CYCLIN D1 GENETIC POLYMORPHISM AND CLINICAL PRESENTATION OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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CYCLIN D1 GENETIC POLYMORPHISM AND CLINICAL PRESENTATION OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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ABTRACT

Previous studies reported that cyclin D1 G870A polymorphism was associated with risk, age of onset, and event-free survival in a variety of cancers. This study determined the association between cyclin D1 genetic polymorphism and clinical presentations, outcomes, risk and age of onset of childhood acute lymphoblastic leukemia (ALL). PCR-RFLP method was selected to determine cyclin D1 genotype of 100 childhood ALL patients and 300 healthy controls. The obtained result demonstrated that A allele and G allele of cases and controls occur with equal frequencies, 0.62 and 0.38 for A and G allele, respectively. Cyclin D1 polymorphism was not associated with the clinical presentations including age of onset, gender, hepatosplenomegaly, lymphadenopathy, immunophenotype, risk to relapse, cytogenetics, white blood cell count, hematocrit, platelet count, hyperuricemia, hyperkalemia, hyperphosphatemia and hypocalcaemia. Cyclin D1 polymorphism also had a lack of association with the risk of childhood ALL and therapeutic outcomes. In conclusion, cyclin D1 genetic polymorphism is not associated with clinical presentations and outcomes of childhood ALL.

KEY WORDS: CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA / ALL / CYCLIN D1 / CCND1 / PCR-RFLP

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ความหลากหลายทางพันธุกรรมของ cyclin D1 และการแสดงออกทางคลินิกของผู้ป่วยเด็กโรคมะเร็งเม็ดเลือดขาวเฉียบพลันชนิดลิมโฟบลาสต์ (CYCLIN D1 GENETIC POLYMORPHISM AND CLINICAL PRESENTATION OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA)

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บทคัดย่อ

การศึกษาที่ผ่านมาพบว่าความหลากหลายทางพันธุกรรมของ cyclin D1 ที่ตำแหน่ง G870A มีความสัมพันธ์กับความเสี่ยงในการเกิดโรค, ช่วงอายุที่เกิดโรค และระยะเวลาปลอดโรคในมะเร็งหลายชนิด ดังนั้นการศึกษานี้จึงจะศึกษาความสัมพันธ์ของความหลากหลายทางพันธุกรรมของ cyclin D1 กับการแสดงออกทางคลินิกและความเสี่ยงของเด็กในการเกิดโรคมะเร็งเม็ดเลือดขาวเฉียบพลันชนิดลิมโฟบลาสต์ โดยจะศึกษาในเด็กที่เป็นโรค ALL จำนวน 100 คนและกลุ่มควบคุมจำนวน 300 คน ด้วยวิธี PCR-RFLP จากการศึกษาพบว่า ความถี่ของ A allele และ G allele ในกลุ่มตัวอย่างและกลุ่มควบคุมมีค่าเท่ากันคือ 0.62 และ 0.38 สำหรับ A allele และ G allele ตามลำดับ ความหลากหลายทางพันธุกรรมของ cyclin D1 ไม่มีความสัมพันธ์กับการแสดงออกทางคลินิกของผู้ป่วย เช่น ช่วงอายุในการเกิดโรค, เพศ, ภาวะตับและม้ามโต, ต่อมน้ำเหลืองโต, ชนิดของมะเร็ง, ความเสี่ยงในการกลับเป็นโรคซ้ำ, จำนวนโครโมโซม, จำนวนเม็ดเลือดขาว, ความเข้มข้นของเลือด (Hematocrit), จำนวนเกร็ดเลือด, ภาวะกรดยูริคสูง, ฟอสเฟตสูง, โปแตสเซียมสูงและแคลเซียมต่ำ นอกจากนี้ไม่เพิ่มความเสี่ยงในการเกิดโรคมะเร็งเม็ดเลือดขาวเฉียบพลันชนิดลิมโฟบลาสต์ในเด็กและผลการรักษาของแต่ละกลุ่มก็ไม่แตกต่างกัน สรุป ความหลากหลายทางพันธุกรรมของ cyclin D1 ไม่มีความสัมพันธ์กับการแสดงออกทางคลินิกและผลการรักษาผู้ป่วยเด็กโรคมะเร็งเม็ดเลือดขาวเฉียบพลันชนิดลิมโฟบลาสต์

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LIST OF ABBREVIATIONS

A	=	adenine
ALL	=	acute lymphoblastic leukemia
bp	=	base pair(s)
CALLA	=	common ALL antigen
CCG	=	Children's Cancer Group
CCND1	=	cyclin D1
CD	=	Cluster of Differentiation
CI	=	confidential interval
cIg	=	cytoplasmic immunoglobulin (
^{o}C	=	degree Celsius
CNS	=	Central nervous system
COG	=	Children's Oncology Group
dl	=	deciliter
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide 5'-triphosphate
EDTA	=	ethylene diamine tetraacetic acid
FAB	=	French-American-British
g	=	gram
G	=	guanine
HLA-DR	=	Human leukocyte antigen subclass DR
kb	=	kilobase
l	=	litre
mEq	=	miliequivalent
min	=	minute(s)
mM	=	milimolar
mmol	=	milimole
MMR	=	mismatch repair

LIST OF ABBREVIATIONS (Continued)

mRNA	=	messenger ribonucleic acid
ng	=	nanogram
nm	=	nanometer
µg	=	microgram
µl	=	microlitre
µM	=	micromolar
µmol	=	micromole
OR	=	odds ratio
PCR	=	polymerase chain reaction
PEST	=	proline (P), glutamic acid (E), serine (S) and threonine (T)
Plt	=	platelet
POG	=	Pediatric Oncology Group
PRAD1	=	parathyroid adenomatosis 1
RBC	=	red blood cell
RFLP	=	restriction fragment length polymorphism
SCCHN	=	squamous cell carcinoma of the head and neck
SEER	=	Surveillance Epidemiology, and End Results
SNP	=	single nucleotide polymorphism
sur	=	surface
t	=	translocation
TdT	=	terminal deoxynucleotidyl transferase
TLS	=	tumour lysis syndrome
UV	=	ultraviolet
WBC	=	white blood cell count
WHO	=	World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Background and problems

Acute lymphoblastic leukemia (ALL) is the most common type of leukemia in children among the 12 major types of childhood cancer, accounting for approximately 30% of all cases (1). Recent data from the National Cancer Institute's Surveillance Epidemiology, and End Results (SEER) program shows that about 3,970 new cases of this disease are diagnosed each year in the United States, approximately 75% are children younger than 10 years of age, and 50% are younger than 5 year (1). ALL is a malignant proliferation of lymphoid cells blocked at an early stage of differentiation. The effects are: 1) the uncontrolled and exaggerated growth and accumulation of lymphoblast or leukemic blast cells, which fail to function as normal blood cells, and 2) the blockade of the production of normal marrow cell, leading to a deficiency of red cells (anemia), platelets (thrombocytopenia), and normal white cells especially neutrophils in the blood while the white blood cell (WBC) count is elevated.

A uniform classification system for the acute leukemias and myelodysplastic syndromes was developed by an international group of investigators in 1976. Known as French-American-British (FAB) classification, this system is based on the morphologic appearance of bone marrow and blood leukemic blast in Romanovsky-stained smears, supplemented when necessary with cytochemical stains. Three subtypes of ALL are distinguished on the basis of cell size, nuclear shape, number and prominence of nucleoli, and the relative amount and appearance of cytoplasm. The relative frequency of the morphologic subtypes varies with age. In children with ALL, approximately 70% have L1, 18% have L2 and 3% have L3 morphology. In contrast, 67% of adults with the same disease are classified as L2 (2).

In 2001, the World Health Organization (WHO) classified ALL in 2 categories based on the surface marking characteristics of leukemic blasts by immmunophenotyping; precursor B-lymphoblastic leukemia and precursor T-lymphoblastic leukemia. Precursor T-lymphoblastic leukemia differ form B-lymphoblastic leukemia in that they less frequency express CD10 (the “common ALL antigen” or CALLA) and HLA-DR. Precursor B-cell found about 88% of cases, the most commonly antibodies used are CD19, CD20, CD22, and CD79a which can classified into 4 subtypes; early pre-B, pre-B, mature B-cell and transitional pre-B ALL. For precursor T-lymphoblastic leukemia, the most commonly used antibodies are CD2, CD3, CD5 and CD7 (3).

ALL is a biologically heterogeneous disorder, so that morphologic, immunologic, cytogenetic, biochemical, and molecular genetic characterizations of leukemia lymphoblasts are needed to establish the diagnosis or to exclude other possible causes of bone marrow failure and, finally, to classify ALL subtypes. ALL may be either asymptomatic or acute with life-threatening hemorrhage, infection, or episode of respiratory distress. The signs and symptoms of the child presenting with ALL reflect the degree of bone marrow infiltration with leukemic cells and the extent of extramedullary disease spread. The most common symptoms and clinical findings are usually manifestations of the underlying anemia, thrombocytopenia, and neutropenia, which reflect the failure of normal hematopoiesis. Pallor, fatigue, petechiae, purpura, bleeding, and fever are often present. Lymphadenopathy, hepatomegaly, and splenomegaly are manifestations of extramedullary leukemic spread. Hepatosplen-omegaly occurs in approximately two thirds of the patients and is usually asymptomatic. Lymphadenopathy is usually painless. Other clinical presentation, for example age, gender and tumor lysis syndrome are factors that having prognostic significance.

In humans there are three D type cyclins; D1, D2 and D3. The D type cyclins are required for cells to get to the restriction point. In the absence of D type cyclins, cells are in G_0. Cyclin D1 or parathyroid adenomatosis 1 (PRAD1) was finally isolated in parathyroid adenomas as a locus linked to the parathyroid hormone promoter on a breakpoint of human chromosome 11 on the long arm within 11q13 (4, 5) The CCND1 gene spans approximately 15 kb and includes 5 exons and 4 introns,

and generates two major mRNAs of 1.7 and 4.4 kb (5, 6). This plays a vital role in regulation of the cell cycle through its interactions with the cyclin dependent kinase (7). The levels of D-cyclins are induced by the extracellular environment. Thus, D-cyclins are induced by the mitogenic stimulation and their levels decline when the mitogens are withdrawn (8).

In 1995, a frequent polymorphism (A/G) was identified in the codon 242 at nucleotide 870 of exon 4 of the gene. Although the base change does not alter the amino acids sequence because of both the normal and the alternate transcripts encode a protein that contains the amino acids 55-161, which are thought to be responsible for the cyclin D1 function, the base is integral to the splice donor site between exon 4 and intron 4. This finding led to the discovery of an alternatively spliced cyclin D1 transcript [b]. Transcript [b] differs from the previously reported cyclin D1 transcript (now called transcript [a]); exon 5 is missing, there is no splicing at the exon 4 – intron 4 boundary and the mRNA sequence continues downstream into intron 4. This protein encoded by the alternate transcript is missing the last 55 amino acids at the carboxy-terminus that are replaced by a shorter 43-amino-acid sequence encoded by intron 4. Exon 5 encodes for the polypeptide sequence enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST) which targets proteins for rapid destruction. It was therefore speculated that the protein translated from transcript [b] may have a long half-life and play an important biological role, especially when overexpressed in tumors. This protein could increase the steady - state levels of the protein in patients with the AA and AG genotypes, which may allow cells that are damaged as a result of defective mismatch repair (MMR) to pass through the G1-S phase checkpoint more easily. This would allow them to proliferate rather than to undergo apoptosis (7, 9, 10).

Previous studies reported that the cyclin D1 polymorphism associated with risk, prognosis, and event-free survival in a variety of cancers. The cyclin D1 A allele or AA genotype is associated with an increased risk of familial colorectal cancer (11), sporadic colorectal adenomas (12), urinary bladder cancer (13), sporadic renal cell carcinoma (14), endometrial cancer (15), prostate cancer (16), carcinoma in situ of superficial bladder cancer (17), and lung cancer (18). Otherwise the cyclin D1 GG (homozygous) phenotype is associated with an increased risk of colorectal cancer (19),

and oral squamous cell carcinoma (20). Age of onset to cancer of hereditary nonpolyposis colorectal carcinoma (9), and hepatoblastomas (21) in the homozygous (A/A) earlier which compared to the heterozygous (G/A) or homozygous (G/G) genotype but in colorectal cancer (19) has earlier age of onset in GG genotype. In addition, the cyclin D1 GG phenotype is associated with poor prognosis and reduced disease-free interval in squamous cell carcinoma of the head and neck (SCCHN) (22), hepatocellular carcinoma (23), laryngeal squamous cell carcinoma (24), while the cyclin D1 A allele is associated with a lower event-free survival in acute lymphoblastic leukemia following methotrexate therapy (25), and epithelial ovarian cancer (26).

