CHAPTER I
INTRODUCTION

1.1 Statement of the problem

With more than 10 million new cases every year, cancer has become one of the most devastating diseases worldwide. The causes and forms of cancer vary according to geographic region, but in most country there is hardly family without a cancer victim. The disease burden is immense, not only for affected individuals but also for relatives and friends. At the community level, cancer poses considerable challenges for the health care system in poor and rich countries alike. According to data from WHO (World Health Organization), the death rate worldwide in 2005 was about 58 million, of which 13% (or 7.6 million) were due to cancer. Death rates from cancer are projected to continue to rise, with an estimated 9 million deaths from cancer in 2015 and 11.4 million in 2030 [1]. The National Cancer Institute of America estimates that approximately 10.5 million Americans with a history of cancer were alive in January 2003, about 1,444,920 new cancer cases are expected to be diagnosed in 2007 and about 559,650 Americans are expected to die of cancer in 2007 [2]. According to data and statistics from WHO in 2005, cancer killed approximately 66,000 people in Thailand and about 15.7% all of deaths were cancer related. Cancer is estimated to account for 21.5% all of death, by 2030 [3]. Moreover, cancer was a major cause of death in Chiang Mai province in 2003 and 2004 [4].

Leukemia is a cancer of blood or bone marrow and is characterized by an abnormal proliferation of blood cells, usually white blood cells. It belongs to a broad group of diseases called hematological neoplasms. According to a report from Global Statistics in 2002, leukemia accounts for some 300,000 new cases each year (2.8% of all new cancer cases) and 222,000 deaths (74%) [5]. In 2007, leukemia was expected to occur in men and women in the USA for about 3% of all newly diagnosed leukemias, and the expected percentage of deaths from leukemia for 2007 are about 4% [6]. In Thailand leukemia is one of the top ten causes of death. According to the

annual report for 2006 of The Siriraj Cancer Center, there were 235 cases (3.69%) which was 8th out of the top ten type of cancer seen in Siriraj Hospital [7]. The cause of most leukemias is unknown. However, researchers are investigating the causes. Leukemia occurs in males more often than in females and in white people more often than in black people. People with certain leukemia risk factors are more prone develop to the disease. The risk factors including exposed to radiation, chemicals, chemotherapy agents, viral disease, genetic disorders associated with chromosomal instability, cigarette smoking, and other environmental factors. These factors lead to genetic changes in leukemic cells that in turn effect in the other normal genes, including tumor suppressor genes and oncogenes. Some cases of mutation in oncogenes have provided useful molecular markers for monitoring the cause of the disease during treatment. The detection of overexpression in specific oncogenes or tumor suppressor genes provides information and prognosis of the disease. The overexpression of Wilms' tumor (WT1) protein in leukemia is a good example.

The *Wilm's tumor 1* (*WT1*) gene has been indentified as a tumor suppressor gene involved in the etiology of Wilm's tumor. It was first describe by Max Wilm in 1899. The *WT1* gene is located at chromosome loci 11p13 [8, 9]. WT 1 is expressed during mammalian embryonic development in many tissues, including those of the urogenital system, spleen, certain area of the brain, spinal cord, mesothelial organs, diaphragm, limb, proliferating coelomic epithelium, epicardium and subepicardium mesenchyme [10-12]. Furthermore, WT1 mRNA is expressed in the bone marrow, but not in normal mature blood cells [13], indicating that WT1 plays a role in early hematopoiesis. It had been demonstrated previously that the *WT1* gene is expressed in the leukemic cell lines K562 and HL–60, and that differentiation of these cells in culture is accompanied by downregulation of WT1 protein levels [1, 14] In addition, *WT1* is expressed in acute leukemia of myeloid and lymphoid origin as well as in many acute leukemia cell lines, and expression levels are highest in immature leukemias [15, 16], and heterozygous *WT1* mutations are present in about 10–15% of acute leukemias [17, 18]. These data suggest that *WT1* acts as an oncogene in many types of malignant tumors, especially leukemia [19].

The *WT1* gene has 10 exons. The gene produces a 48 – 52 kD protein with the structure of a transcription factor. Exons 7 to 10 encode four zinc fingers that are able

to bind RNA and DNA with high affinity, whereas the first 6 exons encode a proline/glutamine rich region that is involved in transcriptional activation and repression, nuclear localization, RNA recognition, and homodimerization of the protein [9, 20, 21]. WT1 is a complex gene that can encode at least 24 isoforms as a result of alternative start sites, alternative splicing and RNA editing. There are two alternative splice donor sites, the first one leading to the presence/absence of the 17 amino acid encoded by exon 5 and the second including/excluding a short sequence of three amino acids, lysine, threonine, and serine (KTS) between zinc fingers 3 and 4. Park *et.al* [22] showed that *WT1* gene was mutated in a human mesothelioma, suggesting its contribution to malignancies other than Wilms' tumors. *WT1* transcripts have been detected in a subset of leukemia cells [23]. Hence the expression of the *WT1* gene and its product have been used as biological markers for diagnosis of leukemia and minimal residual disese [15, 24].

At present the progress in treatment and survival for patients with blood cancer is largely due to the development of chemotherapy over the past 40 years. The number of chemotherapy agents available have soared since their first usage 1940s. The goal of chemotherapy is either to kill cancer cells to the point that there is no longer any sign of illness (called remission) or to damage or kill cancer cells to the point that the progress of the diseased is slowed. Chemotherapy can produce long–term remission or outright cure for many people, depending on the specific type and extent of the cancer. However, most chemotherapy drugs also affect normal cells, causing certain side effects. The development of a new therapeutic approach to leukemia remains one of the most challenging areas in cancer research.

Many tropical plants have interesting biological activities with potential therapeutic applications. *Garcinia mangostana* Linn (GM), family Guttiferae, is named in Thailand "the queen of fruits' because many people agree that it is one of the best tasting fruits in the world. The mangosteen is believed to have originated in Sudan Island and the Moluccas. The rind (exocarp) of the edible fruit is deep radish purple when ripe. Botanically an aril, the fragrant edible flesh is sweet and creamy, citrusy with some peach favor. The fruits hull of mangosteen has been used as a traditional medicine for the treatment of skin infections, wounds, and diarrhea in Southeast Asia. The fruit hull contains various xanthone derivatives including α–

mangostine and γ–magostine. In previous studies, these compounds have shown several pharmacological activities such as anti–inflammatory activity [25], and the inhibitory effect of Ca^{2+}-ATPase [26]. Furthermore, inhibitory effects of acidic sphingomyelinase by xanthone compounds isolated from *Garcinia* have been reported by researcher [27]. For leukemic cells, apoptotic effects of α– mangostin have been observed HL-60 cells [28].

In this study, the effects of mangosteen extracts on *WT1* gene expression and WT1 protein in leukemic cell lines were examined.The mangosteen extracts included ethanol, hexane, ethyl acetate, butanol, and methanol fractions and derivative compounds (α-mangostin and xanthone). Each compound was evaluated for its effect on *WT1* gene expression in four types of leukemic cell lines: K562, U937, HL-60, and Molt4.

1.2 Literature review

1.2.1 Cancer

Cancer has long been recognized as a genetic disease in humans in which the incidence rises exponentially in the final decades of life. Tumors may be divided into two main types: benign, where the disease is limited within a well–defined capsule; malignant, where the disease invades surrounding tissue and spreads through around the body. Several lines of evidence support the view that cancer formation in man is a multi–step process involving probably four to seven benign rate–limiting steps, where genetic alterations accumulate and progressively drive changes from normal cells to transformed, invasive, malignant cells (for example as described for colorectal cancer [29]. Typically, cancer cells contain numerous genomic alterations, ranging from gross changes in chromosomes (e.g. translocation or loss of regions) to more subtle point mutations within specific genes. Such genetic alterations may arise from multiple endogenous and/or exogenous factors. There are more than 100 distinct types of cancer illustrative of the complexity of these genetic changes and the wide range of organs affected.

Today, cancer at the cellular level is recognized as a state wherein a variety of normal regulatory processes controlling fundamental cell behavior, such as

proliferation, death and motility, are upset. In recent review [30] the 'hallmarks of cancer' are described in term of six basic acquired properties:

1. Self–sufficiency in growth signals
2. Insensitivity to anti-growth signals
3. Evading cell death or apoptosis
4. Limitless replicative potential
5. Sustained tumor blood vessel formation (angiogenesis)
6. Tumor invasion and metastasis

1.2.1.1 Causes of cancer

Epidemiological studies have established associations between a number of environmental factors and the incidences of different types of cancer. Some of these associations are very strong (e.g. smoking and lung cancer) and almost certainly represent cause and effect relationships. Doll and Peto estimated that about 35% of cancer incidence in the United States may be due to dietary factors and about 30% to smoking. From these epidemiological studies has come the recognition that many types of cancer are potentially preventable.

The most direct evidence for the carcinogenicity of environmental factors is an increase in cancer incidence in animals exposed to these factors. Such exposure has shown that a variety of different and seemingly unrelated factors can cause cancer. These include viruses, a wide variety of chemicals, and both ionizing and ultraviolet radiation. Most of these agents, however, share an important biological property: they can cause damage to or alteration of the DNA in cells. This common property suggests that DNA is the essential target of all carcinogen agents and that cancer arises as a result of changes in cellular DNA. Additional evidence in support of this concept is as follows:

(a) the existence of some (rare) forms of cancer whose incidence is directly dependent on hereditary factors

(b) a high incidence of cancer in individuals who have an inherited deficiency in their ability to repair lesion in DNA

(c) the incidence of several well – defined chromosomal changes in cells of specific types of cancer

(d) the existence of a number of genes (oncogenes) that can transform normal cells into cancer cells

(e) the identification of genes (tumor suppressor genes) whose loss of inactivation leads to malignant transformation.

The genetic analysis of cancer cells is advancing rapidly due to the development of techniques that allow study of the base sequence of DNA and the cloning of genes. These techniques have shown that the transforming genes (oncogenes) that are carried by some tumor viruses are similar in base sequence (but contain specific mutations) to genes found in normal cells (proto–oncogenes). The introduction of such altered genes into cells of immortalized normal cell lines may induce transformation of the cells to a malignant state. Some types of chromosomal rearrangement, such as the translocation that leads to the Philadelphia chromosome in cells of chronic myelogeneous leukemia, have been shown to involve proto-oncogenes, with resultant changes in the protein products of these genes. In hereditary tumors, such as retinoblastoma, a strong association with deletion of a specific cellular gene (not association with any know viral oncogene) exists, suggesting loss of important genetic information in these cancer cells.

Current evidence suggests that the development of cancer involves a number of different genetic alterations, occurring sequentially in a cell, including the deletion of specific genes (tumor suppressor genes), the mutation of proto–oncogenes, and other chromosomal aberrations. These sequential changes are apparently associated with progression to increasing degree of malignancy. The identification of genetic changes in malignant cells, and of the role that the protein products of involved genes play in cancer causation, is a central focus of current cancer research.

The normal development and growth of any multicellular organism requires controlled interactions between cells in the organism. Growth control depends on a variety of signaling mechanisms, which are mediated by hormones and growth factors. The growth of a cancer demonstrates the failure of such a control mechanism. This could occur because the cancer cell does not respond to the control signals, possibly because of damage to its DNA or to the presence of the products of oncogenes.

Alternatively, it may be the homeostatic control mechanisms themselves that are disturbed or inappropriate. For example, loss of growth control may occur as a result of inappropriate secretion of growth factors, either stimulatory or inhibitory, by the tumor cells themselves or by the normal stromal cells surrounding the tumor cells. Alternatively, there may be modification, qualitatively or quantitatively, of growth factor receptors on the cell surface or modification of the response to growth factors by alterations in the way the growth factor receptor complex transmits signals to the cell nucleus.

1.2.1.2 Oncogenes and tumor suppressor genes

1.2.1.2.1 Oncogenes

It is now 25 years since the identification of the first cellular 'cancer gene' or oncogene (*c–SRC*) and 20 years since the discovery of the prototype tumor suppressor genes (p53, *pRB*) [31]. There are now known to be at least 100 oncogenes (Table 1), which broadly can be divided into five functional categories according to their protein products: growth factors, growth factor receptors, cytoplasmic protein kinases (enzymes which add phosphate groups to specific amino acids on substrate proteins), GTP–binding proteins and transcription factors (which bind to specific DNA sequences and activate the transcription of genes).

There are various ways in which oncogenes may be activated. A common mechanism is through chromosomal translocation. An important translocation is that associated with Philadelphia chromosome, Ph1, present in virtually all cases of chronic myeloid leukemia (CML) and involving t(9;22)(q34;q11). This results in the fusion of the *Abl* proto–oncogene on chromosome 9, which encodes a tyrosine kinase, and the *Bcr* (breakpoint cluster region) gene on chromosome 22; the resulting fusion protein processes enhanced constitutive tyrosin kinase activity.

Many translocations involve the *Myc* oncogene on chromosome 8q24, resulting in constitutive activation of this translocation factor. Virtually all cases of Burkitt's lymphoma, a B–cell derived, immunoglobulin producing tumor, invole a translocation of *Myc* with either the Ig heavy chain gene on chromosome 14 or the light chain genes on chromosome 2 or 22. MYC, in association with protein partners MAX (which results in activation) and MAD and MXI1 allow MYC to act as a translocation factor.

Another important chromosomal translocation is that involving the *Bcl–2* gene in chromosome 18 and various immonoluglobulin genes. The translocation is observed in about 80% of cases of follicular lymphoma and 20% of diffuse B – cell lymphomas and results in activation of BCL–2, a protein involved in protecting cells from apoptosis.

A second means of oncogene activation is through gene amplification. DNA amplification may be detected by the classical cytogenetic methods of double minute (DM) chromosome or homogeneously staining regions (HSRs) or by direct DNA analyses (Southern blotting) or by fluorescence *in situ* hybridization (FISH) or comparative genomic hybridization (CGH). Oncogenes where gene amplification has been reported include the Myc family (e.g. *N-Myc* in neutoblastoma, *c–Myc* in small cell lung cancer) and oncogenes associated with the HER family (e.g. *HER1* or *EGFR* in glioblastomas and many epithelial cell carcinomas and *HER2/ErbB2* in breast and ovarion cancer).

Furthermore, some oncogenes, notably those of the Ras family are activated by point mutations. The activating mutations are usually single amino acid changes in specific position which result in mutant RAS proteins with reduced intrinsic GTPase activity, thereby keeping the protein in the 'on' activated GTP–bound form rather than the 'off' GDP–bound state compared to the wild–type normal protein. As shown in Table 1, a number of tumors (about 25% in total) contain Ras mutations.

1.2.1.1.2 Tumor suppressor genes

There are also several tumor suppressor genes now known (Table 2). Many, such as *p53* and *pRB*, play critical roles in the cell cycle. Another important tumor suppressor is *PTEN/MMAC1* (phosphatase, tensin homologue/mutated in multiple advanced cancer) on chromosome 10q23.3 which is deleted or mutated in many tumors, including brain, breast, prostate, endometrium and ovarian [32]. *PTEN* encodes a phosphatase (enzyme opposite in effect to kinase in that they remove phosphate groups, generally leading to inactivation), which *in vivo* dephosphorelates the lipid phosphatidylinositol 3,4,5–triphophate (PIP_3). This form is found in the part of the PI_3K/Akt survival pathway.

