the M5b subtype.

Leukemic cells are usually strongly positive for NSE and lysozyme and express all or some of the monocytic-associated markers such as CD14, CD64, and CD68. They are often positive for CD4, CD11c, CD33, and HLA-DR, and may also express CD34. Abnormalities of chromosome 11(22q23) and trisomy 8 are the most frequent cytogenetic findings.

G) Acute erythroid leukemia (M6 AML)

Most M6 AML cases are preceded by a refractory anemia. Diagnosis of M6 AML is established based on the following bone marrow findings: (a) a reversed myeloid: erythroid (M:E) ratio (more than 50% of the bone marrow cells are erythroid); and (b) 30% or more of the nonerythroid marrow cells are myeloblasts. The erythroid lineage shows marked dysplasia, such as the presence of bi- or multinucleated erythroblasts, giant forms and cells with nuclear fragments. Megaloblastic erythropoiesis is common. An increased number of megakaryoblasts may be present. Blood smears often show abnormal red blood cell (RBC) morphology and marked anisopoikilocytosis. The de novo AML-M6 accounts for about 5% of all AML cases.

The erythroid precursors express glycophorin A, transferrin receptor (CD71), hemoglobin and spectrin, and may show chunk-like cytoplasmic PAS positivity. Myeloblasts are MPO and SBB positive and express CD13, CD33, HLA-DR, and sometimes CD34. Cytogenetic studies may demonstrate structural abnormalities in chromosome 3, 5, and 7 or trisomy 8.

H) Acute megakaryoblastic leukemia (M7 AML)

The cell population in M7 AML is predominantly megakaryoblasts. They are polymorphic and vary in size, amount of cytoplasm, chromatin density, and the number of nuclei. Bone marrow fibrosis, as a consequence of unsuccessful marrow aspiration, is one of the characteristic features of M7 AML observed in over 70% of cases. Megakaryoblasts often appear in clusters trapped within the fibrotic tissue. M7 AML comprises about 5% of all AML cases but is probably the most common type of AML associated with Down's syndrome.

Megakaryoblasts and immature megakaryocytes express CD41, CD42, and CD61, and are positive for factor VIII. They do not express MPO and SBB but demonstrate platelet peroxidase (PPD) activity by immunoelectron microscopy and may show dot-like NSE positivity.

Trisomy 21 and t(1;22) have been reported in *de novo* AML-M7. Moreover, trisomy 8 and structural abnormalities of chromosomes 5 and 7 have been found in therapy-related leukemia.

1.2.2.4.2 Acute lymphoblastic leukemia (ALL)

Acute lymphoblastic leukemia (ALL) is primarily a disease of childhood; most cases occur between the ages of 2 and 10 years. Although ALL is rare in adults, a second peak in incidence occurs around age 40. Treatment of childhood ALL is a triumph of modern hematology. A uniformly fatal disease before 1970, "good-prognosis" ALL has a 90% complete remission rate and 60% of patients are cured. Adults are found to have a

poorer outlook: a 68% to 91% rate of complete remission and a 25% to 41% cure rate. Only half of patients with ALL have leukocytosis, and many may not have circulating lymphoblasts. Neutropenia, thrombocytopenia, and anemia are usually present, leading to fatigue, fever, and mucocutaneous bleeding. Lymphadenopathy, lymph node enlargement, is often a symptom, and a mediastinal mass is present in 5% to 10% of patients. Enlargement of the spleen (splenomegaly) and of the liver (hepatomegaly) may be seen. Bone pain often results from infiltration of leukemic cells into the bone covering (periosteum) [105].

1.2.2.4.2.1 Prognosis

The prognosis for ALL depends on age at the time of diagnosis, lymphoblast burden, and immunophenotype. Chromosomal translocations are the strongest predictor of adverse treatment outcomes for children and adults. The presence of the Philadelphia chromosome, t(9;22), is an indicator of significant adverse effects. The t(12;21) marker is found in many childhood ALL patients. Moreover, t(12;21)(p13;q22) and t(1;19)(q23;p13.3) in B-ALL and del(1)(p32p32) or del(1)(p32p36) and t(7;11)(q35;p13) in B-ALL are translocations in ALL patients [106].

Prognosis varies with age; children rather than infants or teens do the best. Peripheral blood lymphoblast counts greater than 20 to 30 x 10⁹ cells/L, hepatosplenomegaly, and lymphadenopathy all adversely affect the outcome. T cell and mature B cell immunophenotypes are associated with a poor prognosis in children and adults, in contrast to the immature B cell phenotypes. In addition, the presence of an aberrant myeloid surface marker found in ALL (such as CD66) is associated with a poor

prognosis in adults. Other variables possibly associated with poorer prognosis are race (Native Americans have a worse prognosis than Caucasians), the presence of karyotypic abnormalities, and male sex.

1.2.2.4.2.2 The FAB categories and other morphological categories of acute lymphoblastic leukemia

Initially, acute lymphoblastic leukemia is largely a diagnosis of exclusion. Although some cases have characteristic cytological features, others are categorized as “lymphoid” only because they do not show any definite cytological or cytochemical evidence of myeloid differentiation. With the availability of a wide range of monoclonal antibodies directed at antigens expressed on lymphoid cells, the diagnosis of ALL is now based on positive criteria. ALL is classified broadly as B lineage and T lineage. B-lineage ALL includes a small minority of cases with the immunophenotypic features of mature B cells (regarded as non-Hodgkin lymphoma rather than as ALL in the WHO classification) and a large majority of cases with the immunophenotype of B-cell precursors. The latter group includes a major subset designated common ALL, expressing a surface membrane antigen known as the common ALL antigen or CD10 [100]. The surface membrane antigens found in B-lineage ALL include CD 10, CD 19, CD 20, CD 22, CD 24, CD 79a, and CD 79b. The surface membrane antigens found in T-lineage ALL include CD1a, CD2, CD3, CD4, CD5, CD7, CD8, Anti-TCR $\alpha\beta$, and Anti-TCR $\gamma\delta$.

The FAB group have assigned ALL to three cytological categories: L1, L2, and L3. The classification is summarized in Table 6. Apart from a strong correlation between L3 cytological features and a mature B phenotype, there is little relationship between the

cytological features and the immunophenotype. The recognition of L3 ALL is generally straightforward but the categorization of a case as L1 or L2 can be difficult. However, it is of little clinical significance whether the cytological features are those of L1 or L2 ALL.