This thesis hypothesized that patient with cyclin D1 genetic polymorphisms may effect the risk of de novo childhood ALL compared with the control group. Furthermore, cyclin D1 polymorphisms may be associated with clinical presentations of childhood ALL. The polymorphisms analyses are performed by usage polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

CHAPTER 2
OBJECTIVES

Objectives of this thesis are:

1. To study the association of cyclin D1 genetic polymorphism with clinical presentations and out come of childhood ALL.

2. To evaluate the cyclin D1 genetic polymorphism on risk of childhood ALL.

CHAPTER 3
LITERATURE REVIEW

3.1 Childhood acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a cancer of the hematopoietic system which malignant transformation of lymphoid progenitor cells. ALL is the most common cancer occurring in children, approximately 75% are children younger than 10 years of age, and 50% are younger than 5 year in the Unites States. There is a sharp peak in ALL incidence among children ages 2 to 3 years (>80 per million per year), with rates decreasing to 20 per million for ages 8 to 10 years. The incidence of ALL among children ages 2 to 3 years is approximately 4-fold greater than that for infants and is nearly 10-fold greater than that for children who are 19 years old.

3.1.1 Epidemiology and pathophysiology

Morphologic and cell kinetic studies indicate that in acute lymphoblastic leukemia, there is a block in differentiation and that the neoplastic blasts have a prolonged rather than shortened generation time. Thus, the accumulation of blasts results from clonal expansion and a failure of maturation of the progeny into functional mature cells. As blasts accumulate in the marrow, they suppress normal hematopoietic stem cells. This has 2 important clinical implications (27): [1] the major manifestation of acute leukemia result from the paucity of normal RBCs, white cells, and platelets; and [2] therapeutically, the aim is to reduce the population of the leukemic clone enough to allow reconstitution with the progeny of remaining normal stem cells. Physical findings include various combinations of pallor, petechiae or purpura,mucous membrane bleeding, fever, lymphadenopathy, splenomegaly, tenderness over the sternum and other bones, and fundic hemorrhages. Abnormal

organomegaly rarely is symptomatic. Skin involvement is rare; when it occurs, it is associated with the pre-B cell phenotype. Symptoms related to depression of normal bone marrow function. These include fatigue, owing mainly to anemia; fever, reflecting and infection resulting from an absence of mature leukocytes; and bleeding (petechiae, ecchymoses, epitaxis, gum bleeding) secondary to thrombocytopenia. Bone pain and tenderness result from marrow expansion with infiltration of the subperiosteum. Young children, in particular, often present with gait disturbances or a refusal to walk. Vetebral compression fractures may complicate generalized osteoporesis. Generalized lymphadenopathy, splenomegaly, and hepatomegaly reflect dissemination of the leukemic cells; this occurs in all acute leukemias but is more pronounced in ALL. Central nervous system (CNS) involvement by leukemia occurs in approximately 2% of patients at diagnosis and may be manifested by headache, vomiting, and nerve palsies resulting from meningeal spread. Other that weight loss rarely is severe and is seen only in patients with longstanding symptoms.

3.1.2 Classification and differentiation

3.1.1.1 French-American-British (FAB) classification (2)

A uniform classification system for the acute leukemias and myelodysplastic syndromes was developed by an international group of investigators in 1976. Known as French-American-British (FAB) classification, this system is based on the morphologic appearance of bone marrow and blood leukemic blast in Romanovsky-stained smears, supplemented when necessary with cytochemical stains. Three subtypes of ALL (**Table 3-1**) are distinguished on the basis of cell size, nuclear shape, number and prominence of nucleoli, and the relative amount and appearance of cytoplasm. The relative frequency of the morphologic subtypes varies with age. In children with ALL, approximately 70% have L1, 18% have L2 and 3% have L3 morphology. In contrast, 67% of adults with the same disease are classified as L2.

Table 3-1. Morphologic (FAB) Classification of Acute Lymphoblastic leukemia (2)

Morphologic Features	L1	L2	L3
Cell size	Small	Large	Large
Nuclear chromatin	Fine or clumped	Fine	Fine
Nuclear shape	Regular, may have cleft or indentation	Irregular, may have cleft or indentation	Regular, oval to round
Nucleoli	Indistinct or not visible	One or more per cell; large, prominent	One or more per cell; large, prominent
Amount of cytoplasm	Scanty	Moderately abundant	Moderately abundant
Cytoplasmic basophillia	Slight	Slight	Prominent
Cyotplasmic vacuoles	Variable	Variable	Prominent

3.1.1.2 World Health Organization (WHO)

In 2001, a group convened by the World Health Organization (WHO) published a new classification of hematopoietic and lymphoid neoplasms, including the acute leukemais. The directive for the classification was that it be up to date, incorporate genetic and immunophenotypic as well as morphologic information, and that it also be usable throughout the world, not just in Western academic medical centers. The WHO classification does not group the acute lymphoid leukemias together, but separates them into precursor B-cell and precursor T-cell lymphoblastic leukemia. Additionally, in each case it lumps predominantly leukemic disease, based on similar biologic and genetic characteristics of disease rather than clinical presentation.

- **Precursor B-lymphoblastic leukemia/lymphoma**

Precursor B-lymphoblastic leukemias and lymphomas have demonstrable B-lineage differentiation in blasts by immunophenotyping. The most commonly used antibodies are for CD19, CD20, CD22 and CD79a. In difficult or ambiguous cases, cCD22 and cCD79a are considered lineage specific for B differentiation. They are also usually positive for CD10, HLA-DR, and TdT, and may be positive for CD34. They may have FAB L1, L2 or mixed blast morphology. The leukemias and lymphomas presumably differ in homing mechanisms for marrow versus lymph nodes, but share immunophenotype, genetic abnormalities, and response to therapy. The may be further subclassified by genetics into good risk. [t(12;21)(p13;q22), hyperdiploidy > 50], poor risk [t(9;22)(q34;q11.2), and average risk] groups.

- **Precursor T-lymphoblastic leukemia/lymphoma**

Precursor T-lymphoblastic leukemias and lymphomas have demonstrable T-lineage differentiation in blasts by immunophenotyping. The most commonly used antibodies are for CD2, CD3, CD5, and CD7. In difficult or ambiguous cases, cCD3 is considered lineage specific for T differentiation. They are usually positive for TdT, negative for HLA-DR, and may express CD34. They may have FAB L1, L2, or mixed blasts morphology. The leukemias and lymphomas presumably differ in overlapping immunophenotypes, genetic abnormalities, and response to therapy. A large number of recurring translocations have been described, but currently are not used for subclassification of cases for treatment purposes.

3.1.3 Clinical and Laboratory Features at Diagnosis

For children, a number of clinical and laboratory features have demonstrated prognostic value that reliably predict outcome when risk-based treatment assignment. Previous study showed that elevated leukocytosis (>50x10^9/L), male gender, hemoglobin >12 g/dl, platelet count <100x10^9/L, age under 1 year or over 9 years, enlarged nodes, spleen, and liver , T-phenotype and absence of CD10+ cells were predictive of poor outcome (1, 28). Some of these prognostic factors were demonstrated in **Table 3-2**. They are more likely to have massively enlarged livers, spleen and lymph nodes. The white blood cell count is elevated in 60% of patient. Because the number of normal leukocytes is reduced, the WBC count generally mirrors the number of circulating blast cells. Lower hematocrit concentration and platelet count were found higher in this group.

3.1.3.1 White Blood Cell (WBC) count

The total white blood cell (WBC) count at the time of diagnosis is the single most powerful clinical determinant of remission induction, remission duration, and long-term survival for all age groups. The inverse relationship between remission duration and WBC or blast count is linear, although the relationship between WBC count and prognosis is a continuous rather than a step function. Higher WBC counts at diagnosis represent an increase risk for treatment failure in patients with B-precursor ALL. A WBC count of 50,000/µl is generally used as and operational cut point between better and poorer prognosis (29) if a WBC count in excess of 100,000/µl at diagnosis is particularly devastating (30). Elevated WBC count is often associated with other high-risk prognosis factors, including unfavorable chromosomal translocations such as t(4;11) and t(9;22), high risk for relapse in the CNS or testes after attainment and bone marrow remission.

Table 3-2 Frequency of Clinical and Laboratory Findings at Diagnosis in 178 Children and Adults with Acute Lymphoblastic Leukemia

Clinical & Laboratory Features	Percentage
Hepatomegaly	74
Splenomegaly	86
Lymphadenopathy	76
White blood cell (x 10^9/L)	
<10	40
10-49	34
50-99	15
≥100	11
Hematocrit (%)	
<30	65
>30	35
Platelets count (x 10^9/L)	
<50	62
50-150	30
>150	8

(Modified from Whitlock et al. Wintrobe's clinical hematology, 2004)

3.1.3.2 Age

Age at diagnosis has strong prognostic significance, reflecting the different underlying biology of ALL in different age groups. Infants younger than 1 year of age with ALL have a particularly high risk of treatment failure, with the risk of treatment failure being greatest for young infants (younger than 3 months) and those with poor early response to prednisone (31, 32). The poor outcome for infants with ALL is strongly associated with the presence of the MLL gene at chromosome band 11q23 can be detected in the leukemia cells of a large percentage (33). ALL in infants is also associated with a number of other characteristics associated with poor outcome, including elevated WBC count, central nervous system leukemia, lack of CD10 expression, and poor response to initial treatment (31, 34). Young children (1-9 years) have a better disease-free survival in comparison to either older children or adolescents in comparison to infants (29, 35, 36). Adolescents in the age group 10 to 20 years are also at higher risk than younger children (37, 38), and they may be higher risk than adults 20 to 50 years of age (39). Leukemia in adolescents, as in infants, is frequently associated with adverse prognostic factors, including high WBC counts at diagnosis, T-cell immunophenotype, FAB L2 blast morphology, a low DNA index, and ploidy other than hyperdiploidy. Despite these associations, age 10 to 20 years at diagnosis of ALL has independent adverse prognostic significance.

3.1.3.3 Gender

The prognosis for girls with ALL is slightly better than for boys with ALL (40-42). From SEER program in year 2005 found that boys have estimated new cases and estimated death of ALL more than girls. One reason for the superior prognosis for girls is the occurrence of testicular relapses among boys, but boys also appear to be at increased risk for bone marrow and CNS relapse for reasons that are not well understood.

3.1.3.4 Hematocrit concentration

Patient who presented with hemoglobin less than 10 or 11 g/dl or hematocrit less than 30 or 33 % had a higher remission induction rate, a lower relapse rate, and a longer survival rate than those with no anemia in early analyses (43, 44). The hematocrit level appears to be an indirect gauge of the biologic aggressiveness of leukemia. With explosive disease, symptoms evolve before anemia has time to develop, whereas with indolent leukemia, disordered bone marrow function becomes clinically apparent before anemia. Normal hematocrit levels are associated with bulky extramedullary involvement and a high percentage of blasts in the proliferative (S) phase of the cell cycle.

3.1.3.5 platelet count

An association between low platelet count (less than 30 x 10^9/L) and shorter remission duration has been noted in some studies (28, 45). Leukemic seeding associated with petechial hemorrhages may predispose patients to testicular and CNS relapses.

3.1.3.6 Lyphadenopathy and hepatosplenomegaly

A size of peripheral lymph nodes, liver, and spleen provide an indirect measurement of leukemic cell burden. Therefore, several studies demonstrated that massive lymphadenopathy, hepatomegaly, and/or splenomegaly impact adversely on remission duration and survival. However, in multivariate analysis, the prognostic significance of these features is diminished by the level of WBC at diagnosis, early response to therapy, and other factors.

3.1.3.7 Tumor lysis syndrome

Tumor lysis syndrome (TLS) describes the metabolic derangements that occur with tumor breakdown following the initiation of cytotoxic therapy. TLS results from the rapid destruction of malignant cells and the abrupt release of intracellular ions, nucleic acids, proteins and their metabolites into the extracellular space. These metabolites can overwhelm the body's normal homeostatic mechanisms and cause hyperuricaemia, hyperkalaemia, hyperphosphaetemia, and hypocalcaemia (46, 47). TLS can lead to acute renal failure and can be life-threatening, for example, posterior leukoencephalopathy (48). It is most commonly observed after the initiation of cytotoxic chemotherapy such as after hydrocortisone (49, 50) or prednisone therapy (51-53).

- **Hyperuricaemia**

Hyperuricaemia (uric acid ≥ 476 μmol/l or ≥ 8.0 mg/dl) results from rapid release and catabolism of intracellular nucleic acids. Purine nucleic acids are catabolized to hypoxanthine, then xanthine, and finally uric acid by xanthine oxidase (**Figure 3-1**). Uric acid clearance occurs in the kidney, and in normal circumstances approximately 500 mg of uric acid is excreted through the kidneys each day. Uric acid has a pKa of 5.4–5.7 and is poorly soluble in water. At normal concentrations and at physiological blood pH, over 99% of uric acid is in the ionized form. Hyperuricemia can prevent by use alkaline diuresis (54), urate oxadase or rasburicase (55-57) before initiating steroid or other chemotherapy.

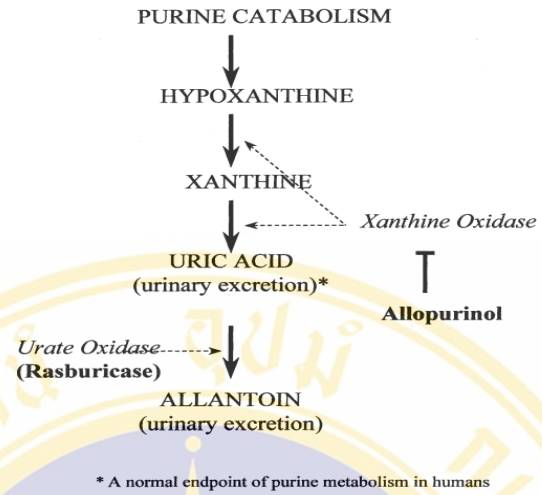

Figure 3-1. The pathway of purine catabolism. Rasburicase is a recombinant form of urate oxidase, an enzyme that converts uric acid to allantoin. In comparison, allopurinol acts by inhibiting the endogenous enzyme xanthine oxidase, thereby inhibiting formation of uric acid. (From Goldman et al, 2001. Copyright American Society of Hematology).