Complementary to the identification of oncogenes and tumor suppressor genes is a better understanding of the normal cell cycle. Oncogenes act in a dominant manner and may be viewed as cell cycle accelerators, whereas tumor suppressors are analogous to cell cycle breaks. A third class of cancer–causing gene is genes involved in the DNA repair processes nucleotide excision repair and mismatch repair. When such genes are mutated, cells acquire more mutations in, for example, oncogenes and tumor suppressor genes, leading to increased tumor formation. With the completion of the human genome and the ongoing cancer genome project, all genes associated with cancer will eventually be known, although it will take some additional time to elucidate how the various gene/proteins function and interact with one another to cause cancer.

Table 1 Some of major tumor suppressor genes

Tumor suppressor genes	Function	Tumor examples
RB1	Cell cycle control	Retinoblastoma
P53	Cell cycle control, apotosis	Many types
WT1	Cell proliferation and	Wilm's
APC	differentiation	Colon
PTEN	Cell matrix adhesion	Many
P16	Phosphatase, cell survival	Melonoma
BRACA1	Cell cycle control	Breast and ovary
MLH1	DNA repair	Colon
	DNA mismatch repair	

1.2.1.3 Cancer chemotherapy

Since the origins of cancer chemotherapy, from observations of the effects of sulphur mustard gas used during World War I, there are now around 50 drugs licensed for the treatment of the disease. In general terms, anticancer drugs may be classified into a limited number of broad categories (Table 3).

1.2.1.3.1 Alkylating agents

The alkylating agents kill cells by direct interaction with DNA. Some are bifunctional (i.e. have two chemically reactive sites and produce two sites of damage or covalent adduct on DNA), whereas others are monofunctional and produce one adducts on DNA. R- CH_2 groups are added to DNA *via* the formation of highly reactive cyclical immonium ions, including cyclophosphamide, ifosfamide, nitrogen mustard, melphalan, thiotepa, busulfan and chlorambucil. Cyclophophamide is inactive as the parent drug but requires metabolic activation in the liver by cytochrom P450 enzyme to generate the active metabolite phospharamide mustard.

Table 2 Some of major oncogenes

Oncogenes	Function	Tumor
Ras – K	GTP/GDP binding	Pancreatic, colon
Ras – H	GTP/GDP binding	Bladder
Ras – N	GTP/GDP binding	Thyloid
Wnt 1	Growth factor	-
ErbB2	Growth factor receptor	Breast, ovary
Kit	Growth factor receptor	-
Abl	Tyrosine kinase	Leukemia
Src	Tyrosine kinase	-
Yes	Tyrosine kinase	Gastric
Lck	Tyrosine kinase	-
Akt	Serine/threonine kinase	-
Mos	Serine/threonine kinase	-
Raf	Serine/threonine kinase	-
Bcl–2	Antiapotosis	Follicular lymphoma
Myc	Transcription factor	Burkitt's lymphoma, neuroblastoma, leukemia
Myb	Transcription factor	-
Fos	Transcription factor	-
Jun	Transcription factor	-
Ets	Transcription factor	Ewing sarcoma

1.2.1.3.2 Antimetabolites

The antimetabolite class of anticancer drugs comprises analogues of natural compounds required for DNA or RNA synthesis. One of the earliest discovered was methotrexate, the first drug, in 1974, to cause complete remissions in children with acute lymphoblastic leukemia (ALL). Methotrexate is a potent inhibitor of the enzyme dihydrofolate reductase (DHFR), which catalyses the reduction of dihydrofolate to tetrahydrofolate. As a consequence, levels of intracellular folate coenzymes are decreased, levels of intracellular folate coenzymes are decreased. These coenzymes are required for thymidylate biosynthesis as well as purine biosynthesis; when depleted, DNA synthesis is inhibited and cell replication stops.

1.2.1.3.3 Antimicrotubule agents

The antimicrotubule agents comprise two distinct classes: the vinca alkaloids (vincristine and Vinblastine isolated from the leave of the Madagascar periwinkle plant) and the taxanes paclitaxel and docetaxel isolated from the Taxus or yew tree. The vinca alkaloid binds to tubulin, which is essential for forming the mitotic spindle fibers along which the chromosomes migrate during mitosis and for maintaining cell structure, and inhibit spindle assembly. In contrast, the taxanes bind to tubulin and stabilize the microtubule structure, thereby inhibiting depolymerization. The drugs are cell cycle specific, acting at the G_2/M phase.

1.2.1.3.4 Topoisomerase inhibitors

Topoisomerase inhibitors comprise two classes of drugs: those inhibiting *topoisomerase II* and other inhibiting *topoisomerase I*. These are enzymes important in maintaining DNA topology and in DNA replication and recombination as they introduce transient double–or single–stranded DNA breaks followed by strand passage and rejoining. *Topoisomerase II* inhibitors form a tenary complex between the enzyme and DNA, which results in the formation of DNA strand breaks. The *topoisomerase I* inhibitors irinotecan and topotecan are analogues of compothecin and act by binding to *topoisomerase I*, thereby resulting in DNA double–strand breaks.

1.2.1.3.5 Anti–hormonal drugs

The anti–hormonal group of drugs exploits the hormone dependence for the growth of some cancers, notably the common requirement for oestrogen in breast cancer and androgens in prostate cancer. One of the main anti–endocrine drugs used to treat breast cancer is the oestrogen receptor antagonist, tamoxifen.

Table 3 Major class of anticancer drugs

Class	Example
Alkylating agents	Melphalan, chlorambucil, cyclophosphamide, BCNU, CCNU, Procabazine, dacarbazine, temozolomide,
Amtimetabolites	Cisplatin, carboplatin, oxaliplatin
Antimicrotubule agents	Methotrexate, 5 – fluoracil, raltitrexed, Ara – C, gemcitabine, 6 – mercaptopurine, pentostatin, 6 – thioguanine, Hydroxyurea
Topoisomerase inhibitors	Vincristine, vinblastine, vinorelbine, paclitaxel, docetacel
Topo II	Doxorubicin, mitoxantrone, Etoposide,
Topo I	m – AMSA
Anti – hormonal agents	Irinotecan, topotecan
Breast	Tamoxifen, anastrozole
Prostate	Goserelin, cyproterone acetate

1.2.2 Leukemia

Leukemias are malignancies that arise from uncontrolled clonal proliferation of hematopoietic cells. The normal cellular control mechanisms are though to be inoperative in leukemia owing to change in the genetic code that are responsible for regulation of cell growth and differentiation. It is often mistakenly believed that acute leukemias are hyperproliferative disorders that result in the production of new cells at a pace. However, it is almost paradoxically true that leukemia cells may actually have

cycling times that are several times slower than normal bone marrow cells. These leukemic cells mature slowly and incompletely and survive longer than their normal marrow counterparts because of the failure to achieve normal and final maturation. It is believed, for example, that AML cells may actually have doubling times of approximately 30 days and require 50 to 70 doubling times before there is a significant accumulation of malignant cells. This final accumulation may occur over a period of 1 to 10 years and thus makes it difficult to truly access the initiation of the disease.

Leukemias undoubtedly arise as a result of multiple transformation step, or 'hits.' Significant evidence has been gathered that strongly implicates cellular oncogenes as key players in this leukemic transformation process. Oncogenes typically play an important role in normal cellular control and regulation and only display their oncogenic potential when their genetic structure or control elements are mutated, rearranged, amplified, or activated. Amplification and promotion of oncogenic activity may occur when the normal transcriptional promoter and control regions of these genes are disrupted. Various retroviruses have also been implicated as a cause or initiator of leukemia. Leukemia may secondarily arise as a result of exposure to a variety of environmental and iattogenic therapies.

Leukemias, in general, are defined as malignant neoplasms of the hematopoietic system arising in the bone marrow. As the bone marrow is replaced with the malignant cells, the excess malignant cells escape into the peripheral blood, hence the derivation of the name leukemia: white (leu-)/ blood (-emia). In a simplistic fashion, it is easier to classified leukemias based on (a) the natural course of disease, i.e. acute versus chronic, and (b) the basic cell type involved (lymphoid versus myeloid). Acute leukemias are the results of a block in maturation of immature lymphoid or myeloid cells. This accumulation of leukemic blast is the result of a block in maturation and differentiation rather than increased rapidity of differentiation. Acute leukemias are characterized by a rapidly fatal course of days or weeks, if untreated. This is in contrast to the chronic leukemias, which are typically associated with an indolent, albeit progressive, course of disease. Chronic leukemia is characterized by a proliferation of differentiated cells, as opposed to the immature blast seen in the acute leukemias. Table 4 outlines the various acute and chronic leukemias; figure 1 is a schematic diagram of their relationship to normal hematopoiesis. The relative

incidence of the four major subgroups of leukemias is follows; acute lymphoblatic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML) and chronic melogenous leukemia (CML).

1.2.2.1 Causes of leukemia

1.2.2.1.1 Inherited factor

The incidence of leukemia is greatly increased in association with some genetic diseases, particularly Down's syndrome (where acute leukemia occurs with a 20–30 fold increased frequency), Bloom's syndrome, Fanconi's anemia, ataxia telangiectasia, Klinefelter's syndrome and Wiskott–Aldrich syndrome. There is also a weak familial tendency in diseases such as acute myeloid leukemia (AML), B–cell chronic lymphocytic leukemia (CLL), Hodgkin's lymphoma and non–Hodgkin's lymphoma (NHL), although the genes predisposing to this risk are largely unknown.

1.2.2.1.2 Environmental influence

Chemical

Of all the chemicals to which people in our society are exposed only benzene is widely accepted as being a definite leukemogen. However, other chemicals have been implicated, for example the insecticide, hexachlorcyclohexane [33].

Various hematologic disorders of obscure cause (such as aplastic anemia) may be followed by acute leukemia [34]. Therefore, any chemical or drug which may cause aplastic anemia, for example, should be held suspect as a possible leukemogenic agent. There is a good deal of circumstantial evidence to implicate phenylbutazone in this regard. In 1960 Bean reported three or four cases of acute leukemia in elderly persons who had been receiving this drug. Subsequently, single cases of acute leukemia following the administration of phenylbutazone were reported by at least six observers [35, 36]. In 1964 Woodliff and Dougan reported five cases in which there was this association [37]. They estimated that in Western Australia, during the period of their study, in 9 percent of the cases of acute leukemia phenylbutazone had been given previously. If there is an increased incidence of leukemia among those receiving

phenlybutazone, those most likely to be affected are elderly patients who have taken the drug for long periods and who have had bone marrow depression

Table 4 Classification of leukemia

Acute leukemia	Chronic leukemia
Acute lymphoblastic leukemia (ALL) FAB: L1, L2, L3 IPh: B – precursor ALL T – cell ALL B – cell (Burkitt's leukemia/ lymphoma) Acute myelogenous leukemia FAB: M_0 – M_7 Acute biphenotypic leukemia Myelodysplastic syndrome (preleukemia) Refractory anemia Idiopathic anemia Refractory anemia with excess blasts Refractory anemia with excess blasts in transformation Chonic myelomonocytic leukemia Myelodysplastic syndrome	Chronic lymphoproliferative disorder (CLPD) B – CLPD B – Chronic lymphocytic Leukemia (CLL) Prolymphocytic leukemia Hairy cell leukemia Variants of CLL Wandenstrom's macroglobulinemia T – CLPD T – CLL Adult T–cell leukemia/ lymphoma Sezary syndrome Chronic myeloproliferative disorder (CMPD) Chronic myeolgenous leukemia Polycythemia vera Essential thrombocythemia Idiophatic myelofibrosis

FAB, French – American – British ; IPh, immunophenotype

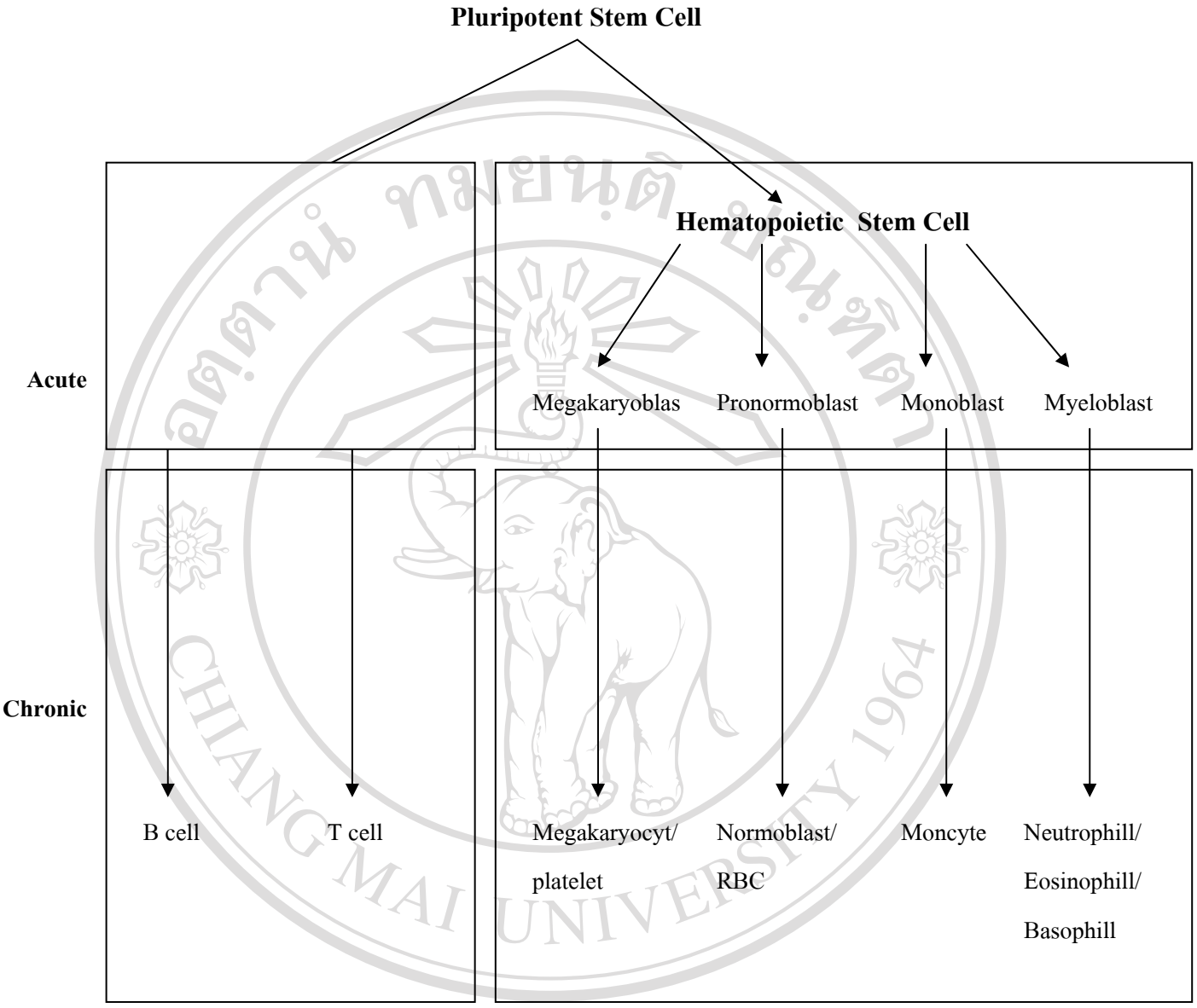


Figure 1 The relationship of the acute and chronic myeloid and lymphoid leukemias to normal hematopoietic and lymphoid development

Drugs

The alkylating agents (e.g. chlorambucil, mustine, melphalan, procarbazine and nitrosoureas–BCNU, CCNU) predispose to AML, especially if combined with radiotheraphy or if used to treat patients with lymphocytic or plasmatic disorders. Epipodophyllotoxins such as etoposide are powerful antileukemic agents but their use is associated with a risk of the development of secondary leukemias associated with balanced translocations including that of the *MLL* gene at 11q23

Radiation

It has been recognized for many years that radiation may be leukemogenic, and many studies have supported this hypothesis. Abbatt and Lea [38] collected reports of cases of ankylosing spondlylitis in England, and did not find leukemia in any of 399 males who had not received x–ray therapy. On the other hand, seven cases occurred in 1627 males who had received x–ray therapy (expected incidence in general population, 0.33 cases).