It should be noted that although myeloblasts do not show any appreciable chromatin condensation, lymphoblasts may do so. This is often noticeable in some of the smaller blasts in common ALL of L1 type. It has also been noted that in a minority of cases of T-lineage ALL, particularly those with a relatively mature immunophenotype, the leukemic cells are difficult to recognize as blasts because of chromatin condensation and inconspicuous nucleoli; immunophenotyping is of importance in these cases.

A) Acute lymphoblastic leukemia of L1 subtype

The L1 ALL is small, up to twice the diameter of a red cell. It has a high nucleocytoplasmic ratio. The nucleus is regular in shape with only occasional clefting. The chromatin pattern is fairly homogenous and nucleoli are small and inconspicuous. The scant cytoplasm is slightly to moderate basophilic. In a minority of cases there are small numbers of azurophilic granules. The L1 ALL category includes the majority of ALL, in childhood ALL 70-80% of cases fall in this category. L1 ALL may be of B or T lineage.

B) Acute lymphoblastic leukemia of L2 subtype

The blasts of L2 ALL are larger and more heterogeneous. The nucleocytoplasmic ratio is variable, indicating a variable amount of basophilia, which may be moderately abundant. The nucleus are irregular in shape with clefting, folding being common, and chromatin pattern is heterogeneity. Nucleoli are usually present and may be large.

Cytoplasmic vacuolation may be present, and there are small numbers of azurophilic granules. About a quarter of cases of ALL fall into the L2 category. L2 ALL may be of B or T lineage.

C) Acute lymphoblastic leukemia of L3 subtype

The blasts of L3 ALL are medium to large and more homogeneous. The nucleocytoplasmic ratio is lower than in L1 ALL. The nucleus is regular in shape, varying from round to oval. The chromatin pattern is uniformly striped or homogenous, with one or more vesicular nucleoli. The cytoplasm is strongly basophilic with variable but prominent vacuolization. L3 ALL constitutes only 1-2% of cases of ALL.

When a patient shows L3 cytological features, further investigation is essential. This should be initially immunophenotyping and, in B-lineage cases, cytogenetic or molecular genetic analysis. Cases with a mature B immunophenotype may have specific Burkitt's lymphoma-related translocations; they do poorly with standard ALL management but have a much more favourable prognosis with specific protocols. Patients with L3 morphology and a mature B immunophenotype may also have t(14;18)(q32;q21); their prognosis is poor and optimal management has not been defined. Rare cases with L3 cytological features have had t(9;22)(q34;q11.2). A B-cell precursor immunophenotype and t(1;19)(q23;p13) may also be found; they do not have an adverse prognosis and should be treated as ALL, not with protocols relevant to Burkitt's lymphoma.

Only when FAB L3 cytological features are associated with a precursor-B (or precursor-T) immunophenotype, can the diagnosis of ALL be sustained. Other cases are usually non-Hodgkin's lymphoma, particularly Burkitt's lymphoma.

Table 6 The FAB classification of acute lymphoblastic leukemia [100]

FAB category	L1 ALL	L2 ALL	L3 ALL
Cell size	Mainly small	Large, heterogeneous	Large, homogeneous
Nuclear chromatin	Fairly homogeneous, may be condensed in some cells	Heterogeneous	Finely stippled, homogeneous
Nuclear shape	Mainly regular	Irregular; clefting and indentation common	Regular; oval to round
Nucleolus	Not visible or small and inconspicuous	Usually visible, often large	Usually prominent
Amount of cytoplasm	Scanty	Variable	Moderately abundant
Cytoplasmic basophilia	Slight to moderate	Variable	Strong
Cytoplasmic vacuolation	Variable	Variable	Often prominent

The primary use of cytochemistry in ALL diagnosis is in helping to distinguish ALL from other non-lymphoblastic leukemias. Secondly, and to a lesser extent, particular cytochemical reactions can help in distinguishing subtypes of ALL, B- or T-lymphocyte precursors. The myeloperoxidase and Sudan black staining reactions are negative in ALL lymphoblasts. However, it has been reported that Sudan black positivity may occasionally be observed in lymphoblasts in cases of common-ALL. Consequently, a negative

myeloperoxidase reaction remains the most satisfactory cytochemical method for diagnosing ALL.

Lymphoblasts show great variability with the PAS reaction. PAS positivity is usually seen as coarse granular or block-like staining. However, in a minority of cases of ALL the PAS reaction produces a fine scattered granular appearance similar to acute non-lymphoblastic leukemia. The acid phosphatase reaction is of considerable importance in identifying the acute T-cell malignancies (T-ALL, pre-T-ALL, and T-LbLy). This reaction pattern contrasts with that of B-cell derived ALL (null, common and B), which have more variable pattern of reactions ranging from negative to weak scattered granular positivity.

1.2.2.4.3 Chronic myelocytic leukemia (CML)

Chronic myelocytic leukemia (CML) is a clonal myeloproliferative expansion of transformed, primitive hematopoietic progenitor cells [107]. It was the first hematological malignancy involved with a specific chromosome abnormality, known as the Philadelphia chromosome [108]. CML accounts for 15% of leukemia in adults, and most cases occur in patients at around 60 years of age or older. There are only 2%–3% of CML diagnosed in patients less than 20 years. The incidence of CML per year in children <10 years is about 1/1,000,000 and increases rapidly to 2/100,000 in adults by age 50 and 1/10,000 at age >80 [107]. The disease is more common in males. In the United States, the incidences are slightly higher in Caucasians than in Blacks or Hispanics. For Asians, its incidence and

median age of onset appear to be lower than the respective 1.5 per 100,000 and 65 years seen in the US [109].

1.2.2.4.3.1 Classification of chronic myelocytic leukemia

A) Chronic phase

Patients with CML in the chronic phase may be asymptomatic; however, more than 50% of patients are identified through routine blood tests. Common presenting symptoms include fatigue, night sweats, and splenomegaly with abdominal discomfort and early satiety; in some cases, patients may present with a hyperviscosity syndrome, stroke, priapism, and stupor or visual changes caused by hemorrhage [110].