- **Hyperphosphataemia**

Hyperphosphataemia (phosphorus ≥ 2·1 mmol/l in children or ≥ 6.5 mg/dl) results from the rapid release of intracellular phosphorous from malignant cells, which may contain as much as four times the amount of organic and inorganic phosphorous as compared to normal cells. Initially, the kidneys are able to respond to the increased concentration of phosphorous from tumor lysis by increased urinary excretion and decreased tubular re-absorption of phosphorous. Eventually, however, the tubular transport mechanism becomes saturated and serum phosphorous levels rise. The development of hyperphosphataemia may be further exacerbated by acute renal insufficiency associated with uric acid precipitation or other complications of tumor therapy. Hyperphosphataemia can lead to the development of acute renal failure after the precipitation of calcium phosphate in renal tubules during TLS (58, 59).

- **Hyperkalaemia**

Hyperkalaemia (potassium ≥ 6·0 mmol/l or ≥ 6.0 mEq/L) may also be a life-threatening consequence of TLS and is partly a result of the kidneys' inability to clear the massive quantities of potassium released by lysed tumor cells. Hyperkalaemia results from initial lysis of tumor cells and then becomes exacerbated by the development of uremia (renal failure) and is occasionally secondary to excess iatrogenic administration of potassium during induction therapy. The rapid rise in serum potassium may result in severe arrhythmias and sudden death.

- **Hypocalcaemia**

Hypocalcaemia (serum calcium ≤ 1·75 mmol/l or ≤ 7.0 mg/dl or an ionized calcium of ≤ ionized institutional limits) may be asymptomatic or symptomatic. Hypocalcaemia results from hyperphosphataemia (see above) and the precipitation of calcium phosphate crystals in the renal tubules. When the calcium phosphorus multiple exceeds 70, there is a significant risk of calcium phosphate deposition in the kidney and other tissues that secondarily leads to systemic hypocalcaemia. Occasionally, a low albumin may suggest hypocalcaemia and, in cases of hypoalbuminaemia, an ionized calcium is required to determine if there is true hypocalcaemia.

3.1.4 Leukemic cell characteristics

3.1.4.1 Immunophenotyping

CD2, CD3, CD4, CD7 and CD8 are useful identification of T cells and their tumors, and CD10, CD19, and CD20 and surface Ig are markers of normal and neoplastic B cells. Immature T and B cells (lymphoblasts) express the enzyme terminal deoxytransferase (TdT), which is useful in distinguishing them from immature myeloid cells (myeloblasts) and mature lymphocytes. CD34 is expressed on pluripotent stem cells and is retained on the earliest lymphoid progenitor cells (see

Figure 3-2) and the phenotypes corresponding to each of these subtypes are summarized in **Table 3-3.**

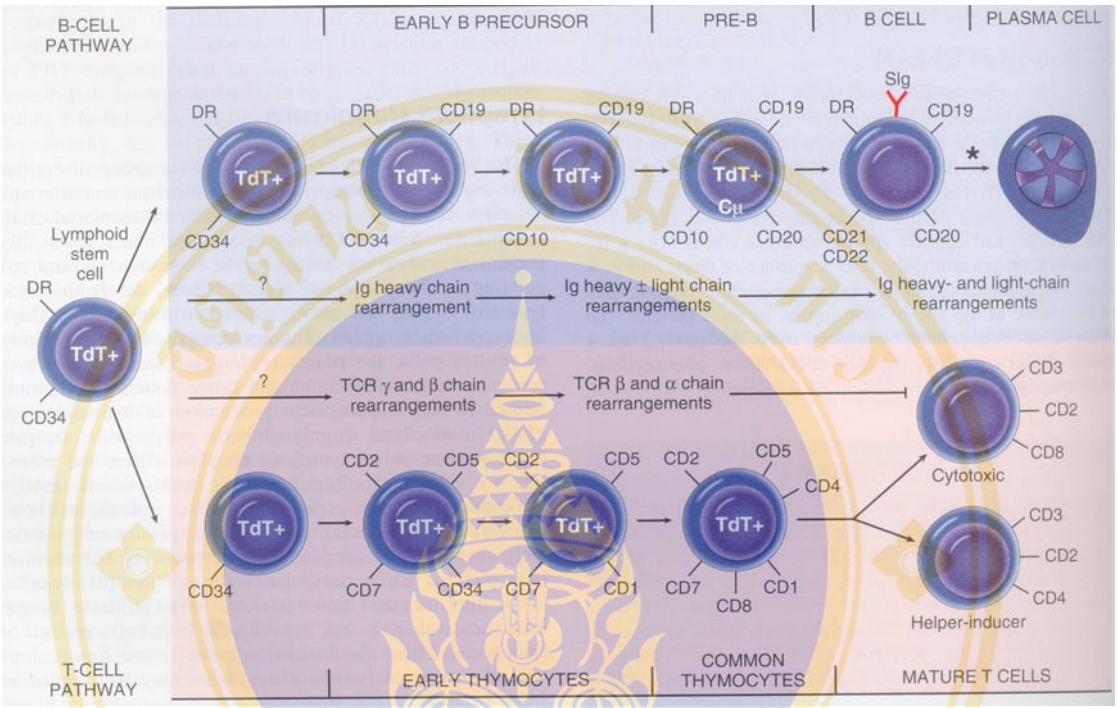

Figure 3-2 Immunologic Classification of Acute lymphoblastic Leukaemia

Early pre-B cell ALL comprises approximately two-third of cases of childhood ALL and is associated with a favorable prognosis. Although most early pre-lymphoblasts are CALLA positive, CALLA expression does not appear to have independent prognostic significance.

Pre-B cell ALL accounts for approximately 20% of childhood ALL and has been associated with a poorer outcome compared to early pre-B cell ALL. The leukemic cells of patients with pre-B ALL contain cytoplasmic immunoglobulin (cIg). This subtype is associated with a nonrandom cytogenetic abnormality, t(1;19)(q23;q13). The presence of this translocation, which is identified at the cytogenetic level in 20 to 30% of pre-B ALL patients is the primary determinant of adverse prognosis in pre-B ALL. Patients without this lesion have a survival rate similar to that of patients with early pre-B ALL.

Mature B cell ALL is uncommon, accounting for only 1 to 2% of ALL cases in children. Both B-cell ALL and Burkitt lymphoma are characterized by FAB L3 morphology, an (8; 14) or related translocations, and overexpression of the *c-myc* oncogene. Children with B-cell ALL are older than those with other B-lineage subtypes and have a higher incidence of CNS involvement. Children with B-cell ALL have a therapeutic regimens; however, dose-intensive regimens of brief duration used for Burkitt lymphoma have led to cure rates of 75% or higher.

Transitional pre-B ALL, characterized by expression of cytoplasmic and surface μ heavy chains without Ig light chain expression, occurs in a small (approximately 1%) subset of childhood ALL. These cases lack FAB L3 morphology or the chromosomal translocations associated with mature B-cell ALL and have a favorable outcome.

T-cell ALL accounts for approximately 15% of ALL in children. It is rare in infants younger than 1 year of age. T-cell ALL occurs more frequently in males and is frequently associated with a high WBC count at diagnosis. A mediastinal mass is present in 50 to 60% of patients, and the incidence of CNS involvement is higher than in other types of ALL. Although T-cell ALL was previously associated with a poor prognosis, the recent use of more intensive therapeutic regimens has resulted in a survival approaching that of non-T cell ALL. The prognostic significance of T-cell ALL is related to clinical features associated with a poor prognosis, such as high initial WBC count, age older than 15 years, massive splenomegaly, FAB L2 blast morphology, and abnormal karyotype; however, some investigators have found that clinical and laboratory prognostic factors are not as predictive for outcome in T-cell ALL as in B-precursor ALL. By multivariate analysis, the T-cell immunophenotype per se does not appear to be an independent prognostic variable. Among T-cell ALL patients, those with the least mature immunophenotype have a significantly sores outcome than those with more mature immunophenotypes. A subset of ALL characterized by expression of the T-cell associated CD7 surface antigen in the absence either CD4 or CD8 is associated with resistance to conventional chemotherapy and poor survival. Absence of CD2 expression identifies a group of T-cell patients with a worse outcome.

3.1.4.2 Cytogenetics; Chromosome number

Hyperdiploidy: Hyperdiploidy (>50 chromosomes per cell or DNA index >1.16) is the presence of additional copies of whole chromosomes and occurs in 20% to 25% of cases of B-precursor ALL but very rarely in cases of T-cell ALL (60). Hyperdiploidy can be evaluated by measuring the DNA content of cells (DNA index) or by karyotyping. Hyperdiploidy generally occurs in cases with favorable prognostic factors (age 1-9 years and low WBC count), and is itself associated with favorable prognosis (35, 61, 62). However, outcome of children with hyperdiploidy is heterogeneous and depends on age, sex, and specific trisomies (63). Hyperdiploid leukemia cells are particularly susceptible to undergoing apoptosis, and accumulate methotrexate and high levels of its active polyglutamate metabolites (64), which may explain the favorable outcome commonly observed for these cases. Certain patients with hyperdiploid ALL and greater than 64 chromosomes may have a hypodiploid clone which has doubled. These cases may be interpretable based on the pattern of gains and losses of specific chromosomes. These patients have an unfavorable outcome (65).

Trisomies: For the treatment approaches utilized by both the former Pediatric Oncology Group (POG) and the former Children's Cancer Group (CCG), extra copies of certain chromosomes appear to be specifically associated with favorable prognosis among hyperdiploid ALL cases. In POG studies, patients whose leukemia cells have extra copies of both chromosome 4 and chromosome 10 appear to have particularly favorable outcome (66). In CCG studies, children with trisomies of chromosomes 4, 10, and 17 have an excellent prognosis (67). In new Children's Oncology Group (COG) trials, the presence of trisomies for 4, 10, and 17 will be utilized to define a potentially favorable (low)-risk group (68). A Medical Research Council study showed that trisomies 4 and 18 were independent favorable prognostic indicators among hyperdiploid ALL cases (63).

Hypodiploidy: Approximately 1% of children with ALL have leukemia cells showing hypodiploidy with less than 45 chromosomes. These patients are at high risk for treatment failure (67, 69). There is a significant trend for

progressively worse outcome with a decreasing chromosome number, such that cases with 24 to 28 chromosomes (near haploidy) have the worst outcome (70, 71).

3.2 Cyclin D1

Cyclin D1 (CCND1) or parathyroid adenomatosis 1 (PRAD1) was finally isolated in parathyroid adenomas as a locus linked to the parathyroid hormone promoter on a breakpoint of human chromosome 11 on the long arm within 11q13. The D type cyclins are required for cells to get to the restriction point. In the absence of D type cyclins, cells are in G_0. In humans there are three D type cyclins; D1, D2 and D3. Cyclin D1, which is a key regulatory protein at the G1-S checkpoint of the cell cycle. It forms complexes with cdk4 or cdk6, and is responsible for the phosphorylation of the retinoblastoma tumor suppressor protein, that results in the release of E2F transcription factors that allow cells to enter into S-phase. The CCND1 gene spans approximately 15 kb and includes 5 exons and 4 introns, and generates two major mRNAs of 1.7 and 4.4 kb. The levels of D-cyclins are induced by the extracellular environment. Thus, D-cyclins are induced by the mitogenic stimulation, and their levels decline when the mitogens are withdrawn.

In 1995, Betticher et al found that a frequent polymorphism (A/G) was identified in the codon 242 at nucleotide 870 of exon 4 of the gene. Although the base change does not alter the amino acids sequence because of both the normal and the alternate transcripts encode a protein that contains the amino acids 55-161, which are thought to be responsible for the cyclin D1 function, the base is integral to the splice donor site between exon 4 and intron 4 (**Figure 3-3**). This finding led to the discovery of an alternatively spliced cyclin D1 transcript [b]. Transcript [b] differs from the previously reported cyclin D1 transcript (now called transcript [a]); exon 5 is missing, there is no splicing at the exon 4 – intron 4 boundary and the mRNA sequence continues downstream into intron 4. This protein encoded by the alternate transcript is missing the last 55 amino acids at the carboxy-terminus that are replaced by a shorter 43-amino-acid sequence encoded by intron 4. Exon 5 encodes for the polypeptide

sequence enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST) which targets proteins for rapid destruction. It was therefore speculated that the protein translated from transcript [b] may have a long half-life and play an important biological role, especially when over expressed in tumors. This protein could increase the steady - state levels of the protein in patients with the AA and AG genotypes, which may allow cells that are damaged as a result of defective mismatch repair (MMR) to pass through the G1-S phase checkpoint more easily. This would allow them to proliferate rather than to undergo apoptosis.