The greatest body of information concerning radiation leukimogenesis has been collected by the The Atomic Bomb Casualty Commission in Japan [39]. The commission's data reveal an increase incidence of leukemia in the people exposed to more than 50 rad, with a linear relationship between dose and incidence of leukemia in those exposed to between 100 and 500 rad. The increased incidence of leukemia was first seen one year after exposure.

Infection

A proportion of cases of childhood acute lymphoblastic leukemia (ALL) are initiated by genetic mutations that occur during development *in utero*. Studies in identical twins have shown that both may be born with the same chromosomal abnormality. This has presumably arisen spontaneously in a progenitor cell that has passed from one twin to the other as a result of the shared placental circulation. Environmental exposure during pregnancy may be important for this first event. One twin may develop ALL early (e.g. at age 4) because of a second transforming event while the other remains well or develops ALL later. The *TEL–AML1* translocation is present in the blood of approximately 10% of newborn infants but only 1 in 100 of

these go on to develop ALL at a later date. The mechanism of the 'second genetic hit' within the tumor cell is unclear but an abnormal response of the immune system to infection is suggested by epidemiological studies. Children with high levels of social activity, notably those attending early nursery daycare, have a reduced incidence of ALL, whereas those living in more isolated communities and who have a reduced exposure to common infections in the first years of life have a higher risk.

Viruses

Viral infection is associated with several types of haemopoietic malignancy. The retrovirus human T–lymphotropic virus type 1 (HTLV–1) is the cause of adult T–cell leukemia/ lymphoma (ATLL) although most people infected with this virus do not develop the tumor. Epstein–Barr virus (EBV) DNA is integrated into the genome of endemic (African) Burkitt's lymphoma cells but rarely in sporadic Burkitt's lymphoma cells. It is also the cause of posttransplant lymphoproliferative disesase (PTLD) which develops during immunosuppressive therapy after solid organ transplantation, of many cases of lymphoma associated with HIV infection and is present in a proportion of patients with Hodgkin's disease. Human herpes virus 8 (HHV–8; Kaposi's sarcoma associated virus (KSHV)) is associated with Kaposi's sarcoma and primary effusion lymphoma (PEL).

HIV infection is associated with an increased incidence of lymphomas at unusual sites such as the central nervous system. The HIV–associated lymphomas are usually of B–cell origin and of high grade histology.

Bacteria

Helicobactor pylori infection has been implicated in the pathogenesis of gastric mucosa B–cell (MALT) lymphoma.

Protozoa

Endermic Burkitt's transformation occurs in the tropic, particularly in malaria areas. It is thought that malaria may alter host immunity and predispose to tumor formation as a result of EBV infection.

1.2.2.1.3 Genetic factors

Malignant transformation occurs as a result of the accumulation of genetic mutations in cellular genes.The genes that are involved in the development of cancer can be divided broadly into two groups: oncogenes and tumor–suppressor genes.

Oncogenes

Oncogenes arise because of gain–of–function mutations in normal cellular genes called proto–oncogenes. Proto–oncogenes are involved in a variety of important cellular processes, often in the pathway by which external stimuli are transduced to the cell nucleus to activate genes. Oncogenic versions are generated when the activity of proto–oncogenes is increased or when they acquire a novel function. This can occur in a number of ways, including translocation, mutation or duplication. One of the striking features of hematologic malignancies (in contrast to most solid tumors) is their high frequency of chromosomal translocations. A subset of proto–oncogenes is involved in control of apoptosis (e.g. *BCL–2* which is overexpressed in follicular lymphoma)

Tumor – suppressor genes

Tumor–suppressor genes may acquire loss–of–function mutations, usually by point mutations or deletions, which lead to malignant transformation. Tumor–suppressor genes commonly act as components of control mechanisms which regulate entry of the cell from the G_1 phase of the cell cycle into the S phase or passage through the S phases to G_2 and mitosis. The most significant tumor–suppressor gene in human cancer is *p53* which is mutated or inactivated in over 50% of malignant disease, including many haemopoietic tumors.

1.2.2.2 Acute lymphocytic leukemia

Acute lymphocytic leukemia (acute lymphoblastic leukemia–ALL) is a malignant disorder resulting from a clonal proliferation and accumulation of progenitors that exhibit cell markers associated with the earliest stage of lymphoid maturation. The leukemia originates in the marrow, and the leukemia clone may exhibit features of either B–cell or T–cell commitment. At the time of diagnosis, the normal marrow cell population has usually been completely replaced by the leukemic

clone and there has been hematogenous dissemination to a variety of extramedullary sites. The blast cells have extremely limited functional maturation. Therefore, limited, if any, disturbance of normal humoral and cellular immunity is associated with ALL. The disappearance of normal hemopoiesis is not associated with malignant involvement of the pluripotential hematopoietic stem cell but results from a secondary effect of the accumulating marrow lymphoblasts.

ALL was the first disseminated malignancy to respond consistently to chemotherapy. Two decades later combined–modality therapy had resulted in long–term disease–free survival in more than half the cases of ALL in children. By three decades it was evident that ALL is a curable malignancy. Enhanced understanding of ALL came with the demonstration that the blast cells can be defined by specific markers. Studies were subsequently amplified to allow definition of lymphoid lineage and degree of maturation, which have clinical significance. Improved cytogenetic techniques permitted the correlation of karyotypic features, leukemic cell immunophenotypes, and responses to treatment. The information derived from studies of therapeutic strategies and biology has assumed an importance beyond the number of patients with this form of leukemia.

1.2.2.2.1 Classification

1.2.2.2.1.1 Morphological features

Morphological classification of leukemias is usually done using the French–American–British (FAB) system [40]. The revised FAB classification of ALL is based on weighing various criteria: nuclear–to–cytoplasm ratio, the number of nucleoli, nuclear membrane irregularity, and cell size (Table 5). The distribution of FAB–L1 and FAB–L2 differ in children and adults. Approximately 80% to 85% of cases of ALL in children are FAB–L1 compared with 35% to 40% in adults. Most of the remaining cases of ALL are classified as FAB–L2. Only 1% to 3% of ALL cases in adults and children are of the FAB–L3 subtype. This latter type of leukemia is a leukemic manifestation of Bukitt's lymphoma and is discussed in a subsequent section. FAB classification was useful for prognostic evaluation in the past; however, with present–day protocols for treatment of high risk patients, the prognostic

significance of morphologic classification is diminished. In fact, a recent proposal by the World Health Organization (WHO) utilizes both immunophenotype and cytogenetic finding for classification of these neoplasms.

In L1 of ALL, most of the leukemic cells are small with little to scant cytoplasm and absent, to inconspicuous at best, nucleoli. The chromatin pattern is clearly homogeneous and is not as finely reticular as those found in other types of leukemia. Nuclear irregularity is minimal. L1 morphology accounts for the vast majority of cases of ALL in children [41] but L2 morphology is the most common class in adults [42].

In L2 of ALL, the most significant difference from L1 is that the leukemic cells are larger and more heterogeneous. L2 cells typically have variable to moderately abundant amounts of cytoplasm and importantly have one or more prominent nucleoli and a finer chromatin pattern compared with L1. Irregular nuclear shapes are commonly seen.

L3 of ALL is characterized by a proliferation of large, homogeneous cells having deeply basophilic cytoplasm and sharply punched–out cytoplasmic vacuoles. The nuclear chromatin is uniform and typically coarser than is found in L1 and L2. This type of ALL is actually the peripheralized version of Burkitt's lymphoma and thus should be considered as an acute leukemia more from a historical than a biological perspective. The WBC may be normal to slightly elevated with only a low percentage of circulating leukemic blasts. The bone marrow typically shows only partial replacement with the leukemic cells. Because of its association with Bukitt's lymphoma, L3 is typically associated tumor masses in Western countries.

1.2.2.2.1.2 Immunophenotype

The ability to classify ALL by lineage commitment and degree of maturation features displayed by leukemic blasts (immunophenotype) began in 1975 [43, 44]. Reagents are available for precise definition of cell lineage characteristics [45-47], including the determination of lineage–specific gene arrangements and cell surface maturation antigens. The earliest indication of lineage commitment is rearrangement of immunoglobulin genes of B–cell leukemia [48, 49] and antigen receptor genes of T–cell leukemias [50]. In a patient both lineage–specific gene arrangements take place but this is associated with minimal acquisition of other markers of maturation. Further

definition of cell lineage characteristics can be achieved with a variety of monoclonal antibodies specific for antigens defining the progressive steps of maturation. Classification by immunophenotyping for ALL, it is possible to further subdivide these groups by the degree of B [51] or T [52, 53] markers displayed, which may have significance in predicting treatment outcome. Occasionally, the blast cells from a patient may display markers characteristic of both myeloid and lymphoid differentiation [54-56].

Characterization of leukemia blast cells by immunophenotyping is usually done with a flow cytometer and a panel of specific monoclonal antibodies that identify antigens with a specific cluster designation (CD). B–cell ALL generally expresses HLA–DR and CD19 and often is positive for CD10 (cALLa). A number of B–cell ALL also may express CD20. T–cell ALL will display CD7 and usually CD5 and CD20. In selecting surface markers to distinguish ALL from acute myelogenous leukemia (AML), CD19 and CD7 are useful in identifying B cells and T cells, respectively, while CD33 and CD13 identify myeloid lineage.

Table 5 Feature of the French–American–British Classification for ALL

Characteristic	L1	L2	L3
Cell features			
Size	Small; uniform	Large; non uniform	Large uniform
Cytoplsm	Scanty; moderate basophil	Variable in amount and degree of basophil	Moderate; abundant deep basophil prominent vacuole
Nucleus	Regular shape; incospicous nucleoli	Irregular shape; prominent nucleoli	Regular shape; prominent nucleoli
Age distribution (%)			
Children	85	14	1
Adult	31	60	9

1.2.2.2.1.3 Cytochemistry

The role of cytochemical staining in the diagnosis of ALL is more relevant to exclude the diagnosis of AML rather than to find supportive evidence for the diagnosis of ALL (Table 6). Historically, the Periodic Acid–Schiff (PAS) reaction has classically been considered a diagnostic stain for ALL. However, only 40% to 60% of ALL cases show a positive PAS, and PAS positive can occasionally be found in AML. Thus, PAS lacks both sensitivity and specificity for ALL. Nonetheless, PAS positive in ALL is characterized by large chunks or blocks of PAS staining cytoplasmic material. This can be distinguished from the diffuse PAS reaction seen in granulocytic cells. This enzyme stains the glycogen present within the cytoplasm of leukemic blasts. Thus, PAS positive in an acute leukemia should be looked at as suggestive, but certainly not diagnostic, of ALL. Acid phosphatase (ACP) has been evaluated as a possible marker for T–cell ALL. T lymphocytes and most T lymphoblasts show focal, punctuate, perinuclear positive with ACP. However, not all T–cell ALL show this pattern of staining, and a significant number of B – precursor ALL show positive with ACP, thus limiting its utility as a stain to differentiate between T–cell and non T–cell ALL. Myeloblasts may also show a focal staining with ACP. Oil red O, which stains lipid material, is an excellent marker for ALL–L3 or Burkitt's leukemia/lymphoma. The oil red O stain distinctly stains the vacuoles that are seen in this subtype of leukemia/lymphoma.

Terminal deoxynucleotidyltransferase (TdT) is a DNA polymerase that contributes to the recombination heterogenerity seen in immunoglobulin and T–cell receptor gene rearrangements. This enzyme is active during lymphoblast development and is expressed in 95% to 99% of ALL cases. ALL–L3 lacks this enzyme, as it biologically represents a maturing lymphocyte rather than a true leukemic blast. Unfortunately, 5% to 10% of AML also express this nuclear antigen, thus limiting the absolute lineage specificity of the TdT assay. TdT may be detected by either immunofluorescence, immunoperoxidase, or enzyme immunoassay methods. TdT can be identified in ALL of either T or B–precursor immunophenotype.

Table 6 Cytochemical staining in acute lymphoblastic leukemia

Stain	L1	L2	L3
MPO	-	-	-
SBB	-	-	-
CAE	-	-	-
NSE	-[a]	-[a]	-
PAS	+ (70%)	+ (70%)	-
ACP	-/+[b]	-/+[b]	-
MGP	-	-	+
ORO	+	+	+
TdT	+	+	-

[a] Faint positive may be seen

[b] Most T–cell acute lymphoblastic leukemia and some B–precursor acute lymphoblastic leukemia, will be positive MPO, myeloperoxidase; SBB, sudan black B; CAE, choroacetate estesrase; NSE, nonspecific esterase; PAS, Periodic Acid–Schiff ; ACP, acid phosphatase; MGP, methyl green pyronine; ORO, oil red O; TdT, terminal deoxynucleotidyltransferase.

1.2.2.2.1.4 Cytogenetic characteristic

The characterization of leukemic lymphoblasts by their cytogenetic pattern become possible during the late 1970s due to the development of better methods for cytogenetic studies in ALL and the development of chromosomal banding techniques [57]. Patients with ALL can be divided into four major groups by the cytogenetic features of the leukemic blast cells [13]. The karyotype may be normal (46 chromosomes without evident structural abnormalities), pseudodiploid (46 chromosomes with structural abnormalities, mostly translocations), or hyperdiploid group I (47 to 50 chromosomes), or hyperdiploid group II (more than 50 chromosomes). In ALL the hyperdiploid karyotypes are uncommon. There are differences between children and adults in distribution of these groupings. The pseudodiploid karyotype is more frequent in adults, and the hyperdiploid is more frequent in children [58]. Cytogenetic findings are currently the most powerful prognostic tool available.