The chronic phase lasts several years and is characterized by accumulation of myeloid precursors and mature cells in bone marrow, peripheral blood, and extramedullary sites. A blood examination of chronic phase shows the white blood cell count is usually more than 50×10^9 cells/L, with the range of 20×10^9 cells/L to 800×10^9 cells/L. The platelet count is elevated and anemia is present in approximately 50% of cases diagnosed as CML. During this phase, leukemic cells can also retain the ability to differentiate normally; a full spectrum of myeloid cells from blast to neutrophils are found in the peripheral blood smear, with blasts less than 5% of all white blood cell differential, and basophilia and eosinophilia are common. The bone marrow examination shows hypercellularity with granulocytic and megakaryocytic hyperplasia, basophilia and fewer than 5% blasts [110].

B) Accelerated phase

After 4-5 years of untreated CML, the disease will progress from the chronic to the accelerated phase, lasting for 4 to 6 months. The accelerated phase is a transitional period with poor prognosis. Patients may have progressive splenomegaly, bone pain and other complaints such as fever, night sweats, or weight loss. Criteria for diagnosing transition into the accelerated phase are variable. The World Health Organization (WHO) criteria, the most widely used criteria, indicate a diagnosis if one or more of the following is present [111]:

- Blasts 10% to 19% of peripheral blood white cells or bone marrow cells
- Peripheral blood basophils at least 20%
- Persistent thrombocytopenia ($<100 \times 10^9$ cells/L) unrelated to therapy, or persistent thrombocytosis ($>1000 \times 10^9$ cells/L) unresponsive to therapy.
- Increasing spleen size and increasing WBC count unresponsive to therapy.
- Cytogenetic evidence of clonal evolution (the appearance of an additional genetic abnormality that was not present in the initial specimen at the time of diagnosis of chronic phase CML.)
- Megakaryocytic proliferation in sizable sheets and clusters, associated with marked reticulin or collagen fibrosis, and/or severe granulocytic dysplasia, should be considered as suggestive of CML-AP. These findings have not yet been analyzed in large clinical studies, however, so it is not clear if they are independent criteria for accelerated phase. They often occur simultaneously with one or more of the other features listed.

The accelerated phase is significant because it signals that the disease is progressing and transformation to blast crisis is imminent. Approximately 20-40% of CML patients progress to the blastic phase without an occurrence of the accelerated phase. Drug treatment often becomes less effective in the advanced stages [110].

C) Blastic phase (Blast crisis)

Blast crisis is the terminal phase of CML. It lasts only a few months and is characterized by the rapid expansion of a population of myeloid or lymphoid differentiation-arrested blast cells.

Cytochemistry of CML shows that Leukocyte alkaline phosphatase (LAP) is usually found to be markedly depressed in CML. However, elevated LAP may be seen in CML during remission, bacterial infection, blast crisis and pregnancy. A high LAP level is usually associated with non-malignant leukemoid reactions and with myeloproliferative syndromes other than CML [101].

The blast crisis of CML is characterized by the presence of blast cells which show very variable morphological and cytochemical characteristics. The most noticeable cytochemical change is the appearance of myeloid precursors containing PAS material.

Frequently mixed features are observed with myeloblasts, monoblasts with their own specific cytochemical reactions, and megakaryocyte precursors. Undifferentiated blast crises with strong basophilic cytoplasm may correspond to the "cryptic erythroleukemia" recently defined as having a very early erythroblastic immunological phenotype. In some blast crises the cells have lymphoblastic cytological appearance with PAS positivity and peroxidase negativity. The lymphoid nature of the blasts in this form of blast crisis has

been confirmed by appropriate immunological studies, mostly showing early B-lineage cell markers [101].

1.2.2.4.3.2 Pathophysiology of CML

The Philadelphia (Ph) chromosome was first discovered in 1960 [108] as the first consistent chromosomal abnormality associated with a specific type of leukemia. It is the hallmark of CML and is found in up to 95% of patients. Besides CML, the Ph chromosome is also found in 5% of children and in 15 to 30% of adults with acute lymphoid leukemia and in 2% of patients with newly diagnosed acute myeloblastic leukemia. It is a shortened chromosome 22 originating from a chromosomal rearrangement between chromosomes 9q34 and 22q11, resulting in reciprocal translocation $t(9;22)(q34;q11)$ [108, 110]. The molecular consequence of this translocation is the fusion of the ABL gene from chromosome 9 to sequences of breakpoint cluster region (BCR) on chromosome 22, giving a novel fusion *BCR-ABL* gene, which encodes the BCR-ABL protein that has constitutive protein tyrosine kinase activity. The pathological defects identified in CML cells include increased proliferation or decreased apoptosis of hematopoietic stem or progenitor cells, leading to a massive increase in myeloid cell numbers. An example of cellular pathway that links to an increased proliferation rate is activation of RAS pathway. STAT-5 mediated upregulation of the anti-apoptotic molecule BCL_{XL} and the phosphorylation and inactivation of pro-apoptotic molecule BAD by AKT. The BCR-ABL activates these pathways by its tyrosine kinase activity [108].

1.2.2.4.4 Chronic lymphocytic leukemia (CLL)

CLL is a neoplastic disease characterized by the accumulation of small mature lymphocytes in bone marrow, blood, and lymphoid tissues. It is the most common adult leukemias in western societies and affects mainly elderly individuals [112]. About a third of patients are less than 60 years of age at diagnosis [113]. Generally, CLL represents a B-cell lineage. However, less than 2 percent of cases represent a T-cell origin of neoplastic cells. CLL follows an extremely variable clinical course with overall survival times ranging from months to decades. Some patients have no or minimal signs and symptoms during the entire disease course and have a survival time similar to age-matched controls. Other patients may experience rapidly deteriorating blood count, organomegaly and suffer from symptoms at diagnosis or soon thereafter. Patients subsequently may develop cytopenia as a result of progressive bone marrow involvement or from autoimmune abnormalities. Bacterial, fungal, and viral infections may occur due to hypogammaglobulinemia and T-cell dysfunction. Moreover, CLL patients may also develop either transformation to a large cell lymphoma (Richter's syndrome) or prolymphocytic leukemia.