Previous studies reported that the cyclin D1 polymorphism associated with risk, prognosis, and event-free survival in a variety of cancers. The cyclin D1 A allele or AA genotype is associated with an increased risk of familial colorectal cancer (11), sporadic colorectal adenomas (12), urinary bladder cancer (13), sporadic renal cell carcinoma (14), endometrial cancer (15), prostate cancer (16), carcinoma in situ of superficial bladder cancer (17), and lung cancer (18). Otherwise the cyclin D1 GG (homozygous) phenotype is associated with an increased risk of colorectal cancer (19), and oral squamous cell carcinoma (20). Age of onset to cancer of hereditary nonpolyposis colorectal carcinoma (9), and hepatoblastomas (21) in the homozygous (A/A) earlier which compared to the heterozygous (G/A) or homozygous (G/G) genotype but in colorectal cancer (19) has earlier age of onset in GG genotype. In addition, the cyclin D1 GG phenotype is associated with poor prognosis and reduced disease-free interval in squamous cell carcinoma of the head and neck (SCCHN) (22), hepatocellular carcinoma (23), laryngeal squamous cell carcinoma (24), while the cyclin D1 A allele is associated with a lower event-free survival in acute lymphoblastic leukemia following methotrexate therapy (25), and epithelial ovarian cancer (26).

Table 3-3 Leukemic Cell Characteristics of Acute Lymphoblastic Leukemia Subtypes

	Early pre-B cell	**Pre-B cell**	**B cell**	**T cell**
Gene rearrangement				
Heavy chain	+*	+	+	-
Light chain	±	±	+	-
Immunologic features				
Cytoplasmic μ	-	+	-	-
Membrane Ig	-	-	+	-
CD10 (CALLA)	+	±	±	±
Ia/HLA-DR	+	+	+	±
CD24, CD19, CD20	+	+	+	-
CD2, CD5, CD7	-	-	-	+
Cytochemical features				
Nuclear TdT	+	+	-	+
5'-Nucleotidase	+	+	-	-
Acid phosphatase	-	-	-	+
Cytogenetic abnormalities		t(1;19)	t(8;14)	t(11;14)
			t(2;8)	
			t(8;22)	
Morphology				
FAB L1	90%	90%	10%	95%
FAB L2	10%	10%	15%	5%
FAB L3	0	0	75%	0

+, usually present or increased; -, usually absent or decreased; ±, may or may not be present

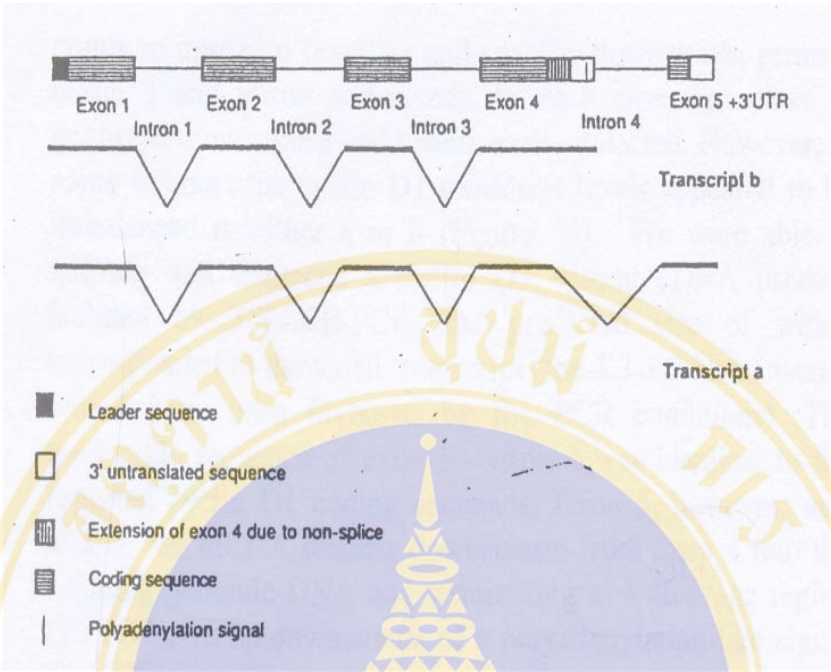

Figure 3-3. CCND1 gene organization, express of cyclin D1 (transcript a) and cyclin D1 variant (transcript b).

3.3 Single nucleotide polymorphisms (SNPs) genotyping methods

Single nucleotide polymorphisms (SNPs) is a human genetic variant at a position which two alternative bases occur an appreciable frequency (>1%) in the human population. In the end of 2000, over 1.5 million SNPs have been found in the human genome and deposited to public databases. There are two ways to detect the SNPs, a sequence-nonspecific and sequence-specific detection. Sequence-nonspecific detection is based on the capture, cleavage, or mobility change during electrophoresis or liquid chromatography of mismatched heteroduplexes formed between allelic DNA molecules or single-strand DNA molecules, that assume slightly different conformations under nondenaturing conditions. While sequence-specific detection relies on four general mechanisms for allele discrimination: allele-specific hybridization, allele-specific nucleotide incorporation and primer extension, allele-specific oligonucleotide ligation, and allele-specific invasive cleavage (72).

3.3.1 Allele-specific hybridization uses two allele-specific probes are designed to hybridize to the target sequence only when they match perfectly, if has a one-base mismatch sufficiently destabilizes the hybridization to prevent the allelic probe from annealing to the target sequence (**Figure 3-4**). This method is the simplest mechanism for genotyping.

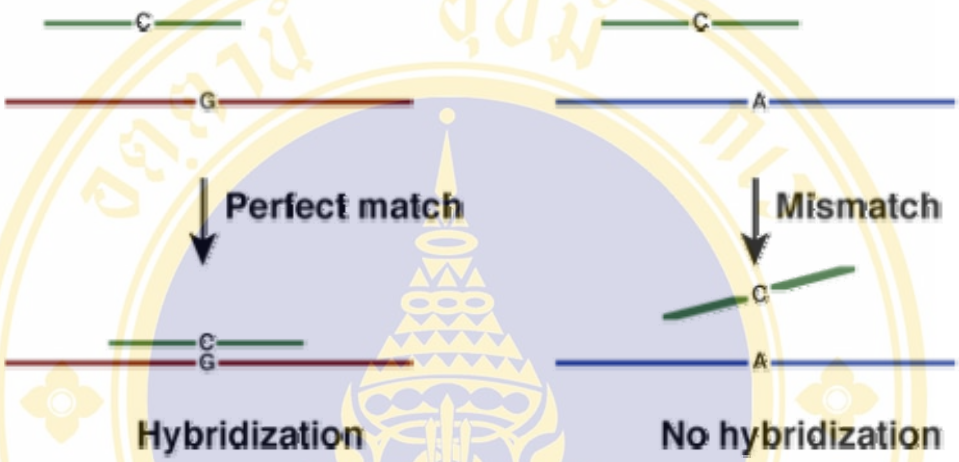

Figure 3-4. Allele-specific hybridization

3.3.2 Allele-specific nucleotide incorporation and primer extension, allele-specific nucleotide incorporation uses to determine the identity of the base(s) incorporated at the polymorphic site in the target DNA (**Figure 3-5**), but the allele-specific primer extension relies on the DNA polymerase to extend a primer when its 3' end is perfectly complementary to the template with polymerase chain reaction (PCR) method. The PCR product is produced when condition is met (**Figure 3-6**). Sequencing is the most common which are based on primer extension using either a) dye-primers and unlabeled terminators or b) unlabeled primers and dye-terminators (**Figure 3-7**).

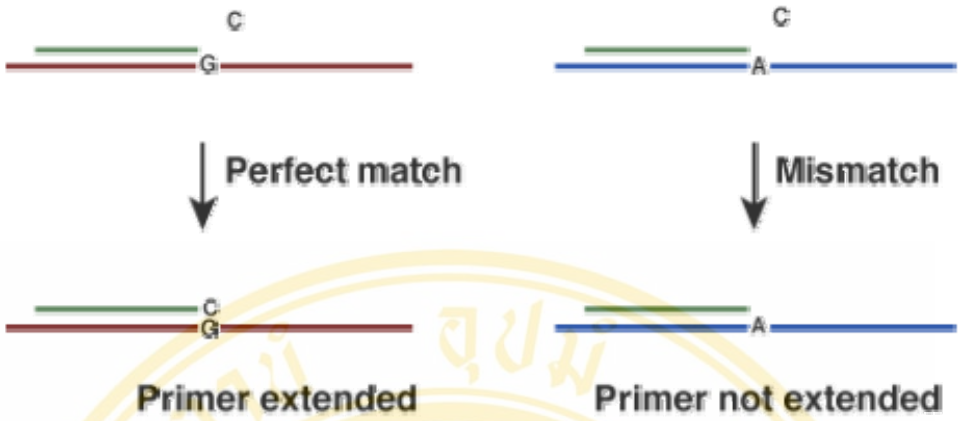

Figure 3-5 Allele-specific nucleotide incorporation

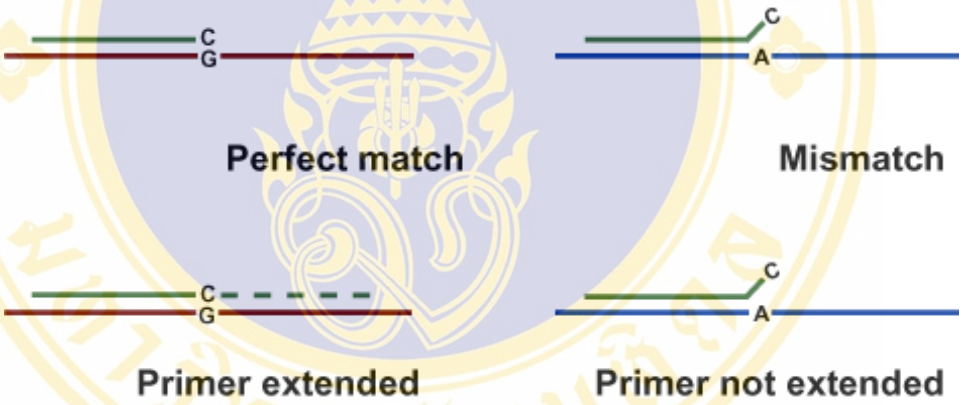

Figure 3-6. Allele-specific primer extension

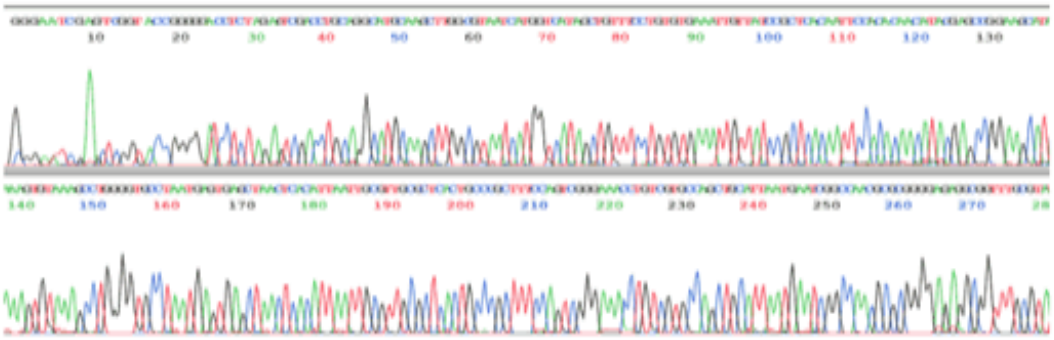

Figure 3-7. Sequencing.

3.3.3 Allele-specific oligonucleotide ligation uses two adjacent oligonucleotides which annealed to a DNA template; they are ligated together only if the oligonucleotides perfectly match the template at the junction (**Figure 3-8**). Although ligation has the highest level of specificity and is easiest to optimize among all allele discrimination mechanisms, it is the slowest reaction and requires the largest number of modified probes.

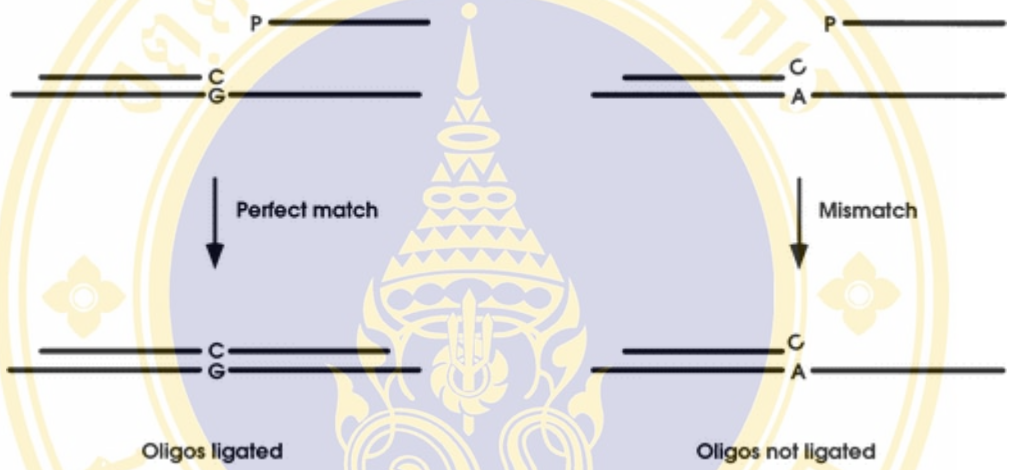

Figure 3-8 Allele-specific oligonucleotide ligation

3.3.4 Allele-specific invasive cleavage, structure-specific enzymes cleave a complex formed by the hybridization of overlapping oligonucleotide probes. When probes are designed such that the polymorphic site is at the point of overlap, the correct overlapping structure is formed only with the allele-specific probe but not with the probe with a one-base mismatch (**Figure 3-9**). Elevated temperature and an excess of the allele-specific probe enable the cleavage of multiple probes for each target sequence present in an isothermal reaction. For example, they are used in a second reaction where a labeled secondary probe is cleaved. This method uses in the isothermal nature of the reaction and the potential for genotyping without PCR amplification.