Nonrandom chromosomal translocations have clinical significance [13, 59]. These specific translocations include the t(8;14), which is a consistent finding in the B–cell leukemia of L3 morphology with surface immunoglobulin; the t(9;22), or Philadelphia, chromosome; the t(4;11); the t(1;19) associated with pre B–cell ALL; and the t(11;14) associated with E rosette + T–cell ALL. The t(4;11) translocation is frequently found to be associated with blast cells that have mixed lymphoid and myeloid antigenic markers [60]. Infants with ALL are likely to have molecular rearrangements on chromosome 11q23. Chromosome translocations are more frequently found in infants [61] and adults [58].

The Philadelphia chromosome is found in about 5% of children with ALL [62] but in 17 to 25% of adults with ALL [58]. A more sensitive method of detecting this specific translocation is the polymerase chain reaction to demonstrate the resultant chimeric *BCR–ABL* gene. With this sensitive technique, 55% of adult B-lineage ALL patients expressing the common ALL antigen (CD10) are *BCR–ABL* positive [63]. Although this translocation when it occurs in ALL is called Philadelphia chromosome, the breakpoint region on chromosome 22 is different in ALL and chronic myelocytic leukemias [64]. The t(9;22) results in a fusion of the oncogene *ABL* on chromosome 9 with the *BCR* (breakpoint cluster region) on chromosome 22. The hybrid gene is expressed, with the gene product being a protein of p190 or p210 in size that has tyrosine kinase activity [65, 66].

Karyotype aneuploidy can also be detected by flow cytometry. With this technique a DNA index (the ratio of the DAN content or leukemic in comparison to normal non dividing cells) can be obtained [67]. Hyperdiploidy is present when the DNA index is greater than 1.15.

The L3 morphology is consistently found with the B–cell leukemia immunophenotype and the t(8;14) translocation. The L1 or L2 morphology, however, has no consistent correlations. The finding of specific translocation patterns with characteristic immunophenotypes has already been mentioned [13]. Hyperdiploidy is more common in children with early pre–B ALL, with translocations being less common in this group. Pre–B ALL is less likely to include hyperdiploidy, and translocations are found more frequently [68]. The t(9;22) ALL patients are most

usually cALLa (CD10) positive, while infants with the molecular rearrangements at 11q23 are cALLa (CD10) negative.

The biological significance of chromosomal translocations and molecular rearrangements in ALL is beginning to emerge [69]. The hybrid genes that are formed appear to result in inappropriate expression of transcription factors that are associated not only with leukemogenesis but also with the leukemic phenotype.

1.2.2.3 Acute myelogenous leukemia

Acute myelogenous leukemia (AML) is a heterogenous group of malignancies originating in the hematopoietic, or stem cells. The neoplastic proliferation seen in AML consists of myeloblasts or partially differentiated myeloid cells. This failure of complete maturation by the neoplastic clone results in an accumulation of immature precursor cells and gradual replacement of normal bone marrow elements. To restate an earlier conclusion, AML is not a disorder of rapidly proliferating cells but rather an accumulation of incompetent, long–surviving cells. The various subgroups or variants of AML basically reflect the existence of the various points in hematopoietic/myeloid differentiation.

The first well – documented case of acute leukemia is attributed to Friedrich, but it was Estien who used the term acute leukemia in 1889, and this lead to the general appreciation of the clinical distinctions between acute myelogenous leukemia and chronic myelogenous leukemia. The availability of polychromatic stains as a result of the work of Ehrlich, the decription of the myeloblast and myelocyte by Naegli, and the earliest appreciation of the common origin of red cells and leukocytes by Hirschfield laid the foundation of our current understanding of the disease.

The exact cause of this form of leukenmia is as yet unknown. However, various risk factors for the development of AML are known, including chemical exposures, such as to benzene; alkylating agent chemotherapy; ionizing radiation; preceding myelodysplastic syndrome (MDSs); aplastic anemia; paroxysmal nocturnal hemoglobinuria; syndromes with chromosome instability, such as Fanconi's syndrome; and rare forms of familial inheritance.

1.2.2.3.1 Classification

Variants of AML can be identificatified by morphologic features of blood film using polychromatic stains and histochemical reactions, monoclonal antibodies against surface markers [47, 70, 71], or the presence of specific chromosome translocations [72]. There is overlap in the epitopes on the progenitor cells of several phenotypic variants, and several monoclonal antibodies are required to make specific distinctions between cell types [73]. There is a poor correlation between morphologic and immunologic phenotyping of AML, as would be expected since the former method is more subjective, is subject to observer variation, and is based on qualitative factors, whereas the latter method, which characterizes surface molecular features, is more accurate and reproducible.

1.2.2.3.1.1 Morphology and FAB Classification

The French–America–British (FAB) group on acute leukemia has morphologically subclassified the AMLs into seven major subtypes: FAB–M1 to FAB–M7. The FAB classification of AML basically relies on the degree of granulocytic, monocytic, erythroid, and megakaryocytic differentiation. This is based on the morphologic appearance of cells, the number of leukemic blasts, and cytochemical findings with myeloid–associated enzymatic stains. Important variants of these seven subtypes also exist. The National Cancer Institute has proposed revised standards for the classification of AML, including other subtypes based on immunophenotyping (M0).

The importantance of the FAB classification of AML lies not in its ability to provide important prognostic information to the patient but rather its ability to provide precise and consistent classification of AML subtypes. The ability to produce the various FAB subgroups morphologically and cytochemically is well proven and allows for consistency between institutions and physicians.

The basic definition of an AML is a leukemia that has more than 30% myeloblasts in the bone marrow. However, in the most recent WHO classification, the required percentage of blasts for diagnosis of AML has been decreased to 20% in the bone marrow and peripheral blood.

1.2.2.3.1.1.1 FAB–M0 AML without mutation

The FAB–M0 group of AML is myeloid leukemia with a minimal differentiation that has a demonstrable myeloid lineage by immunophenotyping or ultrastructural studies. These AMLs consist of small–to intermediate–size leukemic blasts without evidence of granulocytic differentiation. Cytochemical staining with MPO, Sudan black B (SBB), and nonspecific esterase (NSE) is negative. Thus, from a strict morphologic and cytochemical analysis, these cases would have been classified as ALL in the past. However, by flow cytometric immunophenotyping, these cases show reactivity with one or more myeloid–associated markers, such as CD17, CD13, CD33, or CD15, while lacking expression of lymphoid–associated markers.

1.2.2.3.1.1.2 FAB–M1 AML without mutation

In these AMLs without mutation, there is minimal evidence of cytoplasmic granulation and minimal numbers of Auer rods. The blasts are intermediate in size with finely reticular chromatin, small amounts of grayish – blue cytoplasm, and typically one or more prominent nucleoli. Basic FAB criteria for AML–M1 include the following;

(a) the sum of blasts is greater than 90% of the bone marrow cells

(b) less than 10% of bone marrow cells shows evidence of granulocytic differentiation at or beyond the promyelocyte stage

(c) at least 3% of leukemic blasts demonstrate MPO and/or SBB positively

The differential diagnosis in this disorder includes ALL–L2, AML–M5a, and AML–M7. Immunophenotyping and cytochemical staining are usually necessary to make the distinction between these subtypes of leukemias.

1.2.2.3.1.1.3 FAB–M2 AML with mutation

The AMLs with matation are the most common subtype of AML. These leukemias show clear evidence of differentiation at or beyond the promyelocytes stage, and Auer rods are commonly identified. These cells usually have more cytoplasm than is found in FAB–M1 leukemic cells, and fewer undifferentiated blasts are seen. From a diagnostic viewpoint, this subtype of AML is easy to diagnose and

creates little diagnostic consternation. The basic FAB criteria for FAB–M2 include the following

(a) the sum of blasts is 20% or greater but less than 90% of bone marrow cells

(b) more than 10% of the bone marrow cells show evidence of granulocytic differentiation

(c) monocytic cells constitute fewer than 20% of the bone marrow cells

It should also be noted that dysplastic features may be identified in the granulocytic, erythoid, and/or megakaryocytic series. Frank panmyelosis is uncommon but can be seen in some cases. Although most bone marrow specimens are hypercellular and show frank replacement of all bone marrow elements, some patients, mainly the elderly, may have bone marrow cells that are moderately hypercellular.

The differential diagnosis of FAB–M2 would include a leukemiod reaction, a MDS that does not meet the criteria of AML and possibly other types of AML having a granulocytic component such as FAB–M3, FAB–M4, or possibly FAB–M6

A specific bone marrow chromosome abnormality, t(8;21), has been observed in some cases of FAB–M2. The percentage of patients with the t(8;21) translocation has varied from 30% to less than 5% of FAB–M2 in various reports in the literature. Patients having AML–M2 with a t(8;21) generally to have good prognosis. The morphologic features of FAB–M2 with a t(8;21) include large meloblasts, easily identified and numerous Auer rods, and a distinctive dysmyelopoiesis in developing granulocytic cells. This dysmyelopoiesis has been described as a “crushed” orange granularity in the cytoplasm of the granulocytic cells. These patients usually aberrantly express CD19 and/or CD56

1.2.2.3.1.1.4 FAB–M3 Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) can be diagnosed when 20% or more of the bone marrow cells are abnormal promyelocytes. These promyelocytes have abnormally dense and heavy granulation. One of the characteristic features of APL is the so–called faggot cells, which are cells that contain multiple Auer rods that may be bundled, intertwined, or fused together. The granules of the promyelocytes are large and darker staining than normal and times may be so numerous as to obscure nuclear

borders. Intensely basophilic cytoplasm may be present in some cells. The nuclear features, which are frequently ignored or even obscured by the granulation, typically have a monocytoid, bilobed, or kidney–bean shape. It is uncommon for this type of AML to have a significant percentage of blasts. In a large series of APLs, the median blast count was only 12%. These patients are typically leukopenic at presentation and are clinically characterized by disseminated intravascular coagulation (DIC) and bleeding.

Approximately 20% of APLs are a variant from of FAB–M3 and designated as microgranular or hypogranular APL (M3v). The leukemic cells in this FAB–M3 variant are characterized by sparse and/or fine granulation a strikingly irregular nuclear shape. Their identity as abnormal promyelocytes may be obscured by the scarcity of granulation and the nuclear shape. Cells containing multiple Auer rods are usually present, but they may be extremely difficult to identify because they are certainly less abundant than in the typical hypergranular FAB–M3. The variant form of APL has the same incidence of DIC described previously but will more likely present leukocytosis at the time of diagnosis.

APL may occur at any age but is most common in young adults, with a median age of diagnosis of 35 to 40 years. The most outstanding clinical feature associated with APL is the high frequency of DIC. This is due to the release of procoagulant factors from the cytoplasmic granules, which leads to activation of the coagulation cascade. In many patients there are severe DIC and hemorrhage before or during induction of therapy, when the malignant cell contents are lysed and released. Hemorrhage is the cause of death in many patients. Thus, it is essential that this subgroup of AML be accurately diagnosed rapidly so that appropriate chemotherapy and supportive care can be initiated. If DIC and hemorrhage are adequately controlled and treated, patients with this subgroup of AML probably have the best prognostic outcome of any AML group. Recognition of this particular subgroup is especially important, because a specific regimen including all–*trans*–retinoic acid (ATRA) causes leukemic cells to overcome maturation arrest.

The differential diagnosis of APL includes other types of AML with granulocytic components, such as FAB–M2, FAB–M4/M5, and benign agranulocytosis with a promyelocytes arrest. In cases of benign agranulocytosis, the

platelet count and hemoglobin are generally normal, in contrast to the pancytopenia seen in APL. In addition, in the diagnosis of APL is the distinction with granulocytic components, such as FAB–M2, FAB–M4/M5, and benign agranuloctosis with a promyelocyte arrest. In cases of benign agranulocytosis, the platelet count and hemoglobin are generally normal, in contrast to the pancytopenia seen in APL. In addition, in agranulocytosis, the bone marrow is not hypercellular and Auer rods are not identified. The major difficulty in the diagnosis of APL is the distinction of microgranular APL (M3v) from FAB–M4 or M5b. Evaluation with cytogenetic and molecular studies typically resolves this issue. Demonstration of t(15;17)(q22;q12 -21) or t(11;17) is diagnostic of APL and essential for diagnosis. The immunophenotypic studies in typical APL include myeloid phenotype (CD13, CD33) and lack of HLA-DR, CD34.

1.2.2.3.1.1.5 FAB–M4 Acute Myelomonocytic Leukemia

Acute myelomonocytic leukemia and FAB–M2 are the most common AMLs, together accounting for approximately two thirds of all AML cases. The FAB–M4 subgroup of AML is probably the most difficult subgroup of AML in which to perform a reliable differential count, as this is a very heterogeneous–appearing leukemia. Both granulocytic and monocytic differentiations are present in varying proportions in the bone marrow. It may be difficult to identify the granulocytic and monocytic components, as hybrid cells clearly exist in this type of leukemia. The criteria for the diagnosis of FAB–M4 include the following elements:

(a) the sum of all blasts is greater than 20%
(b) the sum of myeloblasts and granulocytic precursor cells account for less than 80% of the bone marrow cells
(c) more than 20% of bone marrow cells are of the monocytic lineage as demonstrated by morphology, NSE cytochemical stain, or elevated serum lysozyme level (three times normal).

Granulocytic and monocytic precursors coexist in proportions varying reciprocally from 20% to 80%. Auer rods may also be identified in approximately half of the cases. Because of the difficulty in the morphologic identification of both promonocytes and hybrid granulocytic/monocytic cells in bone marrow, additional

diagnostic criteria utilizing NSE and elevated serum lysozyme levels have been used. Organomegaly, lymphadenopathy, and other sites of tissue infiltration may commonly be encountered.

A variant of FAB–M4 exist, with has been called FAB–M4 with eosinophilia (M4e). Criteria for diagnosis include the usual diagnostic criteria for FAB–M4 and an increased number of atypical, immature bone marrow eosinophils. The immature and atypical eosinophils contain an abundance of large basophilic–staining granules in addition to the large, red eosinophilic granules that characterize a mature eosinophil. Abnormalities of chromosome 16, including inversion of 16(p13;q22) or deletion of 16q22, are consistently identified in this variant. This type of leukemia has a high rate of remission after the initial induction of therapy compared with other types of AMLs, and diagnosis of this variant is considered a good prognostic sign.