In most case of CLL, the Bcl-2 protein is overexpressed [114]. This protein is produced from the bcl-2 proto-oncogene and functions as an inhibitor to apoptotic cell death. Although the etiology of CLL is unknown, chromosomal abnormalities are present in approximately 80% of cases. Most common are deletions of chromosomes 13q14 and 11q22-23 as well as trisomy 12 [115].

1.2.2.4.4.1 Classification of chronic lymphocytic leukemia

The CLL comprise a very heterogeneous group of neoplastic disorders. Until the publication of the FAB proposals, there was no uniform classification that considered morphological as well as immunological issues. Thirteen different subtypes of CLL were described with detailed morphological/immunological profiles for each disorder. The MIC proposals emphasized the importance of cytogenetics. Recently, the WHO proposals incorporated all of the above and added molecular genetics. The final result is a comprehensive diagnostic approach that should permit recognition of the vast majority of cases and permit appropriate therapeutic choices.

For the first classification of CLL, the FAB Cooperative Leukemia Study group employed flow cytometry to separate CLL into two broad categories: B-CLL and T-CLL [116]. It was recognized that certain cytomorphological features were so strongly associated with a specific diagnosis that markers were of minor interest. In addition, the significance of cytogenetic studies was beginning to impact on the recognition of basic molecular defects. For the B-CLL, nine different disease groupings were described, including typical B-CLL, prolymphocytic leukemia (B-PLL), mixed cell CLL (CLL-PLL), Hairy Cell leukemia (HCL), follicular lymphoma, mantle cell lymphoma, splenic lymphoma with villous lymphocytes (SLVL), plasma cell leukemia, and Waldenstrom's macroglobulinemia. For T-CLL, four types of diseases were classified include typical T-CLL, T-PLL, adult T-cell leukemia lymphoma (ATLL), and Sezary's syndrome.

Concerning MIC classification, the fourth meeting focused on the classification of CLL. Conclusions from this meeting included: replacing the term "T-CLL" with "large

granular lymphocyte leukemia LGLL”. A variety of clonal and nonclonal karyotypic changes were discussed, including trisomy 12, 13q aberrations, 14q+, and t(11;14); in B-PLL and inv (2) in T-PLL.

In 1994, there appeared proposals from the international Lymphoma Study Group [117]. Referred to as the REAL classification, this system rapidly became universally accepted. However, it incorporated virtually all of the chronic B- and T- CLLs. One suspects that the rationale for this was the identical lymph node histology in CLL whenever this material was available and the common evolution of many of the non-Hodgkin lymphomas (NHL) to a leukemic terminal phase.

The WHO proposals follow the FAB chronic T-cell leukemia proposals, with two notable exceptions. First, Sezary’s syndrome is considered under the cutaneous lymphomas, rather than as leukemia; even though circulating cerebriform T-cells are always present. Secondly, a relatively new disorder described by Chang, *et al.* in the early 1990s was included, namely aggressive NK-cell lymphoma [119].

In addition, CLL is classified by two cytological staging systems, which are known as the Rai Classification [118] and Binet Staging [120], respectively.

Rai Classification

The Rai Classification separates chronic lymphocytic leukemia into low-, intermediate-, and high-risk categories, which correspond with stages 0, I & II, and III & IV, respectively.

Rai Stage 0: patients are at low risk and have lymphocytosis, a high lymphocyte count defined as more than 15,000 lymphocytes per cubic millimeter.

Rai Stage I: patients are at intermediate risk and have lymphocytosis plus enlarged lymph nodes (lymphadenopathy).

Rai Stage II: patients are also at intermediate risk but have lymphocytosis plus an enlarged liver (hepatomegaly) or enlarged spleen (splenomegaly), with or without lymphadenopathy.

Rai Stage III: patients are at high risk and have lymphocytosis plus anemia, a low red blood cell count (hemoglobin < 11 g/dL), with or without lymphadenopathy, hepatomegaly, or splenomegaly.

Rai Stage IV: patients are also at high risk but have lymphocytosis plus thrombocytopenia, a low number of blood platelets (< 100 x 10³ cells/μL).

Binet classification

Binet Staging classifies CLL according to the number of lymphoid tissues that are involved (i.e., the spleen and the lymph nodes of the neck, groin, and underarms), as well as the presence of low red blood cell count (anemia) or low number of blood platelets (thrombocytopenia).

Binet Stage A: patients have fewer than three areas of enlarged lymphoid tissue. Enlarged lymph nodes of the neck, underarms, and groin, as well as the spleen, are each considered “one group,” whether unilateral (one-sided) or bilateral (on both sides).

Binet Stage B: patients have more than three areas of enlarged lymphoid tissue.

Binet Stage C: patients have anemia plus thrombocytopenia (platelets $< 100 \times 10^3$ cells/ μ L)

1.2.3 *Wilms' tumor 1 (WT1) gene and Wilms' tumor 1 (WT1) protein*

The *WT1* gene was first described in 1899 by Dr. Max Wilms. It was discovered in pediatric patients with nephroblastoma, malignant neoplasm of the kidney, also known as Wilms' tumor. Wilms' tumor arises from abnormal pluripotent embryonic renal precursors [13]. It affects 1 in 10,000 children, usually around age 5, and accounts for approximately 8% of all pediatric malignancies [121]. This tumor occurs both as sporadic and inherited forms, affecting one or both kidneys. Wilms' tumor itself is composed of many kinds of tissue, but it often reflects the presence of epithelial and blastomal components, or undifferentiated mesenchymal cells [13]. The genetic abnormalities underlying most cases of Wilms tumor remain unknown; however there are three genes that have been implicated in a subset of tumors, including *Wilms' tumor (WT)*, *Insulin-like growth factor 2 (IGF2)*, and *β -catenin* [121]. Inactivating mutations in *WT1* are responsible for 10-15% of neoplasms [122]. Many hereditary cases of Wilms' tumor show an association with congenital developmental abnormalities such as aniridia, genitourinary malformations and mental retardation in WAGR syndrome, associated with deletions on chromosome 11p13 [121, 123]. Although Wilms' tumor may also develop according to many mutations at other chromosome sites, *WT1* on chromosome 11p13 is the only gene that can be cloned and proven to inhibit tumor growth [21].