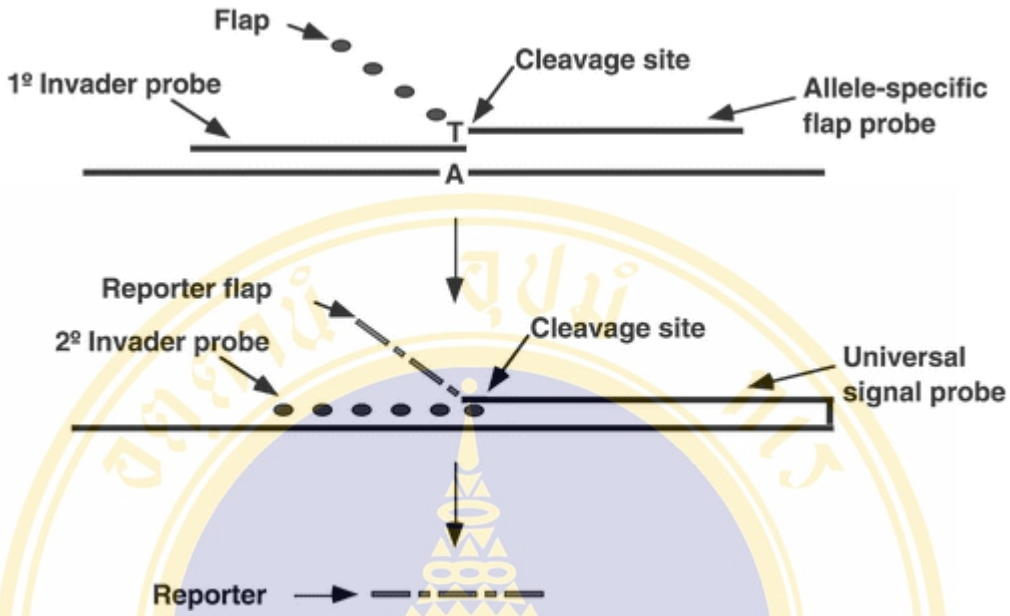

Figure 3-9 Allele-specific invasive cleavage.

Detection of a positive allele discrimination reaction is done by monitoring the light emitted by the products such as luminescence or fluorescence, measuring the mass of the products (mass spectrometry), or detecting a change in the electrical property when products are formed.

CHAPTER 4
MATERIALS AND METHODS

4.1 Materials

4.1.1 Subjects

The case group of the study consisted of 100 patients with childhood ALL. They were diagnosed in the Division of Pediatric Hematology-Oncology, Ramathibodi Hospital, Department of Pediatrics Faculty of Medicine, Mahidol University. The criteria for inclusion in this group were complete clinical history and availability of biological material.

A general population control group composed of 300 samples was collected from healthy volunteer in Blood Bank Department. The criteria for inclusion in control group were unrelated with malignancy and availability of blood sample. Informed consent was obtained from all subjects.

4.1.2 Instruments

1. GeneAmp® PCR System 2004 (Applied Biosystems, USA)
2. GeneAmp® PCR System 2007 (Applied Biosystems, USA)
3. Autoclave (HA-3D, Hirayama, Japan)
4. Automatic pipette; size 10 , 100, 1000 µl (Eppendorf Germany)
5. Analytical Balance (1507MP8, Sartorius, Germany)
6. Centrifuge; Hermle Z283K, Berthold Hermle GmbH, Germany)
7. Freezer-70^{o} C (Frost free, White Westing House USA)

8. Hot air oven (TV460, Memmert, Germany)
9. Hot plate with magnetic stirrer (Thermolyne Type 1000, Sybron/Thermolyne, USA)
10. Hood
11. Vortex Mixer (Vortex-2 Genie, Scientific Industries, USA)
12. Microwave Oven (Sanyo, Japan)
13. Electrophoresis systems (e-power 100, Pacific Science Inc., Thailand)
14. pH meter
15. Refrigerator 4-8^{o} C (GE, USA)
16. UV transluminator (Bio Imaging System, Syngene, UK.)
17. Water bath (Model W350T(2000W), Memmert, Germany)

4.1.3 Chemical and reagents

- Chemical and reagent for DNA isolation

1. Absolute ethanol (BDH Laboratory, UK.)
2. Chloroform (BDH Laboratory, UK.)
3. EDTA (Merk, Germany)
4. Gracial acetic acid (Merk, Germany)
5. Hydrochloric acid (conc.) (Merk, Germany)
6. 8-Hydroxyquinolone (Sigma, USA)
7. Isoamyl alcohol (Merk, Germany)
8. Nonidet (NP40) (Sigma, USA)
9. Phenol (Merk, Germany)
10. Proteinase K (Gibco, BRL)
11. Sodium dodecylsulfate (Sigma, USA)
12. Sodium acetate anhydrous (Merk, Germany)
13. Sodium hydroxide (Merk, Germany)
14. Soduim chloride (Merk, Germany)
15. Sucrose (Ultra pure, Bio Basic Inc., USA)

16. Sterile distilled water
17. Trizma base (Ultra pure molecular biology grade, Research Organics Inc., USA)
18. Triton X-100 (Research Organics Inc., USA)
19. Tris-HCl (Sigma, USA)

- Chemical and reagent for PCR-RFLP

1. Steriled distilled water
2. dNTP Mixture (Bio Basic Inc., Canada)
3. $MgCl_2$ (Promega, USA.)
4. Oligonucleotide primers (Bio Basic Inc., Canada)
5. PCR 10X reaction buffer (Promega, USA.)
6. *Taq* DNA polymerase (Promega, USA.)
7. Nci I restriction enzyme (New England Bio Lab Inc., USA)
8. Nci I restriction enzyme reaction buffer (New England Bio Lab Inc., USA)

- Chemical and reagent for gel electrophoresis and staining

1. Agarose (Molecular biology grade Research Organics Inc., USA)
2. DNA molecular weight markers (SibEnzyme, USA.)
3. Ethidium bromide
4. Loading dye
5. 1X TBE buffer

4.1.4 Supplies

1. Aliminium foil
2. Autoclave tape
3. Cylinder, size 100 ml, 1000 ml
4. Duran bottle

5. Erlenmeyer flask, size 250 ml
6. Gloves
7. Microcentrifuge tube, size 1.5 ml
8. Magnetic stirrer
9. Separating funnel
10. Gel casting platform
11. Thin-walled PCR tube, size 0.2 ml
12. Pipette tips, size 10, 100, 1000 µl

4.2 Methods

4.2.1 DNA isolation

Genomic DNA was isolated from peripheral leukocyte using phenol-chloroform methods (73). This method produces very pure DNA, which can be stored for long periods of time. The DNA isolation is first extracted with a phenol-chloroform mixture to remove protein contaminants, then precipitated with 100% ethanol. The DNA is pelleted after precipitation step, washed with 70% ethanol to remove salts and small organic molecules, and resuspended in buffer at a concentration suitable for further experimentation

4.2.1.1 Cell lysis and protein digestion

DNA isolation from whole blood needs to prepare white blood cells from anticoagulated whole blood by red blood cell lysis. Transfer 300 µl aliquots of whole blood to each of two microcentrifuge tube. Add 900 µl of red blood cell lysis buffer to each tube and invert the capped tubes to mix the contents. Incubate the solution at room temperature for 10 minutes, occasionally inverting the tubes. Then, centrifuge the tubes at maximum speed for 20 seconds at room temperature in a microcentrifuge tube. Discard all supernatant but retain 20 µl of each tube. Resuspend the pellets of white cells in the small amount of supernatant left in each tube. Combine

the resuspended cell pellets in a single tube. Furthermore, transfer the resuspended white blood cell pellets to a microcentrifuge tube containing 600 µl of ice-cold cell lysis buffer. Homogenize the suspension quickly with 30-50 strokes of a microcentrifuge pestle. After that, add 3 µl of protein K solution to the lysate to increase the yield of genomic DNA. Incubate the digest for at least 3 hours but no more than 16 hours at 55°C.

4.2.1.2 Extraction with Phenol:Chloroform

The standard way to remove proteins from nucleic acid solutions is to extract first with phenol:chloroform (optionally containing hydroxyquiniline at 0.1%) and then with chloroform. This procedure takes advantage of the fact that deproteinization is more efficient when two different organic solvents are used instead of one. Furthermore, although phenol denatures proteins efficiently, it does no completely inhibit RNase activity, and it is a solvent for RNA molecules that contain long tracts of poly(A) (Brawerman et al 1972). Both of these problems can be circumvented by using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1). The subsequent extraction with chloroform removes any lingering traces of phenol from the nucleic acid preparation. Extraction with ether, which was widely used for this purpose for many years, is no longer required or recommended for routine purification of DNA. Transfer the sample to a polypropylene tube and add an equal volume of phenol:chloroform. Mix the contents of the tube until an emulsion forms. Centrifuge the mixture at 80% of the maximum speed that the tubes can bear for 1 minute at room temperature. If the organic and aqueous phases are not well separated, centrifuge again for a longer time. Use a pipette to transfer the aqueous phase to a fresh tube. For small volume (< 200 µl), use an automatic pipettor fitted with a disposable tip. Discard the interface and organic phase. Repeat that steps until no protein is visible at the interface of the organic and aqueous phases. Add an equal volume of chloroform and repeat steps 2 through 4. Recover the nucleic acid by precipitation with ethanol.

4.2.1.3 Concentrating nucleic acids

Precipitation with ethanol is the standard method to recover nucleic acids from aqueous solutions. It is rapid, virtually foolproof, and efficient:Subnanogram amounts of DNA and RNA can be quantitatively precipitated with ethanol, collected by centrifugation, and redissolved within minutes.

Ethanol depletes the hydration shell from nucleic acids and exposes negatively charged phosphate groups, Counterions such as Na^+ bind to the charged groups and reduce the repulsive forces between the polynucleotide chains to the point where a precipitate can form. Ethanol precipitation can therefore only occur if cations are available in sufficient quantity to neutralize the charge on the exposed phosphate residues. The most commonly used cations are ammonium acetate, lithium chloride, sodium chloride and sodium acetate.

4.2.1.4 Quantitation of DNA

Two types of methods are widely used to measure the amount of nucleic acid in a preparation. If the sample is pure (i.e., without significant amount of contaminants such as proteins, phenol, agarose, or other nucleic acids), spectrophotometric measurement of the amount of ultraviolet (UV) irradiation absorbed by the bases is simple and accurate. If the amount of DNA is very small or if the sample contains significant quantities of impurities, the amount of nucleic acid can be estimated from the intensity of fluorescence emitted by ethidium bromide.

For quantitating the amount of DNA with spectrophotometric determination, readings are taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to ~ 50 µg/ml for double-stranded DNA, 40 µg/ml for single-stranded DNA and RNA, and ~ 33 µg/ml for single-stranded oligonucleotides. The ratio between the readings at 260 nm and 280 nm (OD_{260}:OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}:OD_{280} values of 1.8 and 2.0, respectively. If there is contamination with protein

or phenol, the OD_{260}:OD_{280} will be less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible.

Ethidium bromide fluorescent quantitation of the amount of double-stranded DNA. Sometimes there is not sufficient DNA (<250 ng/ml) to assay spectrophotometrically, or the DNA may be heavily contaminated with other substances that absorb UV irradiation and therefore impede accurate analysis. A rapid way to estimate the amount of DNA in such samples is to utilize the UV-induced fluorescence emitted by ethidium bromide molecules intercalated into the DNA. Because the amount of fluorescence is proportional to the total mass of DNA, the quantity of DNA in the sample can be estimated by comparing the fluorescent yield of the sample with that of a series of standards. As little as 1-5 ng of DNA can be detected by this method.

4.2.2 Genotyping

4.2.2.1 Genotyping for detect cyclin D1 codon 242 polymorphism

The method for genotyping cyclin D1 polymorphism was modified from Betticher et al.(5). A 167-bp PCR product was amplified using two primers were 5' –GTG AAG TTC ATT TCC AAT CCG C-3' and 5' -GGG ACA TCA CCC TCA CTT AC-3' for forward and reverse primer, respectively. PCR condition for this method consisted of 100 ng of genomic DNA, 0.2 mM of dNTPs, 2.0 mM $MgCl_2$, 0.2 µM of each primers and 1.0 unit of Taq DNA polymerase in 50 µl PCR reaction volume.