1.2.2.3.1.1.6 FAB–M5a: Acute Monoblastic Leukemia and FAB–M5b: Acute Monocytic Leukemia

FAB–M5a is characterized by a predominance of monoblasts that are large and have relatively abundant cytoplasm. Some azurophilic granules may be identified in the cytoplasm that are MPO negative. The nucleus is typically round to oval with finely reticular chromatin and prominent nucleoli. Typically, the nucleus is displaced to one side with an ample amount of cytoplasm wrapping around the nucleus. The sole criteria for diagnosis of FAB–M5a are the existence of more than 80% monoblasts in the bone marrow. This differs slightly from the criteria for FAB–M5b, in which more than 80% of the marrow cells are monoblasts, promonocytes or monocytes but less than 80% of the marrow cells are monoblasts. In other words, FAB–M5b shows more differentiation than FAB–M5a. Nuclear folding and irregularity are common in FAB–M5a. Both FAB–M5a and FAB–M5b are associated with a high incidence of extramedullary infiltration; DIC may also develop in patients with FAB–M5, second in incidence only to FAB–M3 among classes of AML. NSE cytochemical stains are positive in the FAB–M5 leukemia. Auer rods may be seen in a small percentage of monoblasts but are certainly much less frequent than in the granulocytic types of AML. AML–M5a is more commonly diagnosed in the pediatric age group.

1.2.2.3.1.1.7 FAB – M6: Erythroleukemia

Acute erythroleukemia is a relatively uncommon variant of AML and may have multiple presenting appearances. One form, which has previously been called erythemic myelosis, is characterized by bizarre and markedly megaloblastoid changes accompanied by extreme erythroid hyperplasia within the bone marrow. Few, if any, myelogblasts can be identified. Normal granulocytic and megakaryocytic precursors are not identified and are replaced by giant, multinucleated, and markedly dysplastic erythroblasts. In other cases of FAB–M6, the marrow contains more differentiated, albeit dysplastic, erythroblasts at the time of presentation along with a definite population of granulocytic cells, including myeloblasts. The following criteria were established by the FAB group for the diagnosis of erythroleukemia in determining blasts percentage:

(a) 50% or more of all nucleated bone marrow cells must be erythroblasts
(b) dyserythropoiesis is prominent
(c) 20% or more of the nonerythroid cells in the bone marrow are myeloblasts.

Erythroleukemia will frequently evolve into other types of AML such as FAB–M1, M2, or M4. Auer rods may be identified in the myeloblasts in these cases. The leukemic erythroblasts frequently contain a “frothy” or “foamy” vacuolation in the cytoplasm. Progression of the disease is frequently marked by an increase in myeloblasts and a decrease in erythroblasts. A striking erythroblastemia may be identified in the peripheral blood. It is quite common for erythroleukemia to evolve from MDSs or as a secondary leukemia in patients who have received prior radiation and/or alkylating–agent chemotherapy. The differential diagnosis for erythroleukemia include B_{12} /folate deficiency, heavy metal intoxication (such as arsenic), drug effects (such as with antineoplastic agents or chloramphinicol), congenital dyserythropoietic syndromes, MDSs, and potentially other types of AML. The dysplastic erythroblasts are typically PAS positive, which reflects a cytoplasmic maturation defect. This cytochemical finding is not restricted to leukemia and can be identified in benign disorders, such as β-thalassemia, iron deficiency, sideroblastic anemia, and heavy metal intoxication.

1.2.2.3.1.1.8 FAB – M7: Acute Megaloblastic Leukemia

Acute megaloblastic leukemia has only recently been added to the FAB classification. The diagnostic criteria for diagnosis include:

(a) more than 20% blasts in the bone marrow

(b) definitive identification of megakaryoblastic involvement by a platelet peroxidase reaction by electron microscopy or reactivety with megakeryocyte – specific monoclonal antibodies.

The differential diagnosis of megakeryoblastic leukemia includes ALL–L2, AML–M0, AML–M1, and AML–M5a. As can be seen from this differential diagnostic list, there is a morphologic heterogeneity to FAB–M7. The blasts may vary from small–to medium–sized to large, bizarre blasts typically having a high nuclear:cytoplasmic ratio. The nuclear chromatin may be dense and homogeneous or fine and reticular. There is typically scanty basophilic cytoplasm, which may or may not be vacuolated. Indeed, the degree of basophilia may be comparable with that found in erythroleukemia, reflecting the close developmental relationship between erythroid and megakeryoblastic precursors. An irregular cytoplasmic border may be noted in some of the megakeryoblasts resrmbling pseudopods. Very fine granulation can be identified in the cytoplasm of some blasts. Intermediate forms between undifferentiated blasts and definitive micromegakeryocytes may also be seen. Some cases may show little if any differentiation and resemble the lymphoblast of FAB–L2.

The diagnosis of FAB–M7 relies on multiple criteria, including peripheral blood and bone marrow aspirate smear morphology, bone marrow trephine section histology, routine cytochemical stains, immunophenotyping, and ultrastructural studies. In some cases, the peripheral blood and bone marrow aspirate morphology may be sufficient to make the diagnosis, whereas in other cases, more sophisticated diagnostic modalities may be needed to confirm the diagnosis of FAB–M7. Some cases of FAB–M7 have morphologic features suggestive of a megakeryoblastic process, including the presence of circulationg micromegakaryocytes, atypical megakaryocytes in the bone marrow section.

Cytochemically, megakaryoblasts show no reactivity with MPO or SBB. A diffuse, coarse granular positivity is typically seen with PAS, not to be confused with the chunky staining seen in ALL. Megakaryoblasts show no reactivity with α –

naphthyl butyrate esterase but can manifest strong reactivity with the acetate substrate of NSE. This latter discrepancy between the butyrate and acetate substrates of NSE can be a very useful diagnostic feature of FAB–M7. Immunophenotyping with monoclonal antibodies reactive with platelet glycoprotein (Gp) IIb/IIIa or Gp IIIa (CD41 and CD61) has provided a more sensitive and reproducible method of detecting megakaryoblasts. These antibodies are discussed in more detail in the subsequent section on immunophenotyping.

Acute megakaryoblastic leukemia is commonly associated with patients that have Down's syndrome, therapy – related acute leukemias and blast crises of chronic myelopeoliferative disorders. Clinical features of FAB – M7 are variable and can occur in both children and adults. A marked leukocytosis is relatively uncommon in M7 and typically is not associated with extramedullary involvement, lymphadenopathy, or hepatosplenomegaly.

1.2.2.3.1.1 Cytochemistry

MPO is an enzyme present within the primary granules of the granulocytic series, including granulocytes, eosinophils, and some basophils. Monocytes also contain peroxidase granules but are present in very small numbers and never pose a serious problem when interpreting such a stain. Lymphoid and erythroid cells do not stain with MPO. MPO is the most sensitive marker for granulocytic diffentiation; however, MPO stains will fade with time; for this reason SBB is also commonly used and is a good alternative to MPO. SBB stains intracellular lipids that are within the primary granules of the granulocyte series. This is easily detected within myeloblasts and the rest of the granulocyte series. Lymphoblasts, however, may also, show an occasional rare, small positive granule. Thus, one must interpret SBB with some caution. CAE, or specific esterase/Leader stain, is confined to the primary granules of differentiated granulocytes. Myeloblasts are typically lacking in this enzyme. Monocytes, lymphocytes, and erythroid cells are negative with this enzyme stain.

NSE is a particularly useful stain for the monocytes series. Little, if any, staining of the granulocytic series will be found. T lymphocytes and T lymphoblasts typically show a distinct punctate, perinuclear positivity that is easily distinguished from monocyte staining. The butyrate esterase gives a clearer and more definitive stain

compared with the acetate substrate of NSE and is the preferred monocyte – associated enzyme stain. It gives a deep, brick – red stain that is easy to interpret and distinguish from background staining, as opposed to the faint positivity seen with the acetate substrate of NSE. The acetate NSE activity can also be found in megakaryocytes, platelets, and some basophils and plasma cells. Fluoride sensitivity is used by some laboratories to help differentiate between different NSE – positive cells. The NSE reactivity found in monocytes is blocked by fluoride and is partially blocked in megakeryocytes. NSE staining found in lymphocytes and lymphoblasts, however, is not sensitive to fluoride treatment. Occasional cases of B – precursor ALL also show a faint bluish in the Golgi area of such lymphoblasts; this is also fluoride resistant.

As mentioned in the ALL section, PAS may stain some cases of AML, thus limiting its diagnostic use as a specific ALL marker. TdT reactivity can also be found in as many as 20% of AML cases, although it is usually dimmer and positive in a lower percentage of cells than in typical ALL.

1.2.2.3.1.3 Immunophenotype of AML

The diagnosis of AML is usually a straightforward diagnosis made based on morphology and cytochemical staining. Immunophenotyping of potential cases of AML should be done for the following reasons

(a) to distinguish AML from ALL (the most practical reason to analyze a potential AML)

(b) provide correction with the FAB morphologic subtype of AML

The two situations that commonly pose the most difficult problem for diagnosing an AML are leukemias with no or minimal evidence of diffentiation (FAB–M0 and FAB–M1) and those of megakaryoblastic differentiation (FAB–M7). Pan myeloid monoclonal antibodies, CD13, CD33 and CD117, have been universally used to distinguish AML from ALL on an immunologic basis. Overall, 90% to 98% of AMLs will react with these markers. Moreover, these reagents have appeared to be highly specific for myeloid cells and are expressed in all FAB morphologic subtypes of AML. Some FAB–M0 and FAB–M1 AMLs tend to express CD33 only, suggesting that those leukemias are of earlier myeloid development than other AML. In addition, acute megakaryoblastic leukemias (FAB–M7) may not express either CD13 or CD33

or tend to express CD13 and/or CD33 in a lower percentage of blasts with weaker intensity than in more classic AML.

The use of a panel of myeloid – associated monoclonal antibodies is necessary to access any possible correlations between an immunophenotype and the FAB morphologic classification. Reports in the literature have demonstrated that myeloid monoclonal antibody reactivity may correspond to broad categories of morphologic differentiation: myeloblastic (FAB-M1 and M2), promyelocytic (FAB–M3), monocytic (FAB–M4 and M5), erythroid (FAB–M6), and megakaryocytic (FAB–M7). FAB–M1 and M2 acute leukemias react with the pan–myeloid markers CD13, CD33 and CD117. CD15 also react with most cases of FAB–M1 and M2. Monocyte–associated monoclonal antibodies (CD11b, CD14, CD36 and CD64) are usually non-reactive in cases of AML M1 and M2; CD14, however, has been detected in as 15% of such AML cases. Monoclonal antibodies more restricted to the mature stages of granulocytic differentiation, such as CD10, CD16 and CD24 do not react with the myeloblasts in these leukemias.

HLA–DR expression is a common finding in AML. This reflects the normal distribution of HLA–DR or myeloid precursor cells up to, but not including the promyelocytes stage. The lack of reactivity of HLA–DR antibodies in most cases of APL is consistent with the observation that the normal promyelocyte is HLA–DR negative. However, occasional genuine cases of APL may be HLA–DR antigen positive. The hypogranular variant of APL has a pattern of reactivity similar to that of the typical granular form. HLA–DR negativity is not exclusive to APL because as many in APL, it does not appear to be a 100% sensitive and specific diagnostic feature of this unique subtype of AML.

The monocytic leukemias (FAB–M4 and M5) are also reactive with the pan myeloid monoclonal antibodies, CD13, CD15 and CD33 and uniformly express the HLA–DR antigen. CD11b, CD11c, CD14, CD36 and CD64 antigens are also displayed by the majority of acute myelomonocytic and monocytic leukemias. CD14 expression is thought to be acquired at some point after the development of the monoblasts and before maturation to the monocyte stage. This suggests that CD14 reactivity would be expected to be most prominent in the FAB–M4 and FAB–M5b leukemias. However, CD14 is commonly found in almost all cases of monoblastic

leukemias (FAB–M5a). Interestingly, CD14 has recently been identified in a significant number of B–cell, non–Hodgkin's lymphomas, confirming that this antigen is not myeloid restricted.

No specific and sensitive marker of early erythroid development is currently available for the diagnosis of erythroleukemia (FAB–M6). The transferrin receptor CD71 is reactive with some myeloblasts in addition to all erythroblasts in the erythroleukemias. However, labeling of blasts with CD71 can also be observed in other types of AML and may be detected in some cases of ALL as well. Antiglycophorin antibodies appear to react only with cells that are morphologically obvious as erythroblasts, beginning at the basophilic normoblasts stage, and thus will not label the early pronomoblasts. This finding is consistent with studies showing that these glycophorin antibodies label only the post–CFU–E stages of erythrocyte development.

The diagnosis of acute megakaryoblastic leukemia by immunophenotyping with monoclonal antibodies directed against megakaryoblast–associated antibodies has allowed for a rapid and easier method of diagnosis of this poorly recognized subtype of acute leukemia. Monoclonal antibodies against platelet glycoprotein IIb/IIIa or IIIa (CD41 and CD51) are routinely used in the diagnosis of acute megakaryloblastic leukemia (FAB–M7). Although factor VIII related antigen activity may be observed in rare cases of megakaryoblastic leukemia, the reactivity of megakaryoblasts with platelet glycoprotein IIb/IIIa or IIIa is more sensitive than labeling for factor VIII–related antigen.

Although immunophenotyping is generally accepted as the standard for diagnosing acute megakaryoblastic leukemia, it must be realized that some false positivity can be seen with these antibodies. This is most generally seen in myeloid leukemia that have a monocytic component. This increased reactivity of these megakaryoblast–associated glycoproteins appears to be nonspecific background labeling, reaction with platelet membrane components adhering to monocytic cells.

Other antigens not typically associated with myeloid cells can also be identified in AML. CD34 can be found in 30% to 40% of all AML and has been associated with a poorer prognosis than CD34 negative AML. The CD34 antigen can be useful in

evaluating a specimen containing a mixture of myeloblasts and differentiated myeloid of lymphoid cells.

CD38 has classically been used as an early thymocyte marker but is now recognized to be present on activated T cells as well as being an excellent plasma cell marker. Not surprisingly, it can also be identified in AML.

CD4 is the receptor for HLA class II antigen that characterizes the T–helper subset or T lymphocyte. This antigen is not restricted to T cells and can be identified in monocyte, monocyte precursors, and monocytic leukemias.

CD7 is a pan–T–cell antigen that can be found in 5% to 10% of all AML cases; CD7 can be found in all subtypes of AML. Although some have suggested that the presence of CD7 reflects an early bone marrow precursor, other data do not support this concept.

CD45, the common leukocyte antigen, is found in virtually all cases of AML.

1.2.2.4 Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is a neoplastic disease characterized by the accumulation of small mature–appearing lymphocytes in blood, marrow and lymphoid tissue. The first descriptions of patients with CLL were published in the early nineteenth century. In the 1954, Tivey published the survival data of 685 patients with CLL, observing that as median survival time was approximately 3 years from the sign of symptoms related to CLL [74]. In 1967, Dameshek hypothesized that CLL was an accumulative disease of immunologically incompetent lymphocytes [75]. In the early 1970s, the leukemic cells from most cases of CLL were observed to express surface immunoglobulin, indicating that the neoplastic cells were of B–cell origin [76]. The monoclonal nature of CLL was established by the light–chain restriction [77] and idiotypic uniformity [78-80] of the immunoglobulins expressed by the leukemic cells from any one patient, and by the expression of a single isoenzyme in the lymphocytes of female patients with CLL who are hererozygous for glucose–6–phosphate dehydrogenase [81]. A clinical staging system for patients with CLL was introduced in 1975 by Rai and colleagues [82], delineating the adverse implications of anemia or thrombocytopenia on patient survival. In the late 1980s, purine analogs, such as fludarabine or 2–chlorodeoxyadenosine (cladribine), were found to be

effective in the treatment of CLL. Other treatment modalities are being examined, including immunotherapy or ablative chemotherapy with marrow transplantation, as the disease still is not considered curable.