1.2.3.1 Structure and function of *WT1* gene and WT1 protein

The organization of the *WT1* locus is quite complex. The *WT1* gene is located at chromosome 11p13 and spans approximately 50 kb of genomic DNA [124, 125]. The gene consists of 10 exons and generates a 3 kb mRNA [124, 125], which encodes the WT1 protein. The amino acid sequence of WT1 revealed a presumptive transcription factor with a C-terminal four Kruppel-like Cys₂-His₂ zinc fingers domain (exons 7–10) and an N-terminal proline/glutamine-rich domain (exons 1–6) [126]. The C-terminal zinc finger domain has a high degree of amino acid homology to those of the immediate early gene early growth response 1 (EGR1) [127], involved in mediating sequence specific DNA and RNA binding and harbors multiple nuclear localization signals (NLS). Two nuclear localization signals have been identified in the zinc finger domain of WT1, one being in zinc finger 1 and the other in zinc fingers 2 and 3 [128]. The N-terminal includes a transcriptional repression domain followed by a transactivation domain, implicating in transcriptional repression or activation [14, 16, 129], suggesting the role of WT1 as transcription factor. The use of WT1 deletion constructs indicated that residues 84–179 were required for the transcriptional repression, and residues 180–294 contained a transcriptional activation domain, while deletion of the first 84 residues had no effect on transcriptional regulation [130]. Besides binding to other proteins, WT1 can also self associate, this domain has mapped to the first 182 amino acids of WT1 [130]. An RNA editing event, which alters the amino acid at position 280 from Leu to Pro, is indicated on the WT1 protein as well as the boundaries for the transcription regulation domains and the self-association domain (Figure 1) [131].

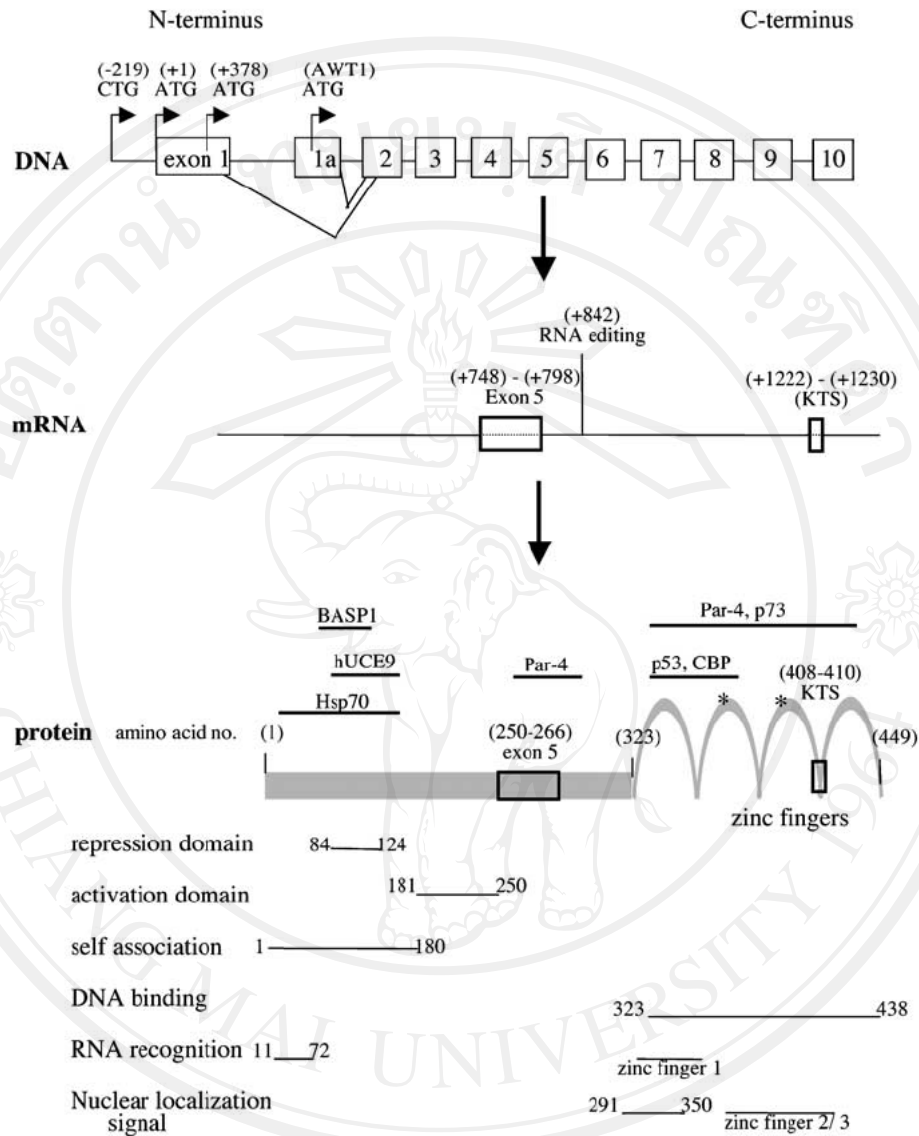


Fig. 1 Schematic diagram of the WT1 structure at the DNA (exon only), mRNA and protein level [18]. WT1 can be translated from four initiation start sites. WT1 RNA is edited at nucleotide position 843 and subject to RNA splicing of exon 5 and of KTS between exon 9 and 10 yielding the major isoform of WT1. WT1 protein has several functional domains. N-terminal domain contains transcriptional repression and activation domain, self-association, and RNA recognition motifs. The C-terminal domain contains four C₂H₂ zinc-fingers, which in addition to binding DNA and some proteins, can regulate RNA targets and mediate nuclear localization.

The activity of these domains may depend on cell type. Among the target genes of WT1 protein are those important for cellular growth and metabolism, including extracellular matrix components, growth factors, and other transcription factors, and thus the gene may be involved in regulating cellular differentiation and proliferation. The WT1 protein can repress a large number of potential target genes such as *EGR1*, *WT1* itself, *PDGF-A* [18, 130], *insulin-like growth factor II (IGFII)*, *insulin-like growth factor receptor (IGFR)*, *Pax2*, *colony stimulating factor 1 (CSF1)*, *transforming growth factor- β (TGF- β)*, *retinoic acid receptor- α* , *C-myc*, and *N-myc* [16, 18, 132-133]. Additional target genes that may be activated for transcription by WT1 include *Amphiregulin* [134], *Dax1*, *Bcl-2*, *Syndecan-1*, and *E-cadherin* [16, 18]. The promoter of *Bcl-2* is either repressed or activated by WT1. WT1 can function as an activator of transcription and repression mediated by WT1 may be due to its physical association with the p53 protein [135]. Ectopic expression of WT1 has different effects on cells, including arrest in the G1 phase of the cell cycle and induction of apoptosis [136].