Amplification procedure: prepare master mixture by pipetting water first, followed by the other ingredients except the DNA template. The master mixture must be fresh prepared according to the number of samples and immediately used after preparation. The remaining reagent may be combined to form a master mixture that may then be liquoted into the reaction tubes. To make the master mixture, multiply the number of reaction being done by the following:

1.	Sterile distilled water	37.8	µl/reaction
2.	10X PCR reaction buffer	5.0	µl/reaction
3.	50 mM $MgCl_2$	2.0	µl/reaction
4.	10 mM dNTP	1.0	µl/reaction
5.	10 µM Forward primers	1.0	µl/reaction
6.	10 µM Reverse primers	1.0	µl/reaction
7.	Taq DNA polymerase	0.2	µl/reaction

When running many samples it is helpful to make a master mixture that is slightly larger than required, (e.g., multiplying by 11 when only doing 10 reactions). Aliquot 48 µl of PCR master mixture into each tube and place 2 µl of each genomic DNA into each PCR reaction tube. Gently tap the tubes to mix all reagents. Briefly centrifuge tubes to deposit all of the liquid at the bottom of the reaction tube, place the PCR tubes into the PCR Themal Cycler. After initial denaturation step (94°C, 5 mins), followed by 35 cycles of denaturation (94°C, 1 min.), annealing (60°C, 1 min.), and extension (72°C, 1 min.), with the final extension at 72°C by 7 mins.

Restriction enzyme digestion was performed on the PCR fragment in 5 µl of PCR product, 1.0 10X buffer4, 5 units of NciI enzyme and 4.75 µl of water in a total volume of 10 µl and incubated at 37°C overnight. The digestion fragments were separated by 2.5% agarose gel and it was staining with ethidium bromide. Bands were then visualized directly by UV transluminator. The Guanine (G) allele creates a NciI site in the 145-bp and 22-bp products but the Adenine (A) allele cannot digest by this enzyme so the NciI site of A allele is 167-bp product (Figure 5-3).

4.2.3 Agarose gel electrophoresis and ethidium bromide staining

Agarose gel electrophoresis is a simple and highly effective method for separating and identifying DNA fragment. DNA is negative charged due to the phosphate backbone and moves to a positive pole in the electric field. This movement of charged particles in various media due to an applied electric field in called electrophoresis. The rate of migration is inversely proportional to size. The larger

fragments move slower than smaller ones. Therefore, DNA fragment of different size can be separated and identified with this method. The migrated DNA fragments were detected by using ethidium bromide which is a fluorescent dye that intercalates double strand DNA. DNA bands can be made visible upon exposure to ultraviolet light.

In brief, prepared adequate volumes of electrophoresis buffer (1X TBE) to fill the electrophoresis tank and prepare the gel. Add the desired amount of electrophoresis-grade agarose to a volume of electrophoresis buffer sufficient for constructing the gel. The 2.5% agarose gel was melted in a microwave oven. Pour in the melted agarose and insert the gel comb, make sure that no air bubbles are trapped underneath the combs and all bubbles on the surface the agarose are removed before the gel sets. After the gel has hard, withdraw the gel comb, place the gel casting platform containing the set gel in the electrophoresis tank. Add sufficient electrophoresis buffer to cover the gel to a dept of about 1 mm. DNA sample should be prepared in a volume (10 µl) that will not overflow addition of the appropriate amount of 10X loading buffer (2 µl). Molecular weight marker (M) (5 µl) was used to differ the size of base pair of PCR products. The gel was electrophoresed in 1X TBE at 100 volt. Then the gel was stained with ethidium bromide. The PCR products and digested PCR products were visualized by UV transaluminator.

4.3 Statistical analysis

The Chi-square test or Fisher's Exact test was used to examine difference in frequencies between cyclin D1 genotype in cases and controls and the association between cyclin D1 genotype with clinical presentations of childhood ALL. The level of significance was calculated at p-value < 0.05. Crude Odd ratios (ORs) are given with 95% confidence interval (CI) was used to determine the risk of cyclin D1 genotype on childhood ALL. The age of onset and outcome of 10-year diseased-free survival rate were analysed using the Kaplan-Meier method. The log-rank test was applied to compare the age of onset and outcome between genotype groups. All analyses were carried out using the SPSS statistic software package, version 11.5 for Windows (SPSS Inc., Chicago, Illinois, USA).

CHAPTER 5
RESULTS

5.1 Optimize of the PCR amplification for cyclin D1

This protocol was designed to optimize the reaction component and condition. The first stage determined the optimal $MgCl_2$ concentration. The master mixture for optimizing reaction components consisted of 5.0 µl of 10X PCR buffer, 0.2 mM of dNTP, 0.2 µM of each primers, and 1 unit of Taq DNA polymerase. The PCR reaction used ~ 100 ng of DNA in a total volume of 50 µl. The $MgCl_2$ concentrations were varied from 1.5, 2.0 and 2.5 mM. The result was shown in **Figure 5-1**. The optimal concentration of $MgCl_2$ was 2.0 mM per reaction.

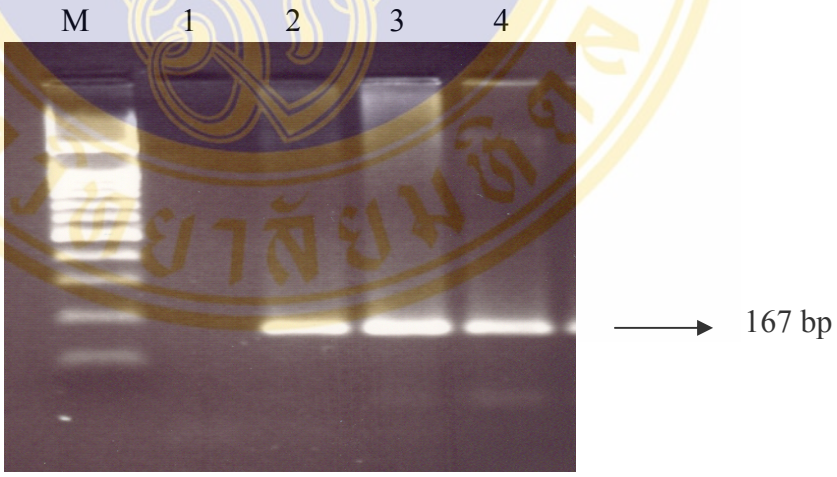

Figure 5-1 The optimization for $MgCl_2$ concentration of CCND1 G870A.
The PCR products using different $MgCl_2$ concentration, 1.5 mM (lane 2), 2.0 mM (lane 3), 2.5 mM (lane 4) were demonstrated. A DNA ladder marker (M) 100 base pair was used as a size marker. Lane 1 was negative control (blank). The intensity of PCR band using 2.0 mM (lane 3) was stronger than the bands of 1.5 and 2.5 mM (lane 2 and lane 3).

5.2 The Optimization of NciI enzyme digestion

The enzyme reaction tubes were added with 1.0 µl of 10X buffer. Then, the NciI concentrations were varied from 3 units, 5 units and 7 units for each tube of the reaction and adjusted to 5.0 µl with distilled water. Finally, 5.0 µl of PCR product was added. The total volume of 10 µl was incubated at 37°C overnight. The products were detected by 2.5% gel electrophoresis. The PCR amplification of the wild-type (GG genotype) allele created an NciI cleavage site [5' CC↓SGG 3', S for C or G]. The result was shown in **Figure 5-2**. The PCR products using NciI enzyme concentration between 3 units to 7 units (lane 1-6) were completely digested by NciI enzyme. The optimal NciI enzyme concentration for this study was 5 units for 5.0 µl of the PCR products from genomic DNA concentration about 100 ng.

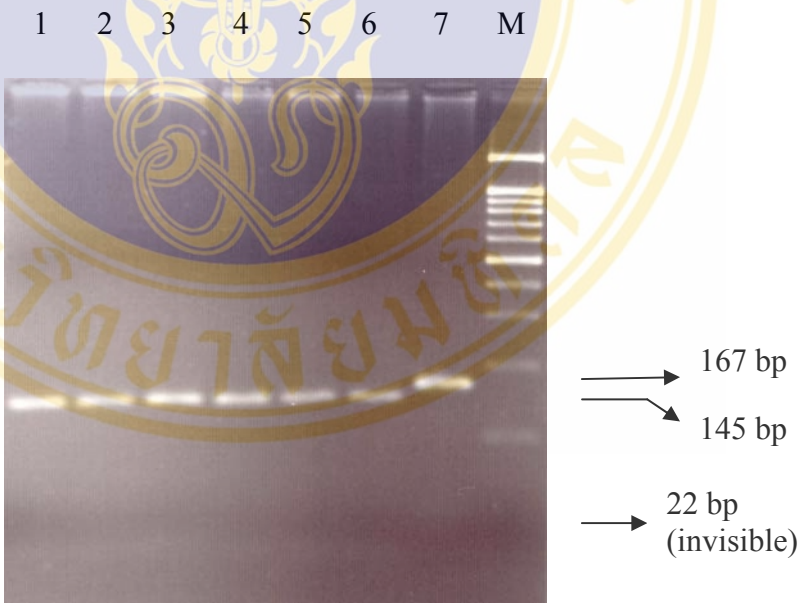

Figure 5-2 The optimization of NciI enzyme concentration.
The PCR products were digested by different NciI enzyme concentration, 3 units (lane 1 and lane 2), 5 units (lane 3 and lane 4), and 7 units (lane 5 and lane 6). A DNA ladder marker (M) 100 base pair was used as a size marker. Lane 7 was negative control (uncut PCR product). The PCR products using NciI enzyme concentration between 3 units to 7 units (lane 1-6) were completely digested by NciI enzyme.

5.3 PCR-RFLP for the detection of cyclin D1 polymorphism by NciI enzyme

Restriction enzyme digestion was performed on the PCR fragments in 5.0 µl of PCR product. The mixture consisted of 1.0 µl of 10X buffer 4, 0.25 µl (5 units) of NciI enzyme and 4.75 µl of water in a total volume of 10 µl and incubated at 37°C overnight. The digestion fragments were separated by 2.5% agarose gel electrophoresis and it was staining with ethidium bromide. Bands were directly visualized by UV transluminator. The AA homozygous gave only one band of the NciI site at 167-bp. The AG heterozygous consists of A allele and G allele so it has 2 sites in the 167-bp and 145-bp products. The GG genotype completely digested by NciI enzyme, so this genotype has only 145-bp product of the NciI site. While the 22-bp of the G allele was invisible (**Figure 5-3**).

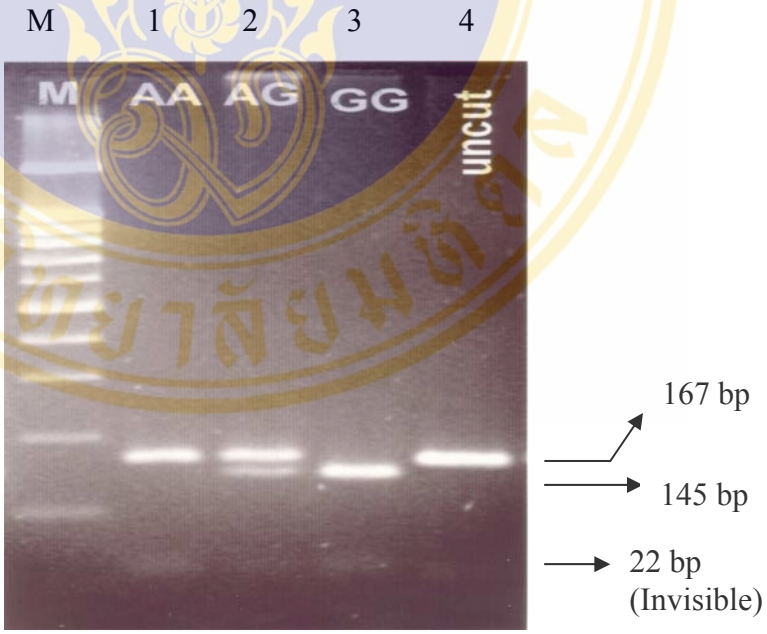

Figure 5-3.The cyclin D1 genotypes. The PCR products were digested by 5 units of NciI enzyme. Lane 1, lane 2 and lane 3 are the AA homozygous, the AG heterozygous and the GG homozygous cyclin D1 genotype, respectively. A DNA ladder marker (M) 100 base pair was used as a size marker. Lane 4 was negative control (uncut PCR product).

5.4 The clinical data of ALL patients and controls.

The childhood ALL case was comprised of 56 (56%) males and 44 (44%) females, and the control group included 155 (51.7%) males and 145 (48.3%) females. Mean age ± SD in the case group was 6.2±3.7 years (range 0.8-14.8 years) and control group aged range from 15-55 years.

Differences in the frequencies of clinical and laboratory characteristics of childhood ALL at diagnostic were shown in **Table 5-1** and **Table 5-2**. This study found that clinical characteristics frequencies of childhood ALL were hepatomegaly 74.2%, splenomegaly 51.6% and lymphadenopathy 22.6%, respectively **(Table 5-1**). Laboratory characteristics frequencies were showed in **Table 5-2**.

The distribution of cyclin D1 genotypes in ALL patients and controls were shown in **Table 5-3**. The frequency of cyclin D1, AA genotype was observed in 38% of the cases compared to 38% of the controls. The frequency of AG genotype was 49% of the cases and 47.7% of the controls and the frequency of GG genotype was 13% of the cases and 14.3% of the controls. The frequencies of cyclin D1 genotypes were not statistically significant difference ($p = 0.940$) among the cases and the controls. The genotype distribution of both groups was in the Hardy-Weinberg equilibrium ($p = 0.81$).