Table 7 Classification of acute myelogeneous leukemia

FAB Subtype	Diagnostic criteria
M0 Acute leukemia, undifferentiated; Myeloid immunophenotype	No morphologic evidence of differentiation Negative cytochemical staining with MPO/SBB/NSE Positivity with myeloid markers (CD13,CD33, etc) and negative lymphoid markers
M1 Acute myelogenous leukemia (AML) without maturation	90% blasts in bone marrow <10% of marrow showing granulocytic differentiation >3 of blasts with MPO or SBB positivity
M2 AML with maturation	≥20% blasts and <90% blasts in marrow ≥10% of marrow showing granulocytic differentiation ≤20% monocytic cells
M3 Acute promyelocytic leukemia (APL)	≥20% of marrow or abnormal promyelocytes
M3v Hypogranular variant of APL	Same as M3 except composed of hypogranular variants
M4 Acute myelomonocytic leukemia	≥20% blasts ≤80% myeloblasts and granulocytic precursor in marrow >20% monocytic cells in marrow (morphology/NSE stain)
M4e M4 with eosinophilia	Same as M4 with increased number of atypical/immature marrow eosinophils
M5a Acute monoblastic leukemia	≥80% monoblasts
M5b Acute monocytic leukemia	≥80% of monoblasts/promonocytes/monocytes <80% monoblasts in marrow
M6 Acute erythroleukemia	≥50% nucleated RBC Prominent dyserythropoiesis >20% myeloblasts in nonerythroid cells
M7 Acute megakaryoblastic leukemia	≥20% blasts Identification of megakaryoblasts by ultrastructural cytochemistry or by immunophenotyping

CLL involves the blood and bone marrow, and also frequently the lymph nodes, spleen, and liver. It is a disease with a male predominance and a median age of approximately 65, being rarely seen in individuals less than 40 years of age. It usually follows an indolent course, and many patients are diagnosed by finding an elevated lymphocyte count incident through evaluation for another disease. Most cases of CLL are of B- cell type, and the following discussion applies to B-cell CLL. T–cell ALL, which is very rare, is biologically distinctly different from B–cell CLL

B–cell CLL is the only major adult leukemia that is not associated with exposure to ionizing radiation, drugs or chemicals [83-85]. Also, there is no evidence for viral etiology [86]. One study of uncertain significance found that the relative risk for CLL in Sweden correlated with magnitude of exposure to low–frequency electromagnetic fields [87, 88]. More convincingly, the epidemiology of B–cell CLL suggests that sex and genetic factors influence disease susceptibility.

1.2.2.4.1 The feature of CLL

To diagnose CLL, there should be a peripheral blood lymphocytosis greater than 5,000/mm^{3}and a marrow lymphocyotosis greater than 30%. At diagnosis, most patients have a white blood cell count of greater than 15000/mm^3 with a median count of about 30,000 to 40,000/mm^3. Some cases may have a WBC well over 100,000/mm^3. Because CLL is a clonal process, there is frequently a monotonous lookalike appearance to the lymphocytes on smears. Cytoplasm usually is small to moderate in amount, pale blue, and agranular; the nuclear chromatin frequently shows exaggerated chromatin clumping compared with a normal lymphocyte.

The bone marrow in CLL usually aspirates freely, and marrow section may range from normocellular to hypercellular. When the marrow is less extensively infiltrates, the lymphocytes in the marrow may be present in focal aggregates or may be diffusely intermixed with residual normal marrow elements. This latter pattern is called an interstitial infiltrative pattern. Frequently, a mixed focal and interstitial pattern may be seen. As the marrow becomes more extensively infiltrated, focal or interstitial patterns are gradually lost, and the infiltrate diffusely obliterates the marrow space.

When the blood and marrow counts are near the lower limits described above, and the diagnosis is in question, immunologic cell surface marker studies may be helpful in establishing the diagnosis and in ruling out post–plenectomy lymphocytosis, a chronic reactive lymphocytosis or other chronic lymphoproliferative disorders. Clonality, as evidenced by weakly reacting surface immunoglobulin with a restricted light chain, and CD5 and CD23 positivity are characteristic of CLL. In cases with a low WBC in the range of 5,000 to 15,000/ mm^3, it may be easier to make the diagnosis using peripheral blood immunology than with bone marrow examination, because the bone marrow in these cases also may show a low percentage of lymphocytes, and characteristic spreading focal aggregrates may be absent. If immunologic marker studies are available, immunologic evaluation of the peripheral blood is the method of choice to confirm the diagnosis, especially if therapy is not contemplated.

1.2.2.5 Chronic Myelogenous leukemia

Chronic myelogenous leukemia (CML) is a hemopoietic stem cell disease that is characterized by anemia, extreme blood granulocytosis and granulocytic immaturity, basophilia, often thrombocytosis and splenomegaly. The hemopoietic cells contain a reciprocal translocation between chromosome number 9 and 22 in over 90 percent of patients, which leads to overtly foreshortened long arms of one of the chromosome pair number 22 (i.e. 22.3q-) referred to as the Philadelphia (Ph) chromosome. A rearrangement of the breakpoint cluster region, a segment of the long arm of chromosome 22, is probably present in all subjects with CML, even the 10 percent without an over 22q- abnormality. The disease has a very high propensity to evolve into an accelerated, rapidly fatal phase resembling acute leukemia.

In 1960 Nowell and Hungerford reported that two patients with the disease had an apparent loss of the long arm of chromosome number 21 or 22 an abnormality that was quickly confirmed [89-91] and designated the Philadelphia chromosome. This observation led to a new approach to diagnosis, a marker to study the pathogenesis of the disease, and focus for future studies of the molecular pathology of the disease. The availability of more sensitive banding techniques to define the structure of chromosomes [92, 93] led to the discovery, by Rowley, that the apparent lost chromosomal material on chromosome 22 was part of a reciprocal translocation

between chromosome 9 and 22 [94]. The discovery that the cellular oncogene, *ABL,* on chromosome number 9 and a segment of chromosome 22, the breakpoint cluster region, fuse as a result of the translocation has provided a focus for the study of the molecular pathology of the disease [95].

The *BCR–ABL* hybrid protein, which is produced as a result of the characteristic translocation, is a constitutively active, cytoplasmic tyrosine kinase [96, 97]. This protein could upregulate tyrosine kinase activity with multiple signal transduction pathways by phosphorylation. Cytokines that regulate the growth of normal hematopoietic cells utilize the same signal pathways. As a result, CML cells escape normal cellular control of growth and differentiation.

Laboratory studies also have shown decreased adhesion of CML cell to bone marrow stromal cells [98, 99]. This observation may account for the peripheral circulation of immature granulocytes and may indicate that the stromal cells have lost control of the growth of hematopoietic cells. The *BCR–ABL* hybrid protein has been demonstrated to transform hematopoietic cells to become independent of cytokines and growth factors [100]. In addition, some protection from apoptosis or programmed cell death may be conferred on the CML cells by the *BCR–ABL* on coprotein.

Patients with CML have developed the Ph chromosome during the course of the disease, have had periods of the disease when the Ph chromosome disappeared [101], or have had Ph–positive and Ph–negative cells concurrently [102-106]. Most, if not all, patients with CML have hemopoietic stem cells which, after treatment [107] or culture *in vitro* [108-110] or using special cell isolation techniques [111, 112], do not have the Ph chromosome [113, 114]. Nearly patients with CML have an abnormality of chromosome 22 at a molecular level. Thus, the absence of a Ph chromosome is not a valid measure of normality of chromosome 22. The cytogenetic abnormality in CML has variably been thought to be cause of the chronic phase of the disease or alternatively, responsible for the clonal instability and progression that results in the development of the later accelerated phase.

1.2.2.5.1 Chronic Phase

In peripheral blood, CML manifests as a primary proliferation of granulocytic elements. CML is indicated in the differential diagnosis whenever a granulocytosis is

encountered in a complete blood count. Granulocytosis is defined as an absolute granulocyte count greater than 7.5 x 10^9 /L. Granulocytes include not only segmented neutrophils, but also bands, metamyelocytes, myelocytes, promyelocytes and myeloblasts. Before considering the possiblility of CML, reactive causes of neutrophilia must be excluded. With the widespread use of colony stimulating factor (CSF) therapy, it is uncommon to encounter a peripheral leukocytosis with a granulocytic left shift [115]. A neutrophilia is expected in certain, "normal" physiological states and responses to infections that may be associated with a leukemoid reaction, pregnancy, neonates, stress/exercise, and acute hemorrhage/hemolysis.

Peripheral blood finding in CML is rather characteristic and many times diagnostic. The white blood cell count is increased, but is variable and ranges between 20 and 1000 x 10^9 /L (median 150 to 200 10^9 /L). The two most important findings are basophilia (greater than 0.2 x 10^9 /L basophils) and granulocytosis at various stages of maturation. Peripheral blood smear shows segmented neutrophils, bands, metamyelocytes, myelocytes and promyelocytes without significant dysplasia. Often occasional blasts are seen, but usually no more than 1% to 2%. The presence of blasts in adults almost always indicates a hematologic malignancy, except in patients receiving CSF. Other features of peripheral blood smears in CSF therapy include very intense granulation in the granulocytes, but basophilia is often not present. In addition, occasional blasts in the peripheral blood smears of infants and children are not unusual, especially in tertiary medical settings.

Other peripheral blood findings in the chronic phase of CML include eosinophilia in 90% of cases and some degree of monocytosis [116]. Thrombocytosis of variable degree usually is present, and levels as high as 1,000 x 10^9 /L have been observed in some patients. Thrombocytopenia is extremely rare at diagnosis, and its presence would indicate an accelerated or blasttic phase [117]. Most patients are anemic at presentation with normal red cell morphology.

In regard to bone marrow, the findings in the bone marrow biopsy do not provide significant information for diagnostic purposes. However, a bone marrow biopsy is still recommended [118]. Cytogenetic analysis for confirmation of the diagnosis of CML and for prognostic purposes is much better on bone marrow

aspiration material than peripheral blood. A baseline picture of the bone marrow allows comparison with future changes, assessing initial degree of reticulin fibrosis, and looking specifically for foci of blasts in the core biopsy to indicate focal blastic transformation.

The cellularity is markedly increased, and a marked granulocytic hyperplasia is present with a myeloid to erythroid ratio of 10:1 or greater. Megakaryocytes are increased and typically small and hypolobated, in contrast to other CMPDs. Reticulin stain may show mildly increased reticulin fibrosis. Pseudo–gaucher cells may be seen in 10% to 20% of the cases, representing the high release of sphingolipids from the leukemic granulocytes.

1.2.2.5.2 Accelerated Phase

The diagnostic criteria for the accelerated phase are vague and include both morphological and clinical findings [119]. Clinically, the patient may be refractory to therapy with increasing splenomegaly or may require higher doses of drugs to control the disease. In the peripheral blood, the white cell count may increase or decrease with increasing blasts, but blasts constitute less than 30%. Peripheral blasts greater than or equal to 15% (but less than 30%) or peripheral blasts and promyelocytes greater than or equal to 30% (but blasts less than 30%) have been used as criteria for the accelerated phase. Increasing basophilia greater than or equal to 20% and morphologic evidence of dysplasia and platelet counts may drop with platelet counts less than 100 x 10^9 /L found to be a poor, though independent prognostic factor. In the bone marrow, increasing numbers of blasts (but less than 30%) and increasing basophilia may be seen. A reticulin stain to evaluate for increasing myelofibrosis is useful. Clonal cytogenetic evolution with additional cytogentic abnormalities is indicative of progression of CML. Common additional abnormalities include a second Ph chromosome, trisomy 8(+8), and isochromosome 17q.

1.2.2.5.3 Blastic Transformation Phase

A diagnosis of blastic transformation of CML can be made if any of the following three criteria are met [120]. First, there are 30% or more blasts in the peripheral blood or bone marrow. The development of an extramedullary myeloid

tumor or lymphoblastic lymphoma also indicates a blast crisis. The extramedullary disease may be the first and only evidence of blastic transformation with no increased blasts in the bone marrow and peripheral blood [121]. Finally, blastic transformation has occurred if blasts in the peripheral blood and bone marrow are less than 30%, but the bone marrow core biology shows large intramedullary foci of blasts. This latter finding is usually referred to as focal blasts transformation.

About two thirds of the blasts at crisis are myeloid in origin and one third are lymphoid in origin. It is clinically and prognostically important to determine the lineage of these blast crises. Although blast crises of CML in general are clinically very aggressive with survival usually less than 6 months, several studies have found that the lymphoid blast crises in CML are associated with longer median survival and better response to therapy when compared with the myeloid blast crisis. The lymphoid blast crisis appears to respond to acute lymphoblastic leukemia therapeutic regimens containing vincristine and prednisone. Although the prognosis is better with the lymphoid blast crisis, the overall survival advantage is of short duration with median survival of 9 to 12 months for a lymphoid blast crisis versus 3 to 4 months for a nonlymphoid blast crisis. As a results, allogenic BMT is recommended for patients with a lymphoid blast crisis after induction of a second chronic phase [122].

Other characteristics associated with the lymphoid blast crisis in CML include abrupt transformation without a preceding accelerated phase, less frequent organomegaly, lower white blood cell count with lower blast percentage, fewer peripheral basophils, lesser degree of anemia, more extensive involvement of bone marrow by blasts, and more frequent extramedullary involvement [123, 124]. The overwhelming majority of the lymphoid blast crises show a B – lineage with only rare cases of T–cell blast crisis [125]. Aberrant expression of myeloid antigens in these lymphoid blast crises appears to be relatively common.

Immunohistochemical analysis should be performed. For an extramedullary myeloid tumor (granulocytic sarcoma), myeloperoxidase and lysozyme are good myeloid markers. For lymphoblastic lymphoma, TdT is probably the most useful in confirming the diagnosis. For the purpose of determining lineage, CD79a and CD10–immunopositivity indicate B–cell lineage. CD20 is not as useful, given that most precursor B–acute lymphoblastic leukemia is negative for CD20. For T– cell lineage,

antibody against CD3 can be used to detect both surface and cytoplsmic CD3 expression. Although CD43 is a nonspecific T–cell marker, it also marks myeloid cells, and many times it may be the only marker initially positive if an extramedullary myeloid tumor was not considered in the differential diagnosis. CD34 is a marker of progenitor hematopoietic cells, and its expression is consistent with acute leukemia, but it does not indicate myeloid or lymphoid origin.