As a result of alternative splicing, The WT1 proteins have molecular masses of 52–54 kDa depending on the inclusion or exclusion of the two splice inserts [137].

Although several transcriptional modifications can occur, there are two predominant alternative splicing events. One alternative splicing is inclusion or exclusion of a stretch of 17 amino acids (± 17 aa) in exon 5 at the N-terminal of the four zinc fingers. The other alternative splicing involves a splice acceptor site in exon 9, resulting in presence or absence of a three amino acid insert (lysine-threonine-serine or \pm KTS). Alternative splicing of these two sites gives rise to four different protein isoforms, including the WT1

splice variant that lacks both inserts as the WT1(-/-) isoform, the splice variant containing only the 17aa insert as the WT1(+/-) isoform, the splice variant containing only the KTS insert as the WT1(-/+) isoform, and the splice variant that contains both inserts as the WT1(+/+) isoform, or A, B, C, and D, representing WT1 (-/-), (+/-), (-/+), and (+/+), respectively [138].

Under normal physiological conditions, the expression of KTS(+)/KTS(-) ratio is maintained at approximately 2:1 [138]. The +KTS proteins showed higher affinity for RNA than the -KTS forms. Furthermore, the WT1 (+KTS) products are found to be co-localized with and bound to nuclear splicing factors. These findings strongly support the possibility that the WT1 (+KTS) proteins play a role in mRNA splicing rather than transcriptional control. Recently, a second translational initiation site has been identified resulting in WT1 proteins with a higher molecular weight. 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 [139].

The four WT1 protein isoforms described above of molecular masses between 52 and 54 kDa are generated through the use of the major initiator AUG. Larger WT1 protein isoforms have been identified that are generated by translation initiation at an in-frame CUG codon upstream of the initiator AUG and results in WT1 protein isoforms of 60–62 kDa [140]. Internal translation initiation at an in-frame AUG codon downstream of the initiator AUG generates smaller WT1 protein isoforms with molecular masses of 36–38 kDa [141]. Therefore, taking into account all possible modifications, WT1 protein is

generated in up to 24 different isoforms, which seem to serve distinct but also overlapping cellular and developmental functions.

1.2.3.2 Expression of *WT1* gene and WT1 protein in normal cells and tissues

WT1 is expressed during mammalian development in many tissues, including the urogenital system, spleen, certain areas of the brain, spinal cord, mesothelial organs, diaphragm, limb, proliferating coelomic epithelium, epicardium and subepicardial mesenchyme [142-144]. WT1 may contribute either to the induction or to the response to such developmental signals. The targeted disruption of the *WT1* gene in mice leads to gonadal and renal agenesis, and severe heart, lung, spleen, adrenal and mesothelial abnormalities [144-146]. Moreover, WT1 also plays an important role in neuronal development [147]. In normal adult tissue, it is expressed in mesothelium, glomerular podocytes and mesangial cells of the kidney, CD34-positive hematopoietic stem cells, sertoli cells of the testis, stromal cells, surface epithelium and granulosa cells of the ovary, and myometrium and endometrial stromal cells of the uterus [148].

1.2.3.3 Expression of *WT1* gene and WT1 protein in malignant tissues

WT1 was initially discovered as a tumor suppressor in Wilms' tumor, a pediatric kidney malignancy [13]. During normal renal development, WT1 functions to suppress an IGF-II/IGF-IR autocrine loop that effects differentiation of the renal epithelium. The loss of WT1 function contributes to Wilms tumorigenesis through constitutive activation of this loop [150]. Moreover, it has been reported that the *WT1* gene is also expressed in

other cancers such as ovarian tumors, renal cell carcinomas, mesotheliomas, tumor of the stomach, prostate, biliary and urinary systems, and malignant melanomas [149].

The WT1 protein has been reported to operate as a tumor suppressor and oncoprotein in different cells. Silbstein *et.al.* (1997) reported that WT1 would be a candidate for a breast cancer tumor suppressor, based on lack of immunodetectable WT1 in breast cancer [150]. The *WT1* gene expression also was detected in 3 of the 4 gastric cancer cell lines, all of the 5 colon cancer cell lines, 12 of the 15 lung cancer cell lines, 2 of the 4 breast cancer cell lines, the germ cell tumor cell line, the 2 ovarian cancer cell lines, the uterine cancer cell line, the thyroid cancer cell line, and the hepatocellular carcinoma cell line, in 82% of the cancer cells, examined. However, the growth of 3 cell lines expressing *WT1* (gastric cancer cell line AZ-521, lung cancer cell line OS3, and ovarian cancer cell line TYK-nu) was significantly inhibited in association with a reduction in WT1 protein levels. These results indicated that the *WT1* gene plays an essential role in the growth of solid tumors and performs as an oncogene rather than a tumor suppressor gene [151]. Overexpression of the *WT1* gene was also demonstrated in 83% of de novo small cell lung cancers by immunohistochemistry. Furthermore, *WT1* gene mutations were not present. These results suggested that the nonmutated, wild-type *WT1* gene plays an important role in tumorigenesis of de novo lung cancers and may indicate new therapeutic strategies for lung cancer via targeting the *WT1* gene and its products [152].

1.2.3.4 Expression of *WT1* gene and WT1 protein in normal and malignant hematopoiesis cells

The expression of *WT1* has been detected in the fetal spleen, liver, and thymus, tissues in which hematopoiesis take place during embryonic development. In adult tissues, *WT1* expression is found in tissues involved in hematopoiesis, including the bone marrow and lymph nodes [16]. In normal human bone marrow, *WT1* is expressed at extremely low levels and is confined to the primitive CD34⁺ population of cells [153, 154]. Inoue *et.al.* (1997) showed that *WT1* expression in the CD34⁺ cell populations in bone marrow and umbilical cord blood, normal hematopoietic progenitor cells, was at least 10 times less than those in leukemic cells [155]. A study with chimeric mice which are showed that 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 [196]. This suggests that *WT1* plays a role in the self-renewal of early murine hematopoietic cells [156]. The studies of Nishida *et al.* (1996) reported that placing WT1 downstream from the tec promoter in hematopoietic progenitor cells can increase bone marrow cellularity and increased numbers of BFU-E, CFU-GM and CFU-GEMM; production of mature cells was normal in these mice [157], which showed that WT1 plays a role in cell proliferation and differentiation of immature blood cells in early hematopoiesis.