Table 5-1 Frequencies of Clinical Characteristics of Childhood ALL at diagnosis.

Clinical Characteristics	n(%)
Hepatomegaly	
Present	69(74.2)
Absent	24(25.8)
Splenomegaly	
Present	48(51.6)
Absent	45(48.4)
Lymphadenopathy	
Present	21(22.6)
Absent	72(77.4)
Immunophenotype	
B-lineage	58(82.9)
T-lineage	12(17.1)
Risk	
High	14(14.4)
Standard	35(36.1)
Low	48(49.5)
Cytogenetics	
Normal	52(86.7)
Hyperdiploid and other	8(13.3)

Table 5-2 Frequencies of Laboratory Characteristics of Childhood ALL at diagnosis.

Laboratory Characteristics	n(%)
WBC x 10^9/L	
<10	44(46.3)
10-49	29(30.5)
>50	22(23.2)
Hematocrit (%)	
≤30	67(71.3)
>30	27(28.7)
Platelet x 10^9/L	
<50	39(41.9)
50-100	26(28.0)
>100	28(30.1)
Uric Acid (mg/dl)	
≥8.0	17(18.9)
<8.0	73(81.1)
Phosphate (mg/dl)	
≥6.5	16(17.8)
<6.5	74(82.2)
Potassium (mg/dl)	
≥6.0	1(1.1)
<6.0	89(98.9)
Calcium(mg/dl)	
≤7.0	6(6.7)
>7.0	84(93.3)

Table 5-3 Distribution of cyclin D1 genotype in childhood ALL and control

Characteristics	Total (n)	Genotype frequency			p-value
		AA(%)	AG(%)	GG(%)	
Groups					
Case group	100	38(38.0)	49(49.0)	13(13.0)	0.940
Control group	300	114(38.0)	143(47.7)	43(14.3)	
Total	400	152(38.0)	192(48.0)	56(14.0)	
Case group					
Male	56	21(37.5)	26(46.4)	9(16.1)	0.576
Female	44	17(38.6)	23(52.3)	4(9.1)	
Control group					
Male	155	60(38.7)	72(46.5)	23(14.8)	0.905
Female	145	54(37.2)	71(49.0)	20(13.8)	

5.5 Cyclin D1 genetic polymorphism and clinical presentation of childhood ALL.

The cyclin D1 polymorphism was not associated with clinical presentations of childhood ALL including clinical and laboratory characteristics and tumor lysis syndrome (hyperuricemia and hyperphosphatemia) (p –value > 0.05). (**Table 5-4**, **Table 5-5** and **Table 5-6**)

The other clinical presentations such as immunophenotype, risk to relapse, cytogentics, and tumor lysis syndrome (hyperkalemia, and hypocalemia) had a small size in some subgroup therefore Fisher's Exact test was used for analyzing the association between cyclin D1 genotype an these characteristics. This study found that immunophenotype, risk to relapse, cytogenetics, hyperkalemia and hypocalcemia were not associated with AA and AG+GG genotype (p-value > 0.05) (**Table 5-7**).

5.6 Cyclin D1 genetic polymorphism and the risk to childhood ALL

The distributions of the cyclin D1 genotypes of cases compared to controls were shown in **Table 5-8**. Using the GG genotype as the reference genotype, this study found no association between cyclin D1 polymorphisms and the risk to childhood ALL with Odd ratios (ORs) of 1.13 (95% CI, 0.56-2.28, p = 0.73) , 1.10 (95% CI, 0.54-2.27, p = 0.79) and 1.12 (95% CI, 0.58-2.18, p = 0.74) for AA, AG and AA+AG, respectively. While using AG and GG genotypes as the reference in recessive model, there was no association between AA genotype and the risk to childhood ALL with an OR of 1.00 (95% CI, 0.63-1.59, p = 1.00).

Table 5-4 Clinical characteristics of childhood ALL at diagnosis and cyclin D1 genotype.

Characteristics	Total (n)	Genotype frequency			p-value
		AA(%)	AG(%)	GG(%)	
Age (year)					
≤ 10	79	32(40.5)	35(44.3)	12(15.2)	0.16
>10	21	6(28.6)	14(66.7)	1(4.8)	
Gender					
Male	56	21(37.5)	26(46.4)	9(16.1)	0.58
Female	44	17(38.6)	23(52.3)	4(9.1)	
Hepatomegaly					
Present	69	27(39.1)	32(46.4)	10(14.5)	0.80
Absent	24	8(33.3)	13(54.2)	3(12.5)	
Splenomegaly					
Present	48	15(16.1)	25(26.9)	8(8.6)	0.39
Absent	45	20(44.4)	20(44.4)	5(11.1)	
Lymphadenopathy					
Present	21	7(33.3)	10(47.6)	4(19.0)	0.73
Absent	72	28(38.9)	35(48.6)	9(12.5)	

Table 5-5 Laboratory characteristics of childhood ALL at diagnosis and cyclin D1 Genotype.

Characteristics	Total (n)	Genotype frequency			p-value
		AA(%)	AG(%)	GG(%)	
WBC x 10^9/L					
<10	44	17(38.6)	23(52.3)	4(9.1)	0.65
10-49	29	11(37.9)	12(41.4)	6(20.7)	
>50	22	7(31.8)	12(54.5)	3(13.6)	
Hematocrit(%)					
≤30	67	27(40.3)	30(44.8)	10(14.9)	0.45
>30	27	8(29.6)	16(59.3)	3(11.1)	
Platelet x 10^9/L					
<50	39	17(43.6)	13(33.3)	9(23.1)	0.09
50-100	26	8(30.8)	16(61.5)	2(7.7)	
>100	28	10(35.7)	16(57.1)	2(7.1)	

Table 5-6 Laboratory characteristics for tumor lysis syndrome of childhood ALL at diagnosis and cyclin D1 genotype.

Characteristics	Total (n)	Genotype frequency			p-value
		AA(%)	AG(%)	GG(%)	
Uric Acid (mg/dl)					
≥8.0	17	5(29.4)	9(52.9)	3(17.6)	0.72
<8.0	73	29(39.7)	34(46.6)	10(13.7)	
Phosphate (mg/dl)					
≥6.5	16	7(43.8)	8(50.0)	1(6.2)	0.58
<6.5	74	27(36.5)	35(47.3)	12(16.2)	

Table 5-7 Characteristics of childhood ALL at diagnosis and cyclin D1 genotype by Fischer's Exact test

Characteristics	Total (n)	Genotype frequency		p-value
		AA(%)	AG+GG(%)	
Immunophenotype				
B-ALL	58	25(43.1)	33(56.9)	0.11*,#1
T-ALL	12	2(16.7)	10(83.3)	
Risk to relapse				
High	14	4(28.6)	10(71.4)	0.67
Standard	35	13(31.7)	22(62.9)	
Low	48	20(41.7)	28(58.3)	
Cytogenetics				
Normal	52	21(40.4)	31(59.6)	1.00*,#2
Hyperdiploid and other	8	3(37.5)	5(62.5)	
Potassium (mg/dl)				
≥6.0	1	0(0)	1(100)	1.00*,**
<6.0	89	34(38.2)	55(61.8)	
Calcium(mg/dl)				
≤7.0	88	33(37.5)	55(62.5)	1.00*,**
>7.0	2	1(50.0)	1(50.0)	

*, **,# Test by Fisher's Exact test

** p-value was similar when compare between AA+AG and GG genotype

#1 p-value = 0.59 when compare between AA+AG and GG genotype

#2 p-value = 0.58 when compare between AA+AG and GG genotype

Table 5-8 Association between cyclin D1 genotype and ALL risk.

Genotype	Cases (%) n=100	Control (%) n = 300	OR(95%CI)	p-value
General genotype				
GG	13(13.0)	43(14.3)	1.00(Reference)	-
AG	49(49.0)	143(47.7)	1.13(0.56-2.28)	0.73
AA	38(38.0)	114(38.0)	1.10(0.54-2.28)	0.79
Dominant genotype				
GG	13(13.0)	43(14.3)	1.00(Reference)	-
AA+AG	87(87.0)	257(85.7)	1.12(0.58-2.18)	0.74
Recessive genotype				
GG+AG	62(62.0)	186(62.0)	1.00(Reference)	-
AA	38(38.0)	114(38.0)	1.00(0.63-1.59)	1.00

5.7 Cyclin D1 genetic polymorphism on age of onset and outcome of childhood ALL

The mean age of onsets in children with ALL were 5.6±3.7, 6.7±3.7, and 5.8±3.6 for AA, AG and GG genotype, respectively. There was no statistically significant difference between age of onset and those genotypes (p = 0.290, KW test).

5.8 Association of cyclin D1 genotypes and the different ethnicities.

Allele frequencies of CCND1 G870A of Japanese, Korean, Singaporean, Taiwanese, Chinese, Caucasians, African-Americans, and Hispanics population have been previously described (**Table 5-9**). The frequencies of G allele an A allele were 38% and 62% in a Thai populations. Our allele frequencies were similar to Singaporean, Taiwanese and Portuguese. Asians seem to have a higher A allele frequency compared to Caucasians, except Portuguese populations.

Table 5-9 Comparison of A-allele and G-allele in different ethnicities

Asia	**A allele**	**G allele**	**Europe & USA**	**A allele**	**G allele**
Japanese (13)	0.43	0.57	German Caucasians (22)	0.45	0.55
Japanese (14)	0.43	0.57	English Caucasians (77)	0.42	0.58
Korean (15)	0.58	0.42	Portuguese (74)	0.65	0.35
Korean (76)	0.49	0.51	American Caucasians (12)	0.38	0.62
Singaporean(19)	0.63	0.37	African Americans (21)	0.21	0.79
Taiwanese (23)	0.64	0.36	Hispanics (21)	0.41	0.59
Chinese (75)	0.79	0.21			
Thais (this study)	0.62	0.38			

5.9 10-year diseased-free survival.

The Kaplan-Meier survival plots were showed in **Figure 5-4**. It can be seen there was no difference of 10-year diseased-free survival rate between AA, AG, GG (**Figure 5-4 A**). Unsurprisingly **Figure 5-4 B** showed similar result that no difference of 10-year diseased-free survival rate between AA+AG and GG genotype.

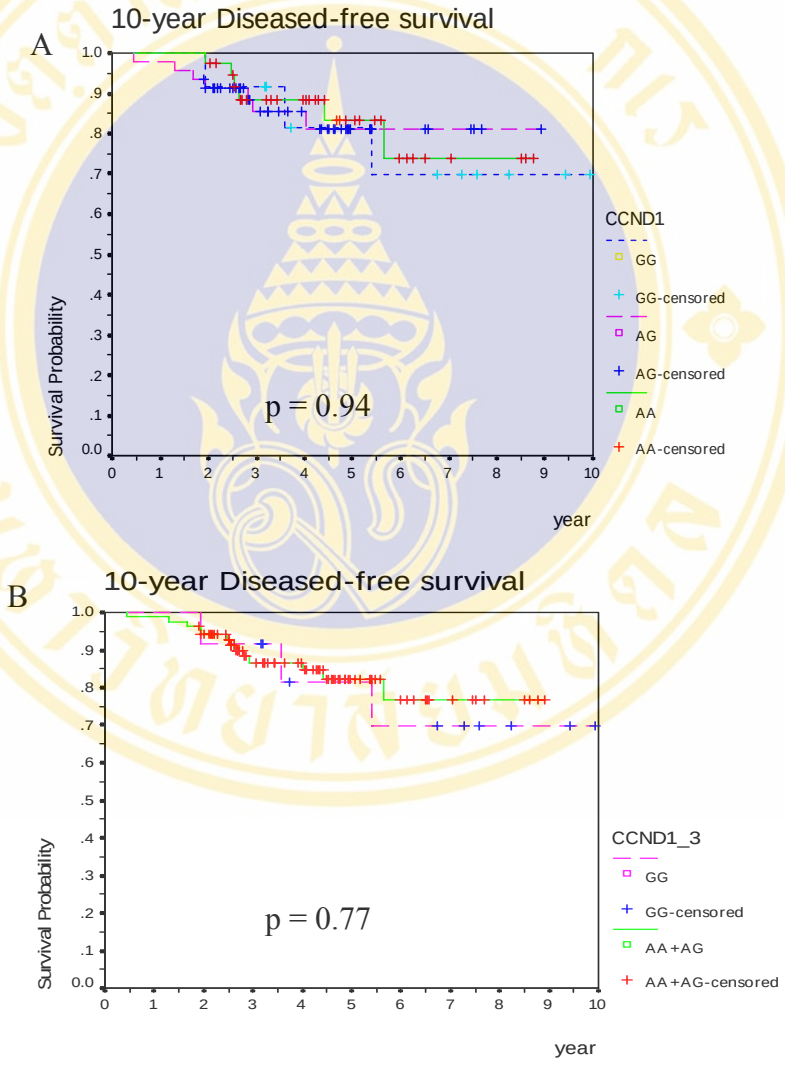

Figure 5-4 Kaplan-Meier survival analysis.
Kaplan-Meier survival plot of the association between the CCND1 genotype and 10-year diseased-free survival of childhood ALL.

CHAPTER 6
DISCUSSION

The study of cyclin D1 polymorphism and childhood ALL has showed no correlation between any particular prognostic factors (genders; aged <1 year, 1-10 years and >10 years and WBC count below and above $50x10^9$/l). This study also showed some similarities to the previous study on cyclin D1 polymorphism and childhood ALL (25).