1.2.3 *Wilms' tumor (WT1)* gene and its product (WT1 protien)

In 1899, Max Wilms made a significant discovery when he first described a young patient with malignant neoplasm of the kidney. This condition affects about 1:10,000 children usually below the age of 5 years, and accounts for approximately 8 % of all childhood tumors [126]. Inactivation of a tumor suppressor gene, *Wilm's tumor 1* (*WT1*), is responsible for 10–15% of the neoplasms. Although nephroblastoma may also develop in response to mutations at other chromosomal sites, *WT1* on human chromosome 11p13 is so far the only gene that has been cloned and classified as a tumor suppressor gene.

The human *WT1* genes span 50 kb and consists of 10 exons and encodes mRNAs of approximately 3 kb [8, 9]. As shown in Figure 2, it encodes a protein that shares a high degree of structural homology with the early growth response family of transcription factors [127] The *WT1* gene product contains four COOH–terminal C_2H_2 zinc fingers for nucleic acid binding. Its NH_2 terminus includes both transcriptional repression and activation domains. Addition motifs in the WT1 protein are essential for self association, nuclear localization, and RNA recognition (Figure 3). More than 20 different *WT1* gene products with molecular masses of 52–65 kb are generated by a combination of alternative mRNA splicing [128], initiation of translation at variable start codons and RNA editing [129].

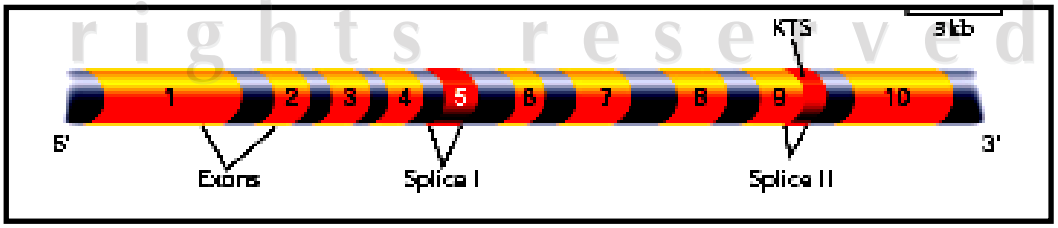


Figure 2 The *WT1* gene span 50 kb on human chromosome 11p13 and consists of 10 Exons [130]

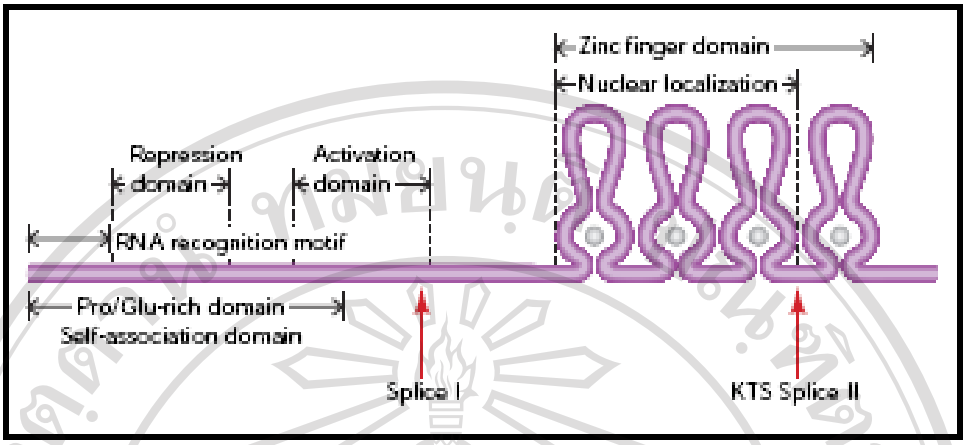


Figure 3 Structure of WT1 protein with variant functional domains [130]

In mammals, exons 5 and 9 of *WT1* are alternatively spliced, giving rise to four different splice isoforms (Figure 3). In all other vertebrates tested , exons 5 is not present in the *WT1* gene, so that only two different mRNA transcripts are generated [131]. Inclusion of exon 5 inserts 17 amino acids between the proline and glutamine–rich amino terminus and the zinc finger domain of WT1. Alternative splicing at the end of exon 9 results in incorporation of three additional amino acids (lysine, threonine, and serine or KTS) between the third and the fourth zinc finger. The corresponding proteins are designated as WT1(-KTS) and WT1(+KTS) respectively. In the thesis, WT1 (-/), WT1 (+/-), WT1 (-/+), WT1 (+/+) isoforms are used to refer to the WT1 splice variant which lacks both inserts, which only contains the 17 aa insert, which only contains KTS insert, and which contains both inserts, respectively. Computer modeling [132] and *in vitro* studies [133] revealed a higher affinity for RNA of +KTS protein compared with the –KTS forms. Furthermore, the WT1(+KTS) products are localized with and bound to nuclear splicing factors [134-136]. These findings strongly support the possibility that the WT1(+KTS) proteins play a role in mRNA splicing rather than transcription control.

The ratio of the different *WT1* splice variants was found to be conserved between normal fetal kidney, Wilm's tumors and several tissues of the murine genitourinary system. The mRNA isoform containing both splice inserts is the most prevalent variant in both human and mouse, whereas the least common is the transcript missing both inserts. These findings were later extended by a report describing the

splicing of exon 5 which is differentially regulated within a species, tissue and developmental stage specific manner, while the +KTS/-KTS ratio is maintained in all cell types tested [137]. Additional WT1 mRNAs are generated through RNA editing at nucleotide 839 of the WT1 mRNA that replaces leucine 280 in WT1 proteins by proline [129], although the frequency of RNA editing might be significantly lower than initially published [138]. The *WT1* gene may thus produce eight different mRNA isoforms, suggesting that each isoform has a distinct contribution to the function of the *WT1* gene and that balanced expression of the isoforms is essential for proper *WT1* function.

Depending on the absence or presence of the two splice inserts, the WT1 proteins have molecular masses of 52–54 kDa [139]. The WT1(-/-) protein with a mass of 52 kDa lacks both splice inserts and the WT1(+/+) protein with a mass of 54 kDa contains both splice inserts. In addition to the eight WT1 protein isoforms generated through translation initiation at the initiator AUG of the eight mRNAs describe above, larger and smaller WT1 isoforms have been identified (Figure 4). Translation initiation at an in–frame CUG codon upstream of the initiator AUG results in WT1 protein isoforms with molecular masses of 60–62 kDa [140]. Internal translation initiation at an in–frame AUG127 codon downstream of the initiator AUG generates smaller WT1 isoforms with apparent molecular masses of 36–38 kDa [141]. Both the larger and the smaller WT1 isoforms can be detected in different mammalian tissues. Since the in–frame, downstream AUG is conserved in the *WT1* genes of all species sequenced so far, it appears that it may function as an alternative translation initiation site in all of these species. When these novel isoforms are included, 24 WT1 protein forms have been describe to date, generating an enormous potential for regulation and functioning of WT1 proteins. From the primary structure of the WT1 proteins, it was predicted that they could function as transcription factors. The WT1 proteins contain a proline and glutamine–rich region, which mediates transcriptional regulation and homodimerization, and four Cys2–His2–types zinc fingers at their C–termial. The zinc–finger domain can bind to several DNA sequences and contains two nuclear lacalization signals, one in zinc finger 1 and another zinc fingers 2 and3 [142]. In 1995, a first report suggested that, in addition to its function as a transcription factor, WT1 protein may also be involved in post–transcriptional processing of RNA

[136]. Structural modeling revealed a potential RNA recognition motif in the N-terminus of WT1, and it has been demonstrated that the zinc finger domain in the C–terminus part of WT1 can bind to IGF–II RNA.

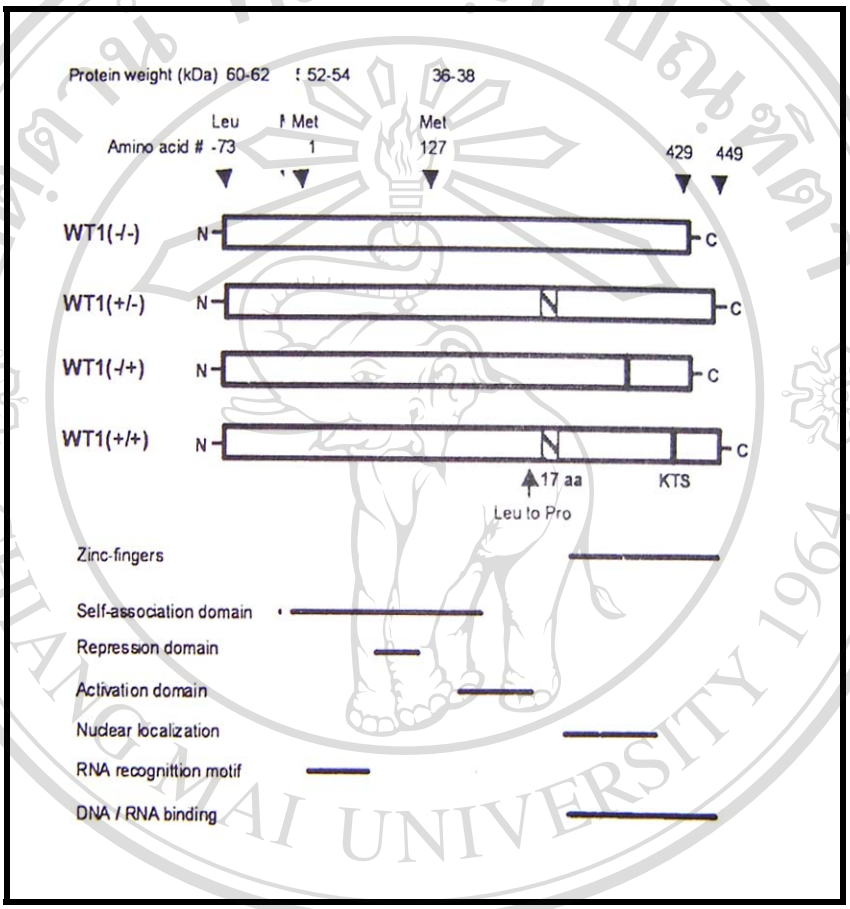


Figure 4 Schematic represent of the WT1 protein isoform. Insertion or exclusion of the two splice insert (17 amino acids and KTS) generates the four main WT1 protein isoform starting at the first initiator AUG(Me) of the WT1 mRNAs. Translation initiation at an upstream, in–frame CUG codon generates four larger WT1 proteins, while translation initiation at in – inframe AUG 127 codons downstream of the initiator AUG leads to four smaller WT1 proteins. Moreover, RNA editing at codon 280 raise the total number of WT1 protein to 24 isoforms [141].

1.2.3.1 Function of WT1 (Tumors suppressor gene versus oncogene)

WT1 is widely accepted to function as tumor-suppressor gene in the formation of Wilm's tumors. However, over the past few years, data have accumulated on the expression of WT1 in adult tumors from different origin, including colorectal [143], breast [144], and brain tumors [145]. As these tumors arise in tissues that normally do not express WT1, and no mutations in the gene have been identified, it has been suggested that expression of WT1 might play an oncogene role in these tumors. In fact, evidence using antisense oligonucleotides shows that WT1 is required for proliferation while inhibiting appoptosis of tumors cell in culture [146]. *WT1* expression in the adult appears to be limited to the kidney podocytes, therefore, oncogene *WT1* expression might be a relatively tumor–specific target for therapeutic intervention. Indeed, trials using peptide vaccines against WT1 in patients with leukemia, breast or lung cancer were promising [147]. If WT1 is functionally active in the tumorigenic process in these tumors, additional therapeutic schemes can be envisioned.

These observations will need further study. First of all, so far there are no clear data on the isoforms expressed in these tumors. As *in vitro* data on the isoforms that result from the alternative start site (up or down stream) suggest domain negative effects for theses isoforms, this might be an important aspect of the role of WT1 in these tumors. Second, all publications on WT1 expression in adult cancers show mainly, if not only, cytoplasmic localization of the protein. This might be part of an oncogenic role for WT1, as normally only 10–25% of the protein is found in the cytoplasm [148]. Third and finally, it is not known whether *WT1* is expressed during development of the tissues where the tumors are found, if so, the expression found in the tumors might reflect either de–differentiation of cells or the cancer stem cell origin of the tumor. New gain and loss–of–function mouse models will need to be developed to fully analyse the oncogenic potential of WT1 isoforms as oncogens.

The apparent contrasting roles of WT1 in inducing differentiation versus inhibiting differentiation of cell with mesenchymal–epithelial fate might partially explain how WT1 can function as a tumour suppressor gene in some tissues and as a potential oncogene in others. The adult cancers where *WT1* is expressed are generally derived from epithelial cells. These tumurs will undergo an EMT during their

development, and this is often linked to a poor prognosis. If WT1 is involved in maintaining the mesenchymal–epithelial balance in cells, activating its expression might help establishing or maintaining this mesenchymal status in these tumour cells. In contrast, Wilm's tumors are derived from mesenchymal cells and WT1 normally guides the cells towards an epithelial state. In this case losing WT1 keeps the cells in the desired mesenchymal state.

The role of WT1 loss in the development of Wilm's tumours was further complicated by the observation that *WT1*- mutant tumours select for oncogenic activation of β–catenin [149]. Somehow, the two genetic aberrations must be linked. The obvious candidate for this would be Wnt4 if it would signal *via* the canonical, β–catenin–mediated, pathway. However, the *BAT*–gene reporter mouse for β–catenin activity shows no activity in the mesenchyme where Wnt4 is active [150], strongly suggesting that Wnt4 in the kidney cannot signal *via* β–catenin. Yet, there clearly is β–catenin activity in the ureteric bud, so the reciprocal interactions between bud and mesenchyme in the kidney development might still provide a more indirect link between *WT1* and β–catenin mutations in Wilm's tumor.

Interestingly, expression of activated β–catenin can force cells into premature EMT [151]. And although a role for β–catenin in the epicardila EMT where WT1 is active has not been studied, other EMT processes in the developing heart are clearly linked to canonical Wnt signaling [152]. Again, there is a contrast between WT1, β–catenin and regulation of the mesenchymal–epithelial balance, which might explain the role of WT1 in both Wilm's tumors and adult cancers. It might be relevant that a subset of many of the adult cancers where *WT1* is found to be activated is known to have oncogenic mutations in β–catenin. It would be interesting to see whether there is any overlap in the tumors with β–catenin mutation and *WT1* activations in these tumors, comparable with the situation in Wilm's tumors.

1.2.3.2 WT protein and WT1 mRNA in the human hematopoietic system

A developmental role for WT1 in the human hematopoietic system is indicated by a number of observations. WT1 is expressed in a small subset of dormant as well as lineage–committed progenitor cells, but is not detectable in peripheral mature blood cells [13, 153-155]. Its function is in early hematopoietic development. In addition,

WT1 is expressed in acute leukemias of myeloid and lymphoid origin as well as in many acute leukemia cell lines, and expression levels are highest in immature leukemias. Most acute leukemias show high expression of WT1, the level of which correlates with a poor outcome [15, 23, 156-158], and although the number of analyzed cases in the literature is low, AML with heterozygous mutations of WT1 may be associated with a poor response to chemotherapy [17, 159, 160], arguing for effects of WT1 on viability, proliferation, and differentiation. Results from forced expression of WT1 in leukemic cell lines, showing interference with the differentiation response [144, 161-165] and the observation that murine bone marrow cells transduction under certain culture conditions [166], lend further support for the notion of WT1 having a functional role in the differentiation of hematopoietic tissue.