Early evidence that WT1 may be involved in leukemia was reported in 1990. Initial reports using the Northern blot technique revealed that 14–68% of acute leukemia cells overexpressed *WT1* gene [158, 159]. *WT1* transcripts have been detected in a subset of leukemic cells. Significant levels of *WT1* gene were expressed in all acute leukemia

patients (AML, ALL and AMLL) and for CML the levels increased as the clinical phase progressed. In striking contrast with acute leukemia, the levels of *WT1* gene expression for non-Hodgkin's lymphoma were significantly lower or even undetectable [19]. Lack of *WT1* expression in chronic lymphocytic leukemia, hairy cell leukemia and plasma cell leukemia suggests that *WT1* expression is limited to immature leukemia [158]. The quantitation of the *WT1* gene expression can be used for detecting minimal residual disease (MRD) in acute leukemia and monitoring of *WT1* expression levels in bone marrow and peripheral blood makes it possible to rapidly assess the effectiveness of individual treatment and diagnose clinical relapse in the early stage for all leukemia patients, regardless of the presence or absence of tumor-specific DNA markers [19, 160]. It is known that *WT1* participates in leukemogenesis; the antisense oligomers were used to elucidate the role of *WT1* in this process. *WT1* antisense oligomers can suppress *WT1* gene expression and inhibit the cell growth of both leukemic cell lines and fresh leukemic cells from patients with acute or chronic myeloid leukemia, but not normal colony-forming unit granulocyte-macrophage (CFU-GM), suggesting that *WT1* involves in leukemogenesis by regulating cell growth and differentiation [161, 162].

Although the mechanisms remain unknown, *WT1* protein dysregulates the transcription of the target genes and interacts with cellular proteins that regulate cell growth and differentiation, resulting in the development of leukemia in cooperation with other oncogenes. *WT1* gene expression has been detected in many leukemic cell lines such as K562, Molt4, and HL60 [17, 163, 164]. These several cell lines have been used to investigate the function of *WT1*. For example, *WT1* protein expression was

downregulated during the differentiation of HL60 cells by dimethyl sulfoxide or retinoic acid [164] and during induction of erythroid and megakaryocytic differentiation of K562 cells [163]. K562 treated with WT1 antisense can stop growing and subsequently endure apoptosis. All of these results support the suggestion that *WT1* gene expression may influence the proliferation, differentiation, and apoptosis of human blood cells.

1.2.4 Kaffir lime (*Citrus hystrix* DC.)

Citrus hystrix DC. or commonly known as kaffir lime is the a citrus plant, belonging to the Rutaceae family. Kaffir lime is widely distributed and cultivated in Southeast Asia. It has many names depending on the area of origin such as gondhuraj (East Indian Language, Bengali), limau purut (Malaysia) or makhrut (Thai) [165]. The kaffir lime trees are shrubs to medium-size trees up to about 5 m (16 ft) in height. They are single-trunked with very hard wood with thin, smooth, and gray-brown bark with short spines [166]. The leaves of the kaffir lime tree are a dark green color with a glossy sheen. They come in two parts, the top leaflet is lightly pointed at its tip and is attached to another leaflet beneath that is broader on its upper edge [161]. The size of the leaves can vary quite a bit, from 2.5 to 4 cm. wide and 4 to 7 cm. long [168]. Under the leaves, there are many oil glands, making the leaves very fragrant [168]. Flowers are clustered into 3 to 5 with white petals. The pollen is yellow and aromatic [168]. The fruits are dark green and round with a distinct nipple on the stem end. They have a thick rind, knobby, and wrinkled. As the fruit becomes older, the color fades to a lighter, yellowish green. The

leaves and rind have a perfume unlike any other citrus, which results from the combination of lemon/lime aroma (Figure 2).



Figure 2 Kaffir lime tree, leaves, flowers, and fruits

Kaffir lime is commonly used in Southeast Asian cuisine, especially in Thailand.

The kaffir lime leaves and peels are aromatic and used as a spice for various flavoring purposes such as seasoning or savory curry paste. The well-known Thai dishes garnished

with kaffir lime leaves and peels are Tom-yum, Green curry and Red curry. The kaffir lime juice is infrequently used in cooking because it is very sour. In the past, in Thailand, kaffir lime herb has been widely used in traditional medicine. The leaves can be used to maintain healthy teeth and gums and remedy scurvy. The juice has properties to clean the blood, dispel gas, relieve giddiness, and also increase appetite due to the sour taste. In addition, kaffir lime juice and peels can prevent dandruff and hair loss and make hair look healthy.

Kaffir lime has great potential in research and commercialization [169]. The usages of kaffir lime include aromatherapy and spa practices and making shampoos, cosmetics and beauty products [170-172]. The key element for such application is the oil contained in kaffir lime. Lawrence *et al.* (1970) reported that the main chemical constituents in kaffir lime peel were β -pinene (30.6%), limonene (29.2 %) and sabinene (22.6%), and the main compound in leaves is citronellal (65.4%) [173]. However, reports concerning the amount of each chemical contents are slightly different, for example, Chantaphon *et al.* (2008) reported β -pinene (30.48%), sabinene (22.75%), and citronellal (15.66%) as major components of the hydrodistilled essential oil of kaffir lime peel [174], and Kasuan *et al.* (2009) showed that the steam distilled essential oil of kaffir lime included limonene (27.97%), citronellal (15.31%), and α/β -pinene (9.82%) for the peel and citronellal (71.55%) for the leaf [171]. Weikedre *et al.* (2010) found that the main constituents of essential oil of kaffir lime leaf growing in New Caledonia were also monoterpenes with terpinen-4-ol (13.0%), β -pinene (10.9%) [175] as principal compounds. Besides hydrodistillation technique, essential oil from peels and leaves of

kaffir lime may be extracted by using solvent extraction. The major constituents of ethyl acetate extracts from kaffir lime peel were limonene (31.64%), citronellal (25.99%), and β -pinene (6.83%) [173]. Whereas the study of Yunas (2009) showed that citronellal (2.80%), limonene (0.31%), and pinene (0.13%) were major components of essential oil of the ethyl acetate extract from peel, and citronellal (73.08%) from leaves [176]. The variation of each component amount depended on several parameters such as growing region, ripeness of fruits, vegetative stage of plant, storage condition, and extraction method [177].