This study focused on various prognostic factors such as hepatosplenomegaly, lymphadenopathy, hematocrit concentration, platelet count, hyperuricemia, and hyperphosphatemia whilst the previous one concentrated on B or T cell type and DNA index above or below 1.16. Surprisingly, the result from both studies showed no association between these factors and cyclin D1 genotypes.

This study divided the risk of relapse into different categories i.e. low, standard and high. The study reviewed that the prognostic factors were not associated with AA and AG+GG genotype ($p = 0.667$). On contrary, the previous study showed some associations between cyclin D1 genotypes and risk of relapse ($p = 0.02$)in all group; standard, high and very high.

Because the sample size of immunophenotype, cytogenetics, risk to relapse, hyperkalemia and hypocalcemia were limited, therefore Fisher's Exact test was selected to examine the association between the factors and AA and AG+GG genotypes of cyclin D1. The selected test demonstrated that there was lack of association among them.

Furthermore, this study indicated no significance in 10-year disease-free survival rate between AA, AG, GG and AA+AG genotypes. On the other hand the previous study revealed that AA genotype had a lower probability of 5-year post-treatment diseased-free survival compared to AG+GG genotype.

However, there are several possible reasons for the discordance between this study and Costea et al. (25); sample size, population and protocol. Firstly,

this study had limitation in the sample size but the Hardy-Weinberg equilibrium showed no significance deviations of observed genotype frequencies ($p = 0.81$). Secondly, ethnic or environmental variation in populations might confound the result. Finally, the differences in treatment protocols resulted in different outcomes.

Previous studies reported that cyclin D1 polymorphisms were not associated with an increased risk or susceptibility of squamous cell carcinoma of uterine cervix in Korean woman (76), breast cancer (78, 79), colorectal cancer (79), and esophageal squamous cell carcinoma (80). By contrast, many previous studies found the association between cyclin D1 polymorphism and risk of various cancers (10-20, 81). However other than gene polymorphism, mechanisms such as post transcriptional or post translational, rearrangements and gene amplification can also result in abnormal protein levels for instance, protein overexpression and impaired cyclin D1 function.

Cyclin D1 overexpression and CCND1 amplification have been reported in a variety of cancers including breast, head and neck, esophageal, colorectal, genitourinary, lung, and skin cancers and hepatocellular carcinoma (21, 82, 83). Furthermore, Coqueret et al. (4) and Arnold and Papanikolaou (84) were indicated that cyclin D1 can act via two different pathways; a cdk activator to regulate cell cycle progression and a transcriptional regulator to modulate the activity of transcription factors. The first pathway, cdk/cell cycle-related; this pathway has many mechanisms such as activate cdk4/6 via Ras Raf-1MAPK/ERK (MEKs) kinase, dequester cdk inhibitors, deregulated pRB, E2F pathway and abnormal cell cycle progression by clonal selection. The second pathway of cyclin D1 is a transcriptional regulator through the cdk-independent. The transcription of the cyclin D1 gene is regulated by serum and number of growth factors, including colony-stimulating factor-1, platelet-derived growth factor, insulin-like growth factor and steroid hormone (85). Previous studies (86-89) illustrated that the interplay between cyclin D1 and the estrogen pathway had an important role in regulated activation of transcription activity, for example to bind directly and activate the estrogen receptor (ER) alpha, while cyclin D1 interacted with nuclear receptors such as the androgen (90, 91) and thyroid hormone receptors, the repression of transcriptional activity was

observed. However, the mechanisms underlying the transcriptional regulation of the cyclin D1 gene are largely unknown.

According to this study, the frequencies in 100 cases and 300 controls for each allele A and G were identical; 62% for A allele and 38% for G allele. These frequencies were also similar to Zang et.al. (23), Hong et al. (19) and Catarino et al. (74), which were found in Taiwanese, Singaporean and Portuguese population, respectively. Furthermore, the A allele has more frequencies than G allele in Asian populations; Taiwanese (23), Chinese (75), Singaporean (19) and Thai ethnicities except Portuguese (74). Although they were found A allele had more frequency than G allele but cancers associated with GG genotype more than AA or AG genotype. Zang et al., Hong et al. and Catarino et al. stated that the GG genotype associated with poorly differentiated tumor, increase risk or advance in hepatocellular carcinoma, colorectal cancer and invasive cervical cancer, respectively. Moreover, Shu et al. found that the AA and AG genotype had low hazard ratios diseased-free survival among female with late stage or estrogen/progesterone receptor negative or tumor-node-metastasis stage III to IV post menopausal breast cancer. In the other word, there were several possible mechanisms; first GG genotype tended to be associated with more transcript b (7, 19) whereas previous studies reported that the G allele associated both transcript a and transcript b and the A allele associated with transcript b (5, 77). Second, a reduction in the proportion of CCND1 expression cells within the tumors of patients with CCND1 GG (74, 81) so human papillomavirus (HPV) E6 protein binding to pRB and causing the nonphysioligical release of active E2F for cell proliferation. The last, when the cyclin D1 activates the estrogen receptor through the cdk-independent it caused protein overexpression but postmenopausal breast cancer patients had no estrogen receptor.

This thesis was forced to use an unmatched control group, because it was not possible to recruit blood donors younger than 15 years of age. From the study, it can be conducted that there was no statistically difference in the allele distribution between patients and controls. Likewise, age of onset in 3 genotypes of cyclin D1 showed no association with cyclin D1 polymorphism in childhood ALL.

It was important that control DNA samples of known genotype are analyzed in parallel with test samples regardless of the method being used for

genotyping. Although, this study did not have DNA samples of known genotypes to parallel the test samples, but internal control cutting site of each restriction enzymes are checked specific restriction site in each PCR products. However, the genotyping for all test samples indicated corrected polymorphism patterns; wild-type, heterozygous and variant-type.

Genomic DNA was extracted by using a phenol-chloroform standard method, which produced pure DNA. Purified DNA can be stored for long periods of time. Using phenol can be very hazardous and time consuming even there are easier methods available for good yield reproducibility DNA but this method requires standard equipment and it's expensive.

This study used PCR-RFLP to determine specific genotype. PCR-RFLP is relatively simple, required inexpensive equipment and it has many restriction enzyme patterns, which can be recognized. It's time consuming, low throughput and must be used carefully because it required ethidium bromide staining to detect specific genotype. Alternatively, this detection can be monitored under the light of luminescence or fluorescence that emitted by the products such as real time PCR of the TagMan™ and Molecular Beacon. These methods are based on a principle of energy transfer. The fluorescence is detected as a result of a change in physical distance between a reporter fluorophore and quencher molecule on hybridize of the allele specific oligonucleotide probe to its perfectly matched target sequence, measuring the mass of the products (mass spectrometry), or detecting a change in the electrical property when products are formed or can determine the precise sequence of nucleotide in the sample of DNA like that DNA sequencing. But, they are expensive and must use specific equipment.

In summary, this study showed no association of cyclin D1 genotypes with clinical presentations and risk to childhood acute lymphoblastic leukemia. The contribution of genetic polymorphisms to the risk for childhood ALL may be dependent on the variations in environmental and factors that influenced the studied population. Geographical or ethnic differences have been reported regarding the genotype frequency of several polymorphisms. These results are consistence with a recently published study in Thai population. Further studies may include the analysis of other genetic polymorphisms that have been already associated with cancer risk.

This can be characterized the genetic profile of childhood ALL susceptibility such as thymidylate synthase (TS) and methylenetetrahydrofolate reductase (MTHFR) which enzymes are requisite for maintaining DNA synthesis and DNA repair or Phase I and Phase II xenobiotic-metabolizing enzymes which are eliminated environmental toxicants from the body.

Larger-scale molecular studies are needed to confirm the role of A870G polymorphism of cyclin D1 in childhood ALL. The results can lead to a better understanding of cyclin D1 influence on childhood ALL, the biologic mechanisms of childhood ALL and in the definition of a genetic profile for this disease.

CHAPTER 7
CONCLUSION

The purpose of this study was to determine the association of cyclin D1 genetic polymorphism with clinical presentations and outcomes of childhood ALL and to evaluate the cyclin D1 genetic polymorphism on risk of childhood ALL. The childhood ALL 100 cases were comprised of 56 (56%) males and 44 (44%) females, and 300 healthy control group was included of 155 (51.7%) males and 145 (48.3%) females. Mean age ± SD in the case group was 6.2±3.7 years (range 0.8-14.8 years) and control group aged range from 15-55 years. The frequency of AA genotype was observed in 38% of the cases compared to 38% of the controls. The frequency of AG genotype was 49% of the cases and 47.7% of the controls and the frequency of GG genotype was 13% of the cases and 14.3% of the controls. The A and G allele in cases and controls were equally frequencies, 0.62 and 0.38 for A and G allele, respectively. This frequencies were not statically significance different (p >0 05) among the cases and the controls. Although the genotype distribution of both groups were in the Hardy-Weinberg equilibrium (p = 0.81). Cyclin D1 genetic polymorphisms were not associated with clinical presentations and outcomes of childhood ALL and they were not increased risk for this disease.

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APPENDIX

APPENDIX

Preparation of buffer and solution

1. DNA isolation solution

1.1	10% SDS	50	ml
	Sodium dodecylsulfate (SDS)	5	g
	DW	50	ml
	Keep in room temperature		
1.2	Proteinase K (5 mg/ml)	20	ml
	Proteinase K	100	mg
	DW	20	ml
	Aliquot the solution 1.0 ml per vial and keep in freezer below -20°C		
1.3	1M Tris-HCl pH 8.0	1000	ml
	Tris base	121.1	g
	DW	800	ml

Adjust pH to 8.0 with HCl 42 ml, allow the solution to cool to room temperature before making final adjustments to the pH and adjust the volume to 1 liter with DW. Dispense into aliquots and sterile by autoclaving.

(Note: use 250 ml at step 1.5, to make saturated phenol pH 8.0 then dilute the rest with 1:4 pf water to make 0.1 Tris-HCl pH 8.0 adjust enough for step 1.6 and 1.7.

1.4	Saturated Phenol pH 8.0	250	ml
	Phenol (melt at 60°C)	250	g
	8-Hydroxyquinolone	0.25	g
	Add while Phenol still at 60°C.		
1.5	0.5 M Tris-HCl pH 8.0	250	ml
	Approximately used and pore buffer in phenol bottle, continuously shake for 15 min. Let solution separate into 2 layers, the top should be transparence within a few min, otherwise shake again. Discard the top layer by suction gently, leave the least possible.		
1.6	0.1 M Tris-HCl pH 8.0	250	ml
	Mix with phenol, shake for 15 min then follow the rest of step 1.5		
1.7	0.1 M Tris-HCl pH 8.0	250	ml
	As step 1.6, pH should be 8.0 and keep in the brown bottle or clean glass bottle wrapped in aluminium foil at 4-10°C		
1.8	Chloroform-Isoamyl alcohol (24:1 v/v)	1000	ml
	Chloroform	960	ml
	Isoamyl alcohol	40	ml
1.9	3 M Sodium acetate pH 5.6	100	ml
	Sodium acetate	24.61	g
	DW	80	ml
	Adjust pH to 5.6 with glacial acetate and to make a final volume to 100 ml with DW, sterile by autoclave and keep in 4-10°C.		

1.10	70% Ethanol	250	ml
	Absolute ethanol	175	ml
	DW	75	ml
	Keep in freezer		
1.11	Lysis buffer	1,000	ml
	Triosma base	12.1	g
	Sucrose	109.5	g
	DW	800	ml
	Adjust pH to 7.5 with conc. HCl		
	Triton X 100	10	ml
	To make a final volume to 1,000 ml with DW, then sterile by autoclave and keep at 4-10°C		
1.12	10X STE buffer	100	ml
	Tris base	6.05	g
	EDTA	0.37	g
	NaCl	5.84	g
	DW	80	ml
	Adjust pH to 7.5 with conc. HCl, and to make a final volume 100 ml with DW, then sterile by autoclave and keep at 4-10°C		
1.13	TE buffer pH 7.4	500	ml
	Tris-HCl	6.06	g
	EDTA	0.19	g
	Adjust pH to 7.4 by conc. HCl, to make a final volume 500 ml with DW, then sterile by autoclave and keep at room temperature.		

1.14	0.1% Nonidet	1,000	ml
	Nonidet (conc.)	1	ml
	DW	1,000	ml

Keep in sterile bottle at 4-10°C.

2. Electrophoretic analysis solution and Ethidium bromide staining

2.1	5X TBE buffer (stock solution)	1,000	ml
	Tris base	54.0	g
	Boric acid	27.5	g
	EDTA	3.7	g

Dissolved the Tris base and Boric acid in 700 ml DW, then add the EDTA and adjusted the volume to 1,000 ml with DW, keep at room temperature. Working solution (1X TBE) was prepared by adding 200 ml of 5X TBE with 800 ml of DW.

2.2	50 mg% Ethidium bromide solution	100	ml
	Ethidium bromide	0.5	g

Dissolved in 100 ml of DW (5 mg/ml) and sterile on magnetic stirrer for several hours to ensure that the dye was dissolved. Stored in dark bottle in aluminium foil and stored at 4°C.

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