Recently, forced expression of WT1 by retroviral infection of human hematopoietic progenitor cells has been reported, demonstrating anti–proliferative effects on myeloid and the particular on erythroid clonogenic cells [167, 168]. No impairment has been observed, but rather an enhanced differentiation of committed myelomonocytic progenitors was indicated. The mechanisms for the observed effects have not been explained, but expression of cdk inhibitor $p21^{Waf1/Cip1}$ (p21), a defined target gene for WT1, was not sufficient by itself for the effects on proliferation and differentiation exerted by WT1, indicating interactions of WT1 with other proteins. The molecular mechanisms by which WT1 affects hematopoietic proliferation and differentiation are therefore presently unclear.

Mutations of WT1 are observed in adult as well as childhood leukemia, most common in AML. In four studies, in total investigating more than 100 leukemia samples, mutations in WT1 were found in 10-12% of the cases. AML–associated mutations of WT1 are most often small insertions causing frameshifts or missense mutations, encoding WT1 proteins with deletions of most of the Zn–fingers, respectively. Heterozygous point mutations in the zinc–finger region, potentially affecting binding to DNA, underlie the congenital malformation syndromes WAGR and DDS. Both WAGR and DDS predispose for the development of Wilms' tumor, but while the mutations are dominant for developmental disturbances, they are most often recessive for tumor development, arguing for dominant functions in the first cases and loss of function in the latter. While some cases of AML show homozogous

or compound mutations of WT1, the majority of WT1 mutations in leukemia are heterozygous, suggesting a dominant negative effect or acquisition of novel gain–of–function of mutant WT1 in leukemogenesis.

WT1 mRNA is expressed in the bone marrow, but not in normal mature blood cells [13, 169], suggesting a function in early hematopoietic development. In addition, *WT1* is expressed in acute leukemia cell lines, expression levels are highest in immature leukemias [15, 16]. Although in apparent contrast, a mutation in the remaining allele of *WT1* was found in a leukemia that occurred as a secondary malignancy in a WAGR syndrome patient [170] and heterozygous *WT1* mutations are present in about 10–15% of acute leukemias [17, 18]. In retrospect, the first clue that abnormal *WT1* expression could be involved in leukemia was that one of the two groups that first cloned the *WT1* gene obtained a *WT1* cDNA clone from a pre – B cell line, in which a serine important for DNA binding [171] was replaced by a phenylalanine [8].

Since *WT1* is expressed in many acute leukemias, WT1 mRNA expression levels are now used as a prognostic factor in myelodysplastic syndrome and acute leukemia. Inoue et al (1994) established a clear correlation between the relative levels of *WT1* expression and the prognosis for acute leukemia, while Tamake *et al*. (1999) demonstrated that *WT1* expression levels increase during progression of myeloidysplastic syndromes to acute myeloid leukemia. In both reports, patients with relatively low WT1 mRNA expression had a better prognosis than patients with high *WT1* mRNA levels. Thus, in most leukemias the WT1 protiens appear to act as survival factor, although the presence of mutations in the *WT1* gene some cases would suggest a tumor suppressor function.

1.2.4 *Garcinia mangostana* Linn.

In Thailand there are many tropical plants that have interesting biological properties with potential therapeutic applications. *Garcinia mangostana* Linn, family Guttiferae, is named ‘the queen of fruits’ because many people agree that it is one of the best tasting fruits in the world. It can be cultivated in the tropical rainforest such as foud in Indonesia, Malasia, the Philipines and Thailand. In Thailand the people called this fruit mangosteen (Figure 5). The mangosteen fruit is composed of the rind (skin of

the fruit), the pulp (four to eight segments) and the seeds (usually two to three seeds). The chemical components of mangosteen include tannin, which is obtained from the bark. The fruit shell contains 7–13% tannin and the seeds contain 3% oil. The rind of the fruit contains tannin, a resin and a bitter principle called mangostin. The edible aril contains saccharose, dextrose and kerrelose [172]. The rind contains 5.5% of tannin, and a resin as well as a yellow crystalline bitter principle, mangostin ($C_{20}H_{22}O_5$) or mangosim [25] isolated from the rind. It was reported that the flesh of the fruit (aril) contains 10.8% saccharose, 1% dextrose and 1.2% kerrelose. The seeds are reported to contain vitamin C [173]. From a methanolic extract of mangosteen leaves a new compound, 2–ethyl–3–methylmaleimide N–beta–gludopyronoside was found [174]. The rind is rich in pectin

Figure 5 *Garcinia mangostana* Linn. [190]

A new xanthone with a geranyl group, named mangostinone, and 7 known xanthone (α-, β-, γ- mangostins, gartanin, garcinone E, 1,5–dihydroxy–2–(3–methylbut–2–enyl) –3–methoxyxanthone and 1,7–dihydroxy–2–(3–methylbut–2–enyl) –3–methoxyxanthone), were isolated from pericaps of *G. mangostana* [175]. Twelve xanthones were isolated from the hexane extract of the heartwood of *Garcinia mangostana* from Myanmar [176].

A new polyxygenated xanthone, magostanol, was isolated from fruit hulls of *G. mangostana,* along with known xanthones, alpha–mangostin, gamma–mangostin, gartanin,8–deoxygartanin,5,9–dihydroxy–2-,2–dimethyl–8–methoxy–7–(3–methybut–

2–enyl)–2H,6H–pyrano(3,2– b) xanthone and epicatechin [177]. Two novel xanthones have also been isolated from hulls of *Garcinia mangostana* [178].

Three new xanthones, mangostenol, mangostenone A, and magostenone B, were isolated from green fruit hulls of *Garcinia mangostana* along with the known xanthone, trapzifolixanthone, tovophyllin B, alphs–and beta–mangostins, garcinone B, mangostinone, mangostanol, and the flavonoid epicatechin [179].

Investigation of the constituents of *Garcinia mangostana* has led to the isolation of four new compounds: three minor xanthone, garcimangosone A, garcimangosone B, and garcimanosone C and garcimangosone C, and a benzophenone gluscoside, garcimangosone D [180].

Mangostin is obtain by boiling the rind in water, and tannin is removed by boiling in alcohol and evaporating ; the resulting product is mangostin and resin; resin is precipitated by redissolving it in alcohol and water, and evaporating the water. It occurs in small yellow scales, insoluble in water, but readily soluble in alcohol and ether.

The pericarps of mangosteen have been used as a traditional medicine, for example as an antifungal: the activity of several xanthones isolated from fruit hulls of *Garcinia mangostana* and some derivaties of mangostin against *Fusarium oxysporum* f.sp. vasinfectum, *Alternaria tenuis* and *Drechslera oryzae* have been evaluated. The natural xanthones inhibited the growth of all the fungi. Substitution in the A and C ring modified the bioactivities of the compounds [181]. Extracts of *Garcinia mangostana* have shown inhibitory effects against the growth of *Staphylococcus aureus* and some of the components had activity against methicillin–resistant *Staphylococcus Aureus* (MRSA). One active isolate, α–mangostin, a xanthone derivative, had a minimum inhibitory concentration (MIC) of 1.57–12.5 μg/ml. Other related xanthones have also been examined to determine their anti–MRSA activity. The strong *in vitro* antibacterial activity of xanthone derivatives against both methicilin–resistant and methicillin–sensitive *S. aureus* suggests that the compounds might find wide pharmaceutical use [28]. *Garcinia mangostana* fruit hulls are also used as an anti-inflammatory agent, as an astringent and to treat diarrhea. The fruit hull of mangosteen, *Garcinia mangostana* has been used as a Thai indigenous medicine for many years. The 40% ethanol extract of mangosteen has potent

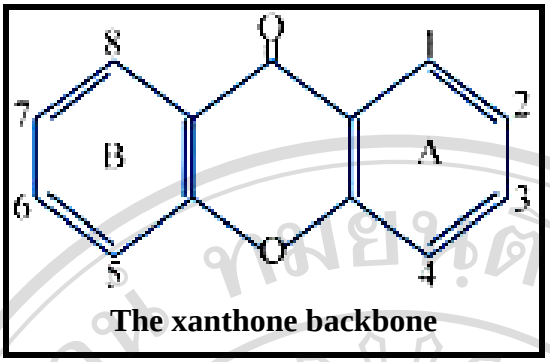
inhibitory activities of both histamine release and prostaglandin E2 synthesis [182]. Additionally, the rind of the fruit which contains resin, is used for diarrhea and dysentery. The bark and young leaves are also used for the same purpose and for ailments of the genito–urinary tracts. In Colombia, the bark and the rind of the fruit are used for diarrhea and dysentery. It has been found very useful in chronic diarrhea in children. The value of the rind lies in the yellow resin which may act as a stimulant to the intestines. The mangosteen also has anti–tubercular action, with α- and β–mangostin and garcinone β, which have exhibited strong inhibitory effects against *Mycobacterium tuberculosis* with the minimum inhibitory concentration value of 6.25 μg/ml [179]. Mangosteen has been used for many years as a medicine for treatment of skin infection, wounds and diarrhea in Southeast Asia. The effect of γ–mangostina a tetraoxygenated dipenylated xanthone contained in mangosteen has been examined, on arachidonic acid (AA) cascade in C6 rat glioma cells. The study demonstrated that γ–mangostin, a xanthone derivative directly inhibited COX activity. In the course of a search for natural antioxidants, the methanol extract of the fruit hulls of *Garcinia mangostana* originating in Vietnam was found to exhibit a potent radical scavenging effect. By monitoring this radical scavenging effect, two xanthones, α– and γ–mangostin, were isolated and the antioxidant activities of the two xanthones were measured by the ferric thiocynate method. γ–mangostin was more active than butulhuanisol and α–tocopherol.

Additional, biological studies on the xanthones obtained from *Garcinia mangostana*. have demonstrated interesting biological activities [183]. Studies have been conducted to examine the anticancer properties of the extracts or xanthones obtained from the fruit hulls of this plant species against colon preneoplastic lesions [184], DNA topoisomerases I and II, heptoma (HCC36, TONG, HA22T, Hep 3B, HEpG2, and SK–Hep–1), lung (NCI–Hut 125, CH27–LC–1, H2981, and Calu-1), gastric carcinomas (AZ521, NUGC–3, KATO–III, and AGS) [185], human breast cancer SKBR3 cells [186, 187], and human leukemia (HL-60, K562, NB4, U937, P3HR1, and Raji) [28, 188].

Xanthone is an organic compound with the molecular formula $C_{13}H_8O_2$. It can be prepared by the heating of phenyl salicylate. In 1939, xanthone was introduced as an insecticide and it currently finds uses as an ovicide for codling moth eggs and as a

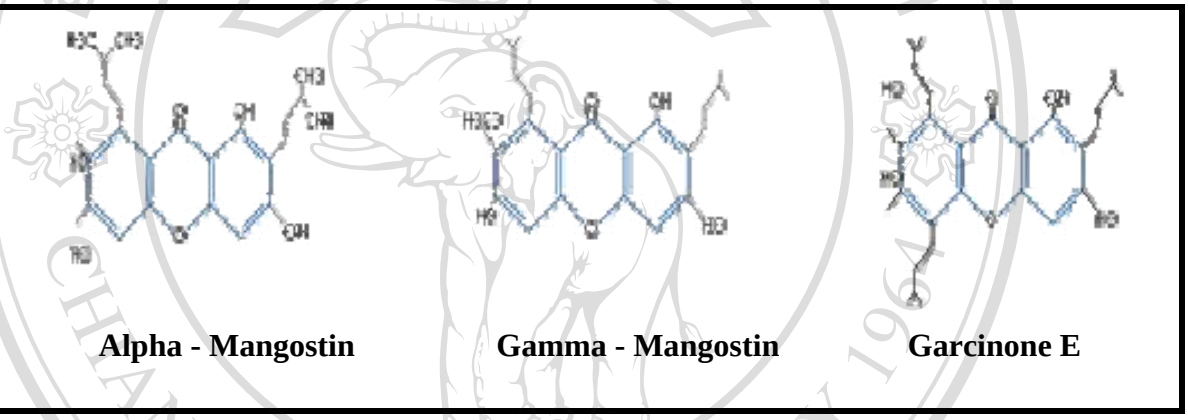
larvicide. Xanthone is also used in the preparation of xanthydrol which used in the determination of urea levels in the blood. The chemical structure of xanthone forms the central core of a variety of naturally occurring organic compounds, such as mangostin, which are sometimes collectively referred to as xanthones. Over 200 xanthones have been identified. Xanthones are a biologically active group of molecules and possess a six-carbon conjugated ring structure with multiple double carbon bonds (Figure 6) that make the xanthone molecule very stable. Each xanthone contains the same chemical backbone, but they also possess unique chemical accessories known as sidechains which alter the molecules chemical activity and produce versatility. For example, α-mangostin serves as an anti-oxidant, γ-mangostin as an anti-inflammatory and Garcinone E as an anti-tumor agent (Figure 7). Xanthones are natural constituents of plants in the families Bonnetiaceae and Clusiaceae and are found in some species in the family Podostemaceae. Many of these xanthones are found in the pericap of the mangosteen fruit (*Garcinia mangostana*), which can be found in the region of Southeast Asia. Synthetic derivatives of xanthone can be added during the polymerization of polyester, to form a plastic that has a greater resistance to degradation by ultraviolet light. The most useful derivative is tetrahydroxyxanthone. Polyester film can be used for the production of third generation printed solar cells, to make them a cost effective alternative to silica-based solar energy generation. It was originally intended that the additive be used for polyester greenhouses in hot climates, where the plastic would degrade after a few years from UV exposure. The xanthone-treated product has an extended useful lifetime of ten years instead of three.

Mangostin is a natural organic compound isolated from various parts of the mangosteen tree (*Garcinia mangostana*). It is a yellow crystalline solid with a xanthone core structure Mangostin and a variety of other xanthones from mangosteen have been investigated for biological properties including antioxidant, anti-bacterial, anti-inflammatory, and anticancer activities. The rind of partially ripe mangosteen fruit yields mangostin, also β-mangostin. That of fully ripe fruits contains the xanthones gartanin, 8-disoxygartanin, and normangostin. A derivative of mangostin, mangostin-3,6-di-O-glucoside, is a central nervous system depressant and causes a rise in blood pressure



The xanthone backbone

Figure 6 Chemical structure of Xanthone [191]



Alpha - Mangostin **Gamma - Mangostin** **Garcinone E**

Figure 7 Chemical structure of Mangostin [192]

1.3 Objectives

1. To study the effect of mangosteen peel extracts on *WT1* gene expression in leukemic cell lines.
2. To study the effect of mangosteen peel extracts on WT1 protein level in leukemic cell lines.