Many studies have demonstrated various biological activities of kaffir lime. An anti-oxidative property of kaffir lime was reported by Tachakittirungrud *et al.* (2007) [178]. The kaffir lime peel and leaf is a source of phenolic compound [179], antioxidative substance [182]. Furthermore, the extracts from leaf and peel of the Kaffir lime exert the strongest effect on protection of deoxyribose from OH \cdot , suggesting the free radical scavenging and anti-inflammatory activities of kaffir lime [27]. Most of the studies of kaffir lime bioactivities are related to the antimicrobial effect. The essential oil and extracts of kaffir lime exhibit antimicrobial activities. Kaffir lime peel has an inhibitory effect on the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Saccharomyces cerevisiae* var. *sake*, and *Aspergillus fumigatus* TISTR 3180 [175] whereas kaffir lime leaf extracts fail to inhibit *Bacillus cereus*, *Bacillus megaterium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus ochraceus*, and *Cryptococcus neoformans* [181]. However, the studies of Jantarach *et al.* (2006) and Waikedrei *et al.* (2010) showed that oil and extract of the leaf could inhibit *Aspagillus flavus* [182],

Staphylococcus epidermidis, *Candida albicans*, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* [178]. Nanasombat *et al.* (2005) suggested that both the essential oil and extract of kaffir lime peel and leaf exhibited antimicrobial activity against salmonellae and enterobacteria test strains, but the peel showed greater action, compared to the leaf [183]. The mechanistic processes of plant extracts on antibacterial properties are poorly understood. Some researchers have demonstrated the antimicrobial activity of the most common terpene compounds, such as thymol, carvacrol, linalool, eugenol, α -pinene, and β -pinene in spices against several microbial strains [184-186]. Hirasa *et al.* (1998) reported that β -pinene and limonene had greater inhibitory activity against *S. Enteritidis* than citronellal [184]. This might be the reason why kaffir lime peel showed greater antibacterial activity than kaffir lime leaf [183] in some strains of microorganisms. Moreover, kaffir lime also exhibits repellency activity, although it is less effective than turmeric, citronella grass or hairy basil [187]. The kaffir lime oil also has been reported to possess anti-viral activity.

In regard to cancer research, bioactive compound in many kinds of citrus fruits are capable of inhibiting cancer cell proliferation. Limonoids, a family of triterpenoids with putative anticancer properties in citrus fruits, exert a strong multifaceted lethal action against human neoblastoma and colon cancer cells [188]. The volatile oil of *Citrus aurantifolia* containing two major compounds, D-limonene and D-hydrocarvone, can inhibit proliferation of colon cancer cells by apoptosis mediation [189]. It has been demonstrated that D-limonene and the oils isolated from citrus fruits can inhibit the formation of pulmonary adenoma and the occurrence of forestomach tumours induced by

carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [190]. Flavones, active compounds detected in *Citrus reticulata*, were found to have *in vitro* anti-tumour activity against human promyelocytic leukemia cells and murine myeloid leukemic cells [191, 192]. Murakami *et. al.* (1995) reported that two glyceroglycolipids, 1,2-di-*o*- α -linolenoyl-3-*o*-galactopyranosyl-sn-glycerol and a mixture of two compounds, 1-*o*- α -linolenoyl-2-*o*-palmitoyl-3-*o*-galactopyranosylsn-glycerol and its counterpart, extracted from kaffir lime leaves were potent inhibitors of tumor promoter-induced Epstein-Barr virus (EBV) activation and 12-*o*-tetradecanoylphorbol 13-acetate (TPA), a skin carcinogen, activities in mice [29]. The essential oils of kaffir lime leaf and peel prepared by steam distillation had been shown to have anti-proliferative activity on KB (cervical cancer) and P388 (mouse leukemia) cell lines [25]. The crude ethanolic extracts of kaffir lime leaf and peel showed cytotoxic effects on U937, K562, HL60 and Molt 4, and crude kaffir lime leaf extract had strong cytotoxic effects on these human leukemic cell lines, and non-toxic to normal PBMCs [193].

Taken together, essential oil and extracts from kaffir lime, especially the leaf, are probably a potential source of anticancer agents. However, the difference in methods and solvents used in kaffir lime extraction resulted in the differences of the contents in extracts including amounts and types of active compound. This also resulted in differences in biological effects of crude kaffir lime leaf in each fractional extracts. Cytotoxic effects of ethanolic extract and essential oil from kaffir lime leaf on leukemic cells have been reported in previous studies. To investigate the most effective kaffir lime leaf extract, kaffir lime leaf fractional extract were used in this study. The goal is to provide new basic

knowledge on the group of active compounds in kaffir lime leaf extracts that have anti-leukemic activity.

Table 7 Examples of main compounds in essential oil of kaffir lime by different extraction techniques [169-176]

Extraction techniques		Main compounds in essential oil of kaffir lime	
		Peel	Leaf
Hydrodistillation		<i>α/β</i> -pinene Limonene Sabinene Citronellal	Citronellal Terpinen-4-ol <i>β</i> -pinene
Solvent extraction	Ethyl acetate	Citronellal Limonene Pinene	Citronellal

1.3 Objectives

1. To study the cytotoxic effects of crude kaffir lime leaf fractional extracts on K562, Molt4, U937, and HL60 cell lines
2. To study the inhibitory effects of crude kaffir lime leaf fractional extracts on *WT1* gene and WT1 protein expression in K562, U937, HL60, and Molt4 cell lines
3. To study the inhibitory effect of the most effective crude kaffir lime leaf fractional extract on *WT1* gene and WT1 protein expression in K562 cell line by dose and time dependent manner